INVESTIGATION OF THE FUNCTIONAL ROLES OF SPECIFIC PROTEIN KINASE C ISOFORMS IN 3T3-F442A ADIPOCYTE DEVELOPMENT AND FUNCTION

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Summary

It is clear, from a wide range of studies, that the protein kinase C (PKC) family has an important role in the regulation of cell growth, differentiation and function. However, as of yet, relatively little is known about the functions of the individual members of the PKC family in any system.

The aim of this study was to investigate the roles of specific PKC isoforms in the regulation of adipocyte development and function using 3T3-F442A cells which differentiate in culture into cells with the charateristics of adipocytes. Initially, the PKC complement of 3T3-F442A cells was thoroughly characterised using a panel of isoform-specific antibodies with application of strict criteria to ensure the appropriate identification of PKC isoforms. Both undifferentiated 3T3-F442A preadipocytes and fully differentiated 3T3-F442A adipocytes were found to express PKC α, γ, δ, ε, ζ and μ but not PKC β, η, θ or ι/λ. The PKC complement of 3T3-F442A adipocytes was found to be identical to that of rat adipocytes. This supported the suitability of using 3T3-F442A cells as a model system of adipocyte development and function.

Initially, in order to gain an indication of the functional roles of individual PKC isoforms in the process of preadipocyte differentiation, the temporal changes in cellular levels of PKC isoforms were examined throughout the time-course of differentiation of 3T3-F442A cells. The α, γ and δ isoforms displayed similar temporal patterns of expression during the differentiation of 3T3-F442A cells; all increased rapidly, peaking at day two of differentiation. Subsequently, the expression of these isoforms decreased, resulting in markedly reduced levels in fully differentiated adipocytes as compared to preadipocytes. The expression of PKC ε increased steadily during differentiation, resulting in markedly elevated levels in adipocytes. Although expression of PKC μ increased during differentiation, this was attributable to prolonged confluence rather than to the differentiation process *per se*. No change was observed in the expression of PKC ζ during adipocyte development. That selective changes in PKC isoform expression accompanied the differentiation process in 3T3-F442A cells implied distinct functional roles for PKC isoforms in the regulation of adipocyte development and function.

To define the dependence of 3T3-F442A preadipocyte differentiation on PKC

isoforms, antisense oligodeoxynucleotides (ODNs) were used to deplete the individual PKC subtypes selectively from 3T3-F442A preadipocytes prior to the induction of differentiation. Each of the ODNs depleted effectively the appropriate PKC isoform and did not affect the expression of other PKC subtypes. By this approach it was demonstrated that PKC ζ is not essential for the differentiation of 3T3-F442A preadipocytes. PKC α, δ and μ each exert an inhibitory influence upon the early stages of adipocyte development and are therefore likely to modulate this stage of the differentiation process. PKC ε and PKC γ are both essential for 3T3-F442A preadipocyte differentiation. Further antisense studies revealed that PKC γ is necessary for the clonal expansion of differentiating cells. PKC ε is not required for clonal expansion but is essential for later stages of the differentiation process, when its expression is markedly elevated, for the attainment of the adipocyte phenotype.

In order to gain an indication of the functional roles of PKC isoforms in adipocytes, their involvement in the signalling mechanisms by which two important hormonal regulators of adipocyte metabolism, growth hormone (GH) and insulin, alter the rate of lipid synthesis was examined in 3T3-F442A adipocytes. By selective depletion of individual PKC isoforms from 3T3-F442A adipocytes using antisense ODNs, it was established that PKC α, δ and ζ are not essential for the stimulation of lipogenesis by insulin or the suppression of basal or insulin-stimulated lipogenesis by GH. PKC ε is one of a number of components required by insulin to increase the rate of lipogenesis. PKC γ is an essential component of the signalling mechanism utilised by GH to antagonise the stimulation of lipogenesis by insulin. PKC μ is, at least in part, required for the suppression of basal lipogenesis by GH. PKC μ is also an essential component of the signal transduction mechanism by which insulin stimulates lipogenesis in 3T3-F442A adipocytes.

The results of these studies clearly demonstrate that individual PKC subtypes assume distinct functional roles in 3T3-F442A cells and are therefore likely to target different cellular substrates. These findings may, therefore, have relevance to our understanding of the diseased state of obesity which involves both adipocyte hyperplasia and hypertrophy and selective changes in expression of PKC isoforms.

Declaration

This thesis has been compiled by myself and is based on work carried out on an original line of research. This thesis has not been offered in any previous application for a degree. All sources of information are referenced and help provided by other people has been duly acknowledged.

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List of Abbreviations

ACC	acetyl coA carboxylase
ADD1	adipocyte determination- and differentiation-dependent factor
AKAP	A-kinase-anchoring protein
BSA	bovine serum albumin
C/EBP	CCAAT/enhancer binding protein
cdk	cyclin-dependent kinase
CRE	cyclic AMP response element
CREB	cyclic AMP-response element binding protein
DAG	diacylglycerol
DHAP	dihydroxyacetone phosphate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethyleneglycol-bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid
FCS	foetal calf serum
GH	growth hormone
GPDH	glycerol-3-phosphate dehydrogenase
GSK	glycogen synthase kinase

Hepes	(N-[2-hydroxyethyl]piperazine-N'-[4-butanesulphonic acid])
HRE	hormone response element
HRP	horseradish peroxidase
HSL	hormone sensitive lipase
IBMX	3-isobutyl-1-methyxanthine
IGF-I	insulin-like growth factor-I
INS	insulin
IP3	inositol trisphosphate
IRS	insulin receptor substrate
LPL	lipoprotein lipase
MAP kinase	mitogen-activated protein kinase
mcv	mean cell volume
MEK	mitogen-activated protein kinase kinase
NADH	α-nicotinamide adenine dinucleotide
ODN	oligodeoxynucleotide
oGH	ovine growth hormone
PA	phosphatidic acid
PBS	phosphate-buffered saline
PC	phosphatidylcholine
PDE	phosphodiesterase
PDGF	platelet-derived growth factor

PDH	pyruvate dehydrogenase
PDK	3-phosphoinositide-dependent protein kinase
PI 3-kinase	phosphatidylinositol 3-kinase
PI	phosphoinositide
PICK	protein interacting with C-kinase
PIP2	phosphatidylinositol bisphosphate
PIP3	phosphatidylinositol trisphosphate
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PKD	protein kinase D
PKM	protein kinase M
PLA	phospholipase A
PLC	phospholipase C
PLD	phospholipase D
PMSF	phenylmethylsulphonyl fluoride
PPAR	peroxisome proliferator activator receptor
PS	phosphatidylserine
RACK	receptor for activated C-kinase
Rb	retinoblastoma protein
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SEM	standard error of the mean
SIE	sis-inducible element
STAT	signal transducers and activators of transcription
T_3	triiodothyronine
TBS	Tris-buffered saline
TPA	12-O-tetradecanoylphorbol 13-acetate
Tris	(tris[hydroxymethyl]aminomethane)
VLDL	very low density lipoprotein
α-rGH	antiserum to rat growth hormone

List of Publications

Iona Millar - née FLEMING

Refereed papers

MacKenzie, S., FLEMING, I., Houslay, M.D., Anderson, N.G. and Kilgour, E. (1997) Growth hormone and phorbol esters require specific protein kinase C isoforms to activate mitogen-activated protein kinases in 3T3-F442A cells. *Biochem. J.*, **324:** 159-165.

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Abstracts

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FLEMING, I.M., MacKenzie, S.J., Houslay, M.D., Vernon, R.G. and Kilgour, E. (1997) Antisense technology reveals distinct roles for protein kinase C isoforms during 3T3-F442A preadipocyte differentiation. *Biochem. Soc. Trans.*, **25;4:** S665.

Vernon, R.G., Arana, A. and FLEMING, I.M. (1998) Growth hormone signalling in ruminant adipose tissue. *J. Animal Sci.*, **76:** Suppl.1/*J.Dairy Sci.*, **81:** Suppl.1: 112.

Chapter 1

Introduction

1.1 General introduction

White adipose tissue depots are widely distributed throughout the body and are the major sites of lipid storage and metabolism. Hence, adipose tissue represents the body's largest energy reservoir. Vertebrate animals store excess metabolic energy in the form of triglycerides. Adipocyte cells in adipose tissue take up fatty acids from blood, or synthesise them *de novo* from glucose and other precursors, when the energy balance is positive. The fatty acids are then esterified with glycerol-3-phosphate and stored intracellularly as triglycerides (lipogenesis). When needed, this stored energy can be mobilised by the hydrolysis of triglycerides (lipolysis) and by the release of fatty acids into the blood stream.

Obesity is perhaps the single most important cause of morbidity in the developed world and is strongly linked with the state of insulin resistance (Reaven, 1988; DeFronzo *et al*, 1992). Thus, resolution of the hormonal, cellular and molecular processes that control adipocyte development and function may lead to a better understanding of these disorders and reveal possible targets for therapeutic intervention. Considerable commercial interest also exists in the ability to manipulate adipose tissue development with a view to the possibility of producing animals with a higher muscle to fat carcass composition.

1.2 Adipose tissue metabolism

The key features of adipocyte metabolism are the synthesis of triglycerides and their subsequent hydrolysis. These processes are under the control of an array of strictly regulated enzymes and are summarised in Figure 1.1. The synthesis of lipids requires a source of fatty acids. These are either liberated from the triglycerides transported in the blood as very low density lipoprotein (VLDL) particles or chylomicrons, by the action of the action of lipoprotein lipase (LPL), or synthesised from glucose, acetate or other precursors (Vernon, 1992). Fatty acids are synthesised from glucose in a series of reactions catalysed by hexokinase, the enzymes of the glycolytic pathway, pyruvate dehydrogenase (PDH), fatty acid synthase (FAS) and acetyl coA carboxylase (ACC). Lipid synthesis also requires a source of glycerol-3-phosphate (Vernon, 1992). This is formed mainly by the reduction of dihydroxyacetone phosphate (DHAP), catalysed by glycerol-3-phosphate dehydrogenase (GPDH).

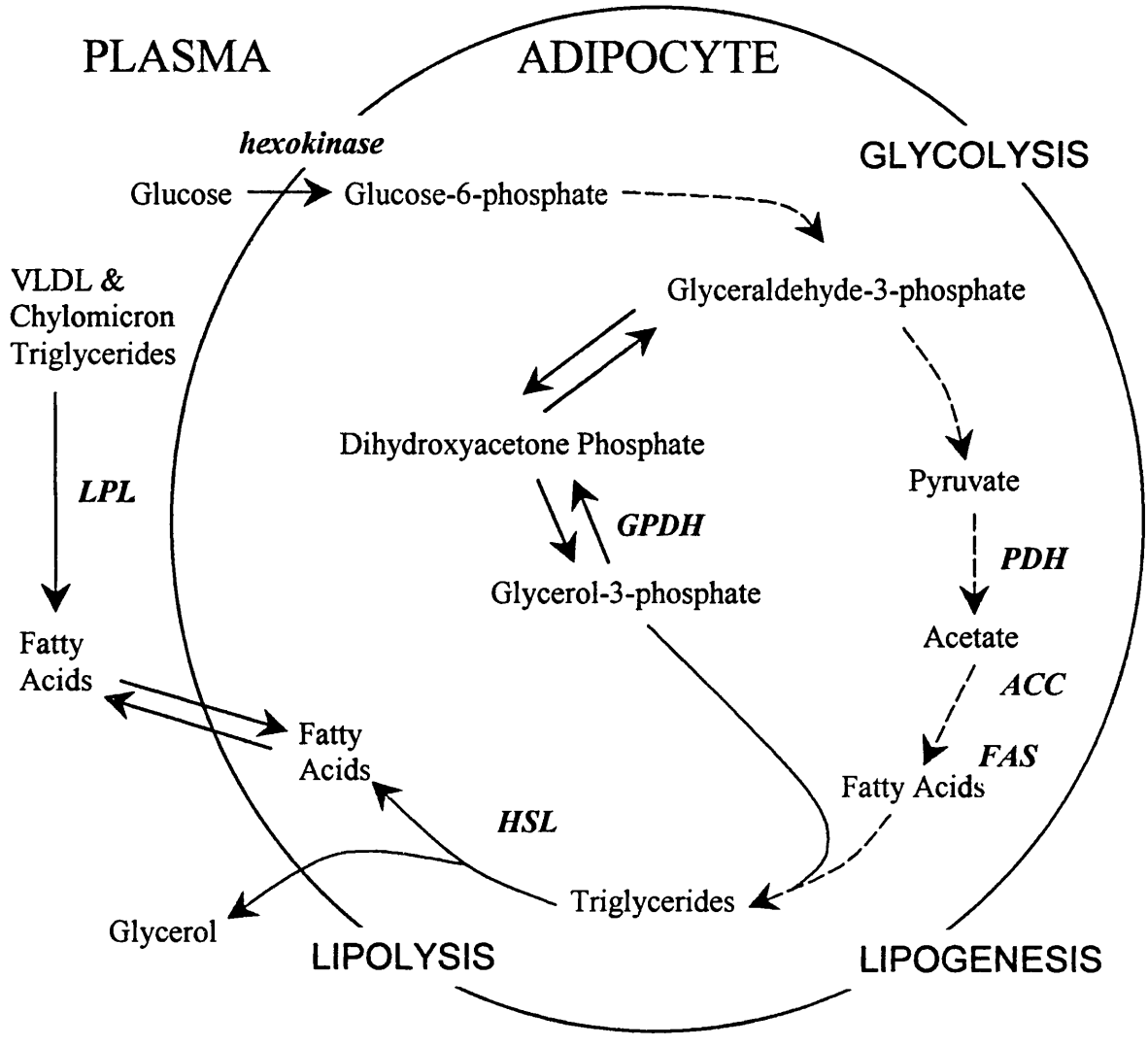


Figure 1.1 Lipid metabolism in the adipocyte

A summary of the lipogenic and lipolytic pathways in the adipocyte, indicating a number of the key enzymes involved.

LPL, lipoprotein lipase; GPDH, glycerol-3-phosphate dehydrogenase; PDH, pyruvate dehydrogenase; ACC, acetyl coA carboxylase; FAS, fatty acid synthase; HSL, hormone sensitive lipase

Lipids are subsequently synthesised from glycerol-3-phosphate and fatty acids in a further set of enzyme reactions.

The crucial step in triglyceride breakdown is the activation of the hormone-sensitive lipase (HSL), which is mediated *via* the adenylate cyclase system (Belfrage, 1984). This results in the generation of fatty acids and diacylglycerol, the latter being rapidly hydrolysed to fatty acids and glycerol. Although fatty acids can be partially re-esterified for triglyceride synthesis, glycerol cannot be re-utilised due to lack of significant glycerokinase activity and is released from the cell (Vernon, 1992).

Lipid metabolism rates are depot-specific (Budd *et al*, 1994). Lipogenesis and lipolysis are occurring continuously, therefore, the relative rates of the two processes determine whether there is net synthesis or deposition of lipid. Hypothalamic centres of the brain are thought to play an important coordinating role in this respect through effects on hunger and satiety, energy expenditure and the secretion of hormones that regulate metabolism (Hubel, 1979). The adipocyte itself is thought to play an active role in regulating energy homeostasis and body composition by secreting a number of products, including leptin (Halaas *et al*, 1995; Ahima *et al*, 1996), the product of the *ob* gene (Zhang *et al*, 1994b). Leptin is absent from *ob/ob* mice, resulting in profound obesity, often accompanied by diabetes (Friedman and Lehel, 1992; Halaas *et al*, 1995). Expression of the *ob* gene itself is controlled by the nutritional status of the animal and the anabolic hormone, insulin (Frederich *et al*, 1995; MacDougald *et al*, 1995; Saladin *et al*, 1995; Kim *et al*, 1998). Leptin reduces adiposity by a number of mechanisms. Leptin lowers food intake and increases metabolism in lean and obese mice (Mistry *et al*, 1997). *In vitro* addition of leptin to adipocytes induces lipolysis (Fruhbeck *et al*, 1997), inhibits binding of the anti-lipolytic hormone insulin (Walder *et al*, 1997) and suppresses the expression of lipogenic enzymes and lipogenesis (Bai *et al*, 1996). The precise mechanisms of leptin action are not known, although it may have a direct action on the hypothalamus (Lee *et al*, 1996; Mistry *et al*, 1997).

1.2.1 Hormonal regulation of adipocyte metabolism

Adipocyte metabolism is regulated by a number of hormones *via* interaction with specific receptors on the surface of adipocytes. It is well established that insulin is the most important regulator of adipocyte metabolism with regard to anabolic functions

(increasing glucose transport and the uptake of other metabolites, stimulating triglyceride synthesis and inhibiting lipolysis) and that catecholamines are the major, acutely acting, counterparts of insulin action (Saggerson, 1984). Other hormones, such as growth hormone (GH), glucagon and glucocorticoids have also been reported to exert a variety of significant modulatory effects on adipocyte metabolism (reviewed in Lager, 1991).

The importance of GH in the modulation of insulin action was initially implied by clinical extremes of GH secretion. Insulin responses are enhanced in GH-deficient patients (Taylor *et al*, 1991) and high serum levels of GH characterise many pathological states in which insulin resistance occurs (Altszuler *et al*, 1974). In addition, insulin resistance is regularly seen during late pregnancy and a contributing reason for this may be the elevated levels of GH that are characteristic of this condition (Eriksson *et al*, 1989). Furthermore, elevated numbers of GH receptors are observed on the surface of diabetic adipocytes (Solomon *et al*, 1990). GH, therefore, appears to have insulin-antagonistic effects and these may be important for the development of the insulin-resistance that is associated with obesity and diabetes mellitus (Fowelin *et al*, 1991). Hence, there has been increasing interest in the modulation of adipocyte metabolism by GH. The metabolic effects of insulin are well documented, therefore, this review will concentrate on the role of GH in the modulation of adipocyte metabolism.

1.2.1.1 Modulation of adipocyte metabolism by growth hormone

The role of GH in the regulation of glucose and lipid metabolism in adipose tissue has been controversial, mainly due to discrepancies between *in vivo* and *in vitro* studies. Clinical observations clearly indicate that excess GH is associated with impaired glucose tolerance and insulin-resistance, whereas GH-deficiency enhances the responsiveness of adipocytes to insulin (Section 1.2.1). In addition, chronic administration of GH to patients with GH-deficiency increases the level of circulating free fatty acids and decreases the lipid content of adipose cells, probably due to increased lipolysis and reduced fat deposition (Bonnet *et al*, 1974; Van Vliet *et al*, 1987). These observations are consistent with GH decreasing adiposity and antagonising insulin action.

In vitro studies of the action of GH in adipose cells initially produced conflicting results. This is mainly due to the fact that, *in vitro*, GH can have transient insulin-like effects that are followed by the classical diabetogenic effects of the hormone if treatment is continued (Vernon and Flint, 1989). The physiological significance of these transient insulin-like effects of GH is not known since cells have to be deprived of GH for a significant period for the effects to manifest themselves, a situation unlikely to normally occur *in vivo*. The dual effects of GH treatment on adipocyte metabolism are summarised in Table 1.1.

It is now well established that, chronically, GH decreases adiposity in many species (Vernon and Flint, 1989). *In vitro* studies have demonstrated that chronic exposure of adipocytes to GH suppresses glucose transport and lipogenesis and promotes lipolysis (Ku Tai *et al*, 1990; Maloff *et al*, 1980; Boyd and Bauman, 1989; Vernon and Flint, 1989; Goodman, 1993). Although the effects of GH on many tissues are mediated *via* insulin-like growth factor-I (IGF-I), this is not the case for adipocytes upon which GH exerts direct effects mediated by its cell surface receptor (Vernon and Flint, 1989). The fact that chronic GH treatment decreases adiposity by a mechanism that appears to involve changes in lipid synthesis and mobilisation is consistent with an insulin-antagonistic role. Chronic GH treatment suppresses the ability of fat cells to respond to insulin in the absence of any change in their capacity to bind the hormone (Foster *et al*, 1988). It is therefore believed that GH induces one or more post-receptor defects in insulin signalling cascades.

The mechanisms by which GH modulates adipocyte metabolism have not been fully elucidated, but undoubtedly the modulation of expression of key proteins, known to be involved in metabolic pathways and cross-talk with insulin and other signalling pathways are important. Changes in gene transcription are also likely to be involved (Goodman *et al*, 1987; Goodman, 1993). Current knowledge of the mechanisms by which GH modulates adipocyte metabolism is outlined below.

1.2.1.1.1 Growth hormone and glucose transport

Chronic exposure to GH restricts glucose utilisation by adipose tissue (Ku Tai *et al*, 1990; Maloff *et al*, 1980). Glucose enters the fat cell by facilitated diffusion across the plasma membrane and this process is mediated by the transporter proteins GLUT 1

Table 1.1 The dual effects of growth hormone treatment on adipocyte metabolism

Effects of growth hormone treatment	
Acute, insulin-like effects	Chronic, insulin-antagonistic effects
Stimulation of glucose transport	Suppression of glucose transport
Enhancement of lipogenesis	Suppression of lipogenesis
Suppression of lipolysis	Enhancement of lipolysis

and GLUT 4 (Gould and Bell, 1990). In adipocytes, the most abundant glucose transporter is GLUT 4, which translocates to the plasma membrane in response to insulin. *In vitro* studies have revealed that chronic GH treatment decreases GLUT 1 protein and mRNA levels in 3T3-F442A adipocytes and that, in contrast, cellular levels of GLUT 4 are not affected (Ku Tai *et al*, 1990). This is consistent with the observation that GH, *in vivo*, down-regulates the amount of both glucose transporters present in rat adipocyte plasma membranes, reflecting a decrease in total cellular GLUT 1 levels and modification of the sub-cellular location of GLUT 4 trasporters (Kilgour *et al*, 1995).

1.2.1.1.2 Growth hormone and lipogenesis

Chronically, GH exerts a small inhibitory effect on basal lipogenesis and antagonises the ability of insulin to maintain or increase the basal rate (Vernon, 1982; Schwartz, 1984; Foster *et al*, 1988). Lipogenesis is subject to control by changes in the expression and activation status of key enzymes (see Figure 1.1). The suppression of lipogenesis by GH has been attributed mainly to GH-induced changes in the levels and activation status of such enzymes. Decreases in the rates of lipogenesis, *in vivo,* have been attributed to GH suppressing the transcription of acetyl CoA carboxylase (Bauman and Vernon, 1993) and fatty acid synthase (Harris *et al*, 1993) mRNA. Decreases in the activities of acetyl CoA carboxylase and fatty acid synthase are also observed following chronic treatment of adipocytes or adipose tissue with GH (Dietz and Schwartz, 1991; Bauman and Vernon, 1993; Vernon *et al*, 1993). In addition, GH inhibits the activation of acetyl CoA carboxylase by insulin, possibly by inhibiting the synthesis of a protein required for mediation of the insulin-induced activation of the enzyme (Vernon *et al*, 1991).

1.2.1.1.3 Growth hormone and lipolysis

The key enzyme in the lipolytic pathway is hormone sensitive lipase (HSL). Lipolytic agents increase cyclic AMP levels, activating cyclic AMP-dependent protein kinase (protein kinase A; PKA) which can phosphorylate and activate HSL (Belfrage, 1984; Allen, 1985). Although GH promotes lipolysis *in vivo*, studies done *in vitro* have failed to produce consistent effects of GH alone on lipolysis in isolated adipocytes (Vernon and Flint, 1989). However, in 3T3-F442A adipocytes, GH promotes lipolysis and increases the activity of HSL (Dietz and Schwartz, 1991). Further evidence

suggests that chronically, GH promotes lipolysis by modulating the ability of acutely acting hormones, especially anti-lipolytic agents, to regulate the process (Vernon and Finley, 1988). This effect appears to involve a lesion in the coupling between receptor, the inhibitory protein G_i and adenylyl cylase (Doris *et al*, 1994).

Increases in cyclic AMP phosphodiesterase (PDE) activity and subsequent hydrolysis of cyclic AMP, lead to decreased lipolysis (Manganiello *et al*, 1987). Indeed, certain PDE isoforms play a key role in the anti-lipolytic actions of insulin in rat adipocytes (Eriksson *et al*, 1995). *In vivo*, GH suppresses the activity and insulin-responsiveness of these PDE isoforms in adipocytes (Schoenle *et al*, 1981). This is interesting in view of reports that, *in vitro*, chronic GH attenuates the anti-lipolytic effect of insulin in 3T3-L1 adipocytes (Glenn *et al*, 1992).

A number of studies indicate the expansion of adipose tissue and, hence, the development of obesity, is due to both an increase in the size and number of adipocytes (Greenwood and Hirsch, 1974; Hager, 1981; Bourgeois *et al*, 1983). An increase in the size of adipocytes is primarily due to the accumulation of lipid and is therefore closely linked to the metabolic pathways controlling lipid accumulation and mobilisation. The factors affecting adipocyte number are discussed below.

1.3 Adipose tissue development

It is now generally accepted that mature, lipid-filled adipocytes do not divide and there is evidence for loss of various components required for mitosis during preadipocyte differentiation (Roncari *et al*, 1984). Increases in fat cell number, therefore, arise as the result of the proliferation and differentiation of adipose precursor cells. The processes involved in adipocyte development are summarised in Figure 1.2.

Studies to date suggest that the adipocyte lineage derives from pluripotent mesenchymal cells with the capacity to differentiate into adipocytes, chondrocytes and myocytes (Green *et al*, 1985; Sager and Kovac, 1982; Grigoriadis *et al*, 1988). Adipose precursor cells are thought to proliferate and differentiate under multi-hormonal control into preadipocytes and, subsequently, adipocytes. Studies with

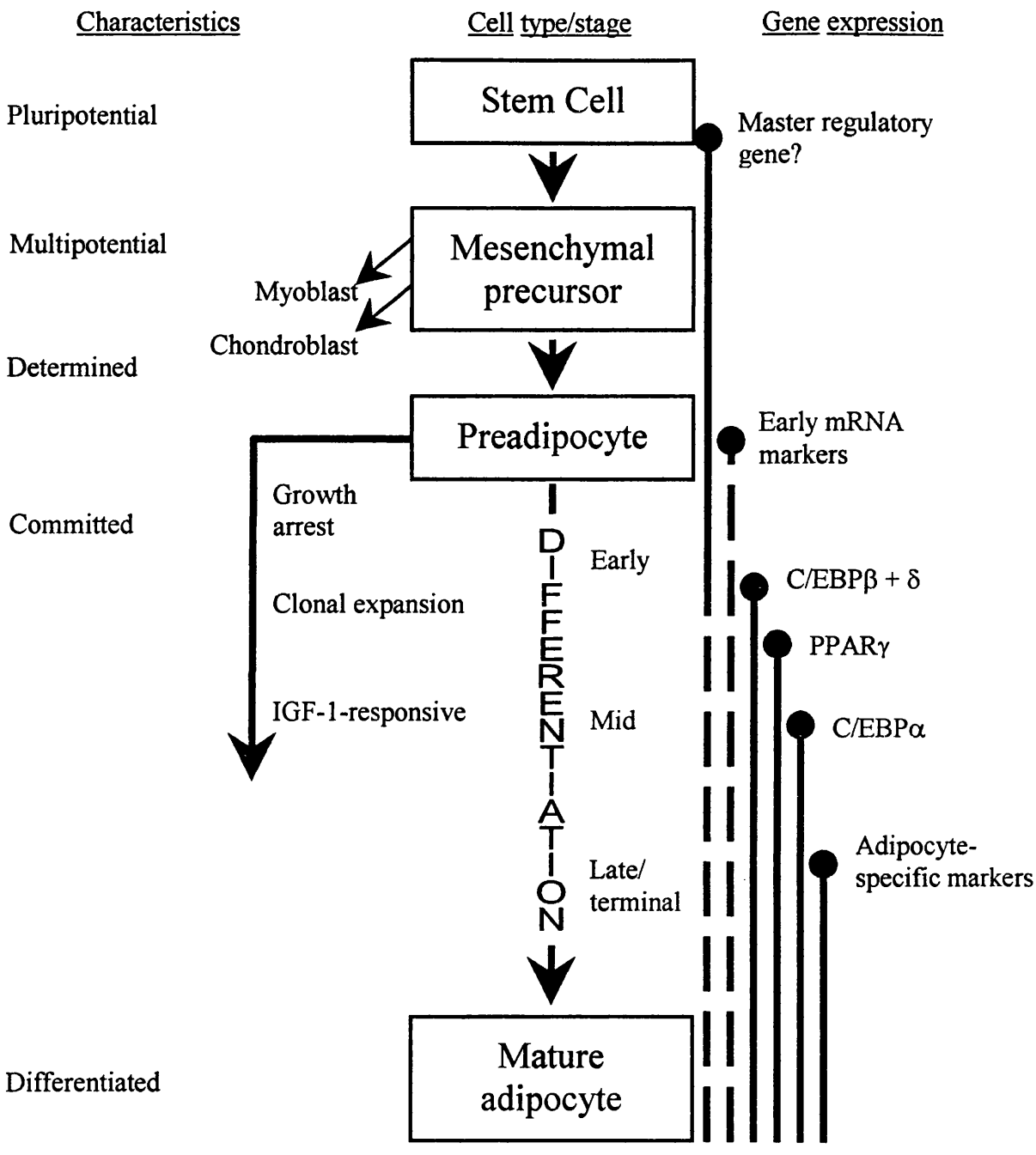


Figure 1.2 Summary of the preadipocyte differentiation process

(Smas and Sul, 1995).

multipotent stem cells suggest that commitment to a specific lineage results from the activation of a small number of regulatory genes by hypomethylation (Konieczny and Emerson, 1984; Lassar *et al*, 1986; Taylor and Jones, 1979). Genes of the MyoD transcription factor family control myoblast differentiation *in vitro* (reviewed in Edmondson and Olson, 1993), however, attempts to identify a so-called "master regulatory" gene for adipocyte differentiation have, as yet, been unsuccessful. Most of the progress in the understanding of the molecular basis of adipose differentiation has, therefore, come from the study of the differentiation of preadipocytes into adipocytes. This is largely due to the development of immortalised preadipocyte cell-lines that differentiate in culture into white adipocyte-like cells.

1.3.1 Cell culture models of preadipocyte differentiation

A number of cellular models have been developed to facilitate the study of the factors controlling preadipocyte differentiation *in vitro*. These include primary cultures of preadipocytes and cell-lines capable of converting to the adipocyte phenotype. Although cell strains that behave reproducibly in culture have been obtained from the stromal fraction of adipose tissue from a variety of species (Ailhaud, 1982), such adipose precursor cells have a limited life span in culture. Consequently, most of the information on preadipocyte differentiation to date has been gained from the study of established cell lines.

The most extensively studied cell culture models are 3T3-L1 and 3T3-F442A preadipocytes. These cells have undergone commitment to the adipose lineage, can only further differentiate into adipocytes and were originally selected from disaggregated mouse embryo cells for their ability to accumulate cytoplasmic lipid (Green and Kehinde, 1974; Green and Kehinde, 1975; Green and Kehinde, 1976). Other common cell lines include Ob 17 and Ob 1771, isolated from obese mice (Doglio *et al*, 1986) and TA1 (Chapman *et al*, 1984) and 30A5 (Konieczny and Emerson, 1984), isolated from an established, multipotential mouse fibroblast line treated with a nucleotide analogue to generate cells committed to the adipose lineage (Taylor and Jones, 1979).

The process of adipose conversion is phenotypically similar in the various preadipocyte cell lines. During proliferation, cultured preadipocytes are biochemically

and morphologically indistinguishable from fibroblasts (Hiragun *et al*, 1980). When induced to differentiate, cells convert to a spherical shape and accumulate small lipid droplets that later fuse into one large droplet. The shape change occurs prior to lipid accumulation and is due to alterations in the complement of cytoskeletal proteins (Spiegelman and Farmer, 1982). The mature adipocyte produced by *in vitro* differentiation has many characteristics of adipose cells *in vivo*. Cells acquire virtually all of the enzymes responsible for *de novo* fatty acid synthesis, lipogenesis and lipolysis and responsiveness to the hormones that regulate these processes (Rubin *et al*, 1978; Spooner *et al*, 1979; Reed and Lane, 1980; Bernholer *et al*, 1985; Cook *et al*, 1988; Guest *et al*, 1990). Furthermore, subcutaneous injection of certain preadipocyte cell lines into nude mice gives rise to fat pads that are histologically indistinguishable from white adipose tissue (Green and Kehinde, 1979; Vannier *et al*, 1985).

1.3.2 The preadipocyte differentiation programme

Preadipocyte differentiation is a multi-step process involving a cascade of molecular events culminating in the terminally differentiated phenotype. The molecular events involved in adipocyte development have not yet been fully elucidated, but studies using preadipocyte cell lines have revealed a number of the factors involved.

Terminal differentiation to adipocytes involves changes in the levels of over one hundred proteins (Sidhu, 1979), mainly at the transcriptional level (Moustaid and Sul, 1991). The initiation of differentiation and the subsequent expression of adipocyte-specific genes requires exit from the mitotic cell cycle. From *in vitro* studies, it appears that growth arrest during G1 (G0 entry), rather than simply cell confluence, is necessary to trigger initiation of the differentiation programme (Ailhaud *et al*, 1989). Cells in G0 begin to express early markers of differentiation such as the mRNAs for the α–2 chain of type VI collagen and lipoprotein lipase (LPL) (Amri *et al*, 1986).

Prior to the expression of late markers, early marker-expressing cells undergo post-confluent mitoses (Kuri-Harcuch and Marsch-Moreno, 1983; Zezulak and Green, 1986). This process of replication has been proposed to lead to the clonal expansion of committed cells (Pairault and Green, 1979) and is thought to allow cells to reach a

unique growth-arrested state that is permissive for subsequent differentiation (Pairault and Green, 1979; Scott *et al*, 1982; Amri *et al*, 1986). Clonal expansion is limited both in magnitude and duration (Ailhaud *et al*, 1989; Gamou and Shimizu, 1986; Kuri-Harcuch and Marsch-Moreno, 1983) and is stimulated by IGF-I and/or insulin (Hauner, 1990; Zezulak and Green, 1986).

Mitogens, such as PDGF, can block adipose conversion at this stage by stimulating cell cycle re-entry (Corin *et al*, 1990). Progression to the terminal stages of differentiation, therefore, requires the induction of an anti-mitogenic state in growth arrested cells. This is induced by GH, which primes cells at G0 to become resistant to the actions of mitogens and responsive to the promoters of terminal differentiation (Corin *et al*, 1990). The mechanisms by which mitogens, such as PDGF, and anti-mitogens, such as GH, control cell cycle entry and exit are not yet fully understood, but probably involve the modulation of proteins that regulate the G1 phase of the cell cycle (Sherr, 1994). For example, cyclin D expression is suppressed in 3T3-F442A preadipocytes treated with GH, which may contribute to the priming action of GH in these cells (Timchenko *et al*, 1996).

As clonal expansion slows, the expression of proteins that give rise to the adipocyte phenotype is initiated. The process of terminal differentiation is characterised by the induction of late markers, including the enzymes responsible for lipogenesis and triglyceride synthesis (Paulauskis and Sul, 1988; Spiegelman *et al*, 1983). The late genes also include those for hormone receptors that confer responsiveness to lipogenic and lipolytic hormones (de Herreros and Birnbaum, 1989; Zou *et al*, 1997). Among the first of these proteins to be expressed is the transcriptional regulatory protein, C/EBP α (Christy *et al*, 1991). C/EBP α is anti-mitogenic (Timchenko *et al*, 1996) and has been implicated in the termination of clonal expansion and maintenance of the terminally differentiated cell (Freytag and Geddes, 1992).

1.3.3 Transcriptional control of protein expression during preadipocyte differentiation

A number of transcriptional regulatory proteins have been implicated in the process of preadipocyte differentiation. In particular, isoforms of the CCAAT/enhancer binding

protein (C/EBP) family and certain members of the peroxisome proliferator activator receptor (PPAR) family are known to interact with the promoter elements of a number of adipocyte-specific genes (see Yeh and McKnight, 1995). These, and a number of other transcription factors (see Section 1.3.3.2), are thought to contribute critically to the reprogramming of gene expression that allows preadipose cells to clonally expand and develop into mature adipocytes. The temporal expression profiles of the C/EBP and PPAR proteins during *in vitro* preadipocyte differentiation are summarised in Figure1.3.

1.3.3.1 Transcriptional regulation by C/EBP and PPAR proteins

C/EBP α is expressed in adipose tissue *in vivo* and *in vitro* (Birkenmeier *et al*, 1989). Its expression precedes that of adipocyte-specific genes during *in vitro* preadipocyte differentiation (Christy *et al*, 1991). C/EBP α binds to and *trans*-activates the promoters of a number of genes that are coordinately expressed during preadipocyte differentiation (Christy *et al*, 1989; Kaestner *et al*, 1990). In addition, the C/EBP α promoter possesses a C/EBP binding site (Christy *et al*, 1991). This apparently mediates *trans*-activation by its own gene product and probably contributes to the maintenance of steady-state levels of C/EBP α in the mature adipocyte (Legraverend *et al*, 1993; Lin *et al*, 1993). These observations suggest that C/EBP α is an important regulator of adipogenesis. Several lines of evidence support this assumption. Antisense experiments demonstrate that C/EBP α is required for the process of adipogenesis (Samuelsson *et al*, 1991; Lin and Lane, 1992) and ectopic expression of C/EBP α has been shown to induce adipogenesis in otherwise non-differentiating NIH-3T3 cells (Lin and Lane, 1994; Freytag *et al*, 1994). In addition, lipid accumulation in adipose tissue is severely retarded in mice lacking functional C/EBP α (Wang *et al*, 1995).

Although C/EBP α can specify growth arrest and adipogenesis (Freytag and Geddes, 1992), it is not expressed during the initial stages of differentiation (Figure 1.3). Two additional members of the C/EBP family are, however, rapidly induced during the clonal expansion phase of preadipocyte differentiation, prior to the induction of C/EBP α (Cao *et al*, 1991). C/EBP β and C/EBP δ are suggested to play an important transient role in early adipocyte differentiation by relaying the effects of hormonal

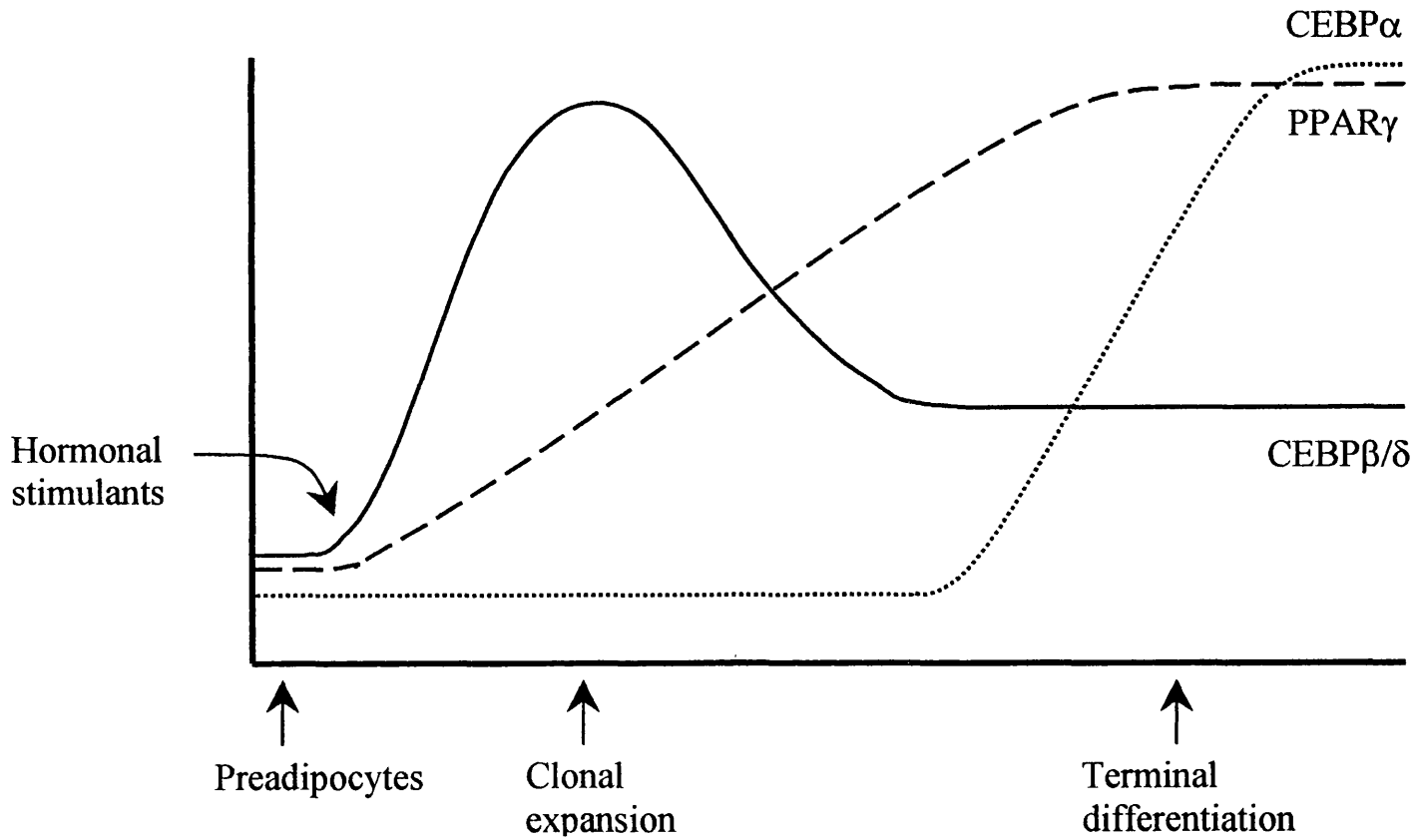


Figure 1.3 Temporal expression profiles of the C/EBP and PPAR transcriptional regulatory proteins during preadipocyte differentiation

(Yeh and McKnight, 1995)

stimulants such as glucocorticoids, GH, insulin and stimulators of the cAMP signalling pathways (Cao *et al*, 1991; Clarkson *et al*, 1995; Yeh *et al*, 1995). C/EBP β and δ are also thought to *trans*-activate the C/EBP α gene (Lane *et al*, 1996). It is therefore thought that C/EBP β and C/EBP δ may relay the effects of adipogenic stimuli, culminating in the production of C/EBP α, the ultimate regulator of terminal adipogenesis (Cao *et al*, 1991).

PPARs are a subgroup of the nuclear hormone receptor subfamily of transcription factors (Isseman and Green, 1990, Drever *et al*, 1992, Gottlicher *et al*, 1992; Keller *et al*, 1993). The PPAR γ isoforms, γ1 and γ2, are abundantly expressed in white adipose tissue (Vidalpuig *et al*, 1996; Zhu *et al*, 1995). Both PPAR γ isoforms are induced very early in several cell culture models of adipogenesis (Amri *et al*, 1995; Chawla *et al*, 1994) and ectopic expression of PPAR γ1 or PPAR γ2 in fibroblasts promotes adipogenesis (Tontonoz *et al*, 1994b). These observations suggest that PPAR γ isoforms play an important role in the process of preadipocyte differentiation. C/EBP β induces PPAR γ expression in the preadipocyte, subsequently triggering differentiation (Wu *et al*, 1995). In addition, PPAR γ synergises powerfully with C/EBP α to stimulate adipogenesis (Tontonoz *et al*, 1994a). It has, therefore, been suggested that PPAR γ isoforms may be important targets of C/EBP β and δ in channeling the adipogenic potential of C/EBP α.

1.3.3.2 Regulation by other transcription factors

ADD1 (adipocyte determination- and differentiation-dependent factor 1), a member of the basic helix-loop-helix family of transcription factors (Tontonoz *et al*, 1993), has also been implicated in preadipocyte differentiation. ADD1 is expressed predominantly in adipose tissue and liver and its expression is induced at a very early stage of adipogenesis (Tontonoz *et al*, 1993; Kim and Spiegelman, 1996). Retroviral expression of dominant-negative ADD1 significantly represses differentiation and the expression of adipocyte-specific genes in 3T3-L1 cells and ectopic expression of ADD1 promotes adipocyte differentiation (Kim and Spiegelman, 1996). In addition, ADD1 can induce expression of fatty acid synthase (FAS) and lipoprotein lipase (LPL), two key proteins involved in lipid synthesis (Kim and Spiegelman, 1996). The effects of ADD1 on preadipocyte differentiation appear to be due, at least in part, to

induction of PPAR γ activity (Kim and Spiegelman, 1996).

Further transcription factors implicated in the process of adipogenesis are the so-called immediate early genes, c-*fos*, c-*jun* and c-*myc*. Transient increases in the expression of these genes precedes the induction of C/EBP β and δ during the early stages of preadipocyte differentiation (Stone and Bernlohr, 1990; Stephens and Pekala, 1992). Homo- and heterodimers of c-*jun* and c-*fos* were the first proposed regulators of adipocyte-specific gene expression (Spiegelman *et al*, 1988). GH rapidly induces the transcription of c-*fos* and c-*jun* in both 3T3-F442A (Gurland *et al*, 1990) and Ob1771 (Barcellini-Couget *et al*, 1993) preadipocytes and this coincides with the induction of C/EBP β and δ by GH (Clarkson *et al*, 1995). In 3T3-L1 preadipocytes, both insulin and IGF-1 induce expression of c-*fos* (Weiland *et al*, 1991). In addition, c-*fos* has been shown to play a role in the modulation of lipoprotein lipase (LPL) gene expression (Barcellini-Couget *et al*, 1993). Therefore, it appears that c-*fos* and c-*jun* may relay specific temporal cues from extracellular stimuli to adipocyte-specific genes. Expression of c-*myc* is induced during clonal expansion of preadipocytes and decreases concomitantly with induction of the C/EBP α gene (Christy *et al*, 1991). The C/EBP α promoter contains a consensus c-*myc*-binding site (Christy *et al*, 1991) that can bind c-*myc in vitro* (Legraverend *et al*, 1993). Therefore the induction of C/EBP α during clonal expansion may involve *trans*-activation by c-*myc*.

Thus, several of the key transcriptional players in adipogenesis have now been identified. Adipogenesis is profoundly influenced by a variety of hormones and nutritional signals (Cornelius *et al*, 1994; Ailhaud *et al*, 1992; Ailhaud *et al*, 1994; Smas and Sul, 1995). A number of these hormones have been shown to affect the activity of adipogenic transcription factors. These observations suggest an important regulatory cascade in which hormonal signals first induce C/EBP β and δ and subsequently activate PPAR γ and C/EBP α, thereby promoting and maintaining adipogenesis.

1.3.4 Hormonal control of preadipocyte differentiation

The precise combination of hormones and growth factors required for the

differentiation of preadipocytes is not known and is likely to vary amongst different cell-lines. Several laboratories have developed serum-free culture conditions and have implicated GH, IGF-I, insulin, triiodothyronine (T_3), glucocorticoids and cyclic AMP-elevating agents as key hormonal regulators of preadipocyte differentiation (Guller *et al*, 1988; Hauner *et al*, 1990; Schmidt *et al*, 1990).

1.3.4.1 Growth hormone and insulin-like growth factor-I

Depending on the cell-line, either GH or IGF-I appear to be obligatory for preadipocyte differentiation. Both 3T3-F442A and Ob1771 preadipocytes require GH for differentiation (Nixon and Green, 1984a; Nixon and Green, 1984b; Catalioto *et al*, 1992; Dani *et al*, 1990). GH has been shown to induce IGF-I gene expression in Ob1771 preadipocytes (Doglio *et al*, 1987) and to increase the sensitivity of 3T3-F442A cells to the actions of IGF-I (Zezulak and Green, 1986). However, IGF-I cannot substitute for GH in the differentiation of either cell type (Nixon and Green, 1984a; Nixon and Green, 1984b; Catalioto *et al*, 1992; Dani *et al*, 1990). At least in 3T3-F442A preadipocytes, it appears that GH first directly stimulates adipocyte differentiation, then the number of young differentiated cells is increased by clonal expansion mediated by the mitotic actions of IGF-I (Green *et al*, 1985). GH does not, however, support the differentiation of 3T3-L1 cells. In this case, IGF-I and pharmacological doses of insulin are required for differentiation (Smith *et al*, 1988).

The effects of GH on preadipocyte differentiation appear to be differential. GH promotes early stages of differentiation and contributes to clonal expansion. However, at later stages of differentiation, GH, through its inhibitory effects on lipid metabolism (see Section 1.2.1.1), can reduce the proportion of newly formed adipocytes and late markers of differentiation (Hausman and Martin, 1989; Wabitsch *et al*, 1996; Fain *et al*, 1985; Goodman *et al*, 1990; Schwartz and Carter-Su, 1988; Wabitsch *et al*, 1996).

The exact mechanism by which GH exerts its adipogenic effects in preadipocytes is understood poorly. However, the transient stimulation of c-*fos* and/or c-*jun* transcription by GH (Doglio *et al*, 1989; Sumantran *et al*, 1992), which coincides with transcriptional activation of C/EBP δ and translational activation of C/EBP β, is likely to contribute to its differentiation-promoting effects (Clarkson *et al*, 1995).

1.3.4.2 Insulin

Developing adipocytes become highly responsive to insulin. This has been attributed to increases in the number of insulin receptors (Rubin *et al*, 1978) and increased expression of metabolic proteins that are regulated by insulin (Park and Kim, 1991). The presence of insulin is obligatory for the differentiation of 3T3-F442A, 3T3-L1 and Ob17 cells (Doglio *et al*, 1986; Smith *et al*, 1988; Guller *et al*, 1988). Although insulin does not appear to be required for commitment to the differentiation process (Steinberg and Brownstein, 1982), it promotes the accumulation of lipid (Smith *et al*, 1988) and the induction of late markers (Dani *et al*, 1986) in differentiating cells. To optimise adipose conversion in preadipocyte cell systems, insulin is often used at supraphysiological concentrations where its effects may be mediated *via* the IGF-I receptor (Guller *et al*, 1988). Therefore, there may exist subtle interplay between the insulin and IGF-I receptors to modulate the differentiation process.

In 3T3-F442A adipocytes, insulin has been shown to increase the expression of PPAR γ1 and γ2 and regulate the transcription of C/EBPs (MacDougald *et al*, 1995; Vidalpuig *et al*, 1996). In addition, insulin is required for maintaining the activity of PPAR γ2 in the adipose tissue of lean and obese mice (Vidalpuig *et al*, 1996). Insulin and IGF-I also increase the transcriptional activity of ADD1 (Streicher *et al*, 1996). As ADD1 has been shown to increase PPAR γ activity (Kim and Spiegelman, 1996), this may be an additional mechanism by which insulin maintains the activity of PPAR γ. Thus, insulin appears to modulate the activity of several critical adipogenic transcription factors during the differentiation process.

1.3.4.3 Epidermal growth factor

Like PDGF, EGF inhibits preadipocyte differentiation by stimulating cell cycle re-entry (Corin *et al*, 1990; Serrero, 1987). However, EGF is required for the maintenance of 3T3-F442A and 3T3-L1 preadipocyte differentiation (Guller *et al*, 1988; Schmidt *et al*, 1990) and elevated EGF levels are associated with adipocyte hypertrophy in obese mice (Kurachi *et al*, 1993). EGF has been shown to promote the insulin-stimulated accumulation of triglycerides in primed preadipocytes, possibly by reducing catecholamine-mediated lipolysis (Taber *et al*, 1993). Thus, although EGF

can prevent initiation of the differentiation programme, it appears to be required for the progression of adipogenesis. The effects of EGF on the adipogenic transcriptional regulatory cascade are, as yet, unclear.

1.3.4.4 Cyclic AMP

The actions of many hormones are mediated at the cellular level by cyclic AMP. Cyclic AMP-elevating agents have been shown to promote the adipose conversion of a number of preadipocyte cell-lines and induce the transcription of several adipocyte-specific genes (Bhandari *et al*, 1991; Park and Kim, 1991; Yang *et al*, 1989). Addition of the cyclic AMP phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX) in the presence of insulin and glucocorticoids, has been shown to increase the differentiation of 3T3-L1 (Rubin *et al*, 1978; Schiwek and Loffler, 1987), rat (Xu and Bjorntorp, 1990) and human preadipocytes (Hauner *et al*, 1989). Agents that increase cyclic AMP have also been shown to promote the differentiation of Ob17 preadipocytes (Vassaux *et al*, 1992) and potentiate the GH-dependent stage of differentiation in 3T3-F442A preadipocytes (Yarwood *et al*, 1998).

Increases in the expression of certain sub units of cyclic AMP-dependent protein kinase (PKA) precede morphological differentiation in TA1 preadipocytes (Kurten *et al*, 1988). PKA phosphorylates members of the CREB (cyclic AMP-response-element-binding protein) family of transcription factors (Gonzalez and Montminy, 1989; Sheng *et al*, 1991) that activate transcription through binding to cyclic AMP response elements (CREs) in target genes (Hagiwara *et al*, 1993). The promotion of adipogenesis by cyclic AMP has also been attributed to the induction of C/EBP β, which has been shown to be regulated by IBMX (Yeh *et al*, 1995). C/EBP family members can form heterodimers with members of the CREB family (Vallejo *et al*, 1993), suggesting that functional "cross talk" between CREB proteins and C/EBPs may contribute to the promotional effects of cyclic AMP on preadipocyte differentiation.

It appears that, like GH, cyclic AMP exerts differential effects on adipocyte development. Cyclic AMP acts both at an early stage to promote preadipocyte differentiation, by inducing adipocyte-specific gene expression, and during the

terminal stages to limit fat cell size, by suppressing lipogenic gene expression (Antras *et al*, 1991) and promoting lipolysis (see Section 1.2.1.1.3).

1.3.4.5 Agents that signal via nuclear receptors

In addition to those growth factors that act through membrane receptors, thyroid and glucocorticoid hormones, fatty acids and retinoids also influence adipocyte differentiation. Their mechanism of action is, as yet, understood poorly. However, in general, these agents diffuse into cells, interact with cognate receptors (Evans, 1988; Green and Chambon, 1988) and exert nuclear effects through *cis*-acting hormone response elements (HREs) in the promoter regions of target genes (Karin *et al*, 1984; Evans, 1988).

1.3.4.5.1 Triiodothyronine

T_3 is essential for the differentiation of Ob17 cells (Grimaldi *et al*, 1982), where it provokes an increase in the activity of certain lipogenic enzymes (Gharbi-Chihi *et al*, 1983). It appears that T_3 acts in synergy with adipogenic factors to promote preadipocyte differentiation (Flores-Delgado *et al*, 1987; Hausman and Martin, 1989). T_3 is thought act transiently, during the early stages of terminal differentiation, to induce the expression of a number of proteins involved in the regulation of lipid metabolism (Ailhaud, 1982; Flores-Delgado *et al*, 1987; Hausdorf *et al*, 1988; Levacher and Picon, 1989; Moustaid and Sul, 1991).

1.3.4.5.2 Glucocorticoids

Addition of the synthetic glucocorticoid, dexamethasone, in the presence of insulin and the cyclic AMP-elevating agent, isobutylmethylxanthine (IBMX) has been shown to enhance the differentiation of preadipocytes from several species (Rubin *et al*, 1978; Schiwek and Loffler, 1987; Hauner *et al*, 1989; Xu and Bjorntorp, 1990). This may be due to the induction of C/EBP β and δ which is regulated by IBMX and dexamethasone respectively (Cao *et al*, 1991; Yeh *et al*, 1995). Dexamethasone also increases the level of PPAR γ expression induced by C/EBP β and δ (Wu *et al*, 1996). In addition, a glucocorticoid response element can be found in the aP2 gene of 3T3-L1 preadipocytes, which may be responsible for transcriptional induction by dexamethasone during adipose conversion (Cook *et al*, 1988).

1.3.4.5.3 Fatty acids and retinoids

When present in low concentrations, fatty acids and retinoids cooperate synergistically to promote the terminal differentiation of Ob1771 preadipocytes (Safonova *et al*, 1994b) and co-regulate 3T3-L1 preadipocyte proliferation and differentiation (Chawla and Lasar, 1994). Both fatty acids and retinoids induce expression of late markers of preadipocyte differentiation, including proteins implicated in fatty acid metabolism (Amri *et al*, 1991; Abumrad *et al*, 1993; Safonova *et al*, 1994b). Fatty acid-activated nuclear receptors, such as PPAR γ2, form heterodimeric complexes with retinoid receptors which can then bind corresponding HREs in adipocyte-specific genes (Amri *et al*, 1995; Tontonoz *et al*, 1994a).

Given the crucial roles of GH and insulin in promoting and maintaining adipogenesis, it is apparent that elucidation of their respective signal transduction pathways will be important for understanding the mechanism of action of these hormones. Although, the signalling mechanisms used by GH and insulin are not fully resolved, a number of key events have been identified and are outlined in Figures 1.4 and 1.5.

1.4 Insulin signalling

Over the past decade there has been a dramatic increase in the understanding of early post-receptor events involved in insulin action. The binding of insulin to the extra-cellular α-sub unit of its receptor stimulates the tyrosine kinase activity located on the cytoplasmic portion of the β-sub unit. This results in autophosphorylation of the receptor, concomitantly enhancing kinase activity towards other protein substrates (White *et al*, 1985). A major substrate is the cytoplasmic protein termed insulin receptor substrate-1 (IRS-1; Sun *et al*, 1991). Tyrosine phosphorylated IRS-1 acts as a docking molecule for multiple proteins that interact *via* phosphotyrosyl-binding domains, termed SH2 (Pawson and Gish, 1992).

Proteins known to associate with IRS-1 are: the lipid kinase, phosphatidylinositol 3-kinase (PI 3-kinase); an adaptor protein, Grb2, which associates with the guanine nucleotide exchange factor Sos; a phosphotyrosine phosphatase, syp and a linker

protein, nck (Skolnik *et al*, 1993; Tobe *et al*, 1993; Kuhne *et al*, 1993; Lee *et al*, 1993). Insulin receptor- and IRS-1-binding proteins are being identified continuously. For instance, hGrb10 (Dong *et al*, 1997), a SH2 domain-containing putative adaptor protein, SH2-B (Wang and Riedel, 1998) and a rho-associated kinase (Farah *et al*, 1998) have recently been found to associate with the insulin receptor and may play an as yet unidentified role in insulin signal transduction. A number of phosphatases, in addition to syp, have also been proposed to modulate insulin signalling (Lammers *et al*, 1997; Lechleider *et al*, 1993; Kuhne *et al*, 1993; Hausdorff *et al*, 1995; Kulas *et al*, 1995). The exact roles of a number of these proteins with respect to insulin signal transduction remain to be determined.

1.4.1 Insulin signalling via phosphatidylinositol 3-kinase

The interaction of PI 3-kinase with IRS-1 results in activation of the lipid kinase (Backer *et al*, 1992). PI 3-kinase catalyses the phosphorylation of inositol lipids at the D3 position (Auger *et al*, 1989), though the exact role of such phospholipids in cellular signalling is uncertain. PI 3-kinase is composed of a 110 kDa catalytic sub unit and a regulatory sub unit. Five regulatory sub units have been identified to date; two 85 kDa sub units, two 55 kDa sub units and a 50 kDa sub unit which is alternatively spliced from the p85α gene (Inukai *et al*, 1997). These five sub units may play differential roles in the various responses induced by the numerous molecules that activate PI 3-kinase.

Activation of PI 3-kinase is thought to be upstream of $p70^{s6}$ kinase activation and hence of mRNA translation (Cheatham *et al*, 1994); $p70^{s6}$ kinase phosphorylates EF-1 and s6, resulting in insulin stimulation of translation elongation (Chang and Traugh, 1997). Some evidence also exists for PI 3-kinase-independent activation of $p70^{s6}$ kinase by insulin (Hara *et al*, 1995).

PI 3-kinase is also upstream of PKB in insulin signalling cascades (Burgering and Coffer, 1995). The α and β isoforms of PKB are activated by insulin in adipocytes (Walker *et al*, 1998). PKB is activated by a kinase termed PDK1 (3-PI-dependent protein kinase, as it is only activated in the presence of the products of PI 3-kinase; Alessi *et al*, 1997). Inhibition of glycogen synthase kinase-3 (GSK3) by serine

phosphorylation *via* PKB (Cross *et al*, 1995) is thought to contribute to the stimulation of glycogen synthesis (Cross *et al*, 1995) and the translation of certain mRNAs (Welsh *et al*, 1994; Welsh *et al*, 1998) by insulin.

PI 3-kinase is required for insulin-stimulated glucose transporter translocation in rat (Okada *et al*, 1994) and 3T3-L1 (Kotani *et al*, 1995) adipocytes and inhibition of lipolysis in rat adipocytes (Okada *et al*, 1994). Insulin activation of the PDE 3 isoform in rat adipocytes, which is involved in inhibition of lipolysis, also appears to be PI 3-kinase-dependent (Rahn *et al*, 1994) and may involve PKB but does not involve $p70^{s6}$ kinase (Wijkander *et al*, 1998). As well as being an important transducing effector, PI 3-kinase may exert a modulatory effect on insulin signalling *via* serine phosphorylation and attenuation of IRS-1 (Tanti *et al*, 1994).

1.4.2 Insulin signalling via mitogen-activated protein kinases

The association of Grb2-Sos with IRS-1 results in the conversion of Ras to its active GTP-bound form (Buday and Downward, 1993). Ras then triggers a protein kinase cascade, involving the sequential activation of two kinases, Raf and MEK (mitogen activated protein [MAP] kinase kinase; Kyriakis *et al*, 1992). This leads to the activation of the p42 and p44 MAP kinases by threonyl- and tyrosyl-phosphorylation (Ray and Sturgill, 1988). MAP kinases are a conserved family of serine/threonine kinases that are activated in all eukaryotic cells in response to a wide range of signals (Lenormand *et al*, 1993; Marshall, 1995). MAP kinases subsequently phosphorylate and activate $p90^{rsk}$ (Haystead *et al*, 1990). This pathway is known to regulate gene transcription *via* phosphorylation of transcription factors by both MAP kinases and $pp90^{rsk}$ (Marshall, 1995).

Additional studies have demonstrated that MAP kinases can also mediate increased protein translation in response to insulin. On insulin stimulation, MAP kinase phosphorylates PHAS-1, a protein which interacts with the eIf-4E component of the translation initiation complex eIF-4F (Lin *et al*, 1994). Both proteins are found in association with mRNA cap structures prior to insulin stimulation (Merrick, 1992). On phosphorylation by insulin, PHAS-1 is released from eIF-4F which, also phosphorylated, is then free to associate with other members of the initiation complex and translation can occur (Lin *et al*, 1994).

MAP kinases appear to be required for insulin-induced mitogenesis, however, as yet, little evidence has been presented to suggest a role for the MAP kinase pathway in the metabolic effects of insulin (Lazar *et al*, 1995). MAP kinases may, however, be required for insulin-induced stimulation of glucose transport (Inoue *et al*, 1993).

1.4.3 Insulin receptor substrate-1-independent signalling

Although IRS-1 undoubtedly plays an important role in mediating the actions of insulin, it is apparent that IRS-1-independent signalling pathways are also involved. Shc has been termed the second major insulin receptor substrate (Pronk *et al*, 1993) and also activates the MAP kinase pathway *via* complex-formation with Grb2-Sos. Insulin activation of MAP kinase and c-*fos* transcription has, in fact, been shown to occur predominantly through Shc-Grb2 interaction (Yamauchi and Pessin, 1994) and it has been suggested that Shc and IRS-1 compete for a limited pool of Grb2 in mediating downstream signalling.

IRS-1 knockout studies led to the identification of a further insulin receptor substrate, termed IRS-2, which can activate PI 3-kinase to a lesser extent than IRS-1 (Araki *et al*, 1994). Thus, glucose transport can still occur in the absence of IRS-1, although at a reduced rate due to the fact that GLUT 4 translocation is PI 3-kinase-dependent. In fact, IRS-2 appears to be a crucial component of the insulin signal transduction mechanism as disruption of the IRS-2 gene in mice results in the development of diabetes (Withers *et al*, 1998). In addition, a third potential member of the IRS family, IRS-3, has recently been identified in rat adipocytes (Smith-Hall *et al*, 1997).

1.5 Growth hormone signalling

On binding, GH induces rapid tyrosine phosphorylation and dimerisation of its receptor but, unlike the insulin receptor, the GH receptor possesses no intrinsic tyrosine kinase activity (Leung *et al*, 1987). Tyrosine phosphorylation is achieved *via* recruitment of the cytosolic protein JAK 2, a member of the Janus family of tyrosine kinases (Argetsinger *et al*, 1993; Wilks *et al*, 1991). Members of the Janus family associate preferentially with the cytoplasmic domain of a number of cytokine receptors (see Lamb *et al*, 1996). Activated JAKs have been shown to phosphorylate both cytokine receptors themeselves and a number of downstream substrates,

particularly members of the STAT (signal transducers and activators of transcription) family of cytosolic transcription factors (see Lamb *et al*, 1996). Studies have demonstrated that GH induces the tyrosine phosphorylation of a number of intracellular proteins (Campbell *et al*, 1993; Anderson, 1992), presumably in a JAK 2-dependent manner (Winston and Hunter, 1995). The identification of these proteins has revealed two pathways that could potentially be used by GH to signal to the nucleus.

1.5.1 Growth hormone signalling via mitogen-activated protein kinases

Anderson (1992) has shown that GH induces rapid and transient activation of p44 and p42 MAP kinases and $p90^{rsk}$ in 3T3-F442A preadipocytes. JAK2, Ras and Raf appear to be required for activation of MAP kinases by GH (Winston and Hunter, 1995). At least in rat liver, GH induces the translocation of MAP kinases to the nucleus where they can phosphorylate various transcription factors (Davis, 1993). This cascade is therefore a potential mechanism for the modulation of gene transcription by GH.

Recently, it has emerged that activation of the MAP kinase cascade is essential for the adipose conversion of 3T3-L1 preadipocytes (Sale *et al*, 1995). In fact, the sequential activation of PI 3-kinase, Ras and MAP kinase appears to be required for differentiation of these cells (Uehara *et al*, 1995). Although these signalling pathways are activated by both GH and insulin, the role of these events in the adipogenic actions of the hormones has not been defined.

1.5.2 Growth hormone signalling via signal transducers and activators of transcription (STATs)

The widely expressed STAT family of cytosolic transcription factors, which are the products of multiple genes and alternative splicing, are known to be phosphorylated by members of the JAK family of tyrosine kinases (see Lamb *et al*, 1996). STAT proteins undergo tyrosyl phosphorylation in the cytoplasm followed by translocation to the nucleus where variations in the complexes formed with other transcription factors confer specificity of interaction with different DNA response elements (Hunter *et al*, 1993). GH phosphorylates and causes nuclear accumumlation of STAT 1α (Kilgour and Anderson, 1994; Meyer *et al*, 1994). Phosphorylation of STAT 1 by GH

results in the formation of complexes that can interact with the sis-inducible element (SIE) of *c-fos* (Meyer *et al*, 1994). Further evidence suggests that GH can also activate STAT 3 and STAT 5, apparently in a cell-specific manner (Meyer *et al*, 1994; Gronowski and Rotwein, 1994; Cambell *et al*, 1995; Gouilleux *et al*, 1995; Thomas *et al*, 1995; Leaman *et al*, 1996; Yi *et al*, 1996).

1.5.3 Growth hormone and additional signalling events

GH has also been shown to induce a number of other signalling events. Like insulin, GH causes tyrosine phosphorylation of Shc and its association with Grb2 (VanderKuur *et al*, 1995) and activates PI 3-kinase (Kilgour *et al*, 1996). A number of other proteins (Rui *et al*, 1997; Kim *et al*, 1998) have been shown to associate with JAK 2 upon GH stimulation, although the roles of these proteins in GH signal transduction remain to be determined.

Thus, by comparison of the signal transduction pathways, it can be seen that GH and insulin induce many of the same signalling events. Much of the data for GH has, however, been derived from studies using preadipocyte cell lines and awaits confirmation in adipocytes. In addition to the events discussed above, GH can induce the tyrosine phosphorylation of IRS-1, -2 and -3 and their association with PI 3-kinase (Souza *et al*, 1994; Argetsinger *et al*, 1996; Yamauchi *et al*, 1998). Indeed, the IRS-1 proteins are now known to be activated by a number of cytokines (Argetsinger *et al*, 1996; Berlanga *et al*, 1997; Chen *et al*, 1997a). Furthermore, it now appears that the JAK-STAT pathway may participate in insulin signalling. In cells over-expressing the insulin receptor, IRS-1 and Shc are found in association with JAK 2 (Maegawa *et al*, 1996). In addition, STAT 5 has been identified as an insulin receptor substrate (Chen *et al*, 1997a) and insulin activates STAT 3 in 3T3-L1 adipocytes (Ceresa and Pessin, 1996) and hepatic cells (Campos *et al*, 1996).

In addition to the activation of individual signal transducers, "cross talk" between individual signalling pathways also appears to be important in modulating the effects of GH. For example, PI 3-kinase is required for the signalling of GH and insulin to MAP kinases (Kilgour *et al*, 1996; Sanchez-Margalet *et al*, 1995). MAP kinase activation itself can be modulated, either positively or negatively, by regulatory "cross

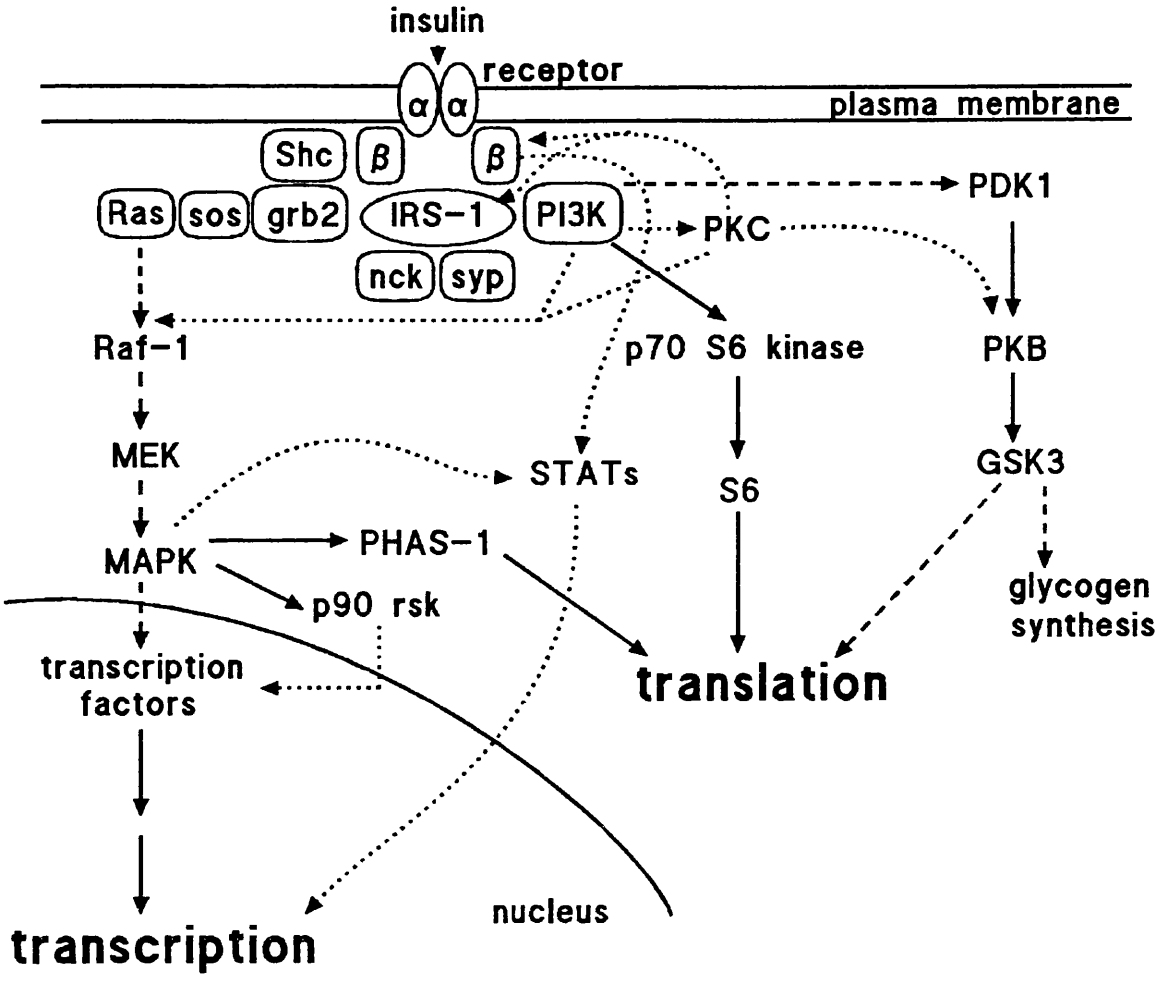


Figure 1.4 Summary of the insulin signalling pathways

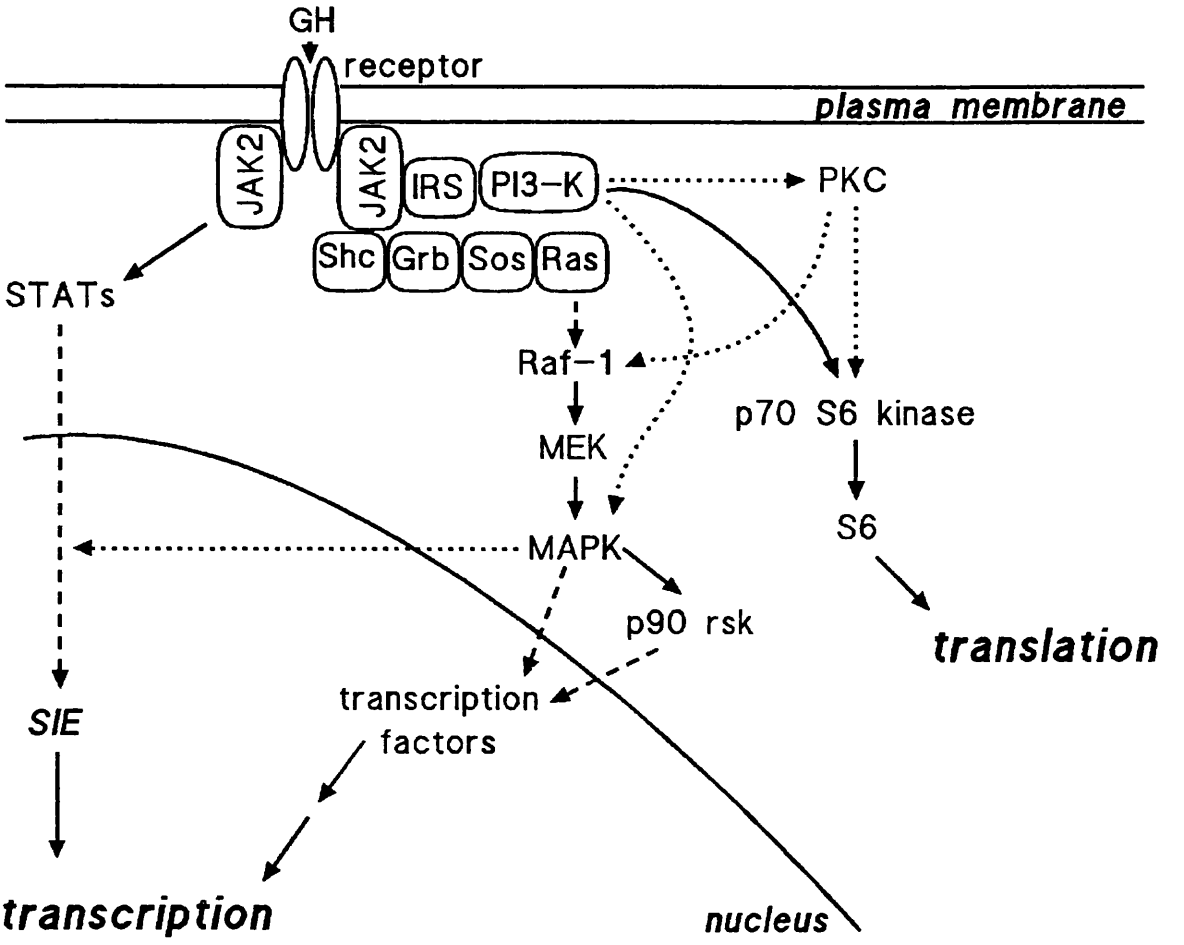


Figure 1.5 Summary of the growth hormone signalling pathways

talk" with other signalling pathways, such as the cyclic AMP cascade (Malarkey *et al*, 1995; Marshall, 1995). In addition, the MAP kinase pathway may regulate signalling *via* the JAK-STAT pathway (David *et al*, 1995; Chung *et al*, 1997; Purcher *et al*, 1997). Thus, there exists subtle interplay between individual signalling pathways in mediating the down-stream effects of hormones.

GH and insulin activate very similar signal transduction systems to exert very different effects. Thus, the timing or duration of activation of signalling proteins may be important in mediating down-stream effects. For example, the duration of MAP kinase activation has been shown to be a determining factor in the decision of cells to differentiate or proliferate; sustained activation provokes differentiation whereas transient activation stimulates cell cycle re-entry (Marshall, 1995). In addition, since a number of the elements of the GH and insulin signalling pathways exist in multiple isoforms, it may be necessary to clarify which isoforms are targeted by which hormone in eliciting particular cellular responses. The protein kinase C (PKC) family of serine/threonine kinases, exists in multiple isoforms and it is thought that individual isoforms may be required for distinct cellular functions. PKCs are known to participate in a number of the signalling pathways stimulated by GH and insulin (see Figures 1.4 and 1.5) and are strongly linked to the regulation of differentiation. Thus, these kinases may play an important role in the regulation of adipocyte development and function.

1.6 The protein kinase C family

PKC was initially identified as a serine/threonine kinase in brain around twenty years ago (Inoue *et al*, 1977; Takai *et al*, 1977). The enzyme was determined to exhibit reversible calcium- and phospholipid-dependent kinase activity which could be stimulated by the second messenger diacylglycerol (DAG) (Kishimoto *et al*, 1980). DAG is produced by phospholipid hydrolysis in response to a variety of extracellular stimuli, thus, the first link between receptor induced phosphoinositide (PI) breakdown and protein phosphorylation was discovered.

PKC is also the major receptor for tumour-promoting phorbol esters, which activate the kinase *in vitro* in a manner very similar to DAG (Gschwendt *et al*, 1991; Bell and

Burns, 1991). Phorbol esters have, therefore, been used widely as a pharmacological tool to investigate the function of PKC in the cell. By this approach it has become clear that PKC is involved in a variety of diverse processes. It has also been established that PKC is not a single entity and that many closely related PKC isoforms exist, perhaps providing an explanation for the range of processes in which PKC has been implicated.

Members of the PKC family of homologous phospholipid-dependent serine/threonine kinases are divided into groups according to their structural properties and kinetics of activation (Figure1.6). The conventional PKCs require DAG and calcium for maximal activation and consist of PKC α, β and γ (Coussens *et al*, 1986, Knopf *et al*, 1986; Parker *et al*, 1986). The PKC β gene generates two isoforms, βI and βII, by alternative splicing (Ono *et al*, 1986; Coussens *et al*, 1987). The novel PKCs, δ, ε, η and θ (Ono and Nishizuka, 1987; Ohno *et al*, 1988; Osada *et al*, 1992; Bacher *et al*, 1991) require DAG for maximal activation but are insensitive to calcium. The atypical PKCs, ζ, ι and λ (Ono *et al*, 1989; Selbie *et al*, 1993; Akimoto *et al*, 1994) are insensitive to both DAG and calcium and are thought to be activated by phospholipid alone, although the regulation of atypical PKS is, as yet, understood poorly. In addition, there exists PKC μ (Johannes *et al*, 1994) and PKD (Valverde *et al*, 1994), which are thought to be human/murine homologues. PKC μ and PKD are structurally quite different from the other isoforms and may, thus, represent a new PKC sub-family.

1.6.1 Protein kinase C structure

PKC isoforms consist of a single polypeptide chain that contains an amino-terminal regulatory region and a carboxy-terminal kinase domain (Figure1.6). These domains can be divided further into regions that are conserved across isoforms (C1-4) and regions that vary between isoforms but are conserved within an isoform across species (V1-5; Nishizuka *et al*, 1988).

The regulatory region contains an auto-inhibitory domain and one or two membrane targeting motifs, the C1 and C2 domains. The auto-inhibitory domain, or pseudo-substrate site, resembles a substrate phosphorylation site but with the

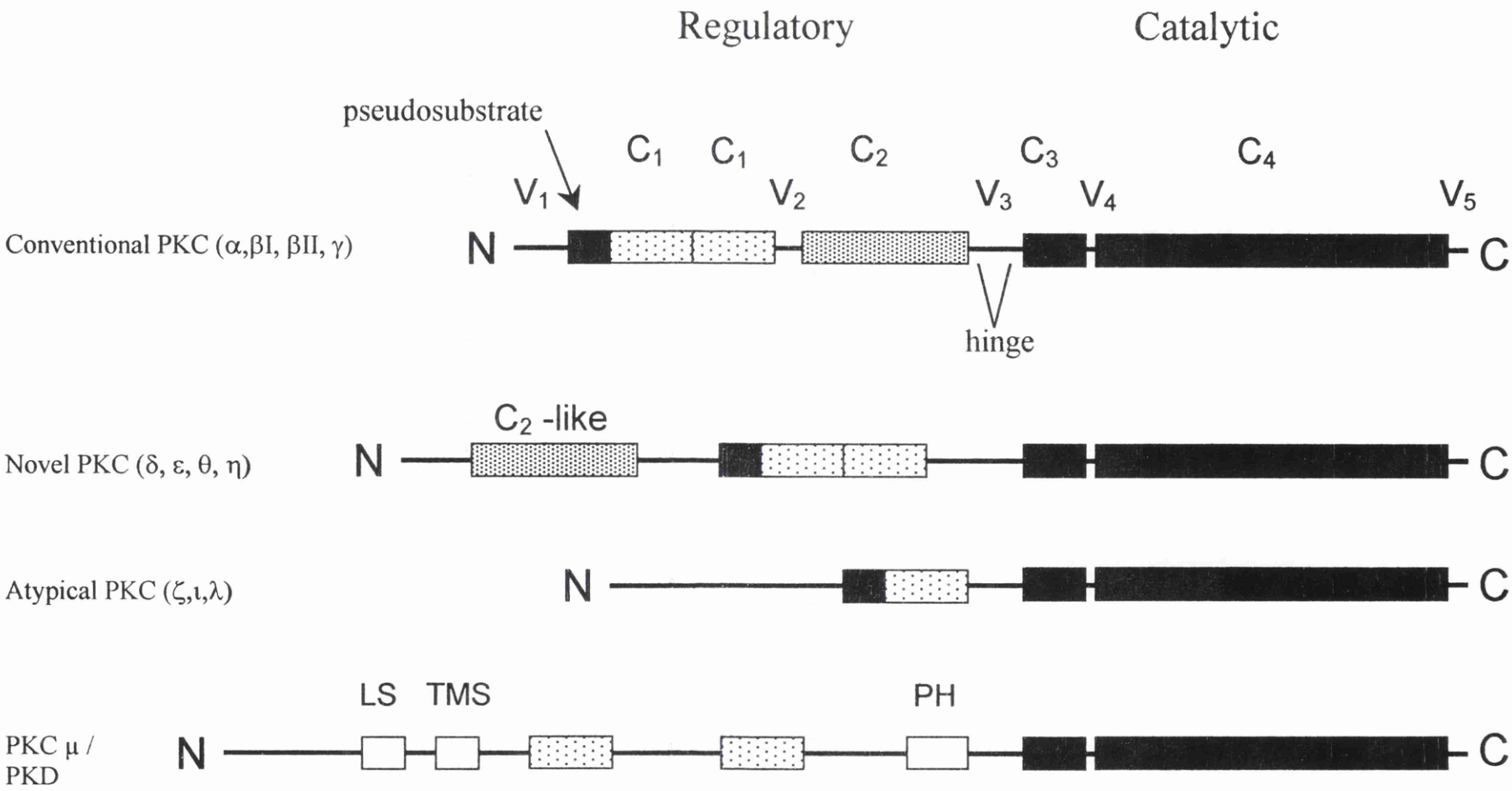


Figure 1.6 Stuctures of the protein kinase C family members

phosphorylatable serine replaced by an alanine residue (House and Kemp, 1987). The pseudo-substrate is thought to prevent activation of the enzyme by binding to the catalytic site (House and Kemp, 1987).

The C1 domain comprises two cysteine-rich regions and is responsible for DAG and phorbol ester binding (Kaibuchi *et al*, 1989; Ono *et al*, 1989). The stoichiometry of binding of phorbol esters to PKC is 1:1, despite the presence of two cysteine-rich domains in most isoforms (Mosior and Newton, 1996). The second cysteine-rich domain appears to be responsible for phobol ester-dependent membrane targeting whereas the function of the first domain remains to be determined (Szallasi *et al*, 1996). Although one cysteine-rich sequence is sufficient for binding of DAG/phorbol ester (Quest *et al*, 1994), the lower cooperativity and affinity of this binding leaves the possibility that two regions are required for efficient binding *in vivo*. This may explain the insensitivity of the atypical PKC isoforms to regulation by DAG/phorbol esters.

The C2 domain is present in conventional PKCs only and thus appears to be responsible for calcium binding. Calcium binding is thought to potentiate the binding of lipid by inducing a conformational change in the C2 domain (Newton, 1995). Similar sequences have been identified in the V1 region of novel PKCs (Sossin and Schwartz, 1993). Novel PKC isoforms display calcium-independent activation as the key aspartates involved in calcium coordination are not present in the C2-type domain in these proteins. (Newton, 1995; Nalefski and Falke, 1996). The C2/C2-like domains in the conventional and novel PKCs also appear to be responsible for acidic phospholipid binding (Newton, 1997; Quest, 1996). It is, as yet, unclear which regions are responsible for phospholipid binding in the atypical PKCs.

The V3 region serves as a hinge that is cleaved by calpain and trypsin to separate PKC into a constitutively active kinase, termed PKM (Parker *et al*, 1986; Kishimoto *et al*, 1989). The other variable regions are highly conserved within an isoform across species and are therefore likely to have a functional role to separate isoforms in terms of substrates, localisation and regulation. The catalytic domain of PKC is similar to regions of other serine/threonine kinases in that it contains consensus sequences for ATP-binding (C3), the phosphate transfer region and the substrate binding site (C4; Bell and Burns, 1991; Hug and Sarre, 1993; Hanks *et al*, 1988). The overall homology

in the kinase region is high between PKC isoforms, with the exception of PKC μ and PKD which do not phosphorylate a number of putative PKC substrates (Valverde *et al*, 1994; Johannes *et al*, 1995; Dieterich *et al*, 1996).

There are a number of other discrepancies between the structures of PKC μ/PKD and the other PKC isoforms (Valverde *et al*, 1994; Van Lint *et al*, 1995; Johannes *et al*, 1995; Dieterich *et al*, 1996; see also Figure 1.6). The pseudo-substrate domain, that is present in all other PKC isoforms, is absent from PKC μ and PKD. These isoforms possess putative leader sequences (LS) and transmembrane sequences (TMS) near the amino-terminus and a pleckstrin homology (PH) domain near the hinge region. In addition, the two cysteine-rich motifs in the C1 domains of PKC μ and PKD are unusually far apart. However, PKC μ and PKD are phospholipid-dependent serine/threonine kinases whose activity is stimulated by DAG and phorbol esters (Valverde *et al*, 1994; Johannes *et al*, 1995; Dieterich *et al*, 1996) and may, thus, represent a new PKC sub-group.

1.6.2 Activation of protein kinase C

Classically, PKC is activated by the concerted action of calcium, phospholipids, particularly phosphatidylserine (PS), and DAG. Enzyme activation requires the removal of the auto-inhibitory pseudo-substrate domain from the active site. This conformational change is achieved by the highly specific binding of DAG and PS to the two membrane-targetting domains, C1 and C2. Binding of ligand to either domain is sufficient to recruit the enzyme to membranes, however, both domains must be membrane-bound for the high-affinity interaction that results in pseudo-substrate removal and maximal activation (Newton, 1995; Newton, 1997). PKC activation by DAG is stereo-specific since only sn-1,2-DAGs (and not 1,3- or 2,3-DAGs) are effective (Sharkey and Blumberg, 1985; Boni and Rando, 1985; Hannun *et al*, 1986). Calcium appears to synergise with DAG/phorbol esters since it increases the affinity of PKC for PS, thus, lower amounts of DAG are required for membrane binding (Luo *et al*, 1995).

In addition to the C1 and C2 domains, the pseudo-substrate domain may also be involved in lipid interactions. Synthetic peptides mimicking the pseudo-substrate

region of PKC contain multiple basic residues that allow binding to acidic phospholipids (Mosior and McLaughlin, 1991, 1992a, 1992b). These interactions between the pseudo-substrate motif and acidic phospholipids may stabilise active PKC at the membrane.

1.6.2.1 Regulation by diacylglycerol and phorbol esters

Although phorbol esters and DAG appear to activate PKC by the same mechanism (Castagna *et al*, 1982; Sharkey and Blumberg, 1985; Hannun and Bell, 1986), phorbol esters are more potent and induce prolonged activation of the enzyme (Castagna *et al*, 1982; Mosior and Newton, 1995). Studies with phorbol esters have revealed that PKCs translocate to the membrane (Kraft *et al*, 1982; Kraft and Anderson, 1983), cytoskeleton (Ito *et al*, 1989; Mochly-Rosen *et al*, 1990; Zalewski *et al*, 1990; Kiley *et al*, 1992) and nucleus (Kiley *et al*, 1995). Subsequently, down-regulation of the protein occurs (Solanki *et al*, 1981). This appears to be *via* proteolytic degradation in the hinge region, by either a calcium-dependent protease, most likely calpain (Pontremoli *et al*, 1990), or a serine protease (Chida *et al*, 1986) and results in the release of the catalytic subunit, PKM (Takai *et al*, 1977). Continued proteolysis presumably results in inactivation of the kinase and to long-term down-regulation of PKC activity in the cell. Whether PKM accumulates to any significant degree in the cell, or is involved in signal transduction, remains to be determined.

Atypical PKC isoforms do not bind DAG or, therefore, phorbol esters. However, species of PKC ζ which are translocationally activated and subsequently down-regulated in response to phorbol ester treatment have been reported (Zhou *et al*, 1994a; Nishikawa *et al*, 1995; Borner *et al*, 1992). These forms of PKC ζ appear to be activated in response to phorbol esters by a mechanism that does not involve binding of the phorbol ester to the PKC. PKC μ contains a DAG-binding site and is, therefore, capable of binding phorbol esters (Johannes *et al*, 1994). However, this isoform is insensitive to acute and chronic modulation by phorbol ester in terms of translocation and subsequent down-regulation (Johannes *et al*, 1995; Rennecke *et al*, 1996). Indeed, PKC μ was originally classified as an atypical PKC, due to lack of apparent phorbol ester responsiveness (Johannes *et al*, 1994), however, enhanced kinase activity in the presence of phorbol ester has since been demonstrated (Johannes *et al*, 1995;

Dieterich *et al*, 1996).

In vitro experiments have shown that DAG increases the affinity of PKC for PS and this is likely to contribute to the redistribution of soluble PKCs to membranes upon activation (Newton, 1995). Thus, the classical model of activation for conventional and novel PKC isoforms involves translocation to cell membranes and interaction there with physiological activators. However, it is becoming apparent that additional mechanisms of regulation exist.

1.6.2.2 Regulation by lipids

The classical regulator of PKC is DAG produced as a consequence of receptor mediated hydrolysis of phospholipids. Cleavage of phosphatidylinositol bisphosphate (PIP2), by the PIP2-specific phospholipase C (PLC) β1, results in the production of inositol trisphosphate (IP3) and DAG. The production of IP3 initiates a rise in intracellular calcium levels (Berridge, 1993) which may help in translocating conventional PKCs to the membrane where they can interact with DAG to become activated. Of course, DAG itself activates PKC by lowering its requirements for PS and calcium to physiological levels (Kishimoto *et al*, 1980). Other lipids can stimulate DAG-dependent activation of PKCs, presumably by mimicking PS and thus increasing PKC's membrane affinity.

PKC is activated by two inositol phospholipids, PIP2 itself and PIP3 (phosphatidylinositol trisphosphate) which is a product of PI 3-kinase (Chauhan *et al*, 1990; Nakanishi *et al*, 1993; Singh *et al*, 1993). PIP2 appears to act in part as a DAG analogue and decreases the requirements of PKC for calcium. However, PIP2 can also satisfy some of the phospholipid requirements of PKC (Lee and Bell, 1991). PIP2 has also been shown to activate PKC in the absence of PS, requiring only calcium and phosphatidylinositol (Kochs *et al*, 1993). PIP3 appears to be a potent activator of novel and atypical PKCs, however, the physiological relevance of this interaction has not been evaluated (Singh *et al*, 1993). Thus, products of the PI cycle may regulate PKC activity in the cell in a complex fashion.

Arachidonic acid, produced by the action of PLA 2, and other *cis*-unsaturated fatty acids directly activate PKC *in vitro* (Blobe *et al*, 1995). Their mechanism of activation is distinct from that of PS/DAG as it may occur in the cytosol, allowing access of PKC to substrates that are not in the vicinity of the membrane. In addition, cleavage of phosphatidylcholine (PC) by PLA 2 also releases lysoPC (Nishizuka, 1992) which activates PKC in the presence of PS and calcium. This activation is synergistic with DAG and additive to fatty acid effects (Oishi *et al*, 1988). LysoPC and fatty acids are produced over long periods of time, thus, they could mediate long term effects of PKC (Nishizuka, 1992). Indeed, exogenous lysoPC can greatly potentiate the effects of DAG on processes that require prolonged activation of PKC, such as cellular differentiation (Asaoka *et al*, 1993).

PLD cleaves PC to form phosphatidic acid (PA) that can then give rise to lysoPA or to DAG. PA has been demonstrated to activate calcium-independent PKCs *in vitro* (Khan *et al*, 1994; Limatola *et al*, 1994). Thus, the PLD pathway may mediate its mitogenic effects through the PKC pathway, either directly, through activities of some PKC isoforms, or indirectly, through further metabolism of PA to DAG.

This diversity in the source of PKC activators allows for the activation of both calcium-sensitive and -insensitive isoforms and the induction of long-term activation of PKC. However, the co-factor regulation between each PKC sub-class is similar, suggesting that mechanisms additional to second messenger binding fine-tune isoform-specific function. These mechanisms could include regulation by phosphorylation and regulation by targeting proteins.

1.6.2.3 Regulation by phosphorylation

PKC is phosphorylated at three positions in the kinase core *in vivo* (Tsutakawa *et al*, 1995; Keranen *et al*, 1995). Most of the PKC in resting cells is in the triple phosphorylated form and these phosphorylations are thought to be involved in the regulation of the kinase since they increase its affinity for calcium and phorbol esters (Huang *et al*, 1986) and putative substrates such as H1 histone (Mochly-Rosen and Koshland, 1987).

In PKC βII, the phosphorylation sites are threonine 500, in the activation loop, and threonine 641 and serine 660, at the carboxy-terminus. Mutation of either threonine residue prevents activation of the enzyme (Cazaubon *et al*, 1994; Orr and Newton, 1994; Zhang *et al*, 1994a). PKC is unable to autophosphorylate at threonine 500 in the activation loop, suggesting that another kinase phosphorylates at this position (Keranen *et al*, 1995). Phosphorylation at the activation loop, by the putative PKC kinase, appears to trigger the subsequent phosphorylation of threonine 641 and serine 660 at the carboxy-terminus (Keranen *et al*, 1995). Phosphorylation of threonine 641 appears to occur by autophosphorylation and to lock PKC in a catalytically competent conformation (Keranen *et al*, 1995). Phosphorylation at serine 660 also appears to occur by autophosphorylation and correlates with release of PKC in to the cytosol (Keranen *et al*, 1995). This suggests that the role of phosphorylation at this site is to direct the sub-cellular location of the enzyme. The activity and stability of PKC α are controlled by similar multi-site phosphorylations (Gysin and Imber, 1997). The sub-cellular location and function of PKC isoforms may also be modulated by dephosphorylation at these key positions. For example, ceramide inactivates PKC α, probably by activating a phosphatase (Lee *et al*, 1996).

Additional phosphorylation at non-conserved residues may provide a mechanism for isoform- and stimulus-specific regulation of PKC isoforms. For example, PKC δ can be phosphorylated on tyrosine which either activates or inhibits the enzyme (Denning *et al*, 1993; Gshwendt *et al*, 1994; Li *et al*, 1994; Soltoff and Toker, 1995; Denning *et al*, 1996). The activity of PKC α is also altered by tyrosine phosphorylation in response to insulin (Liu and Roth, 1994). In addition, PKC α, βI, γ, δ, ε and ζ are tyrosine phosphorylated following treatment of cos7 cells with hydrogen peroxide. This increases activity by a mechanism apparently unrelated to receptor-coupled hydrolysis of inositol phospholipids (Konishi *et al*, 1997).

1.6.2.4 Regulation by binding proteins

A number of proteins that interact with PKC have been identified (Blobe *et al*, 1996; Jaken, 1996; Quest, 1996; Newton, 1997). PKC-binding proteins have three potential functions: i) regulating the activity of the enzyme (activators and inhibitors); ii) mediating down-stream effects of the enzyme (substrates) and iii) localisation of the

protein in particular sub-cellular compartments (anchoring proteins or localisers). In addition to the substrate-binding site in the catalytic domain, the PS-binding sites in the regulatory domain (pseudo-substrate, C1 and C2) appear to be important for interactions with PKC binding proteins (Newton, 1995). For example; deleting the pseudo-substrate domain of PKC α decreases its affinity for binding proteins (Liao *et al*, 1994), the C1 motif of PKC ε and the hinge region of activated PKC α are involved in sub-cellular localisation (Lehel *et al*, 1995a; Lehel *et al*, 1995b; Staudinger *et al*, 1995) and C2 peptides inhibit PKC translocation and binding to putative PKC receptors (Ron *et al*, 1995).

It would seem apparent that isoform-selective phosphorylation of downstream targets should be the basis of isoform-specific functions *in vivo*. However, although a large number of proteins are phosphorylated by PKC, *in vitro* phosphorylation studies have not indicated a large degree of substrate specificity between isoforms (Hofmann, 1997; Kazanietz, *et al*, 1993; Hug and Sarre, 1993; Pearson and Kemp, 1991). Certain proteins are efficient substrates for the conventional isoforms but not the novel PKCs (Hugg and Sarre, 1993). This suggests that the substrate specificity of individual PKC isoforms may be quite different. Indeed, distinct optimal sequences for each PKC isoform have been identified (Nishikawa *et al*, 1997). However, as of yet, the physiological substrates of individual PKC isoforms remain largely unknown. Similarly, although a number of proteins are known to modulate the activity of PKC (Blobe *et al*, 1996; Jaken, 1996; Quest, 1996; Hofmann, 1997), known PKC activators and inhibitors display little specificity for individual isoforms. Therefore, it may be that the intracellular substrate specificity of each PKC isoform is governed by various factors *in vivo*. These may include the concentration of target proteins, regulatory factors and the ability, of at least certain PKC isoforms, to interact with particular anchoring proteins (see below).

It has been established that different PKC isoforms localise to different sub-cellular compartments (Kiley *et al*, 1995; Jaken, 1996). RACKS (receptors for activated C kinase), are a group of proteins found in the particulate fraction of cells that bind to the active conformation of PKC and are thus thought to contribute to sub-cellular targeting (Mochly-Rosen, 1995). In addition, PKC α and βII bind to certain forms of

a multi-enzyme cytoskeletal scaffolding protein family, termed AKAPs (originally named as A-kinase anchoring protein; Klauck *et al*, 1996). Furthermore, PICK1 (protein interacting with C-kinase) is a nuclear protein that specifically interacts with PKC α and may, thus, provide a potential mechanism for selective intracellular targeting of this isoform (Staudinger *et al*, 1997). PKCs are mainly soluble proteins that translocate to membrane, cytoskeletal and nuclear compartments upon activation, therefore, targeting to appropriate domains *via* interaction with RACKS, AKAPS, PICKs and other proteins, may be central to their ability to respond efficiently to second messengers and have ready access to substrates.

1.6.2.5 Additional mechanisms of regulation

Many of the cellular effects of PKC, such as those on proliferation, differentiation and tumourigenesis, require long term changes in PKC activity that cannot be achieved by the short-lived turnover of inositol phospholipids during the PI cycle. To achieve long-term activation of PKC, either the activators of PKC must be elevated for long periods, or the levels of PKC isoforms must be increased. Indeed, both sustained increases in cellular levels of DAG (Fleischman *et al*, 1986; Wolfman and Macara, 1987; Tuominen *et al*, 1993) and increased expression of PKC isoforms (Dobrowsky and Hannun, 1992; Blobe *et al*, 1993a) have been described.

Thus, another mechanism of PKC regulation involves the differential expression of isoforms. A number of studies suggest that differential expression can occur at the transcriptional (Obeid *et al*, 1990; McSwine-Kennick *et al*, 1991), translational (McSwine-Kennick *et al*, 1991; Blobe *et al*, 1993b) and post-translational (Isakov *et al*, 1990; Huwiler et al, 1991) level.

1.6.3 Functions of protein kinase C isoforms

The identification of multiple PKC species has raised important questions with respect to the specific functions of individual isoforms. Isoforms display distinct regulatory properties and tissue distributions (Borner *et al*, 1992; Hata *et al*, 1993; Hug and Sarre, 1993; Selbie *et al*, 1993; Stabel and Parker, 1993; Akimoto *et al*, 1994; Hirai *et al*, 1994; Dieterich *et al*, 1996). This is consistent with the suggestion

that individual isoforms may serve diverse functional roles. Indeed, numerous studies suggest that specific isoforms may control distinct transcriptional events (Kieser *et al*, 1995; Spence *et al*, 1995; Kieser *et al*, 1996; Wang *et al*, 1997). Although the precise functions of different PKC isoforms have still to be elucidated, it is clear, from a wide range of studies, that PKCs function in the regulation of a number of cellular processes including growth and differentiation, apoptosis, tumourigenesis and various cell functions (Hug and Sarre, 1993; Kindregan *et al*, 1994; Li *et al*, 1994; Glazer, 1994; Borner *et al*, 1995; Hundle *et al*, 1995; Blobe *et al*, 1996; Acs *et al*, 1997; Deacon *et al*, 1997). The role of PKC isoforms in the regulation of those processes related to adipocyte development and function are discussed further below.

1.6.3.1 Regulation of signal transduction by protein kinase C

PKC modulates signalling *via* the MAP kinase cascade. PKC may link Ras and Raf-1 in this pathway since interaction of PKC with Ras has been suggested *in vitro* and *in vivo* (Diaz-Meco *et al*, 1994) and PKC has been shown to directly phosphorylate and activate Raf-1 (Kolch *et al*, 1993). PKC may activate Raf-1 only when Raf-1 is bound to Ras-GTP (Marais *et al*, 1998). PKC α and ε function as redundant activators of Raf-1 *in vivo* (Cai *et al*, 1997). In an intact cell assay system, PKC δ activates the MAP kinase pathway in a Ras-independent, Raf-dependent manner (Ueda *et al*, 1996). PKC δ has also been linked to MAP kinase activation in CHO cells (Yamaguchi *et al*, 1995) and 3T3-F442A preadipocytes (MacKenzie *et al*, 1997). Further evidence suggests that PKC α, ε and ζ may also be involved in the activation of MAP kinases (Yamaguchi *et al*, 1995; MacKenzie *et al*, 1997; Berra *et al*, 1995).

Products of the PI 3-kinase reaction have been shown to activate certain PKC isoforms *in vitro* (Nakanishi *et al*, 1993; Toker *et al*, 1994). In 3T3-F442A preadipocytes, both PI 3-kinase (Kilgour *et al*, 1996) and PKC (MacKenzie *et al*, 1997) are required for the full activation of MAP kinases by GH. This suggests that, in 3T3-F442A preadipocytes, PI 3-kinase may lie upstream of PKC, which is upstream of MAP kinases. In addition, PKCs are required for the PI 3-kinase-dependent stimulation of $p70^{s6}$ kinase by GH in these cells (Anderson, 1992 and 1993).

There is also some evidence that PKC is involved in the regulation of the STAT pathway. The hematopoietic Tec kinase family member, Bmx, induces activation of STAT 1, 3 and 5 in both mammalian and insect cells and this is specifically inhibited by PKC δ (Saharinen, 1997). Further evidence suggests that insulin signalling via IRS-1 (De Fea and Roth, 1997; Chin *et al*, 1994) and PKB (Barthel *et al*, 1998) is also modulated by PKC.

1.6.3.2 Protein kinase C and cell cycle control

During the mid to late stages of the G1 phase of the cell cycle, cyclins D and E, acting together with cyclin-dependent kinases (cdks), phosphorylate Rb proteins (Sherr, 1994; Heichman and Roberts, 1994). This releases Rb from E2F transcription factors, allowing E2F to activate S-phase genes (ie cell cycle entry; La Thangue, 1994; Weinberg, 1995; Weinberg, 1996). Inhibition of Rb phosphorylation is an early event leading to terminal differentiation and is essential for maintaining the post-mitotic state of differentiated cells (Chen *et al*, 1989; Gu *et al*, 1993). Phorbol esters stimulate or inhibit S-phase entry in a cell type-specific manner which may be due to the expression of different PKC isoforms (Kaibuchi *et al*, 1985; Rozengurt, 1986; Huang *et al*, 1987; Ohno *et al*, 1994). Indeed, in rat 3Y1 fibroblasts, over-expression of PKC α inhibits E2F and S-phase whereas PKC δ and ε enhance E2F activity and cell growth (Nakaigawa *et al*, 1996).

Association with cyclins and subsequent phosphorylation by an activating kinase are both important for cdk function (Pines, 1994; Nigg, 1996). PKCs can reduce cyclin levels and modulate cdk activation in a number of cells (Kosaka *et al*, 1993; Zhou *et al*, 1993; Zhou *et al*, 1994b; Sasaguri *et al*, 1996; Hamada *et al*, 1996). In addition, PKC can enhance cellular levels of cdk inhibitors, such as $p21^{wafl}$ and $p27^{kipl}$ (Huang *et al*, 1987; deVente *et al*, 1995; deVente *et al*, 1996a; Livneh *et al*, 1996) and p21 induction is strongly implicated in the growth-arrest associated with terminal differentiation (Parker *et al*, 1995; Halevy *et al*, 1995). The importance of cell cycle regulation by PKC isoforms in the differentiation process is demonstrated by ectopic expression of PKC η in NIH 3T3 cells following which, the otherwise non-differentiating cells can be induced to differentiate in response to adipogenic stimuli (Livneh *at al*, 1996). This capacity for differentiation was found to correlate with a

blockage in cell cycle progression due to increased expression of cdk inhibitors and reduced cyclin activity (Livneh *et al*, 1996). This control of the cell cycle by PKC is, therefore, a potential mechanism for regulating preadipocyte differentiation.

1.6.3.3 Protein kinase C and differentiation

A number of other lines of evidence suggest a functional role for PKC isoforms in the regulation of cellular differentiation. Sustained PKC activation is inhibitory to differentiation in 3T3-L1 cells (Sahai *et al*, 1994). Consequently, down-regulation of phorbol ester-sensitive isoforms of PKC by prolonged phorbol ester treatment stimulates adipose conversion of these cells (Yun and Scott, 1983). Indeed, PKC activation is inhibitory to differentiation in a number of cell lines (Minana *et al*, 1989; Felipo *et al*, 1990; Taoka *et al*, 1990) and antisense to PKC δ accelerates the chemically induced differentiation of myeloid erythroleukaemia cells (Pessino *et al*, 1995). In contrast, PKC activators promote differentiation in Ob 1771 preadipocytes (Catalioto *et al*, 1992). Over-expression of PKC δ in 32 D myeloid cells allows differentiation in response to phorbol esters (Mischak *et al*, 1993) and ectopic expression of PKC η in otherwise non-differentiating NIH-3T3 cells, allows cells to differentiate into adipocytes in response to the appropriate stimuli (Livneh *et al*, 1996). Although sustained PKC activation is inhibitory to differentiation in 3T3-L1 cells, levels of PKC ε and PKC θ are increased during 3T3-L1 preadipocyte differentiation (McGowan *et al*, 1996). Collectively, these studies suggest differential roles for individual PKC isoforms at distinct phases of the preadipocyte differentiation process.

1.6.3.4 Protein kinase C and growth hormone signalling

Several studies have produced evidence of a role for PKC in some of the signalling events mediated by GH. For example, GH has been shown to increase cellular levels of DAG, a natural activator of PKC, in various cells (Doglio *et al*, 1989; Catalioto *et al*, 1992). In addition, the inhibition/down-regulation of PKC by prolonged phorbol ester treatment of cells has been shown to attenuate the activation of MAP kinases and two S6 kinases by GH in 3T3-F442A preadipocytes (Anderson, 1992 and 1993). Evidence suggests that in the signalling pathways utilised by GH in 3T3-F442A preadipocytes, PKC lies downstream of PI 3-kinase and upstream of MAP kinases

(Kilgour *et al*, 1996; MacKenzie *et al*, 1997) and $p70^{s6}$ kinase (Anderson *et al*, 1992 and 1993). Studies with Ob1771 preadipocytes suggest that GH stimulates c-*fos* and lipoprotein lipase (LPL) gene transcription and terminal differentiation in general by PKC-dependent mechanisms (Ailhaud *et al*, 1992; Catalioto *et al*, 1992). Similar studies with 3T3-F442A cells suggest that the differentiation-promoting effects of GH involve enhanced transcription of c-*fos* and c-*jun* (Gurland *et al*, 1990) and activation of MAP kinases (Campbell *et al*, 1992) by PKC-dependent mechanisms.

With regard to adipocyte metabolism, at least in the rat adipocyte, data are consistent with GH exerting inhibitory and stimulatory effects on lipogenesis and lipolysis resectively *via* signal transduction which is, at least in part, mediated by the activation of PKC (Smal and De Meyts, 1987; Gorin *et al*, 1990). Thus, one or more isoforms of PKC are likely to be involved in the signalling pathway(s) utilised by GH to regulate adipocyte development and function.

1.6.3.5 Protein kinase C and insulin signalling

Activation of PKC has been associated with states of insulin resistance. PKC appears to counter-regulate insulin action *via* induction of increased serine/threonine phosphorylation of the insulin receptor, which has been correlated with decreased insulin receptor tyrosine kinase activity (see Houslay, 1991) and with decreased ability of the insulin receptor to promote activation of the PI 3-kinase complex (Chin, 1993). PKC activation also results in serine phosphorylation of IRS-1, which contributes to inhibition of insulin signalling (De Fea and Roth, 1997). These effects have been largely attributed to activation of the PKC α isoform (Chin *et al*, 1994; Danielson *et al*, 1995). PKC also suppresses the insulin-mediated increase in PKB activity in 3T3-L1 adipocytes (Barthel *et al*, 1998).

Insulin responses are largely unaffected in cells in which the conventional and novel PKC isoforms have been down-regulated by chronic phorbol ester treatment (reviewed in Blackshear, 1994), implying that PKCs are not involved in the signalling pathways used by insulin to exert its effects. Such studies do not, however, rule out a role for the atypical PKC isoforms in mediating insulin responses. Indeed, atypical PKC ζ appears to be, at least in part, required for the stimulation of glucose transport

by insulin in 3T3-L1 adipocytes (Bandyopadhyay *et al*, 1997).

Although the involvement of PKC in insulin signalling remains controversial, a number of studies suggest a role for PKC in some of insulin's effects. IRS-1 levels are positively regulated by PKC δ (deVente *et al*, 1996), suggesting a possible role for this isoform in insulin signalling. In addition, insulin translocates and activates several PKC isoforms in a number of cell types, including adipocytes (Farese *et al*, 1992; Arnold *et al*, 1993; Bandyopadhyay *et al*, 1997). Furthermore, PKC appears to be, at least in part, required for the stimulation of lipogenesis by insulin in rat adipocytes (Smal and De Meyts, 1987). Further evidence suggests that phorbol ester-sensitive PKC isoforms may play some role in the stimulation of glucose transport by insulin (Grunberger, 1991; Standaert *et al*, 1990b; Yano *et al*, 1993; Chalfant *et al*, 1995). The controversy surrounding the role of PKC in insulin signalling appears to be, at least in part, due to the existence of multiple isoforms.

Thus, a number of studies indicate that PKC may be involved in the regulation of adipocyte development and function. Abnormal regulation or expression of PKC isoforms may, therefore, contribute to the development of diseased states such as obesity and diabetes. Support for this suggestion comes from the observation that altered sub-cellular location and/or expression of PKC isoforms has been observed in association with diabetes in a number of cell types (Babazono et al, 1998; Tang et al, 1993; Considine et al, 1995; Schmitz-Peiffer et al, 1997), including adipocytes (Frevert and Khan, 1996).

1.7 Aims of the project

Due to the central role of the adipocyte in the diseased states of obesity and diabetes, there has been increasing interest in the molecular processes that control adipocyte development and function. Although several lines of evidence implicate the PKC family in adipocyte regulation and the development of obesity, the precise roles of PKC in these processes remain unclear. This is primarily due to the existence of multiple isoforms of PKC.

The aim of this study was to determine the roles of individual PKC isoforms in the regulation of adipocyte development and function using 3T3-F442A cells as a model system. Initially, the PKC complement of 3T3-F442A cells was thoroughly characterised. Attempts were then made to define the roles of the individual 3T3-F442A cell PKC isoforms in the process of preadipocyte differentiation. The involvement of specific PKC isoforms in the signalling mechanisms by which two key hormonal regulators of adipocyte metabolism, GH and insulin, alter the rate of lipid synthesis in differentiated 3T3-F442A cells was then investigated.

Chapter2

Materials and Methods

2.1 Materials

2.1.1 Chemicals and reagents

General laboratory chemicals were obtained from ICN Biomedicals Ltd., Oxon, UK or BDH (Merck Ltd.), Leics, UK unless stated otherwise. All other reagents were obtained from Sigma Chemical Co., Dorset, UK unless stated otherwise.

Tissue culture plastic was purchased from Corning Costar Corp., Bucks, UK. Tissue culture media, supplements and lipofectin reagent were obtained from Life Technologies, Paisley, UK.

Pituitary derived ovine GH was obtained from the National Hormone and Pituitary Program, Bethesda, MD, USA and recombinant bovine GH was a gift from Monsanto, St. Louis, USA.

[^{14}C]-acetate was obtained from ICN. Scintillation cocktails and vials were purchased from Packard, Berks, UK unless stated otherwise.

2.1.2 Cell lines

3T3-F442A and 3T3-C2 cells (Green and Kehinde, 1976) were provided generously by Dr Howard Green, Harvard Medical School, USA. NIH-3T3 cells which had been stably transfected to over-express PKC α, β, γ, δ, ε, ζ and η (Goodnight *et al*, 1995) were a generous gift from Dr JoAnne Goodnight, NCI, Bethesda, USA.

2.1.3 Antibodies

Anti-PKC α, βI/II, γ, δ, ε, ζ, θ, ι, λ and μ mouse monoclonal antibodies were purchased from Affiniti Research Products Ltd., Notts, UK and were raised using 18 - 25.3 kDa fragments of rat (PKC α, δ, ε and γ), mouse (PKC θ and λ) or human (PKC βI/II, ι, μ and ζ) PKC proteins as immunogens. Polyclonal antisera to PKC α, βI/II, γ, δ, ε and ζ were raised previously in-house (Tang *et al*, 1993). Corresponding peptide immunogen for PKC ζ (amino acid sequence; GFEYINALLLSAEESV) was synthesised by Dr Jim Beattie, Hannah Research Institute, Ayr. Polyclonal antisera to PKC βI, η and θ (Marais and Parker, 1989), and corresponding peptide immunogen

for PKC βI (amino acid sequence; SEFLKPEVKS), were supplied kindly by Dr Peter Parker, Imperial Cancer Research Fund, London. An additional polyclonal antiserum to PKC η, raised using a short fragment of mouse PKC protein as immunogen, and corresponding peptide immunogen (amino acid sequence; DEFRNFSYVSPELQL), were purchased from Insight Biotechnologies, Middlesex, UK. HRP-conjugated sheep anti-mouse IgG and goat anti-rabbit IgG were obtained from Amersham International plc, Bucks, UK and Sigma respectively.

2.1.4 Oligodeoxynucleotides

Phosphorothioate-modified s-oligodeoxynucleotides (ODNs) were purchased from Genosys Biotechnologies, Cambs, UK. The antisense sequences designed to hybridise to the PKC isoforms of interest and the corresponding sense (control) sequences are shown in Table 2.1. The antisense ODN sequences for PKC α, ε and ζ were based on the start codons (ATG) plus the 15 (or 12 in the case of PKC ζ) additional downstream bases in the murine PKC sequences. The antisense sequence for PKC δ was based on nucleotides 10-27 of the coding sequence of murine PKC δ. These antisense ODN sequences have been used successfully by others (Xu *et al*, 1994; DeCoy *et al*, 1995; Folgueira *et al*, 1996). The antisense ODN sequences for PKC γ and μ used in this study were, therefore, derived from similar sequences; the start codons plus the 15 additional downstream bases the murine (Bowers *et al*, 1993) and human (Johannes *et al*, 1994) PKC sequences respectively. The antisense sequence for PKD, corresponding to nucleotides 694-711 of the coding sequence of murine PKD (Valverde *et al*, 1994), was derived from a portion of the PKD sequence which is not present within the highly homologous PKC μ sequence.

2.1.5 Rats

Female Wistar rats (A Tuck and Son, Rayleigh, Essex) were fed on SDS CRM(E) non-irradiated diet (SDS, Essex, UK) with water freely available. Age-matched rats of 150-180 g were used for experimental purposes.

Table 2.1 Protein kinase C isoform-specific oligodeoxynucleotide sequences

ODN	Sequence 5' → 3'	Accession No.	Nucleotides
PKC α Antisense	-CGG-GTA-AAC-GTC-AGC-CAT-	M25811	256-273
PKC γ Antisense	-AGG-GCC-CAG-ACC-CGC-CAT-	X67129	68-85
PKC δ Antisense	-GAA-GGA-GAT-GCG-CTG-GAA-	X60304	23-40
PKC ε Antisense	-GCC-ATT-GAA-CAC-TAC-CAT-	AF028009	10-27
PKC ζ Antisense	-GGT-CCT-GCT-GGG-CAT-	M94632	24-38
PKC μ Antisense	-GAC-CGG-AGG-GGC-GCT-CAT-	X75756	236-253
PKD Antisense	-AAA-GCC-AGG-GCT-CAC-AGG-	Z34524	819-836
PKC α Sense	-ATG-GCT-GAC-GTT-TAC-CCG-		
PKC γ Sense	-ATG-GCG-GGT-CTG-GGC-CCT-		
PKC δ Sense	-TTC-CAG-CGC-ATC-TCC-TTC-		
PKC ε Sense	-ATG-GTA-GTG-TTC-AAT-GGC-		
PKC ζ Sense	-ATG-CCC-AGC-AGG-ACC-		
PKC μ Sense	-ATG-AGC-GCC-CCT-CCG-GTC-		
PKD Sense	-CCT-GTG-AGC-CCT-GGC-TTT-		

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Culture of 3T3-F442A preadipocytes

3T3-F442A preadipocytes (Green and Kehinde, 1976) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM glutamine (basic medium) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin (pen/strep) and 10% (v/v) newborn calf serum (growth medium) in a 90% O_2:10% CO_2 atmosphere at 37° C. Stock cultures were passaged routinely at approximately 80 % confluence. For experimental purposes, preadipocytes were seeded at 10^5 cells/dish and grown to confluence in growth medium in 100 mm diameter dishes unless stated otherwise. Cells were deprived of serum for 16-20 h prior to harvesting (or treatment) unless stated otherwise.

2.2.1.2 Differentiation of 3T3-F442A preadipocytes

For conversion to adipocytes, 3T3-F442A preadipocytes were used at passages 6 to 12 and grown to confluence. Two day confluent cultures were induced to differentiate by replacing the growth medium with basic medium supplemented with pen/strep and containing 10% foetal calf serum (FCS) and 1 μM insulin (Sigma Cell Culture, INS). The differentiation medium was replenished after 72 h and insulin removed from the medium on day 8. Under these conditions at least 80% of cells were converted to adipocytes. Adipocytes were typically harvested (or treated) ten days from induction of adipogenesis following a 16-20 h incubation in serum-free medium, unless stated otherwise.

For some experiments, parallel preadipocyte cultures were maintained in growth medium for twelve days. These cells, which displayed no morphological change, are referred to as twelve-day confluent cells (12 DC cells).

2.2.1.3 Culture of NIH-3T3 cells over-expressing protein kinase C isoforms

NIH-3T3 cells which had been stably transfected to over-express PKC isoforms α, β,

γ, δ, ε, ζ and η (Goodnight *et al*, 1995) were grown to confluence in basic medium supplemented with pen/strep and containing 10 % FCS. Cells were deprived of serum for 16-20 h prior to harvesting and preparation of lysates for use as positive controls on immunoblots.

2.2.1.4 Culture of 3T3-C2 cells

3T3-C2 cells (Green and Kehinde, 1976) were treated identically to 3T3-F442A cells, as described in sections 2.2.1.1 and 2.2.1.2

2.2.2 Preparation and treatment of cultured cells

2.2.2.1 Acute stimulation of 3T3-F442A cells with phorbol ester

Stock solutions of TPA were prepared in DMSO and stored at -20° C prior to use. Cells were stimulated with TPA in the absence of serum for 10 min in basic medium; 10 nM TPA for preadipocytes and 100 nM TPA for adipocytes. Control cultures were incubated with diluent. Cells were washed with 10 ml of ice-cold PBS to terminate reactions prior to harvesting and preparation of cytosol and membrane fractions for immunoblotting.

2.2.2.2 Chronic stimulation of 3T3-F442A preadipocytes with phorbol ester

Confluent 3T3-F442A preadipocytes were treated with TPA to down-regulate the phorbol ester-sensitive isoforms of PKC essentially as described by Blackshear *et al* (1985). Briefly, cells were washed three times with basic (serum-free) medium and then incubated in this medium containing 1% BSA (w/v, Sigma fraction V) in the presence of 16 μM or 500 nM TPA for 0-48 h prior to harvesting and preparation of lysates for immunoblotting. Times of initiation of TPA treatments were staggered to allow simultaneous harvesting of cells. Control cultures were treated identically and incubated with diluent.

2.2.2.3 Chronic stimulation of 3T3-F442A adipocytes with growth hormone and insulin

Fully differentiated 3T3-F442A adipocytes were incubated for 24 h in serum-free

basic medium in the presence or absence of 4.5 nM GH or 8.7 nM insulin, either alone or in combination, prior to the assessment of lipogenesis or the preparation of lysates for assessment of immunoreactive PKC levels by immunoblotting.

2.2.2.4 Treatment of 3T3-F442A cells with protein kinase C isoform-specific-oligodeoxynucleotides

2.2.2.4.1 Oligodeoxynucleotide treatment of 3T3-F442A preadipocytes

ODNs were dissolved in water and stored at -20° C until required. 3T3-F442A preadipocytes were cultured in 30 mm diameter wells. At typically 70-80 % confluence, cells were treated with antisense ODNs specific for the individual isoforms of PKC (Table 2.1). The appropriate isoform-specific sense ODNs were used as controls (Table 2.1).

Transfections were carried out in the absence of serum and antibiotics in basic medium which had been pre-filtered through a 0.22 μm Nalgene syringe filter (Fisher Scientific, Leics, UK). Prior to treatment of cells, appropriate dilutions of ODN and lipofectin were prepared in 100 μl volumes in filtered medium and pre-incubated at room temperature for 30 min with occasional mixing. The ODN and lipofectin mixtures were then combined and incubated for 30 min at room temperature with occasional mixing. Preadipocytes were washed twice with 1 ml of filtered medium prior to the addition of the ODN/lipofectin mixture in order to remove traces of serum and antibiotics from the cells. Reagents were added to give a final concentration of 10 μM ODN and 20 μg/ml lipofectin in a total volume of 1 ml. Cells were incubated with the ODN/lipofectin mixture for 6 h at 37° C. Cell monolayers were then washed twice with 2 ml of medium prior to the addition of fresh medium containing 10 % heat-treated (55° C, 30 min) calf serum (pre-filtered through a 0.45 μm syringe filter) and the appropriate concentration of ODN but no lipofectin reagent. After a further 42 h, cells were either assessed for immunoreactive PKC levels by immunoblotting or induced to differentiate by addition of medium containing 10 % heat-treated foetal calf serum (pre-filtered through a 0.45 μm syringe filter) and 1 μM insulin. The appropriate concentration of ODN was present throughout the treatment period with differentiation-inducing agents.

2.2.2.4.2 Oligodeoxynucleotide treatment of 3T3-F442A adipocytes

3T3-F442A cells were grown and differentiated in 30 mm diameter wells. Adipocytes were treated with sense or antisense ODNs specific for the individual isoforms of PKC and PKD at day 8 of differentiation. Adipocytes were washed twice with 1 ml of filtered antibiotic- and serum-free medium prior to the addition of the ODN/lipofectin mixture which was prepared as described in section 2.2.2.4.1 Reagents were added to give a final concentration of 20 μM ODN and 40 μg/ml lipofectin in a total volume of 1 ml. Cells were incubated with the ODN/lipofectin mixture for 6 h at 37° C and then washed twice with 2 ml medium prior to the addition of fresh medium containing 10 % heat-treated foetal calf serum and the appropriate concentration of ODN but no lipofectin reagent. After a further 42 h, cells were either assessed for immunoreactive PKC levels by immunoblotting or chronically incubated with hormones prior to the assessment of lipogenesis.

2.2.2.5 Oil red O staining of 3T3-F442A cells

3T3-F442A preadipocytes were pre-incubated with PKC isoform-specifc ODNs as described in section 2.2.2.4.1 and induced to differentiate with FCS/INS. Oil red O was used to stain cell lipids according to the method of Pearse (1968) at day 7 of differentiation. Cells were washed with 1 ml of PBS per 30 mm well and fixed for 1 h by incubation with 1 ml of 10 % formalin (40% formaldehyde in PBS, 1:4 [v/v]) per 30 mm well. Stock solutions of oil red O were prepared in isopropanol, diluted 6:4 (v/v) in H_2O and filtered through Whatman number 1 filter paper prior to use. Cells were overlayed with 1 ml of Oil red O filtrate for 1 h at room temperature. Stain was then removed and monolayers were washed with H_2O until all excess traces of stain were removed from the washing solution. Cells were then photographed under Phase Contrast Optics (x 24 magnification) using an Olympus IMT3 inverted microscope.

2.2.2.6 Preparation of lysates from cultured cells

Cells were washed with 10 ml ice-cold PBS and harvested by scraping into lysis buffer (25 mM Hepes pH 7.4, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 30 mM Na pyrophosphate, 1% [v/v] triton X-100, 10% [v/v] glycerol, 1 mM Na orthovanadate, 1 mM PMSF and 2 μg/ml each of aprotinin, pepstatin A and leupeptin). Lysates were

prepared by rotation for at least 20 min at 4° C and clarified by centrifugation at 14 000 g for 10 min at 4° C in a Camlab refrigerated microfuge. The supernatant was removed carefully and denatured by adding 0.25 volumes of 5 x concentrated SDS-sample buffer (312.5 mM Tris pH 6.7, 6.25% SDS, 62.5% glycerol, 12.5% β-mercaptoethanol) and boiling for 5 min. Denatured supernatants were stored at -20° C prior to use on immunoblots.

2.2.2.7 Preparation of homogenates from cultured cells and sub-cellular fractionation

3T3-F442A cells were acutely stimulated with phorbol ester as described in section 2.2.2.1. Cells from ten 100 mm diameter plates (preadipocytes), or five 100 mm diameter plates (adipocytes), were harvested in 500 µl of homogenisation buffer (20 mM Tris pH 7.5, 10 mM EDTA, 10 mM EGTA, 255 mM sucrose, 1 mM NaF, 1 mM Na pyrophosphate, 20 mM β-mercaptoethanol, 1 mM PMSF, 1 mM Na vanadate and 20 µg/µl each of leupeptin, aprotinin, and pepstatin A) and homogenates were prepared on ice in a Dounce homogeniser (60 strokes). Following centrifugation at 14 000 g for 10 min at 4° C in a Camlab refrigerated microfuge, supernatants were removed for further fractionation.

Supernatants were spun at 100 000 g for 30 min at 4° C in a Beckman Optima TLX refigerated ultrafuge fitted with a TLA rotor. The cytosolic fraction (supernatant) was removed carefully and boiled in 0.25 volumes of 5 x concentrated SDS-sample buffer for 5 min. The particulate fraction (pellet) was rinsed with 250 µl of homogenisation buffer, in order to minimise contamination from the cytosolic fraction, and re-centrifuged at 100 000 g. The soluble membrane fraction was extracted from the pellet by resuspension of the particulate fraction in 250 µl of homogenisation buffer containing 1% (v/v) triton X-100, using a syringe and 26 guage needle, followed by an incubation on ice for 30 min. A final 100 000 g centrifugation step was used to remove insoluble material (the triton-insoluble cytoskeleton) from the membrane extract which was then boiled in 0.25 volumes of 5 x concentrated SDS-sample buffer for 5 min. Denatured sub-cellular fractions were snap-frozen in liquid nitrogen and stored at -70° C prior to use on immunoblots.

2.2.2.8 Glycerol-3-phosphate dehydrogenase Assay

Cells were washed with ice-cold PBS and homogenates were prepared on ice in GPDH assay buffer (100 mM triethanolamine pH 7.5, 2.5 mM EDTA, 0.1 mM β-mercaptoethanol) by subjecting cell suspensions to eight passages through a 26 guage needle. Samples of homogenate were retained at this point for determination of protein or DNA content as described in sections 2.2.4.1 and 2.2.2.10 respectively, while remaining homogenate was centrifuged at 14000 g for 10 min at 4° C in a Camlab refrigerated microfuge. The resulting supernatant was removed and stored at -20° C prior to the determination of GPDH activity.

GPDH activity was determined by the spectrophotometric method of Wise and Green (1979) in assay buffer containing 0.12 mM β-NADH and 0.2 mM dihydroxyacetone phosphate. Absorbances at 340 nm were read in a CE 5501 double beam UV spectrophotometer at 30 s intervals over an appropriate time period. Enzyme activity was expressed as μmol NADH oxidised/min/mg protein or mg DNA.

2.2.2.9 Lipogenesis Assay

Following the chronic treatment of PKC- or PKD-depleted adipocytes with hormones, the rate of lipogenesis was determined by the incorporation of radiolabelled acetate into cell lipid by a modification of the method described by Vernon and Finley (1988). 3T3-F442A adipocytes, in 30 mm wells, were incubated for 4 h with basic medium supplemented with 2 mM acetate and 1 μCi/ml [^{14}C]-acetate. Adipocytes were lysed by the addition of GIT buffer (0.1 M EDTA pH 8.0 containing 5 M guanidine thiocyanate, Melford Laboratories Ltd, Suffolk, UK). Lysates were transferred to eppendorf tubes and lipids were extracted by addition of an equal volume of water-saturated chloroform followed by mixing and centrifugation at 10 000 g for 5 min in a Camlab refrigerated microfuge. The upper aqueous layer was carefully removed for determination of DNA content as described in section 2.2.2.10. The lipid-containing chloroform layer was transferred to a glass mini-scintillation vial and allowed to dry overnight. Samples were then dissolved in a scintillation cocktail comprised of toluene-based scintillation fluid and EcoLite (ICN), 1:4, and counted in a Packard 1600 TR liquid scintillation analyser. The rate of lipogenesis was expressed as μmol acetate incorporated/h/ mg of DNA.

2.2.2.10 DNA Assay

DNA was assayed according to the method of Labarca and Paigen (1980) using calf thymus DNA as a standard. Standards and samples were incubated with 1 μg/ml Bisbenzimide (H33342; Fluka Chemicals, Dorset, UK) for 20 min and fluorescence read in a Perkin-Elmer LS-5 luminescence spectrometer (excitation 360, emission 475).

Samples of homogenate that had been retained during the preparation of extracts for the assay of GPDH activity were extracted with four volumes of water-saturated chloroform to remove cellular lipid from the samples prior to assay of DNA content.

2.2.3 Preparation and treatment of rat tissues

2.2.3.1 Immunoneutralisation of circulating rat growth hormone

All procedures were conducted strictly according to regulations laid down by the Home Office under the Animals (Scientific Procedures) Act 1986. Animals were handled regularly prior to treatments to minimise stress.

Subcutaneous injections were administered twice daily, at 0900 and 1700 h, for 4 days. Rats received the following: γ-globulin fraction of antiserum to rat growth hormone (α-rGH; 150 mg/injection, equivalent to 4.5 ml serum, reconstituted in sterile saline), either alone, or in combination with ovine GH (oGH; 1 mg/injection prepared in 7.5% bicarbonate:25% polyvinylpyrrolidone [PVP, 1:4]). Control animals received equivalent injections of carrier solution. Details of the preparation and characterisation of α-rGH, which is specific for rGH and does not recognise oGH, have been described previously (Madon *et al*, 1986). This antiserum was provided generously by Dr. David Flint, Hannah Research Institute, Ayr. Between 1000 and 1100 h on day 5, rats were anaesthetised by intraperitoneal injection with Sagatal:Hypnorm (10:1, 0.1 ml per gram body weight) and samples of blood taken for extraction of serum and assessment of IGF-I levels as described in section 2.2.3.7. Parametrial fat pads were removed, weighed rapidly and placed in saline at 37° C prior to the preparation of adipocyte

homogenates as described in section 2.2.3.2.

2.2.3.2 Isolation of rat adipocytes and homogenate preparation

Parametrial fat pads were removed from female Wistar rats under anaesthesia as described in section 2.2.3.1. Fat pads were washed in Krebs-Ringer-Hepes buffer (KRH; 25 mM Hepes pH 7.4, 2 mM glucose, 119 mM NaCl, 4.95 mM KH_2PO_4, 1.19 mM $MgSO_4$, 2.5 mM $CaCl_2$) at 37° C and minced finely. Adipocytes were extracted by digestion with 1 mg collagenase per ml (Sigma type II) in KRH containing 200 nM adenosine. Digestion was carried out for 1 h at 37° C in stoppered flasks shaking at 80 oscillations per min. Cell suspensions were filtered through nylon mesh to remove any undigested material and washed twice in KRH and twice again in TES buffer (20 mM Tris pH 7.4, 1 mM EDTA, 255 mM sucrose). Cells were re-suspended at room temperature in 500 µl TES buffer containing 2 µg/ml aprotinin, pepstatin A and leupeptin and 1 mM PMSF. Homogenates were prepared at room temperature by subjecting cell suspensions to 10 strokes with a syringe and 26 guage needle followed by centrifugation at 300 g for 10 min at 4° C in a Sorvall RC-5B refrigerated centrifuge fitted with an SS-34 rotor. The infranatant was removed carefully, boiled for 5 min in 0.25 volumes of 5 x concentrated SDS-sample buffer and stored at -20° C prior to use on immunoblots.

2.2.3.3 Determination of number of adipocytes per gram tissue

In studies involving the adipocyte preparations from rats treated with α-rGH (and the adipocyte preparations from the appropriate control rats), aliquots of cell suspension, prior to homogenisation, were placed at 37° C until transfer to a warm haemocytometer, where the diameter of one hundred cells was measured using a Projectina microscope (x 145 magnification). The mean cell volume (mcv) was calculated and an estimate of the number of cells per gram tissue was obtained by dividing total lipid content of the tissue by the average lipid content of the fat cells as described by Di Girolamo *et al* (1971). Lipid content of the adipose tissue was taken to be equal to its dry weight and the average lipid content of the fat cells derived from the mcv x density of lipid (density of lipid was taken to be 0.9).

2.2.3.4 Acute phorbol ester treatment of rat adipocytes

Parametrial fat pads were dissected and adipocytes extracted by collagenase digestion essentially as described in section 2.2.3.2 but with the addition of 3% BSA (w/v) to the KRH solution. Cells were then washed four times in BSA-free KRH, re-suspended in 20 ml of this medium plus 200 nM phenylisopropyladenosine (PIA) and allowed to equilibrate for 10 min at 37° C with constant shaking at 110 oscillations per min. Isolated adipocytes were then incubated with 100 nM TPA for 10 min at 37° C. Reactions were terminated by rapidly separating the cells from the incubation medium by centrifugation at 180 g in a Mistral 2000 centrifuge; centrifugation was terminated immediately at 180 g. 0.33 volumes of homogenisation buffer (20 mM Tris-HCl pH 7.5, 10 mM EGTA, 10 mM EDTA, 255 mM sucrose, 1 mM NaF, 1 mM Na pyrophosphate, 20 mM β-mercaptoethanol, 1 mM PMSF, 1 mM Na vanadate and 20 μg/μl each of leupeptin, aprotinin and pepstatin A) was then added to the cells and homogenates prepared by 10 strokes with a syringe and 26 guage needle performed at room temperature. After centrifugation at 300 g for 10 min at 4° C in a Sorvall RC-5B refrigerated centrifuge fitted with an SS-34 rotor, the infranatant was removed for further fractionation. Subcellular fractionation was carried out as described for cultured cells in section 2.2.2.7.

2.2.3.5 Preparation of rat brain homogenate

Rat brain homogenates were prepared essentially according to the method of Kuo *et al* (1980). Following dissection of midbrains, all procedures were carried out at 4° C. Brains were washed in ice-cold PBS and snap-frozen in liquid nitrogen. The tissue was then cryo-pulverised and homogenised in six volumes of homogenisation buffer (25 mM Tris pH 7.5, 2.5 mM EGTA, 250 mM sucrose, 2.5 mM $MgCl_2$, 50 mM β-mercaptoethanol, 1 mM Na vanadate, 1 mM PMSF and 2 μg/ml each of leupeptin, aprotinin and pepstatin A) using a polytron homogeniser at setting 4 for 20 sec. Homogenates were spun at 1000 g for 10 min at 4° C in a Sorvall RC-5B refrigerated centrifuge fitted with an SS-34 rotor. The supernatant was passed through buffer-moistened glass wool and mixed with 1% (v/v) triton X-100 for 1 h at 4° C. Following a second spin at 1000 g the supernatant was boiled in 0.25 volumes of 5 x concentrated SDS-sample buffer for 5 min and stored at -70° C prior to use as a positive control on immunoblots.

2.2.3.6 Preparation of serum from whole blood

Blood samples were taken from rats that had been treated with α-rGH (and the appropriate control rats) and allowed to clot on ice for at least 1 h. Serum was obtained from whole blood by centrifugation at 1800 g for 10 min at 4° C in a Heraeus Sepatech minifuge and stored at -20° C prior to extraction and assay of IGF-I.

2.2.3.7 Assay of insulin-like growth factor-I

Endogenous IGF-I levels were determined by radioimmunoassay using a modified double-antibody technique. Assays were performed in RIA buffer (60 mM NaH_2PO_4 pH 7.4, 0.9 % (w/v) NaCl, 0.5 % (w/v) BSA, 0.1 % (w/v) thimerosal). The major IGF-binding proteins were extracted from the serum by addition of 4 volumes of acid ethanol (2 M HCL:95 % ethanol [1:7 v/v]) followed by mixing and incubation at room temperature for 30 min. After centrifuging at 1700 g for 5 min in a Camlab microfuge, an equal volume of neutralisation buffer (RIA buffer containing 330 mM Tris) was added. Extracted serum samples were then diluted a further 10-fold (100-fold final dilution) in RIA buffer. Diluted sample (100 µl) was mixed with an equal volume of a 1/2000 dilution of anti-IGF-I first antibody (NIDDK and NHPP, University of Maryland School of Medicine, USA) and pre-incubated for 24 h at room temperature before adding 100 µl of [^{125}I]-IGF-I (20,000 cpm/tube) for a further 24 h. Complexed IGF-I antibody was precipitated by adding 300 µl of donkey anti-rabbit IgG precipitating serum (Scottish Antibody Production Unit, Carluke, UK) diluted in RIA buffer containing 10 mM EDTA and 16 % polyethylene glycol (PEG:antibody, 15:1). After incubating at room temperature for 4 h and centrifugation at 1700 g for 30 min in a Heraeus Sepatech Omnifuge, the supernatant was decanted and the radioactivity in the pellets counted in a gamma counter (Cobra Autogamma, Packard). The IGF-I concentration of the serum samples was determined by interpolation from a standard curve (0.625-625 ng/ml IGF-I, Bachem UK, Ltd., Essex, UK). The IGF-I standard was iodinated as described previously (Fraker and Speck, 1978) and treated identically to the serum samples throughout the assay.

2.2.4 Immunoblotting

2.2.4.1 Protein Assays

Protein content of samples was determined by the method of Bradford (1976) using BSA as a standard. Samples were incubated with Bio-Rad Protein Assay reagent (Bio-Rad Labs. Ltd., Herts, UK) and absorbances were read in a Titertek Multiskan at 600 nm.

2.2.4.2 Standard gel electrophoresis

Protein samples were resolved by SDS-PAGE on 16 cm^2 9% gels. Gels were run at 7-30 mA in a Protean II x i vertical electrophoresis system (Bio-Rad). The composition of the running buffer was 49.5 mM Tris pH 8.3, 190 mM glycine and 3.5 mM SDS. Proteins were transferred to Hybond-C Super nitrocellulose membranes (Amersham) in a Transphor electrophoresis-transfer unit with power lid (Hoeffer UK) for 2 h at 400 mA in ice-cold transfer buffer (25 mM Tris pH 8.3, 192 mM glycine, 20% methanol). Electrophoresis and transfer procedures were carried out at room temperature using water-fed cooling systems.

2.2.4.3 Mini-gels

For some experiments, the protein content of samples was examined by Coomassie Blue staining of mini-gels. 5 μg of sample was resolved by SDS-PAGE on 7.5 cm^2 9% mini-gels using a Bio-Rad mini-gel apparatus. Gels were run at 30 mA for approximately 1 h, therefore no cooling system was required. Gels were then incubated with Coomassie Brilliant Blue (1 g/l prepared in methanol:acetic acid:H_2O, 5:1:5 [v/v], pre-filtered through Whatman number 1 filter paper) for 1 h to stain proteins. Excess dye was removed by overnight incubation with de-stain (7 % acetic acid, 5 % ethanol) and further washing with this solution until protein bands could be clearly visualised.

Protein concentration of samples was compared visually and protein assays repeated if samples did not contain equivalent protein concentrations (Figure 2.1). Coomassie staining of proteins was also performed following sub-cellular fractionation to confirm that cytosol and membrane samples had been separated (Figure 2.2).

2.2.4.4 Immunodetection of protein kinase C isoforms

Nitrocellulose was stained with Ponceau S solution (1% acetic acid, 0.5% ponceau S), to allow the position of the non-prestained high molecular weight standards (Sigma) to be marked, then washed several times with distilled water. Active sites were blocked by incubation of membranes in Tris-buffered saline with 0.1% Tween-20 (TBS-Tween; 19.98 mM Tris pH 7.4, 154 mM NaCl, 0.1% Tween-20) containing 3% BSA (w/v). TBS-Tween containing BSA was filtered through Whatman number 1 filter paper prior to use. Membranes were blocked for at least 16 h at room temperature then rinsed twice with TBS-Tween followed by two 5 min washes with vigorous shaking.

A panel of monoclonal antibodies and anti-peptide antisera (See section 2.1.3) was used to screen samples for the presence of PKC isoforms. PKC α, βI/II, γ, δ, ε, ζ, θ, λ, ι and μ mouse monoclonal antibodies (Affiniti) were used at the dilution recommended by the manufacturer, namely: 1:5000 for α and γ; 1:2500 for β; 1:1000 for δ and μ; 1:500 for ε and ζ and 1:250 for θ, λ and ι. Polyclonal antisera to PKC α, β, γ, δ, ε and ζ (Tang *et al*, 1993) were used at a dilution of 1:5000. Polyclonal antisera to PKC βI, η and θ (Marais and Parker, 1989) were used at a dilution of 1:2000. The commercial polyclonal antiserum to PKC η (Insight Biotechnologies) was used at a dilution of 1:500. All antibodies referred to were diluted in TBS-Tween containing 1% BSA (filtered as before) and all washes of the nitrocellulose were with TBS-Tween.

Primary antibodies were incubated with the membranes for 2 h at room temperature with gentle shaking. Membranes were then rinsed twice followed by one 15 min and two 5 min washes with vigorous shaking.

For some experiments, primary antibody incubations were carried out in the presence of excess immunising peptide (1 mg/ml). Antibody and antigen were pre-coupled by incubation of serum and peptide in 100 μl TBS-Tween for 30 min at room temperature. Antiserum was added to give the desired concentration upon final dilution in TBS-Tween containing 1 % BSA. The complexed antibody solution was

then incubated with the membranes as described above. During all peptide competition studies, control (uncomplexed) antiserum was treated identically and immunoblotting in the presence or absence of peptide was carried out simultaneously.

The corresponding secondary antibodies were HRP-conjugated sheep anti-mouse IgG or goat anti-rabbit-IgG and were used at a dilution of 1:5000. Secondary antibodies were incubated with the membranes for 1 h at room temperature with gentle shaking. The membranes were rinsed three times followed by one 15 min and four 5 min washes with vigorous shaking. Immunoreactive bands were visualised using an ECL detection system (Pierce and Warriner [UK] Ltd, Chester, UK). Densitometric analysis of immunoreactive bands was performed using a Molecular Dynamics Personal Densitometer and ImageQuaNT image-analysis software.

2.2.5 Statistical Analysis

Results are presented as means ± S.E.M. and, unless stated otherwise, statistical analysis was by Student's t-test for paired samples.

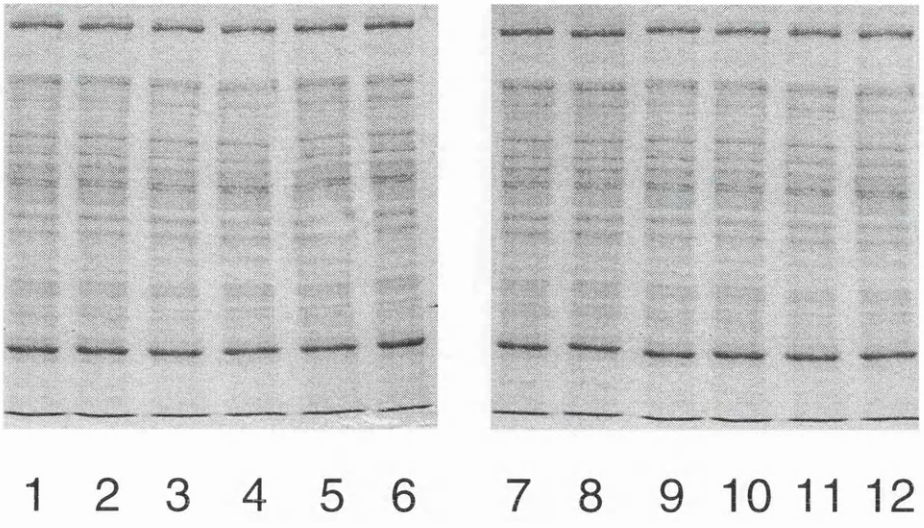


Figure 2.1 Comparing protein concentration of cellular preparations by Coomassie Blue staining

For some experiments the protein content of different cellular preparations was examined, prior to immunoblotting, by Coomassie Blue staining of mini-gels. Equal quantities (5 μg) of cellular protein were resolved by SDS-PAGE and stained with Coomassie Blue as described in section 2.2.4.3. The intensity of staining of the different preparations was compared visually to confirm that the protein content was equal. Representative gels are presented containing 5 μg of lysate protein prepared from just confluent 3T3-F442A preadipocytes (lanes 1-3 and 7-9) and twelve day-confluent 3T3-F442A preadipocytes (lanes 4-6 and 10-12).

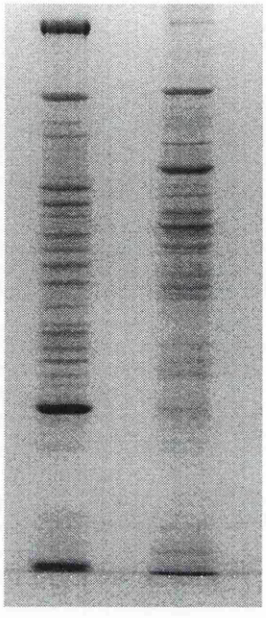


Figure 2.2 Confirming sub-cellular fractionation by Coomassie Blue staining

Sub-cellular fractions were prepared from 3T3-F442A cells and rat adipocytes as described in section 2.2.2.7. Equal quantities (5 μg) of cellular protein were resolved by SDS-PAGE and stained with Coomassie Blue as described in section 2.2.4.3. The protein banding patterns of samples were compared to confirm that the cytosolic and membrane fractions had been separated. Representative gels are presented containing 5 μg of rat adipocyte cytosol (lane 1) and membrane (lane 2) protein preparations.

Chapter 3

Characterisation of protein kinase C isoforms in 3T3-F442A cells and rat adipocytes

3.1 Introduction

Since the discovery that tumour-promoting phorbol esters can substitute for DAG in the activation of PKCs (Castagna *et al*, 1982; Bell and Burns, 1991; Gschwendt *et al*, 1991), there has been increasing interest in the role of these kinases in the regulation of cell growth and differentiation. PKC is a multigene family that, to date, has been shown to consist of twelve members. These have been divided into groups according to their structural and biochemical properties. The fact that PKC isoforms display distinct regulatory properties and tissue distributions is consistent with the idea that individual isoforms serve diverse functional roles. However, although the structural properties of PKC subtypes have been fairly well characterised, as of yet, relatively little is known about the functions of the individual isoforms in any system.

The purpose of this study was to investigate the roles of individual PKC isoforms in adipocyte development and function using 3T3-F442A cells and rat adipocytes. Since the PKC isoform profile of 3T3-F442A cells has not been reported previously and some controversy surrounds the reported PKC profile for rat adipocytes (Farese *et al*, 1992; Messina *et al*, 1992; Stumpo *et al*, 1994), a thorough characterisation of the PKC complement of these cell types was required initially. The complement of PKC isoforms in 3T3-F442A cells and rat adipocytes was, therefore, determined by immunoblotting using a panel of isoform-specific monoclonal and polyclonal antibodies. Strict criteria were applied during immunoblotting to ensure the appropriate identification of PKC isoforms in these cells.

3.2 Methods

3.2.1 Immunoblotting

Cell lysates from NIH-3T3 cells which had been transfected to over-express individual PKC isoforms (Goodnight *et al*, 1995) and a rat brain homogenate (Kuo *et al*, 1980) were prepared as described in sections 2.2.2.6 and 2.2.3.5 for use as positive controls on immunoblots. Cell lysates were prepared from undifferentiated 3T3-F442A preadipocytes (Green and Kehinde, 1976), fully differentiated 3T3-F442A adipocytes and undifferentiated twelve-day-confluent 3T3-F442A preadipocytes and cell homogenates were prepared from isolated rat adipocytes as described in sections 2.2.2.6 and 2.2.3.2 respectively. 3T3-F442A cell and rat adipocyte preparations were analysed for PKC isoform expression, alongside the positive control preparations, by immunoblotting using a panel of isoform-specific monoclonal and polyclonal antibodies as described in section 2.2.4.4. As a further control, when immunoblotting was performed with polyclonal antiserum, this was carried out, where possible, in the presence and absence of the peptide immunogen, as described in section 2.2.4.4.

3.2.2 Modulation of protein kinase C isoforms by phorbol ester

Studies were done to examine the acute modulation of PKC isoforms by phorbol ester. In these experiments, 3T3-F442A preadipocytes were treated with 10 nM TPA, or vehicle alone, and both 3T3-F442A adipocytes and rat adipocytes were treated with 100 nM TPA, or vehicle, for 10 min as described in sections 2.2.2.1 and 2.2.3.4. Cell homogenates were prepared from the treated cells and cytosol and membrane fractions separated as described in section 2.2.2.7. PKC isoform expression in cytosol and membrane fractions was examined, both before and after TPA treatment, by immunoblotting with isoform-specific monoclonal antibodies (Affiniti) to PKC, as described in section 2.2.4.4.

In order to assess the chronic modulation (ie: susceptibility to down-regulation) of PKC isoforms by phorbol esters, 3T3-F442A preadipocytes were treated with either 16 μM or 500 nM TPA for periods of 0, 3, 6, 9 12 or 24 h, as described in section 2.2.2.2, prior to the preparation of lysates for immunoblotting. Down-regulation of individual isoforms during the time-course of TPA treatment was examined by

densitometric analysis of immunoblots.

Densitometric analysis was performed using a Molecular Dynamics Personal Densitometer and ImageQuaNT image-analysis software.

3.3 Results

3.3.1 Protein kinase C isoform expression in 3T3-F442A cells and rat adipocytes

The complement of PKC isoforms in 3T3-F442A preadipocytes and 3T3-F442A adipocytes and in rat adipocytes was examined by immunoblotting using a panel of isoform-specific monoclonal and polyclonal antibodies (see Section 2.1.3). Due to the high degree of sequence homology shared between PKC sub-species, several criteria were employed to ensure correct identification of PKC isoforms in 3T3-F442A cells and rat adipocytes. These criteria exploited the known structural and regulatory properties of the PKC subtypes in order to assess the specificity of the antibodies used for characterisation of the cells. Criteria included:

i) Precise molecular weight determination of immunoreactive species and comparison with the predicted molecular weight of PKC isoforms.

ii) Co-migration of immunoreactive proteins with the appropriate band from a panel of transfected cell-lines over-expressing individual PKC isoforms.

iii) Co-migration of immunoreactive proteins with the appropriate band from a rat brain homogenate.

iv) Screening of samples with more than one PKC isoform-specific antibody where possible.

v) Susceptibility of immunoreactive species to modulation (acute and chronic) by phorbol esters.

The monoclonal (Figure 3.1) and polyclonal (results not shown) anti-PKC α, δ and ε antibodies detected a single major immunoreactive species in the NIH-3T3 cells over-expressing these isoforms of PKC and also in rat brain homogenates. Since most PKC isoforms were originally identified in brain tissue, rat brain homogenate served as a positive control for PKC antibodies when no appropriate over-expressing cells were available and as an additional control for antibodies when the appropriate isoform-over-expressing cell lysate was available. The molecular weights of the immunoreactive proteins detected with the anti-PKC α, δ and ε antibodies were similar to the predicted molecular weights of PKC α, δ and ε (see Table 3.1). That both the monoclonal and polyclonal antibodies specific for these PKC isoforms detected the same sized immunoreactive species led to the conclusion that these

proteins were indeed PKC α, δ and ε. A major immunoreactive band, that co-migrated with that seen in the rat brain homogenates and NIH-3T3 cells over-expressing the appropriate PKC isoform, was detected in 3T3-F442A preadipocytes and adipocytes and in rat adipocytes with antibodies to PKC α, δ and ε. Hence, it was concluded that these PKC isoforms are expressed in each of these cell types.

The predicted molecular weights for PKC βI and βII are 76.6 and 76.8 kDa respectively (Table 3.1). Both a polyclonal antiserum raised in-house to PKC βI/II (Figure 3.1) and a polyclonal antiserum raised to PKC βI (gifted by Dr Peter Parker; Figure 3.2) detected an immunoreactive species migrating at 75 kDa in all cell preparations. However, the major immunoreactive species in both rat brain homogenates and NIH-3T3 cells over-expressing PKC β had a molecular weight of 83 kDa. Since co-migration with the PKC over-expresser and rat brain preparations were criteria used during the screening of samples for PKC isoforms, immunodetection was carried out in PKC β over-expressing-cell lysates and rat adipocyte homogenates in the presence of the immunising peptide. Peptide competition effectively abolished the detection of the 83 kDa protein in the over-expressing cell lysates but had no effect on the 75 kDa band apparent in both the over-expressing-cell lysates and in rat adipocyte homogenates (Figure 3.2). This indicated that the faster migrating species of 75 kDa in size, seen in all blotting studies with the polyclonal PKC β antisera (Figures 3.1 and 3.2), was a non-specific species and that the protein detected in rat brain homogenates and PKC β over-expressing cells, migrating at 83 kDa, was indeed PKC β. Failure to detect an 83 kDa immunoreactive species in the 3T3-F442A cell and rat adipocyte preparations investigated here suggested there was no PKC β isoform present in these cells.

The observation that PKC β was not expressed in rat adipocytes is in contrast to results obtained by Farese *et al* (1992). Because of this discrepancy, a third antibody, a monoclonal antibody capable of detecting both PKC βI and βII (Affiniti), was also used. This antibody detected a single 83 kDa band in PKC β over-expressing-cell lysates and in rat brain homogenates (Figure 3.3) and, in accordance with the polyclonal immunoblots, this band was not apparent in 3T3-F442A cell lysates or rat adipocyte homogenates. This again indicated that there was no PKC β in these cells.

In an attempt to determine whether the PKC β protein was expressed at a low level in the rat adipocyte, a larger quantity (400 μg as opposed to 50 μg) of homogenate protein was immunoblotted using the monoclonal antibody. PKC β was not detected even in this large quantity of rat adipocyte homogenate protein (Figure 3.3).

A monoclonal antibody to PKC γ detected a major immunoreactive species in both 3T3-F442A cells and rat adipocytes which migrated with a molecular weight of 80 kDa, thus corresponding to the predicted molecular weight of this PKC isoform (Table 3.1). Furthermore, this band co-migrated with the major immunoreactive species in the positive antibody control preparations (Figure 3.1). The expression of PKC γ is generally thought to be restricted to the brain and spinal chord (Nishizuka, 1988) and, since a number of PKC isoforms have a similar predicted molecular weight to PKC γ (see Table 3.1), lysates were prepared from NIH-3T3 cells over-expressing either PKC α (76.7 kDa), β (76.7 kDa), γ (78.3 kDa) or δ (77.5 kDa), and immunoblotted with the PKC γ antibody in order to examine the cross-reactivity of the anti-PKC γ antibody with similar PKC sub-species. Wild-type NIH-3T3 cells were also immunoblotted as a control. Figure 3.4 demonstrates the presence of PKC γ in wild type NIH-3T3 cells. Thus, a co-migrating band of similar intensity was detected in NIH-3T3 cells which over-express PKC β and δ. A band of greater intensity was detected in NIH-3T3 cells over-expressing PKC α, suggesting a degree of cross-reactivity between the PKC γ antibody and the PKC α protein. However, that a dramatic increase in intensity of the immunoreactive band in lysates prepared from NIH-3T3 cells transfected to over-express PKC γ was observed, indicated that the PKC γ antibody displayed predominant specificity for the γ isoform of PKC.

Both anti-PKC ζ antibodies (monoclonal and polyclonal) detected a number of immunoreactive species in all samples; the major band detected with each antibody in both the control preparations and in 3T3-F442A cells and rat adipocytes having an apparent molecular weight of 83 kDa (Figures 3.1 and 3.5). From its amino acid sequence, the predicted molecular weight of PKC ζ is approximately 67 kDa (Table 3.1). However, that two different antibodies detected a major immunoreactive protein of 83 kDa suggested that this protein was authentic PKC ζ. To confirm that the 83

kDa species was indeed PKC ζ, lysates prepared from NIH-3T3 cells over-expressing PKC ζ and rat adipocyte homogenates were immunoblotted with the PKC ζ polyclonal antibody in the presence of immunising peptide. Peptide immunogen prevented binding of the PKC ζ antibody to the 83 kDa band in both cell types (Figure 3.5) indicating that the protein detected with the PKC ζ antibody, in both the ζ-over-expressing control cells and in rat adipocytes, was indeed PKC ζ. Thus, under the conditions used, PKC ζ had an apparent molecular weight of 83 kDa and was detected in both 3T3-F442A cells and rat adipocytes (Figure 3.1).

PKC η has a predicted molecular weight of approximately 78 kDa (Table 3.1). In 3T3-F442A cells (results not shown) and rat adipocytes (Figure 3.6A), the major immunoreactive band detected with the PKC η polyclonal antibody was 72 kDa in size and did not co-migrate with the major immunoreactive band of 78 kDa seen in NIH-3T3 cells over-expressing PKC η. Immunising peptide effectively prevented binding of the PKC η antibody to the 78 kDa species in the over-expressing cells indicating that this species was in fact PKC η (Figure 3.6A). In contrast, peptide immunogen did not prevent binding of the PKC η antibody to the 72 kDa species detected in 3T3-F442A cells (results not shown) and rat adipocytes (Figure 3.6A). This indicated that the protein detected in 3T3-F442A cells and rat adipocytes was a non-specific species and was not PKC η.

Both the polyclonal (Figure 3.6 B) and monoclonal (data not shown) anti-PKC θ antibodies detected a major immunoreactive band migrating at the predicted molecular weight of this isoform (82 kDa) in NIH-3T3 cells over-expressing PKC θ. An 82 kDa species was not apparent in preparations from either 3T3-F442A cells or rat adipocytes (Figure 3.6B) indicating that PKC θ was not expressed in these cell types .

The monoclonal antibodies to both PKC ι and λ detected low levels of an immunoreactive protein, displaying an apparent molecular weight of 83 kDa, in both 3T3-F442A cells (Figure 3.7) and rat adipocytes (results not shown). PKC ι and λ are thought to be mouse and human homologues of the same protein (Zhou *et al*, 1994)

and have a predicted molecular weight of approximately 67 kDa (Table 3.1). These isoforms share 72 % homology with PKC ζ (Zhou *et al*, 1994) which displayed an apparent molecular weight 83 kDa under the conditions used during this study (Figure 3.1) and cross-reactivity of anti-PKC ι and λ antibodies with PKC ζ is a common problem (Zhou *et al*, 1994). In an attempt to establish further the specificity of the ι and λ antibodies, lysates prepared from 3T3-F442A preadipocytes and NIH-3T3 cells over-expressing PKC ζ and homogenates prepared from rat heart and spleen were immunoblotted with monoclonal antibodies specific for PKC ι, λ and ζ (Affiniti). Heart and spleen are reported to be rich in PKC ι/λ but not PKC ζ (Zhou *et al*, 1994). Figure 3.7 shows that the PKC ι and λ antibodies detected a distinct 83 kDa band in the PKC ζ over-expressing cells and in 3T3-F442A preadipocytes. Furthermore, the PKC ι and λ antibodies failed to detect a major immunoreactive band in heart and spleen samples. Although it is possible that PKC ι/λ was present in 3T3-F442A cells and rat adipocytes, it is more likely that the monoclonal antibodies to PKC ι and λ are chiefly detecting PKC ζ in these cells. The PKC ζ antibody cross-reacted with PKC ι/λ, which also have an apparent molecular weight of 83 kDa under the conditions used. This was determined by the ability of the PKC ζ antibody to detect immunoreactive species of 83 kDa in heart and spleen homogenates (Figure 3.7). The cross-reactivity data indicates, however, that PKC ι/λ are probably not expressed in either 3T3-F442A cells or rat adipocytes and that the PKC ζ antibody is therefore specific for the PKC ζ protein in these cells.

In rat brain homogenates, the PKC μ monoclonal antibody detected an immunoreactive protein doublet of 116 and 112 kDa (Figure 3.1). A major immunoreactive species was detected with the anti-PKC μ antibody in 3T3-F442A cells and rat adipocytes that co-migrated with the 112 kDa species detected in rat brain homogenates. This corresponds to the predicted molecular weight of the murine homologue of PKC μ, also known as PKD (Van Lint *et al*, 1995), which migrates at 110 kDa and shares 92% sequence homology with the human form of PKC μ to which the anti-PKC μ antibody was raised (see Section 2.1.3). Thus, PKC μ expression was detected in both 3T3-F442A cells and rat adipocytes.

Thus, analysis of 3T3-F442A cells by immunoblotting using a panel of monoclonal antibodies and anti-peptide antisera, applying the defined criteria (see above) to ensure appropriate identification of PKC isoforms, indicated the presence of the PKC α, γ, δ, ε, ζ and μ isoforms. This is demonstrated in the immunoblots shown in Figure 3.1. The same PKC species were found in undifferentiated 3T3-F442A preadipocytes, fully differentiated 3T3-F442A adipocytes and parallel cultures of undifferentiated 3T3-F442A preadipocytes maintained at confluence for twelve days. In addition, the same major immunoreactive bands were detected in rat adipocytes (Figure 3.1).

3.3.2 Modulation of 3T3-F442A cell and rat adipocyte protein kinase C isoforms by phorbol esters

To examine further the specificity and cross-reactivity of the panel of antibodies, they were used to probe the effects of acute and chronic treatment with phorbol esters on the expression of PKC isoforms. Phorbol esters, such as TPA, can substitute for DAG and induce the activation and translocation of conventional and novel PKC isoforms, but not atypical PKCs (Kraft *et al*, 1982; Ito *et al*, 1989; Kiley *et al*, 1995). The acute modulation of PKCs isoforms by phorbol esters was, therefore, assessed by examining PKC isoform expression in cytosol and membrane fractions both prior to and following TPA treatment of cells. According to the structural properties of the PKC isoforms (see Section 1.6; Johannes *et al*, 1994), conventional PKCs α and γ and novel PKCs δ and ε would be expected to translocate from the cytosol to the membrane fraction following such treatment whereas atypical PKC ζ and PKC μ would not. 3T3-F442A preadipocytes were treated with 10 nM TPA for 10 min and immunoreactive PKC isoform levels in both the cytosol and membrane fractions assessed.

The different responses of the PKC isoforms in 3T3-F442A preadipocytes to TPA are shown in Figure 3.8. Prior to stimulation, PKC α was detected only in the cytosolic fraction. Following TPA treatment, expression of PKC α was detected mainly in the membrane fraction, with very low levels of expression in the cytosol. Although TPA induced the translocation of a large proportion of immunoreactive PKC α from the cytosol, a corresponding increase in membrane immunoreactivity was not observed.

This may indicate that TPA induced the translocation of PKC α to other sub-cellular compartments, such as the cytoskeleton and the nucleus. Alternatively, this may indicate that PKC α is rapidly down-regulated following membrane association.

In resting cells, low levels of PKC γ were detectable in membrane fractions, however this isoform was mainly detected in the cytosol prior to stimulation with TPA. TPA treatment induced the translocation of a large proportion of immunoreacte PKC γ from the cytosol to the membrane.

PKC δ was predominantly expressed in the particulate fraction prior to stimulation with TPA, with only low levels of expression in the cytosol. Following TPA treatment, expression of PKC δ was detected exclusively in the membrane fraction.

PKC ε was predominantly detected in the cytosol both prior to and following TPA treatment. However, TPA induced the translocation of a small proportion of immunoreactive PKC ε to the membrane.

Consistent with published data (Selbie *et al*, 1993; Johannes *et al*, 1994), PKC ζ and μ were not induced to translocate by TPA treatment and these PKC isoforms were detected in the cytosol both before and after stimulation of cells with TPA. Considering that PKC μ contains a putative membrane localisation sequence (Johannes *et al*, 1994), it was somewhat surprising that the expression of this isoform was found only in the cytosolic fraction of 3T3-F442A cells and rat adipocytes. However, this may reflect the experimental conditions used. It is possible that during processing of the cells the membrane association of this isoform, or its access to particular binding proteins, is lost.

The sensitivity of PKC isoforms in adipocytes to acute modulation by phorbol esters was also examined. Consistent with the observations of others (McGowan *et al*, 1996), a higher concentration of TPA was found to be required for the stimulation of PKC isoforms in adipocytes. In 3T3-L1 cells (McGowan *et al*, 1996), this has been attributed to the selective down-regulation of phorbol ester-sensitive PKC isoforms during the differentiation of preadipocytes into adipocytes. Both 3T3-F442A and rat

adipocytes were treated with 100 nM TPA for 10 min and immunoreactive PKC levels in the cytosol and membrane fractions assessed. The patterns of PKC isoform expression both prior to and following TPA treatment of 3T3-F442A adipocytes (Figure 3.9) and rat adipocytes (Figure 3.10) were found to be essentially identical, although the patterns were somewhat different from those observed for 3T3-F442A preadipocytes (Figure 3.8).

TPA induced the translocation of a smaller proportion of immunoreactive PKC α and γ from the cytosol to the membrane than was observed in preadipocytes. Thus, a large proportion of immunoreactive PKC α and γ remained in the cytosol following TPA treatment. In addition, PKC γ was not detectable in adipocyte membrane fractions prior to treatment, as observed in preadipocytes.

PKC δ was found only in association with membrane fractions in adipocytes. This was in contrast to preadipocytes, where a proportion of immunoreactive PKC δ was detectable in the cytosol prior to TPA treatment. TPA treatment of 3T3-F442A and rat adipocytes increased the levels of immunoreactive PKC δ in membrane fractions. This may be due to the translocation of PKC δ from cytoskeletal and nuclear compartments rather than the cytosol, as PKC δ is not detectable in the cytosol of either rat adipocytes or 3T3-F442A adipocytes.

Immunoreactive PKC ε was detected both in the cytosol and membrane fractions of adipocytes, whereas this isoform was detected only in the cytosol in preadipocytes, both before and after TPA treatment. TPA induced the translocation of immunoreactive PKC ε from the cytosol, however, a corresponding increase in membrane immunoreactivity was not observed. This may indicate that TPA induced the translocation of PKC ε to other sub-cellular compartments, such as the cytoskeleton and the nucleus, or, that PKC ε is rapidly down-regulated following membrane association.

Consistent with observations in preadipocytes, PKC ζ and μ were not induced to translocate to the membrane by acute phorbol ester treatment of adipocytes.

Thus, the conventional and novel PKC isoforms were induced to translocate by TPA treatment of preadipocytes and adipocytes while atypical PKC ζ and PKC μ were unresponsive, conforming to the known structural properties of the PKC sub-families (see Section 1.6; Johannes *et al*, 1995). Due to the potency of phorbol esters, PKCs are thought to become irreversibly associated with the membrane fractions, resulting in the prolonged stimulation of their activity (Anderson *et al*, 1995; Nelsestuen and Bazzi, 1991). Such prolonged activation results in down-regulation of PKC due to proteolytic degradation (Nishizuka, 1988; Borner *et al*, 1992). The susceptibility of the various immunoreactive PKC species to phorbol ester-induced down-regulation was examined in 3T3-F442A preadipocytes. 3T3-F442A preadipocytes were incubated with 16 μM TPA and PKC isoform expression in cell lysates assessed over a 24 h period by immunoblotting.

Following chronic TPA treatment of 3T3-F442A preadipocytes PKC α, γ and ε were detected as an immunoreactive protein doublet (Figures 3.11 and 3.12). The existence of multiple forms of particular PKC subtypes has been reported previously (Burt *et al*, 1991; Borner *et al*, 1992; Tang *et al*, 1993) in both resting and stimulated cells. Certain PKC isoforms have been shown to be phosphorylated or ubiquitinated in response to phorbol ester treatment (Borner *et al*, 1992; Lu *et al*, 1998). Thus, TPA-induced phosphorylation or ubiquitination of a proportion of PKC subtypes may account for the presence of multiple species of PKC α, γ and ε following the phorbol ester treatment of 3T3-F442A preadipocytes.

Chronic treatment of cells with 16 μM TPA resulted in the rapid down-regulation of PKC α, γ, and δ in lysates prepared from 3T3-F442A preadipocytes. PKC ε was more resistant to TPA-induced down-regulation, with significant levels of this isoform still detectable after 24 h of treatment. (Figure 3.11). Again, PKC ζ and μ were unaffected by TPA treatment. Reduction of the TPA concentration to 500 nM slowed down the down-regulation of the PKC isoforms allowing the individual time-courses to be followed (Figure 3.12). The profiles of down-regulation for each isoform obtained following densitometric analysis of immunoblots (Figure 3.13) indicated that the order of susceptibility to phorbol ester-induced down-regulation was PKC $\delta > \alpha > \gamma > \varepsilon$.

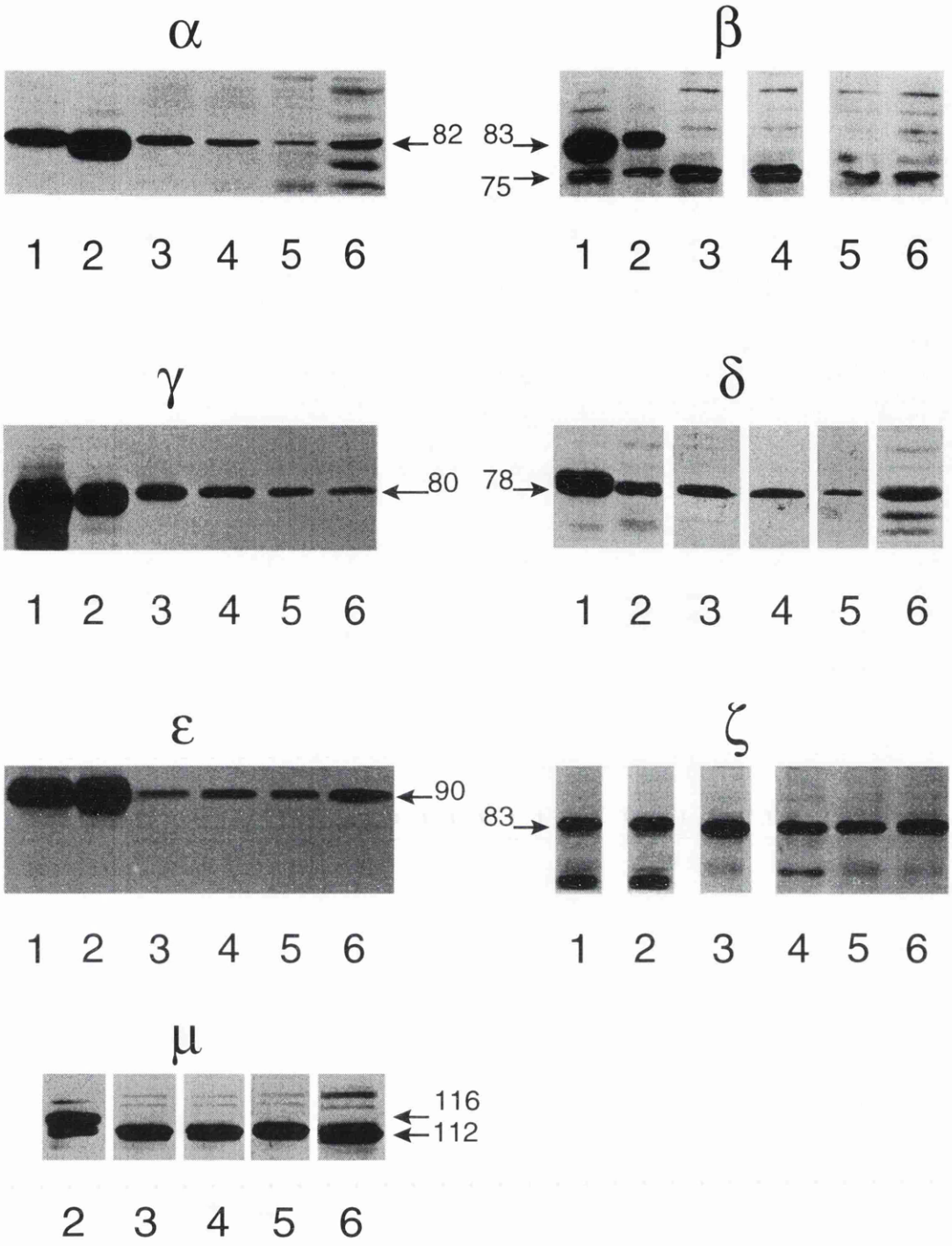
Thus, assessment of the effects of acute and chronic phorbol ester treatment on the immunoreactive PKC species detected by the antibodies used in this study, demonstrated that these species conform to the known properties of the PKC sub-families. Furthermore, that differential responses to these treatments (different relative sub-cellular distributions and time-courses for down-regulation) were displayed by each of the individual PKC isoforms raises confidence that each antibody recognises a distinct PKC isoform.

Table 3.1 Predicted molecular weights of PKC isoforms based on their amino acid composition

PKC Isoform	Predicted Molecular Weight	Reference
Alpha, α	76.7 kDa	Ohno and Suzuki (1995)
Beta I, β I	76.7 kDa	Ohno and Suzuki (1995)
Beta II, β II	76.8 kDa	Ohno and Suzuki (1995)
Gamma, γ	78.3 kDa	Ohno and Suzuki (1995)
Delta, δ	77.5 kDa	Ohno and Suzuki (1995)
Epsilon, ε	83.5 kDa	Ohno and Suzuki (1995)
Eta, η	77.9 kDa	Ohno and Suzuki (1995)
Theta, θ	81.5 kDa	Ohno and Suzuki (1995)
Zeta, ζ	67.6 kDa	Ohno and Suzuki (1995)
Lambda, λ	67.2 kDa	Ohno and Suzuki (1995)
Iota, ι	67.2 kDa	Ohno and Suzuki (1995)
Mu, μ	115 kDa	Johannes *et al.* (1994)
PKD	110 kDa	Valverde *et al.* (1994)

Figure 3.1 Protein kinase C isoforms present in 3T3-F442A cells and rat adipocytes

Cell lysates (75 μg of protein) prepared from "just-confluent" 3T3-F442A preadipocytes (lane 3), twelve-day-confluent 3T3-F442A preadipocytes (lane 4) and fully differentiated 3T3-F442A adipocytes (lane 5) and cell homogenates prepared from rat adipocytes (lane 6) were subjected to SDS-PAGE alongside 50 μg of lysate prepared from NIH-3T3 cells over-expressing the appropriate PKC isoform (lane 1; except for PKC μ) and 50 μg of rat brain homogente (lane 2). PKC isoform expression was assessed by immunoblotting with monoclonal antibodies (Affiniti) to PKC α, γ, δ, ε, ζ and μ and a polyclonal antibody capable of detecting PKC βI as described in section 2.2.4.4. Molecular weights (kDa) of the major immunoreactive bands are indicated. Immunoblots are representative of results obtained in at least six separate experiments.


α
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1 2 3 4 5 6
β
83
75
1 2 3 4 5 6
γ
80
1 2 3 4 5 6
δ
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ε
90
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ζ
83
1 2 3 4 5 6
μ
116
112
2 3 4 5 6

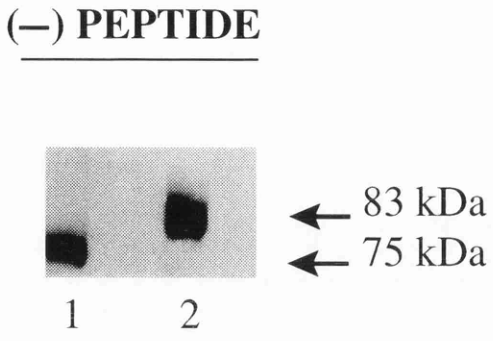


Figure 3.2 Immunoblotting for PKC β in the presence and absence of peptide immunogen

Equal quantities (50 μg of protein) of cell homogenate prepared from isolated rat adipocytes (lane 1) and cell lysate prepared from NIH-3T3 cells over-expressing PKC β (lane 2) were subjected to immunoblotting with polyclonal antiserum raised to PKC βI in the presence (+ peptide) or absence (- peptide) of peptide immunogen. Molecular weights (kDa) of the immunoreactive bands are indicated. Immunoblots are representative of results obtained in at least four separate experiments.

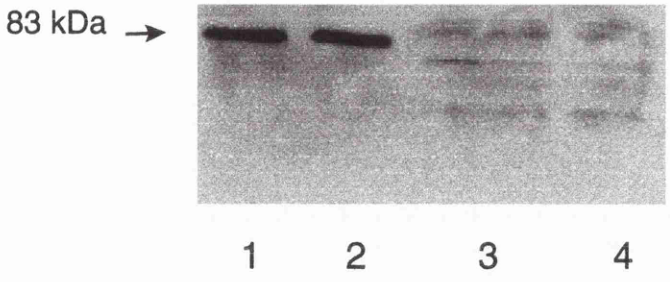
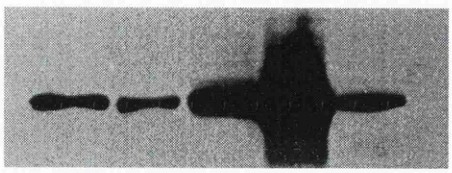


Figure 3.3 Immunoblotting for PKC β using excess protein

50 μg (lane 3) and 400 μg (lane 4) of homogenate protein prepared from isolated rat adipocytes were subjected to SDS-PAGE alongside 50 μg of cell lysate protein prepared from NIH-3T3 cells over-expressing PKC β (lane 1) and 50 μg of rat brain homogenate protein (lane 2). Immunoblotting was carried out using a monoclonal antibody capable of recognising both PKC βI and PKC βII. The position of immunoreactive PKC β is indicated by an arrow. The immunoblot is representative of results obtained in at least three separate experiments.



1 2 3 4 5

Figure 3.4 Assessment of the specificity of the monoclonal PKC γ antibody

Equal quantities of cell lysate (50 μg of protein) prepared from wild type NIH-3T3 cells (lane 5) or from NIH-3T3 cells transfected to over-express either PKC β (lane 1), δ (lane 2), α (lane 3) or γ (lane 4) were subjected to immunoblotting with a monoclonal antibody to PKC γ. The immunoblot is representative of results obtained in at least three separate experiments.

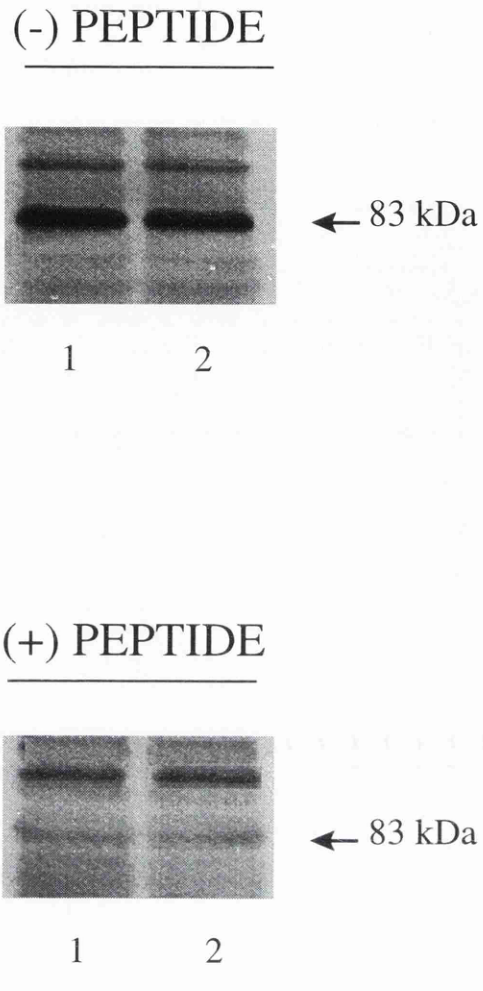


Figure 3.5 Effect of peptide immunogen on detection of protein bands by a polyclonal antiserum to PKC ζ

Equal quantities (50 μg of protein) of cell lysate prepared from NIH-3T3 cells over-expressing PKC ζ (lane 1) and cell homogenate prepared from isolated rat adipocytes (lane 2) were subjected to immunoblotting with polyclonal antiserum raised to PKC ζ in the presence (+ peptide) or absence (- peptide) of peptide immunogen. The molecular weight of PKC ζ is indicated. Immunoblots are representative of results obtained in at least four separate experiments.

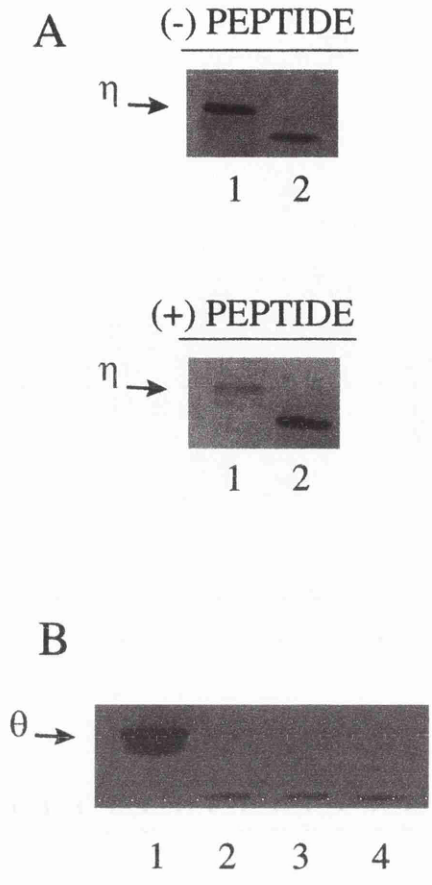


Figure 3.6 Immunoblotting for PKC η and θ

A Equal quantities (50 μg of protein) of a cell lysate prepared from NIH-3T3 cells over-expressing PKC η (lane 1) and of a cell homogenate prepared from isolated rat adipocytes (lane 2) were subjected to immunoblotting with polyclonal antiserum raised to PKC η in the presence (+ peptide) or absence (- peptide) of peptide immunogen. The position of immunoreactive PKC η is indicated. Immunoblots are representative of results obtained in at least three separate experiments.

B Equal quantities (50 μg of protein) of cell lysate prepared from 3T3-F442A preadipocytes (lane 2) or 3T3-F442A adipocytes (lane 3) and cell homogenates prepared from isolated rat adipocytes (lane 4) and rat brain (lane 1) were subjected to immunoblotting with polyclonal antiserum raised to PKC θ. The position of immunoreactive PKC θ is indicated. The immunoblot is representative of results obtained in at least three separate experiments.

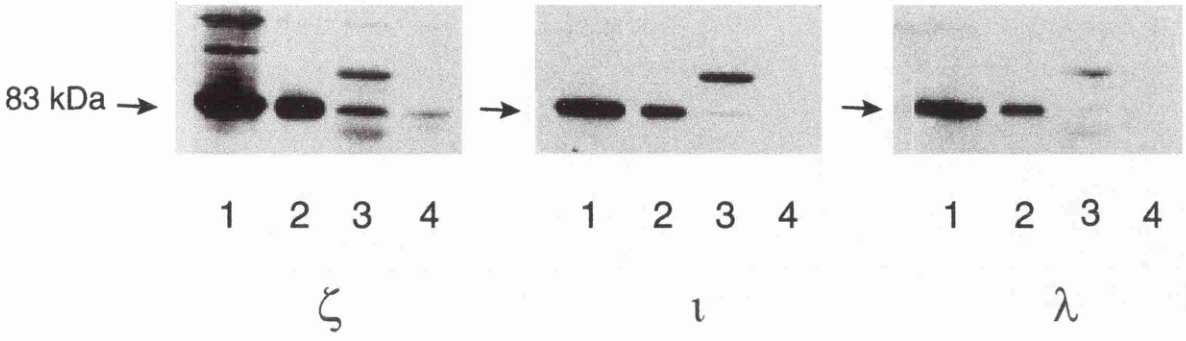


Figure 3.7 Assessment of the specificity of the PKC ζ, ι and λ antibodies

Equal quantities (50 μg of protein) of cell lysate prepared from NIH-3T3 cells over-expressing PKC ζ (lane 1) or from 3T3-F442A preadipocytes (lane 2) and cell homogenate prepared from rat heart (lane 3) and spleen (lane 4) were subjected to immunoblotting with isoform-specific monoclonal antibodies (Affiniti) to PKC ζ, ι and λ as indicated. The molecular weight (kDa) of the major immunoreactive species is indicated. Immunoblots are representative of results obtained in at least four separate experiments.

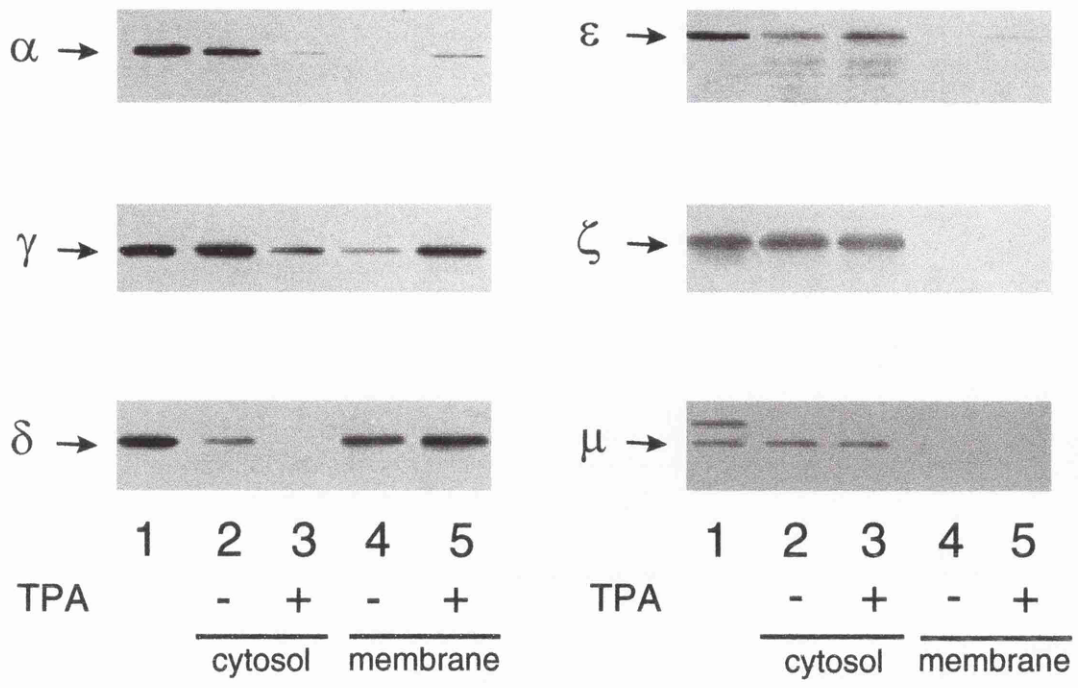


Figure 3.8 Effects of acute phorbol ester treatment of 3T3-F442A preadipocytes on the sub-cellular distribution of protein kinase C isoforms

3T3-F442A preadipocytes were incubated in the presence (lanes 3 and 5) or absence (lanes 2 and 4) of 10 nM TPA for 10 min prior to the preparation of cytosol (lanes 2 and 3) and membrane fractions (lanes 4 and 5) as described in section 2.2.2.7. Equal quantities (50 μg) of cytosol and membrane protein were resolved by SDS-PAGE alongside 50 μg of a rat brain homogenate (lane 1) and analysed by immunoblotting with isoform-specific monoclonal antibodies to PKC as indicated. Immunoblots are representative of results obtained in at least four separate experiments.

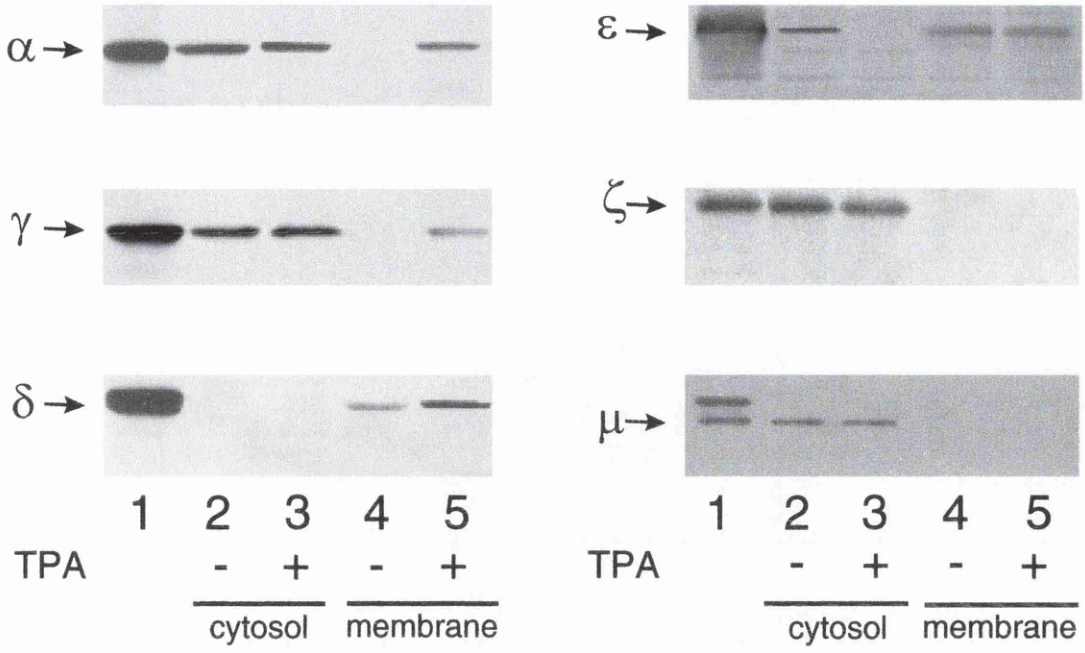


Figure 3.9 Effects of acute phorbol ester treatment of 3T3-F442A adipocytes on the sub-cellular distribution of protein kinase C isoforms

3T3-F442A adipocytes were incubated in the presence (lanes 3 and 5) or absence (lanes 2 and 4) of 100 nM TPA for 10 min prior to the preparation of cytosol (lanes 2 and 3) and membrane fractions. Equal quantities (50 μg) of cytosol and membrane protein were resolved by SDS-PAGE alongside 50 μg of rat brain homogenate (lane 1) and analysed by immunoblotting with isoform-specific monoclonal antibodies to PKC as indicated. Immunoblots are representative of results obtained in at least four separate experiments.

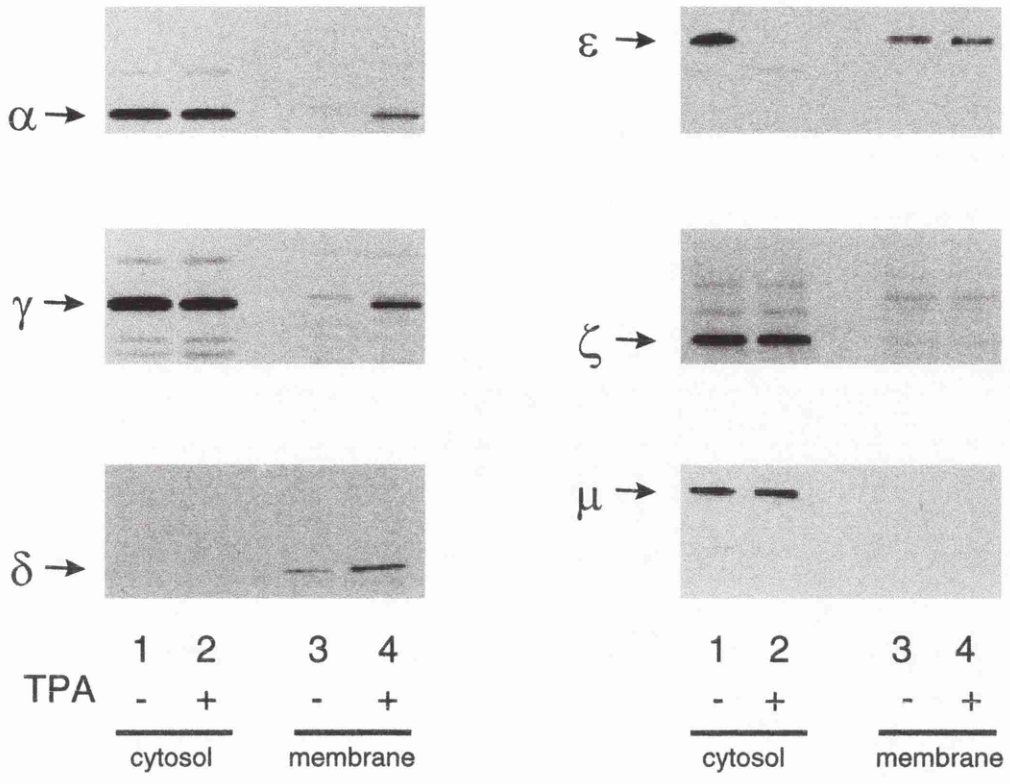


Figure 3.10 Effects of acute phorbol ester treatment of rat adipocytes on the sub-cellular distribution of protein kinase C isoforms

Isolated rat adipocytes were incubated in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 100 nM TPA for 10 min prior to the preparation of cytosol (lanes 2 and 3) and membrane fractions. Equal quantities (50 μg) of cytosol and membrane protein were resolved by SDS-PAGE and analysed by immunoblotting with isoform-specific monoclonal antibodies to PKC as indicated. Immunoblots are representative of results obtained in at least four separate experiments.

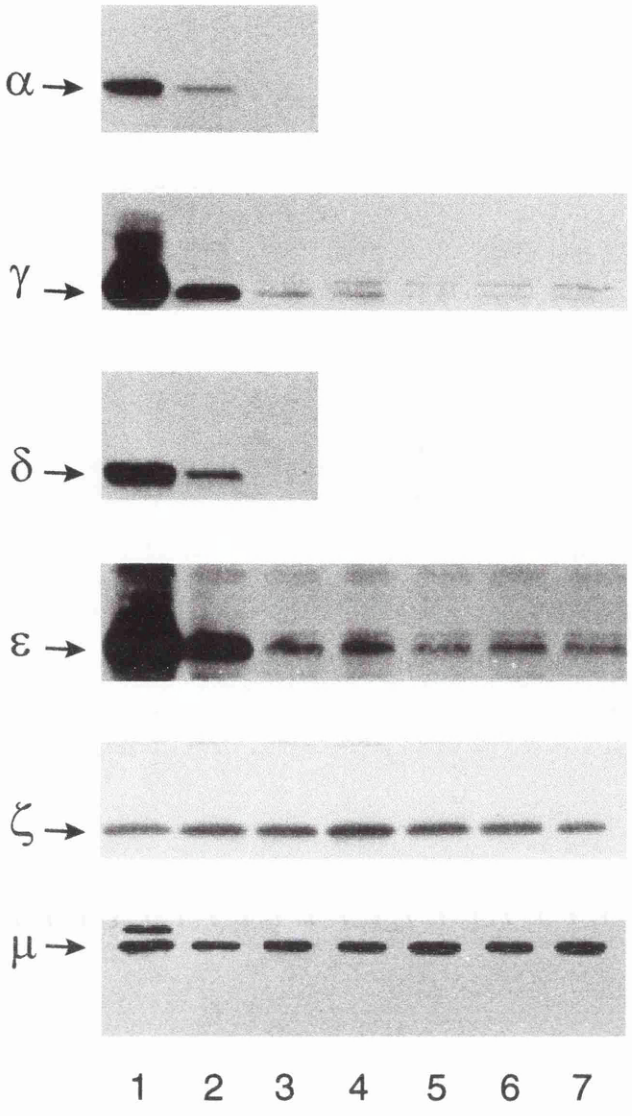


Figure 3.11 Effects of chronic treatment of 3T3-F442A preadipocytes with 16 μM phorbol ester on the cellular levels of protein kinase C isoforms

3T3-F442A preadipocytes were treated with vehicle for 24 h (DMSO, lane 2) or 16 μM TPA for 3 h (lane 3), 6 h (lane 4), 9 h (lane 5), 12 h (lane 6) or 24 h (lane 7) prior to the preparation of cell lysates. Equal quantities (50 μg) of lysate protein were resolved by SDS-PAGE alongside 50 μg of rat brain homogenate (lane 1) and analysed by immunoblotting with isoform-specific monoclonal antibodies to PKC as indicated. Immunoblots are representative of results obtained in at least three separate experiments.

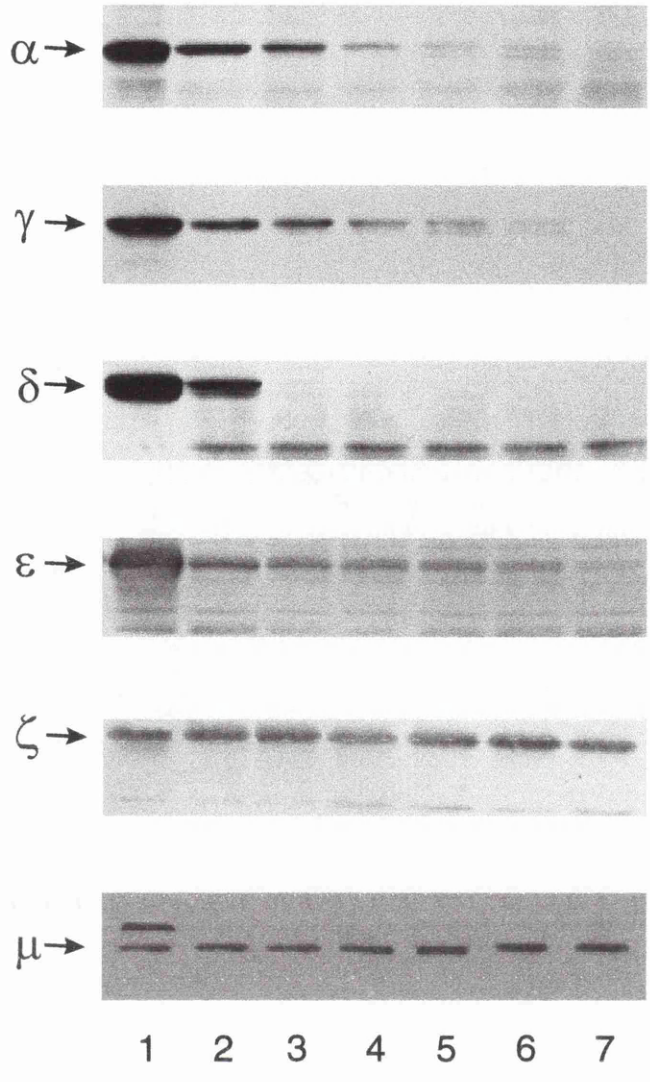


Figure 3.12 Effects of chronic treatment of 3T3-F442A preadipocytes with 500 nM phorbol ester on the cellular levels of protein kinase C isoforms

3T3-F442A preadipocytes were treated with vehicle for 24 h (DMSO, lane 2) or 500 nM TPA for 3 h (lane 3), 6 h (lane 4), 9 h (lane 5), 12 h (lane 6) or 24 h (lane 7) prior to the preparation of cell lysates. Equal quantities (50 μg) of lysate protein were resolved by SDS-PAGE alongside 50 μg of rat brain homogenate (lane 1) and analysed by immunoblotting with isoform-specific monoclonal antibodies to PKC as indicated. Immunoblots are representative of results obtained in at least four separate experiments.

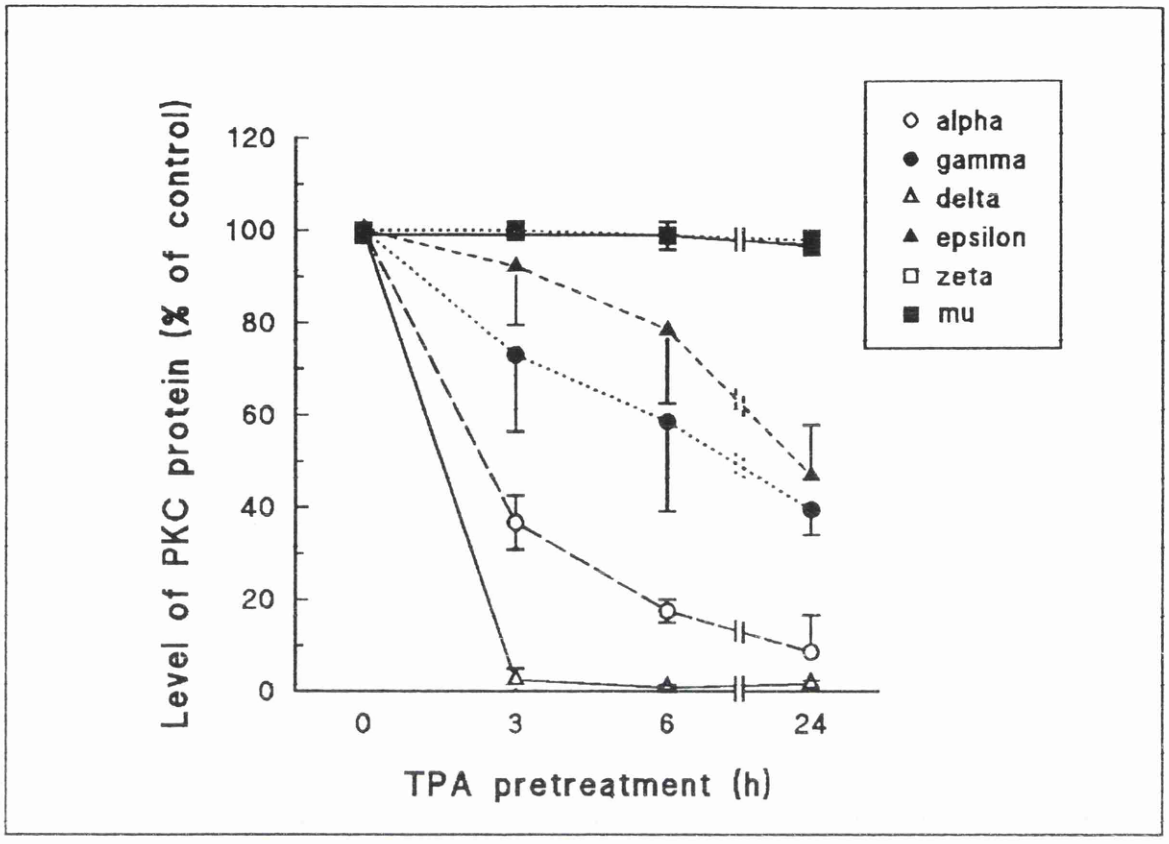


Figure 3.13 Densitometric analysis of effects of chronic treatment of 3T3-F442A preadipocytes with 500 nM phorbol ester on the cellular levels of protein kinase C isoforms

3T3-F442A preadipocytes were treated with vehicle (DMSO) for 24 h (TPA pre-treatment = 0 h) or 500 nM TPA for 3-24 h prior to the preparation of cell lysates for immunoblotting with isoform-specific monoclonal antibodies to PKC. Immunoblots were subjected to densitometric analysis and the level of PKC isoform expression in cell lysates which had been pretreated with TPA expressed as a percentage of the level in cells treated with vehicle alone (control = 100 %). Results are the means ± S.E.M. of four separate observations carried out on different cell preparations.

3.4 Discussion

In this study, the presence of conventional PKCs α and γ, novel PKCs δ and ε, atypical PKC ζ and PKC μ was demonstrated in both undifferentiated 3T3-F442A preadipocytes and differentiated 3T3-F442A adipocytes and in rat adipocytes. Using the antibodies available, expression of the PKC βI/II, η, θ, ι and λ isoforms was not detectable in 3T3-F442A cells or rat adipocytes, despite the fact that the antibodies detected expression of these proteins in the appropriate positive control preparations.

The apparent molecular weight of the PKC ζ isoform detected in 3T3-F442A cells and rat adipocytes differed slightly from the predicted molecular weight of this isoform shown in Table 3.1. This isoform, which has a predicted molecular weight of 67 kDa, was detected as an 83 kDa protein following immunoblotting. Previous studies (see for example Borner *et al*, 1992; Nishikawa *et al*, 1995; Ducher *et al*, 1995; Frevert and Kahn, 1996; Standaert *et al*, 1996a; Gschwendt *et al*, 1992; Ways *et al*, 1992; Tang *et al*, 1993; Nishikawa *et al*, 1995) have indicated, however, that the molecular mass of PKC isoforms deduced from SDS-PAGE may differ somewhat from that calculated from their amino acid composition. Thus, PKC α, γ and δ generally display an apparent molecular weight of ~80 kDa, PKC ε an apparent molecular weight of ~90 kDa and PKC ζ an apparent molecular weight within the range of ~65 to ~85 kDa, with multiple forms of PKC ζ within this weight range often being found within the same cell type (Gschwendt *et al*, 1992; Ways *et al*, 1992; Tang *et al*, 1993; Nishikawa *et al*, 1995). Indeed, the species of PKC ζ expressed in rat adipocytes has been reported previously to display an apparent molecular weight of 85 kDa (Farese *et al*, 1992).

Although expression of PKC β has been reported in rat adipocytes (Farese *et al*, 1992), the three different antibodies used in the present study were unable to detect expression of PKC βI or βII in these cells, even following overloading of gels and over-exposure of autoradiographs. Such anomalies have been reported previously with respect to characterisation of rat H4IIE cells by Farese and co-workers, where the cells were reported to express PKC β (Messina *et al*, 1992) although this result could not be reproduced by Stumpo *et al* (1994). It seems that PKC β is not expressed in the rat adipocytes used during the present study. The rat adipocytes used during this

study were isolated from the parametrial fat pads of female Wistar rats, whereas the rat adipocytes characterised previously were isolated from the epididymal fat pads of male Sprague-Dawley rats (Farese *et al*, 1992). The discrepancy with regard to the PKC complement of rat adipocytes between the two studies may, therefore, simply reflect variation between species, sex or fat depot. Detection of the α, γ, δ, ε and ζ isoforms of PKC in rat adipocytes is in agreement with the findings of Farese *et al* (1992). In this study, expression of PKC μ was also demonstrated in rat adipocytes. The presence of PKC μ, a recently identified member of the PKC family (Johannes *et al*, 1994), in the rat adipocyte has not been reported previously.

Expression of PKC α, δ, ε and ζ has been reported previously for the related Swiss 3T3 cell-line (Olivier and Parker, 1992) and R6 rat embryo fibroblasts (Borner *et al*, 1992). Indeed, expression of these PKC isoforms appears to be fairly ubiquitous. Preadipocytes from the 3T3-L1 cell line, however, which is closely related to 3T3-F442A cells, have been reported to express the α, ε and ζ isoforms of PKC but not PKC δ (McGowan *et al*, 1996; Frevert and Kahn, 1996). 3T3-L1 cells were also found to express PKC θ following differentiation into adipocytes (McGowan *et al*, 1996), whereas this isoform was not detectable in 3T3-F442A adipocytes (or preadipocytes). In addition, 3T3-F442A cells were found to express the same isoforms of PKC in both the undifferentiated and fully differentiated state, therefore, no such phenotype-specific expression of isoforms was observed. Thus, from the studies performed so far, it would appear that 3T3-F442A and 3T3-L1 cells, two closely related cell-lines, display somewhat different complements of PKC isoforms.

Murine adipocytes have been reported to express the novel PKC isoforms δ, ε and η and atypical PKC ζ (Frevert and Kahn, 1996). Expression of other PKC isoforms in murine adipocytes has not been investigated. In the present study, PKC η could not be detected in 3T3-F442A cells, which are a murine cell-line. Thus, although the present study indicated that 3T3-F442A adipocytes express an identical complement of PKC isoforms to that of rat adipocytes, this cell-line may differ, at least with respect to expression of PKC η, from murine adipocytes.

Expression of PKC γ in preadipocyte cell-lines has not been reported previously.

Indeed, expression of this isoform is traditionally thought to be restricted to tissues of the brain and spinal chord (Nishizuka, 1988). Immunoreactive proteins were, however, detected with the anti-PKC γ monoclonal antibody used during this study in both 3T3-F442A cells and rat adipocytes. Affiniti Research Products have indicated that the anti-PKC γ monoclonal antibody cross-reacts with the PKC α protein (personal communication). However, the immunoblotting studies carried out (Figures 3.1 and 3.4), together with the observation that PKC α and γ displayed different susceptibilities to phorbol ester-induced down-regulation (Figure 3.13), indicate that the immunoreactivity of the PKC γ antibody cannot simply be explained by cross-reactivity with PKC α. In addition, expression of PKC γ has been demonstrated previously in the rat adipocyte (Farese *et al*, 1992). Expression of this PKC isoform may, therefore, be more widespread than originally thought.

Having established which PKC isoforms were present in 3T3-F442A cells and rat adipocytes, the panel of antibodies used to characterise the cells were then used to investigate the acute and chronic modulation of these isoforms by phorbol ester. Conforming to their known structural properties (see Section 1.6), the DAG-responsive conventional PKC α and γ and novel PKC δ and ε isoforms were observed to translocate from the cytosol to the membrane and be subsequently down-regulated by treatment of cells with TPA. Conforming to previous reports (Selbie *et al*, 1993; Johannes *et al*, 1994), atypical PKC ζ and PKC μ were not translocated or down-regulated by TPA treatment. The fact that isoforms responded to phorbol ester treatment according to their known structural properties and that the conventional and novel isoforms displayed different susceptibilities to phorbol ester-induced down-regulation, increased confidence regarding the specificty of the antibodies used for characterisation of the cells. The conventional and novel isoforms of PKC have been reported previously (Olivier and Parker, 1992) to display differential sensitivity to treatment with phorbol esters. In Swiss 3T3 cells (Olivier and Parker, 1992), for example, the susceptibility of PKC isoforms to down-regulation following chronic phorbol ester treatment was found to be $\delta > \alpha > \varepsilon > \zeta$, with PKC ε being fairly resistant and PKC ζ showing no response to such treatment. Essentially identical results were observed in 3T3-F442A cells during the present study. In addition, the

susceptibility of PKC γ to TPA-induced down-regulation was found to be between that observed for PKC α and ε. Thus, the order of susceptibility to TPA-induced down regulation in 3T3-F442A cells was found to be $\delta > \alpha > \gamma > \varepsilon > \zeta = \mu$.

During the acute phorbol ester treatment studies, it was established whether specific isoforms were found in the cytosolic or particulate fractions of the cells. This revealed some differences in the sub-cellular distribution of isoforms following differentiation of 3T3-F442A cells. Thus, following differentiation, there was a shift in immunoreactive PKC γ towards the cytosol and PKC δ and ε towards the membrane fraction. In contrast, the relative sub-cellular distribution of PKC α, ζ and μ was unchanged following differentiation. Selective redistribution of PKC isoforms has been observed by others during the differentiation and transformation of various cells as well as in response to different stimuli (reviewed in Kiley *et al*, 1995; Jaken, 1996). Such targeting of PKCs to discrete sub-cellular compartments restricts access to substrates and may contribute to isoform-specific responses. Although the sub-cellular distribution and phorbol ester-responsiveness of PKC isoforms in 3T3-F442A adipocytes was somewhat different from that of 3T3-F442A preadipocytes, it was found to be essentially identical to that found for rat adipocytes. It is, therefore, possible that the altered sub-cellular location of PKC isoforms in differentiated 3T3-F442A adipocytes occurs as a result of the differentiation process.

In summary, both undifferentiated 3T3-F442A preadipocytes and fully differentiated 3T3-F442A adipocytes were found to express PKC α, γ, δ, ε, ζ and μ. These PKC isoforms displayed differential sensitivities to modulation by phorbol esters, which conformed to the results of others. The PKC complement of 3T3-F442A cells was found to be identical to that of rat adipocytes. In addition, the sub-cellular distribution and phorbol ester-responsiveness of 3T3-F442A adipocyte PKC isoforms was found to be similar to that of rat adipocyte PKC isoforms. Together, these results support the suitability of using 3T3-F442A cells as a model system of adipocyte development and function.

It is generally thought that the PKC heterogeneity within cells indicates that

individual isoforms serve diverse functional roles in the regulation of cell growth and differentiation. Knowledge of the PKC isoforms in 3T3-F442A cells and rat adipocytes enabled subsequent evaluation of the roles of these individual isoforms in adipocyte development and function.

Chapter 4

Investigation of the roles of individual protein kinase C isoforms in 3T3-F442A preadipocyte differentiation

4.1 Introduction

The differentiation of preadipocytes is a complex process dependent upon the strict temporal regulation of multiple and interacting signalling events, ultimately leading to the modulation of expression of an array of genes necessary for the attainment of the adipocyte phenotype (Mandrup and Lane, 1997; Smas and Sul, 1995). 3T3-F442A cells have the capacity to undergo differentiation, both *in vitro* (Green and Kehinde, 1976) and *in vivo* (Green and Kehinde, 1979), into cells which exhibit most of the morphological and biochemical characteristics of normal adipocytes and, therefore, provide a good model system of cellular differentiation. As a result of extensive studies using such preadipocyte cell lines, many of the key regulators of the differentiation process have now been identified (see Sections 1.3.2 - 1.3.4). However, the exact intracellular molecular events which regulate the adipocyte differentiation process and which are required to maintain the adipocyte phenotype remain largely unknown.

Although the precise functions of different PKC isoforms remain elusive, it is clear from a wide range of studies that PKCs have an important role in the regulation of cell growth and differentiation (Hug and Sarre, 1993; Kindregan *et al*, 1994; Li *et al*, 1994; Borner *et al*, 1995; Hundle *et al*, 1995; Acs *et al*, 1997). Indeed, a number of lines of evidence implicate this family of kinases in preadipocyte differentiation. For example, PKCs are involved in a number of signalling pathways, including those of MAP kinase (Cobb and Goldsmith, 1995), $p70^{s6}$ kinase (Anderson, 1993) and PI 3-kinase (Nakanishi *et al*, 1993; Toker *et al*, 1994), all of which have been implicated in adipocyte differentiation (Sale *et al*, 1995; Uehara *et al*, 1995; Yeh *et al*, 1995). In addition, phorbol esters, which can activate the diacylglycerol-reponsive PKC isoforms, have been shown to modulate adipogenesis in a number of preadipocyte cell lines (Shimizu *et al*, 1983; Serrero, 1987; Navre and Ringold, 1989; Serrero and Mills, 1991; Hauner *et al*, 1995; Hu *et al*, 1996). Finally, more direct evidence for a role of PKCs in adipocyte development has emerged from studies demonstrating differential changes in the expression of PKC isoforms during the differentiation of 3T3-L1 preadipocytes (Frevert and Kahn, 1996; McGowan *et al*, 1996). These observations suggest distinct functional roles for individual PKC isoforms during preadipocyte differentiation.

In this study, the roles of individual PKC isoforms in preadipocyte differentiation were investigated using the 3T3-F442A cell-model. Initially, in order to gain an indication of the functional roles of PKC isoforms in the differentiation process, the temporal expression patterns of the conventional, novel and atypical PKCs and of the recently identified PKC μ were examined during adipocyte development. Furthermore, by selective depletion of individual PKC subtypes with antisense ODNs prior to the initiation of differentiation, the dependence of the differentiation process on individual PKC isoforms was determined.

4.2 Methods

4.2.1 Assessment of the cellular levels of protein kinase C isoforms during 3T3-F442A preadipocyte differentiation

4.2.1.1 Cell culture

3T3-F442A preadipocytes (Green and Kehinde, 1976) were grown to confluence in 100 mm diameter dishes. Two-day confluent cultures of preadipocytes (subsequently referred to as just-confluent cells) were either harvested or induced to differentiate with foetal calf serum and insulin as described in section 2.2.1.2. Adipocytes were harvested ten days from the induction of differentiation. For some experiments, parallel cultures of 3T3-F442A preadipocytes were maintained in growth medium for twelve days. These cells, which are subsequently referred to as twelve-day confluent cells, displayed no morphological change. As an additional control, cultures of non-differentiating 3T3-C2 cells (Green and Kehinde, 1976) were treated with differentiation medium as described for 3T3-F442A cells in section 2.2.1.2.

4.2.1.2. Immunoblotting

Cell lysates were prepared from 3T3-F442A and 3T3-C2 cells at confluence (day -2), two days post-confluence (just prior to treatment with differentiation-inducing agents; day 0) and at days 2, 5 and 10 of treatment with differentiation-inducing medium. Cell lysates were also prepared from 3T3-F442A preadipocytes that had been maintained at confluence in growth medium for twelve days. The protein content of cell lysates was determined as described in section 2.2.4.1 and confirmed by Coomassie staining of mini-gels as described in section 2.2.4.3. Equal quantities of lysate protein were subjected to SDS-PAGE alongside a homogenate prepared from rat brain (Kuo *et al*, 1980) that served as a positive control for the PKC antibodies. Changes in PKC isoform expression were examined by immunoblotting with isoform-specific monoclonal antibodies to PKC (Affiniti) as described in section 2.2.4.4. PKC isoform expression was quantified by densitometric analysis of immunoblots.

4.2.1.3 Calculating cellular levels of protein kinase C isoforms from immunoblot data

When carrying out direct comparisons of immunoreactive PKC levels in cells at different stages of the differentiation process, cellular lysates were loaded onto gels on the basis of equal protein. To analyse cellular levels of PKC isoforms, values obtained from the densitometric analysis of immunoblots had to be adjusted to reflect the protein content of the cells. This was achieved by setting up parallel cultures for determination of the number of cells per plate. Cell monolayers were washed with 10 ml of PBS and scraped into 1 ml of PBS per 100 mm diameter dish. Aliquots of cell suspension were then counted using a Nebauer Haemocytometer to obtain the number of cells per plate and, hence, the number of cells per lysate. These values, together with the protein content of the cellular lysates, permitted the calculation of the amount of protein per cell that was then used to convert immunoreactive PKC levels, assessed by densitometry of gels, into PKC level per cell.

Parallel cultures were also set up for assay of the adipocyte marker enzyme GPDH, as described in section 2.2.2.8, as a means of assessing adipocyte development following treatment with differentiation-inducing agents.

4.2.2 Effects of depletion of individual protein kinase C isoforms with antisense oligodeoxynucleotides on 3T3-F442A preadipocyte differentiation

4.2.2.1 Treatment of cells with oligodeoxynucleotides

3T3-F442A preadipocytes were grown in 60 mm diameter wells. Typically at around 70-80 % confluence, cells were treated with sense or antisense ODNs to specific PKC isoforms as described in section 2.2.2.4.1. For the first 6 h of treatment, cells were incubated with the appropriate ODN in combination with lipofectin reagent. The lipofectin was subsequently removed from the culture medium and incubations continued in the presence of ODN alone. After a further 42 h, cells were either used immediately for the preparation of lysates for assessment of immunoreactive PKC levels by immunoblotting or, alternatively, cells were induced to differentiate with foetal calf serum and insulin.

4.2.2.2 Assessment of the effects of depletion of protein kinase C isoforms with antisense oligodeoxynucleotides on differentiation of 3T3-F442A cells

3T3-F442A preadipocytes were treated for 48 h with antisense or sense ODNs specific for individual isoforms of PKC, as described in section 4.2.2.1 above, then induced to differentiate with foetal calf serum and insulin. Differentiation medium was replenished every 72 h and the appropriate concentration of ODN was present throughout the entire differentiation treatment period. Following the induction of differentiation, changes in cell morphology were examined on a daily basis, from day 1 to day 7 of treatment with differentiation medium, by photographing the cells under Phase Contrast Optics (x 24 magnification) using an Olympus IMT3 inverted microscope. After 4 and 7 days of treatment with differentiation medium, homogenates were prepared for assay of the adipocyte marker enzyme GPDH as described in section 2.2.2.8. The DNA content of cell homogentaes was determined fluorimetrically, as described in section 2.2.2.10. In addition, at day 7 of treatment, cells were fixed with formalin and stained with Oil Red O, as described in section 2.2.2.5, for assessment of lipid accumulation.

4.2.3 Statistical analysis

Results are presented as means ± S.E.M. and, unless stated otherwise, statistical analysis was by Student's t-test for paired samples.

4.3 Results

4.3.1 Protein kinase C isoform expression is altered following 3T3-F442A preadipocyte differentiation

The results presented in Chapter 3 demonstrate that 3T3-F442A preadipocytes and adipocytes express the same complement of PKC isoforms. To investigate whether PKC isoform expression alters as a result of the differentiation process, cellular levels of PKC isoforms in 3T3-F442A preadipocytes and adipocytes were compared by immunoblot analysis (Figure 4.1). 3T3-F442A preadipocytes maintained in growth medium at confluence for twelve days were used as a control to isolate changes in the expression of PKC isoforms that were specific to differentiation (Figure 4.1).

The protein content of 3T3-F442A preadipocytes is known to increase as a result of differentiation (Lai *et al*, 1981; Kilgour and Anderson, 1993). Therefore, the cellular protein content of lysates was examined during 3T3-F442A preadipocyte differentiation (Table 4.1). In accordance with others (Lai *et al*, 1981), the amount of protein per cell in fully differentiated 3T3-F442A adipocytes was found to be approximately 2.5 times that of preadipocytes (Table 4.1). The twelve day confluent control cells displayed no signs of adipocyte development, either in terms of cell morphology (data not shown) or development of activity of the adipocyte enzyme marker, GPDH (Table 4.1). The cellular protein content of cells maintained at confluence for twelve days, however, was found to be approximately 1.4 fold of that of just-confluent preadipocytes (Table 4.1). These values were used to convert the data obtained from the densitometric analysis of immunoblots, loaded on an equal protein basis (Figure 4.1), to a per cell basis, as described in section 4.2.1.3. (Table 4.2).

Cellular levels of PKC α, γ and δ were decreased significantly ($p < 0.001$, 0.001 and 0.02 respectively) in 3T3-F442A adipocytes as compared to preadipocytes, by 84 ± 3 %, 89 ± 2% and 59 ± 13 % respectively (Figure 4.2, hatched bars). In contrast, cellular levels of PKC ε were increased significantly ($p < 0.001$) in adipocytes by 298 ± 54 % (Figure 4.2, hatched bars). That cellular levels of PKC α, γ, δ and ε were unchanged in twelve-day confluent 3T3-F442A cells (Figure 4.2, plain bars), which displayed no morphological change and did not develop GPDH activity (Table 4.1), suggested that

changes in expression of PKC α, γ, δ and ε were specific to the process of differentiation.

PKC ζ levels were unchanged by differentiation (Figure 4.2, hatched bars) but an elevation ($p < 0.02$) of 160 ± 3 % in expression of this isoform was observed in the twelve-day confluent control cells (Figure 4.2, plain bars). This suggested that PKC ζ levels were unchanged by differentiation of 3T3-F442A cells but increased as a result of the prolonged confluence of growth-arrested preadipocytes.

Cellular levels of PKC μ were increased ($p < 0.01$) by 220 ± 30 % in 3T3-F442A adipocytes as compared to preadipocytes (Figure 4.2, hatched bars), however, a similar increase ($p < 0.02$) occurred in the control cells which were maintained at confluence for twelve days (Figure 4.2, plain bars). That the increase in expression of PKC μ in differentiated cells was matched in cells maintained at confluence for the same period suggested that alterations in expression of PKC μ were not specific to the differentiation process and may be due to prolonged confluence of growth-arrested cells.

4.3.2 Changes in expression of protein kinase C isoforms during the differentiation of 3T3-F442A cells

Comparison of the cellular levels of PKC isoforms in 3T3-F442A preadipocytes and adipocytes suggested that decreased expression of PKC α, γ and δ and increased expression of PKC ε were specific to the differentiation process. To examine the changes in PKC isoform expression more fully, cellular levels of PKC isoforms were examined throughout the time-course of differentiation of 3T3-F442A preadipocytes. The patterns of isoform expression that were observed during differentiation of 3T3-F442A cells were compared to patterns observed for 3T3-C2 cells that had been treated identically with differentiation medium. 3T3-C2 cells are related to 3T3-F442A cells but do not differentiate in response to adipogenic stimuli (Green and Kehinde, 1976) and were therefore employed to separate differentiation-specific effects from other effects of treatment.

Immunoblot analysis of 3T3-C2 cells revealed expression of the same complement of

PKC isoforms as found in 3T3-F442A cells, namely PKC α, γ, δ, ε, ζ and μ (Figure 4.3). Similarly, expression of PKC β I/II, η, θ, λ or ι was not detectable in 3T3-C2 cells (Figure 4.3). Cellular lysates were prepared from 3T3-F442A and 3T3-C2 cells at confluence (day -2), just prior to treatment with differentiation-inducing medium (day 0) and at days 2, 5, and 10 of treatment with differentiation-inducing agents and subjected to immunoblotting with isoform-specific monoclonal antibodies to PKC α, γ, δ, ε, ζ and μ (Figure 4.4). In addition, immunoblotting was performed using polyclonal antiserum raised to PKC β I/II. As reported in Chapter 3, PKC β is not detectable in either 3T3-F442A preadipocytes or adipocytes. Similarly, it has been reported that PKC β is not expressed in either 3T3-L1 preadipocytes or adipocytes (McGowan *et al*, 1996). However, transient expression of this isoform was reported to occur between day 2 and day 5 of treatment of 3T3-L1 cells with differentiation medium (McGowan *et al*, 1996). In the present study, no such temporal expression of PKC β was observed during the differentiation of 3T3-F442A preadipocytes (Figure 4.4).

The protein content of 3T3-F442A and 3T3-C2 cell lysates was examined during treatment with differentiation medium (Table 4.3) to allow levels of PKC isoforms to be expressed on a per cell basis (Table 4.4). By day 2 of treatment, the number of 3T3-F442A cells per plate had increased approximately 2 fold (Table 4.3), reflecting the initial round of cell division found to be necessary for differentiation of the cells (Mandrup and Lane, 1997; Smas and Sul, 1995). The protein content of cell lysates was found to be approximately 1.4 fold that of untreated cells at this time (Table 4.3). At day 5 of treatment, the cellular protein content of the differentiating cells was approximately 2 fold that of preadipocytes and reached 2.5 fold that of preadipocytes when cells were fully differentiated at day 10 of treatment (Table 4.3). GPDH activity was induced between days 2 and 5 of treatment with differentiation-inducing agents in 3T3-F442A cells and increased as differentiation progressed (Table 4.3). No change in either total cell number, cellular protein content, GPDH activity (Table 4.3) nor cell morphology (data not shown) was observed in 3T3-C2 cells treated under identical differentiation-inducing conditions to the 3T3-F442A cells. Values obtained from the densitometric analysis of immunoblots (Figure 4.4) were adjusted accordingly to reflect the levels of PKC isoforms in preparations containing an equal number of cells

(Table 4.4). The changes in cellular levels of PKC isoforms during differentiation of 3T3-F442A cells were then compared to those of 3T3-C2 cells treated identically (Figures 4.5A-F).

Prior to treatment with differentiation-inducing agents, confluent 3T3-F442A preadipocytes were allowed to enter a state of growth arrest (day -2 to day 0). During this period, cellular levels of PKC γ, δ and ε decreased ($p< 0.05$, $p< 0.001$ and $p< 0.05$ respectively) whereas levels of PKC α, ζ and μ were unchanged (Figure 4.5A-F). In 3T3-C2 cells, cellular levels of PKC α, γ, δ, ζ and μ were all unchanged during this period (Figure 4.5A-F), and, in contrast to observations in 3T3-F442A cells, levels of PKC ε were increased ($p< 0.001$; Figure 4.5D).

During the differentiation of 3T3-F442A cells (day 0 - day10), three distinct patterns of change in PKC isoform expression were observed (Figures 4.5A-F.). Prior to decreasing to the levels observed in differentiated adipocytes (Figure 4.2), cellular levels of PKC α, γ and δ increased ($p < 0.001$) between day 0 and day 2 of treatment with differentiation medium (Figures 4.5A-C). Between days 2 and 10 of differentiation, levels of these isoforms fell sharply to values below those observed in day 0 preadipocytes ($p < 0.001$, $p < 0.001$ and $p < 0.01$ respectively; Figures 4.5A-C). Levels of PKC α and γ fell to values below those observed in day 0 preadipocytes ($p< 0.02$ and $p< 0.001$ respectively) between days 2 and 5 of differentiation (Figures 4.5 A&B). PKC δ levels decreased less rapidly after the peak at day 2 of differentiation, but were reduced to values below those of undifferentiated preadipocytes ($p< 0.01$) between days 5 and 10 of treatment (Figure 4.5C). The expression of PKC α, γ and δ followed a similar pattern in identically treated 3T3-C2 cells (Figure 4.5A-C). However, the proportional increases in PKC α and γ between days 0 and 2 of treatment were much larger (Figures 4.5A&B) and cellular levels of PKC α, γ and δ were not reduced between days 2 and 5 of treatment, as observed in 3T3-F442A cells. In addition, cellular levels of PKC α, γ and δ were not reduced below the levels observed in untreated cells (day 0 preadipocytes) at any time during the treatment of 3T3-C2 cells (Figures 4.5A-C). This suggested that, following the transient peak in expression between day 0 and day 2, decreases in expression of PKC α, γ and δ may be important for progression of 3T3-F442A preadipocyte differentiation.

In 3T3-F442A cells, levels of PKC ε increased steadily following the induction of differentiation, peaking at around day 5 of treatment (Figure 4.5D). Although expression of PKC ε followed a similar pattern in 3T3-C2 cells treated identically, the proportional increase (1.92 ± 0.14 fold versus 2.86 ± 0.09 fold at day 5 of differentiation) in expression of PKC ε was much smaller ($p< 0.01$; Figure 4.5D). This fact, coupled to the observation that no significant increase in expression of PKC ε was observed in 3T3-F442A cells held at confluence for twelve days, (Figure 4.2) implied that the proportional increase in expression of PKC ε in 3T3-F442A cells may be important for preadipocyte differentiation to occur.

Cellular levels of PKC μ increased throughout the course of differentiation of 3T3-F442A cells, with the maximal increase observed at day 10 (Figure 4.5F). No significant alteration in PKC μ expression occurred in 3T3-C2 cells over the same period (Figure 4.5F), which suggested that increased expression of PKC μ was an integral part of the differentiation process. However, the increase in expression of PKC μ in differentiating cells was matched in 3T3-F442A cells maintained at confluence for the same period (Figure 4.2) suggesting that PKC μ expression increases in 3T3-F442A cells due to prolonged confluency rather than differentiation *per se*.

Finally, the expression of PKC ζ was unchanged during treatment of either 3T3-F442A or 3T3-C2 cells with differentiation-inducing agents (Figure 4.5E). This implied that PKC ζ was not involved in the regulation of 3T3-F442A preadipocyte differentiation.

4.3.3 Effects of depletion of individual protein kinase C isoforms with antisense oligodeoxynucleotides on 3T3-F442A preadipocyte differentiation

The differential changes in the expression of PKC isoforms during 3T3-F442A preadipocyte differentiation implied distinct roles for PKC isoforms in the regulation of the differentiation process. To investigate further the role of PKC isoforms in adipocyte development, antisense ODNs were used to deplete selectively individual isoforms from 3T3-F442A preadipocytes prior to the initiation of differentiation.

3T3-F442A preadipocytes were treated with the appropriate antisense or sense ODNs for 48 h prior to the induction of differentiation with medium containing foetal calf serum and insulin, as described in section 4.2.2.1. In order to achieve the successful depletion of PKC isoforms, it was necessary for lipofectin reagent, which can form liposomes that complex with nucleic acids and facilitate cellular uptake (Felgner *et al*, 1987), to be present for the first 6 h of the 48 h treatment with ODNs (data not shown). However, treatment with lipofectin alone had no effect on the expression of PKC isoforms (data not shown). Immunoblot analysis of cells that had been preincubated with the specific antisense ODNs revealed that over 90 % of the appropriate PKC isoform was depleted at the initiation of differentiation (Figure 4.6). In contrast, there was no detectable effect following the identical treatment of cells with the appropriate sense ODN (Figure 4.6). Importantly, each antisense ODN was specific for the appropriate PKC subtype and had no effect on cellular levels of other PKC subtypes (Figure 4.6).

Cells were treated with the individual antisense ODNs for 48 h prior to the induction of differentiation. This ensured that the depletion of the relevant PKC subtype was complete at the initiation of differentiation. Further immunoblot analysis, of lysates at days 0, 3, 4 and 7 of treatment with differentiation medium, revealed that maximal depletion of PKC isoforms persisted for at least the first three days of differentiation (Figure 4.7). Between days 3 and 4 of differentiation, however, all PKC proteins recovered from the effects of antisense suppression (Figure 4.7). The rate of recovery, as judged by comparison of levels of expression in cells at day 4 of differentiation to those of cells at day 0, did, however, vary between individual isoforms (PKC isoform expression as a percentage of that observed in untreated cells = 43 ± 8 % for PKC α, 62 ± 3 % for PKC γ, 51 ± 4 % for PKC δ, 25 ± 9 % for PKC ε, 110 ±11 % for PKC ζ and 116 ± 10 % for PKC μ). The fact that ODN suppression of PKC isoforms was transient indicated that the various ODNs were not toxic to the cells and that the suppression of PKC isoform expression was reversible. This allowed the impact of depletion of PKC isoforms during the early stages of differentiation on the attainment of the adipocyte phenotype to be addressed. The effects of preincubation of cells with ODNs on subsequent differentiation were assessed qualitatively, by both examination

of changes in cell morphology and lipid accumulation by staining with Oil Red O, and also quantitatively, by assaying the activity of the adipocyte marker enzyme GPDH.

Antisense treatment of preadipocytes had three main effects on preadipocyte differentiation which were not observed when cells were treated with the appropriate sense ODNs or lipofectin alone (Figures 4.8 - 4.10). From morphological examination of cells, it appeared consistently that depletion of PKC α, δ and μ, enhanced the rate of adipocyte development during the early stages of treatment with differentiation medium (days 2 - 4) compared to cells treated with lipofectin alone (Figures 4.8A, C and F). However, this effect was no longer detectable at the later stages of the differentiation process. This suggested that depletion of PKC α, δ and μ enhanced the rate of adipocyte development. Consistent with these observations, at day 4 of differentiation the specific activity of the adipocyte enzyme marker GPDH was elevated significantly ($p < 0.001$) in cells depleted of PKC α, δ or μ, by 127 ± 2.5 %, 162.9 ± 8.0 % and 153.2 ± 4.0 % respectively, compared to control cells treated with lipofectin alone (Figure 4.9A). However, by day 7 there was no longer any significant difference between GPDH activity in cells depleted of PKC α as compared to control cells and only small increases in GPDH activity, of 108.9 ± 0.5 % and 108.5 ± 1.2 % respectively, were detected in cells depleted of PKC δ and μ (Figure 4.9B).

In contrast, depletion of PKC ε or γ clearly inhibited the differentiation process. Cell rounding was delayed until day 4 of differentiation following suppression of PKC ε and until day 7 following suppression of PKC γ (Figures 4.8B and D). GPDH activity was significantly decreased ($p< 0.001$), by 12.8 ± 1.3 % and 15.3 ± 1.42 % respectively, at day 4 of differentiation in cells depleted of these isoforms (Figure 4.9A). At day 7 of differentiation, the activity of this adipocyte marker enzyme was reduced to 34.8 ± 0.3 % of control in cells depleted of PKC ε (Figure 4.9B) and lipid filling of the cells was retarded compared to control cells treated with lipofectin alone (Figure 4.10). Lipid filling of cells at day 7 of differentiation was completely absent following depletion of PKC γ (Figure 4.10) and GPDH activity was merely 17.3 ± 8.0 % of control (Figure 4.9B); a level of activity comparable to that observed in undifferentiated preadipocytes (results not shown).

Depletion of PKC ζ had no detectable effect upon preadipocyte differentiation assessed either qualitatively or quantitatively by measurement of GPDH activity (Figures 4.8 - 4.10). Thus, it appears that PKC ζ is not essential during the early stages of differentiation in 3T3-F442A cells, PKC α, δ and μ each appear to exert an inhibitory influence while PKC ε and γ are necessary for the differentiation process.

4.3.4 Protein kinase C ε and protein kinase C γ are required for different stages of 3T3-F442A preadipocyte differentiation

Immunoblotting studies revealed that PKC proteins were being expressed by day 4 of differentiation (Figure 4.7). Following depletion of PKC ε, morphological signs of adipocyte development became apparent only when antisense suppression of the PKC ε protein was lost at day 4 of differentiation. Following depletion of PKC γ, however, morphological signs of differentiation were delayed until day 7 of differentiation. That a delay occurred between expression of PKC γ and morphological signs of differentiation suggested a possible role for this isoform in the clonal expansion phase of the differentiation process.

To investigate this possibility, the DNA content of cultures was examined at days 0, 2, 4 and 7 of treatment with differentiation-inducing agents. The clonal expansion that is necessary for terminal differentiation to occur in 3T3-F442A cells (Mandrup and Lane, 1997; Smas and Sul, 1995) is reflected by an approximate doubling of the number of cells per plate that can be observed some two days after exposure to differentiation medium (Table 4.3). This increase in cell number, as observed by a doubling of the DNA content of cell homogenates, was no longer apparent if cells were depleted of PKC γ prior to exposure to differentiation medium (Table 4.5). Indeed, in cultures depleted of PKC γ the increase in DNA content did not occur until between day 4 and day 7 of differentiation (Table 4.5). In contrast, treatment of cells with the corresponding PKC γ sense ODN, or antisense depletion of PKC ε, had no effect upon clonal expansion (Table 4.5). Similarly no effect upon clonal expansion was detected, as measured by an increase in the DNA content of cultures, following depletion of the α, δ, ζ or μ isoforms of PKC (Table 4.5). These results suggest that differential mechanisms account for the differentiative effects of PKC γ and ε. Thus, PKC γ appears to be required for the clonal expansion of differentiating preadipocytes

whereas PKC ε is not and is likely, therefore, to influence another aspect of the differentiation process.

Figure 4.1 Protein kinase C isoform expression following differentiation of 3T3-F442A cells

Equal quantities (75 μg of protein) of lysate protein prepared from just-confluent 3T3-F442A preadipocytes (lanes 1-4), twelve-day-confluent 3T3-F442A preadipocytes (lanes 5-8) and fully differentiated 3T3-F442A adipocytes (lanes 9-12) and homogenate protein prepared from rat brain (lane 13) were subjected to immunoblotting with isoform-specific monoclonal antibodies to PKC. Results are representative of eight separate experiments carried out on different cell preparations.

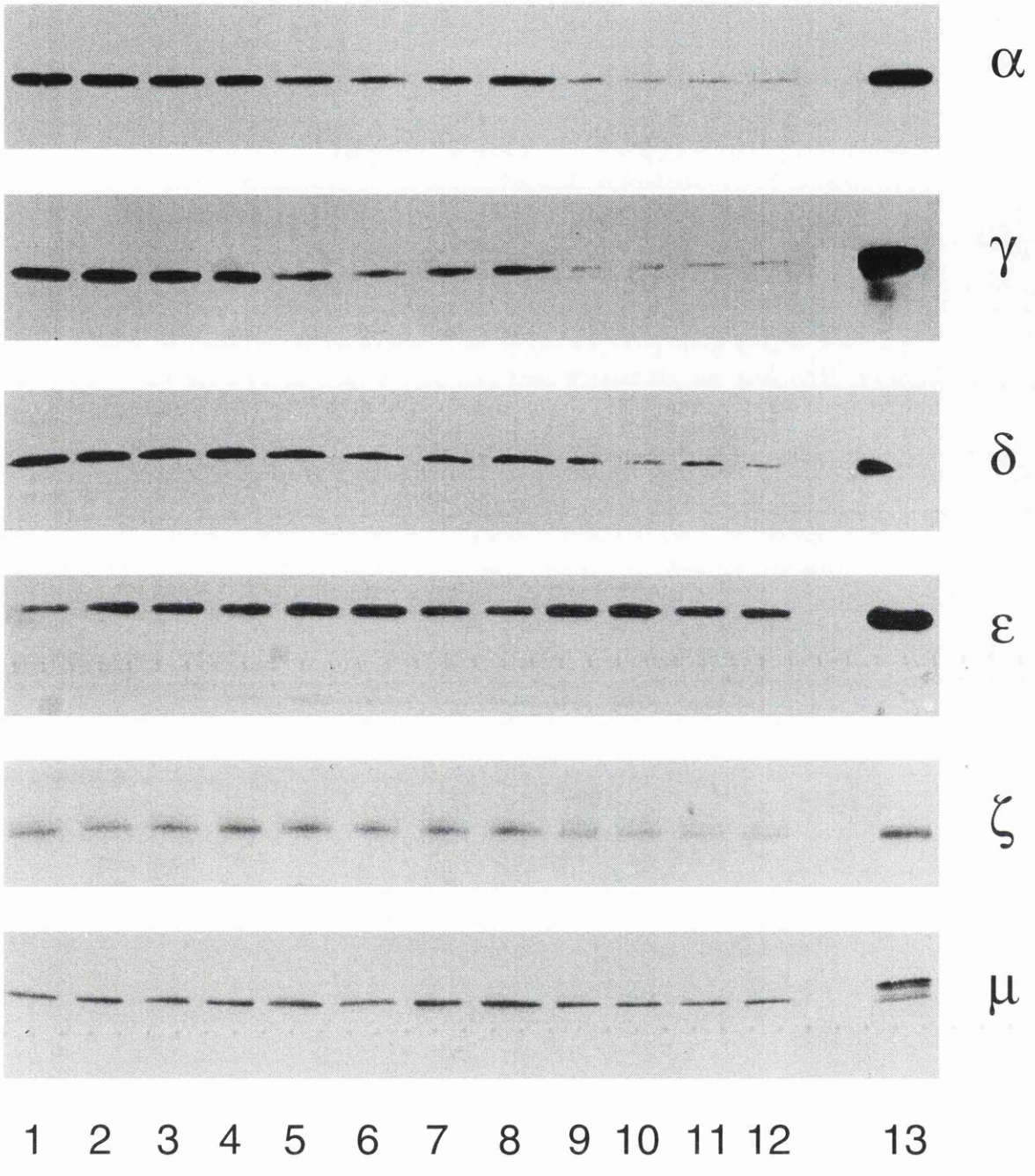

α
γ
δ
ε
ζ
μ
1 2 3 4 5 6 7 8 9 10 11 12 13

Table 4.1 Cellular protein content and glycerol-3-phosphate dehydrogenase activity following differentiation of 3T3-F442A cells

Cell lysates were prepared from just-confluent 3T3-F442A preadipocytes (JC), twelve-day-confluent 3T3-F442A preadipocytes (12 DC) and fully differentiated 3T3-F442A adipocytes (AD) and assayed for protein content as described in section 2.2.4.1. Parallel cultures were scraped into PBS for determination of the number of cells per plate and, hence, the number of cells per lysate. These values were then used to calculate the cellular protein content of the lysates prepared for immunoblotting. Parallel cultures were also set up for the preparation of cellular homogenates for assay of GPDH activity as described in section 2.2.2.8. Data are means ± S.E.M. from eight separate experiments carried out on different cell preparations. * and ** indicate the value differs significantly from that for just-confluent preadipocytes, $p < 0.01$ and 0.001 respectively. Statistical analysis was by Student's t-test for paired samples.

Cell Type	Cells per plate (x 10^6 cells)	Number of plates	Lysate volume (ml)	Cells per lysate (x 10^6)	Cellular protein per lysate (mg)	Protein per 10^6 cells (mg)	GPDH activity (μmol/min/mg protein)
JC	1.0 ± 0.04	3	1.00	3.0 ± 0.12	1.5 ± 0.07	0.5 ± 0.06	< 0.1
12 DC	1.2 ± 0.00	3	1.00	3.6 ± 0.00	2.5 ± 0.30	0.7 ± 0.09*	< 0.1
AD	2.0 ± 0.03	2	1.25	5.1 ± 0.08	6.4 ± 0.83	1.26 ± 0.20**	1.1 ± 0.09**

Table 4.2 Cellular levels of protein kinase C isoforms following 3T3-F442A preadipocyte differentiation

Equal quantities (75 μg) of lysate protein prepared from just-confluent 3T3-F442A preadipocytes (JC), twelve-day confluent 3T3-F442A preadipocytes (12 DC) and fully differentiated 3T3-F442A adipocytes (AD) were subjected to immunoblotting with isoform-specific monoclonal antibodies to PKC (Figure 4.1). PKC isoform expression was quantified by densitometric analysis and the cellular protein content of lysates (Table 4.1) used to convert the values obtained from densitometric analysis of immunoblots into PKC level per cell. Data are means ± S.E.M. from eight separate experiments carried out on different cell preparations. *, ** and *** indicate the value differs significantly from that for just-confluent preadipocytes, $p < 0.02$, 0.01 and 0.001 respectively. Statistical analysis was by Student's t-test for paired samples.

	PKC Expression (arbitrary units)					
	JC cells		12 DC cells		Adipocytes	
Isoform	per unit protein	per cell	per unit protein	per cell	per unit protein	per cell
α	1285 ± 102	1285 ± 102	679 ± 126	951 ± 175	83 ± 19	208 ± 48***
γ	1667 ± 153	1667 ± 153	750 ± 14	1050 ± 20	71 ± 5	177 ± 13***
δ	1305 ± 48	1305 ± 48	932 ± 106	1292 ± 148	218 ± 71	545 ± 173**
ε	1017 ± 23	1017 ± 23	1013 ± 215	1403 ± 302	1213 ± 225	3033 ± 563***
ζ	257 ± 25	257 ± 25	296 ± 25	411 ± 36*	109 ± 25	287 ± 62
μ	288 ± 17	288 ± 17	419 ± 77	587 ± 107*	249 ± 21	633 ± 52**

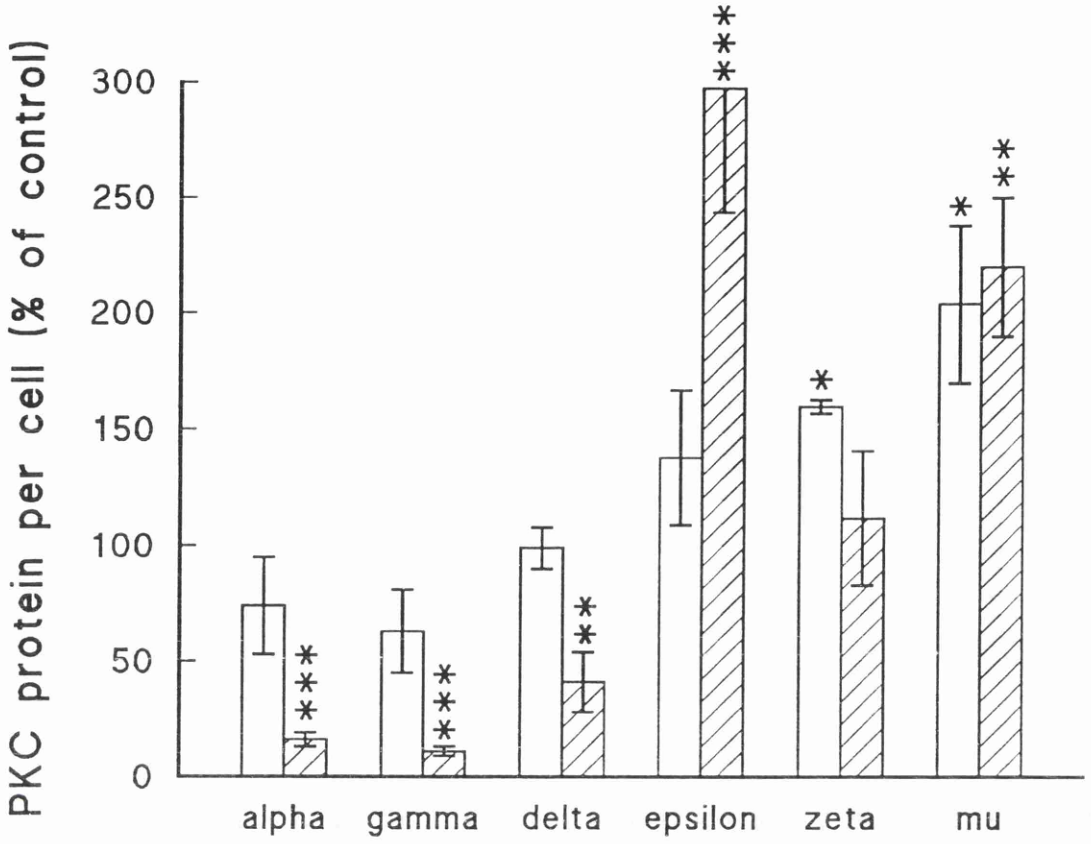


Figure 4.2 The effect of differentiation on cellular levels of protein kinase C isoforms in 3T3-F442A cells

Cellular lysates were prepared from just-confluent 3T3-F442A preadipocytes, 12-day-confluent 3T3-F442A preadipocytes and fully differentiated 3T3-F442A adipocytes and subjected to immunoblotting with isoform-specific monoclonal antibodies to PKC (Figure 4.1). PKC isoform expression was quantified by densitometric analysis of immunoblots and expressed as PKC level per cell (Table 4.2). The changes in cellular levels of isoforms following differentiation (hatched bars) or maintenance of cells at confluence for twelve days (plain bars) are shown as a percentage of expression in just-confluent preadipocytes (control = 100 %). Data are means ± S.E.M. from eight separate experiments carried out on different cell preparations. *, ** and *** indicates the value differs significantly from that for just-confluent preadipocytes, $p < 0.02$, 0.01 and 0.001 respectively. Statistical analysis was by Student's t-test for paired samples.

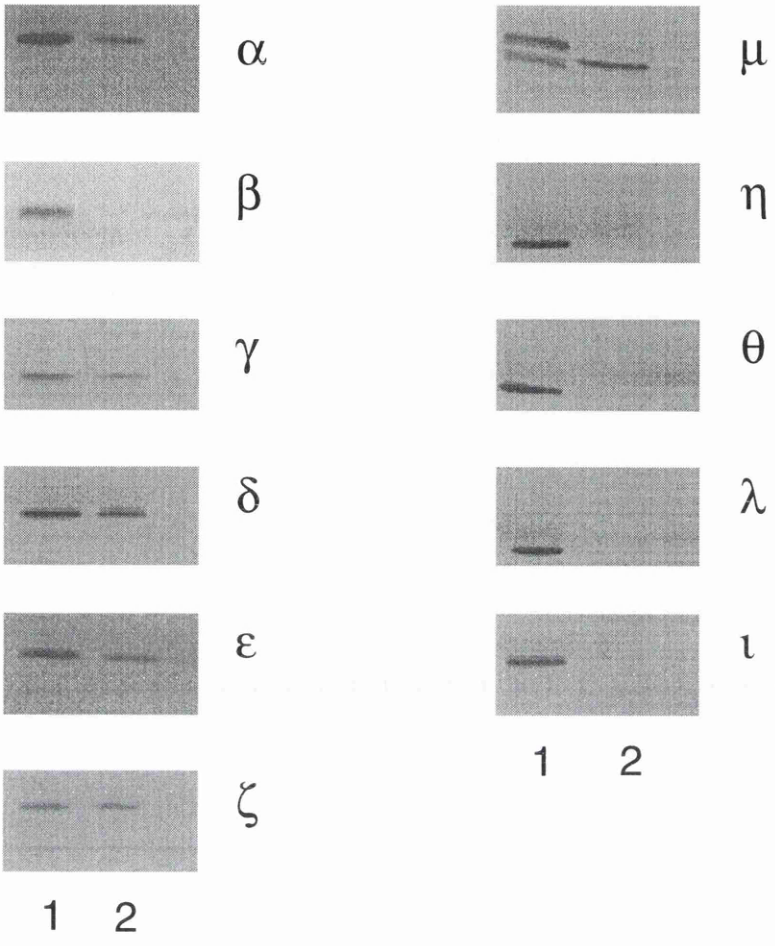


Figure 4.3 Protein kinase C isoforms in 3T3-C2 cells

Equal quantities (75 μg) of lysate protein prepared from confluent 3T3-C2 cells (lane 2) and homogenate protein prepared from rat brain (lane 1) were subjected to immunoblotting with isoform-specific antibodies to PKC as indicated. Results are representative of four separate experiments carried out on different cell preparations.

Figure 4.4 Protein kinase C isoform expression during differentiation of 3T3-F442A preadipocytes and control (non-differentiating) 3T3-C2 cells

Cellular lysates were prepared from 3T3-F442A (lanes 2-6) and 3T3-C2 (lanes 7-11) cells at conflence (day -2; lanes 2 and 7) just prior to treatment with differentiation medium (day 0; lanes 3 and 8) and at days 2 (lanes 4 and 9), 5 (lanes 5 and 10) and 10 (days 6 and 11) of treatment with differentiation-inducing agents. Equal quantities (75 μg) of lysate protein and protein prepared from a rat brain homogenate (lane1) were subjected to immunoblotting with isoform-specific antibodies to PKC as indicated. The molecular weights (kDa) of the species detected with the polyclonal PKC β antibody are indicated. Immunoblots are representative of results obtained from six separate experiments carried out on different cell preparations.

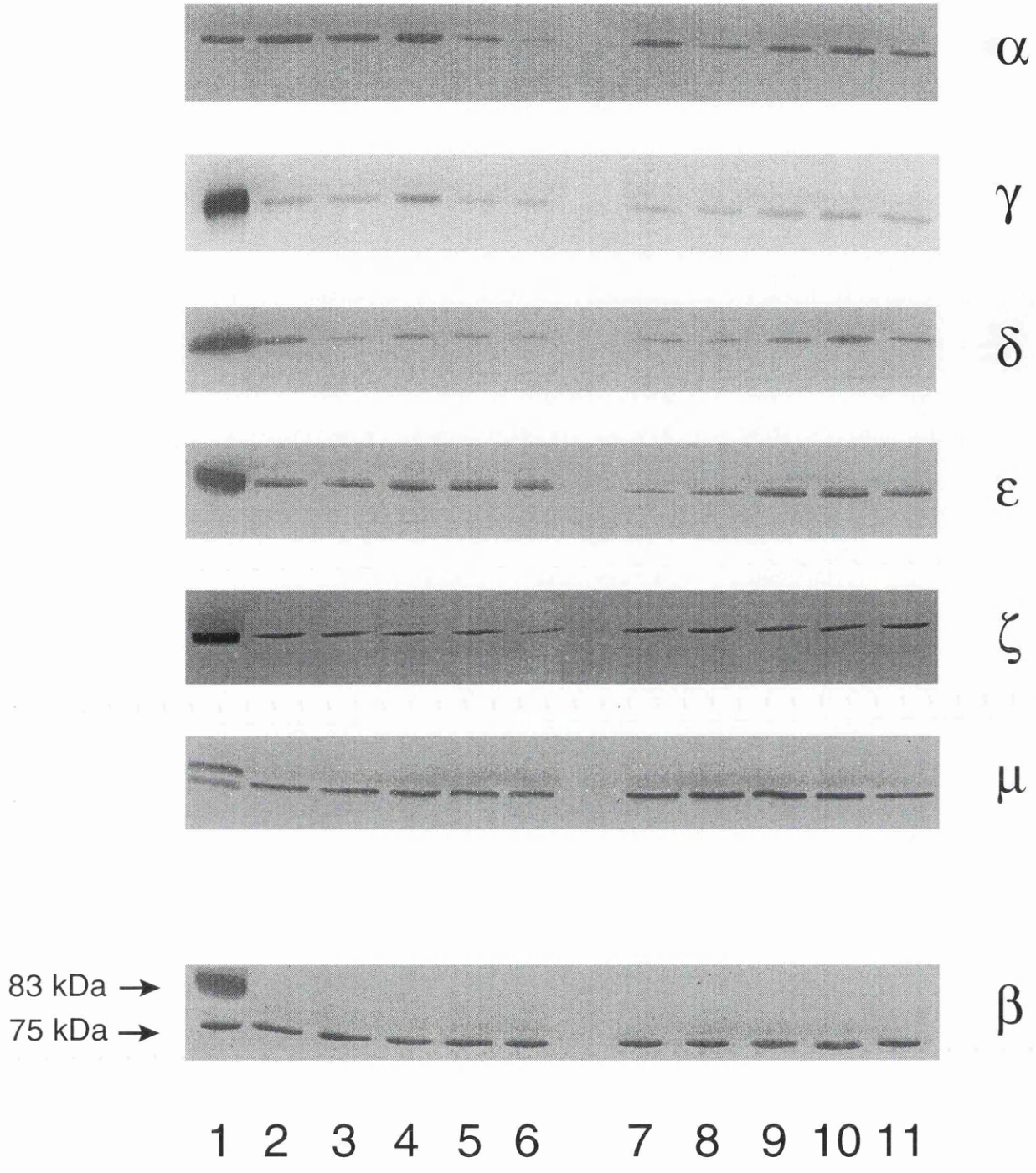

α
γ
δ
ε
ζ
μ
β
83 kDa
75 kDa
1 2 3 4 5 6 7 8 9 10 11

Table 4.3 Cellular protein content and glycerol-3-phosphate activity during differentiation of 3T3-F442A preadipocytes and control (non-differentiating) 3T3-C2 cells

Cellular lysates were prepared from 3T3-F442A and 3T3-C2 cells at confluence (day -2), just prior to treatment with differentiation medium (day 0) and at days 2, 5 and 10 of treatment with differentiation-inducing agents and assayed for protein content as described in section 2.2.4.1. Parallel cultures were scraped into PBS for determination of the number of cells per plate and, hence, the number of cells per lysate. These values were then used to calculate the cellular protein content of the lysates prepared for immunoblotting. Parallel cultures were also set up for the preparation of cellular homogenates for assay of GPDH activity as described in section 2.2.2.8. Data are means ± S.E.M. from six separate experiments carried out on different cell preparations. *, ** and *** indicate the value differs significantly from that for day 0 cells, $p < 0.02$, 0.01 and 0.001 respectively. Statistical analysis was by Student's t-test for paired samples.

Cell Type	Day of differentiation	Cells per plate (x 10^6 cells)	Number of plates	Lysate volume (ml)	Cells per lysate (x 10^6 cells)	Cellular protein per lysate (mg)	Protein per 10^6 cells	GPDH activity (μmol/min/mg protein)
3T3-F442A	-2	0.79 ± 0.007	3	0.75	1.79 ± 0.013	0.90 ± 0.015	0.50 ± 0.012	< 0.1
	0	1.00 ± 0.035	3	0.75	2.26 ± 0.078	1.13 ± 0.020	0.50 ± 0.009	< 0.1
	2	1.90 ± 0.023	3	0.75	4.28 ± 0.052	3.01 ± 0.045	0.71 ± 0.019*	< 0.1
	5	1.98 ± 0.015	2	0.95	3.77 ± 0.033	3.85 ± 0.023	1.02 ± 0.006**	0.40 ± 0.10**
	10	2.02 ± 0.017	2	0.95	3.83 ± 0.033	4.76 ± 0.026	1.25 ± 0.006***	1.10 ± 0.09***
3T3-C2	-2	0.81 ± 0.006	3	0.75	1.80 ± 0.015	0.89 ± 0.015	0.50 ± 0.012	< 0.1
	0	1.00 ± 0.020	3	0.75	2.26 ± 0.046	1.12 ± 0.017	0.50 ± 0.003	< 0.1
	2	1.20 ± 0.009	3	0.75	2.71 ± 0.021	1.35 ± 0.008	0.50 ± 0.012	< 0.1
	5	1.20 ± 0.013	3	0.75	2.70 ± 0.037	1.36 ± 0.009	0.50 ± 0.009	< 0.1
	10	1.20 ± 0.012	3	0.75	2.72 ± 0.032	1.35 ± 0.015	0.50 ± 0.001	< 0.1

Table 4.4 Cellular levels of protein kinase C isoforms during differentiation of 3T3-F442A preadipocytes and control (non-differentiating) 3T3-C2 cells

Equal quantities (75 μg) of lysate protein prepared from 3T3-F442A and 3T3-C2 cells at confluence (day -2) just prior to treatment with differentiation medium (day 0) and at days 2, 5 and 10 of treatment with differentiation-inducing agents were subjected to immunoblotting with isoform-specific monoclonal antibodies to PKC (Figure 4.4). PKC isoform expression was quantified by densitometric analysis and the cellular protein content of lysates (Table 4.3) used to convert the values obtained from densitometric analysis of immunoblots into PKC level per cell. Data are means ± S.E.M. from six separate experiments carried out on different cell preparations. Values for individual PKC isoforms that differ significantly ($p < 0.05$) carry different superscripts. Statistical analysis was by ANOVA.

Isoform	Day of differentiation	PKC Expression (arbitrary units) F442A cells Per unit protein	F442A cells Per cell	C2 Cells Per unit protein/Per cell	S.E.M.
α	-2	817.0	817.0[a]	344.7[c]	72.08
	0	649.3	649.3[a]	203.0[cd]	
	2	938.0	1313.0[b]	392.0[c]	
	5	183.0	365.3[c]	606.0[ad]	
	10	36.3	91.0[d]	285.7[c]	
γ	-2	241.3	241.3[a]	110.3[bd]	23.65
	0	158.3	158.3[b]	48.7[cd]	
	2	207.3	290.0[a]	170.3[be]	
	5	43.0	86.0[cd]	234.7[ae]	
	10	8.5	21.3[c]	89.0[cd]	
δ	-2	356.7	356.7[a]	168.3[bf]	18.49
	0	214.3	214.3[b]	147.0[ef]	
	2	399.1	558.7[c]	318.7[a]	
	5	206.7	413.3[d]	422.7[d]	
	10	38.6	96.4[e]	210.7[b]	
ε	-2	503.7	503.7[a]	154.0[f]	22.90
	0	424.7	424.7[b]	354.0[g]	
	2	570.7	798.7[c]	612.3[h]	
	5	607.3	1214.7[d]	680.7[i]	
	10	428.3	1070.7[e]	540.0[a]	
ζ	-2	177.7	177.7[ab]	184.0[b]	3.80
	0	170.7	170.7[a]	186.7[b]	
	2	128.6	180.0[ab]	183.3[b]	
	5	90.9	181.8[ab]	180.0[ab]	
	10	72.0	180.0[ab]	179.0[ab]	
μ	-2	174.3	174.3[a]	215.3[ad]	28.70
	0	166.7	166.7[a]	270.7[bd]	
	2	196.2	274.7[bd]	343.3[bc]	
	5	166.4	332.7[bc]	322.7[bc]	
	10	147.5	368.7[c]	217.3[ad]	

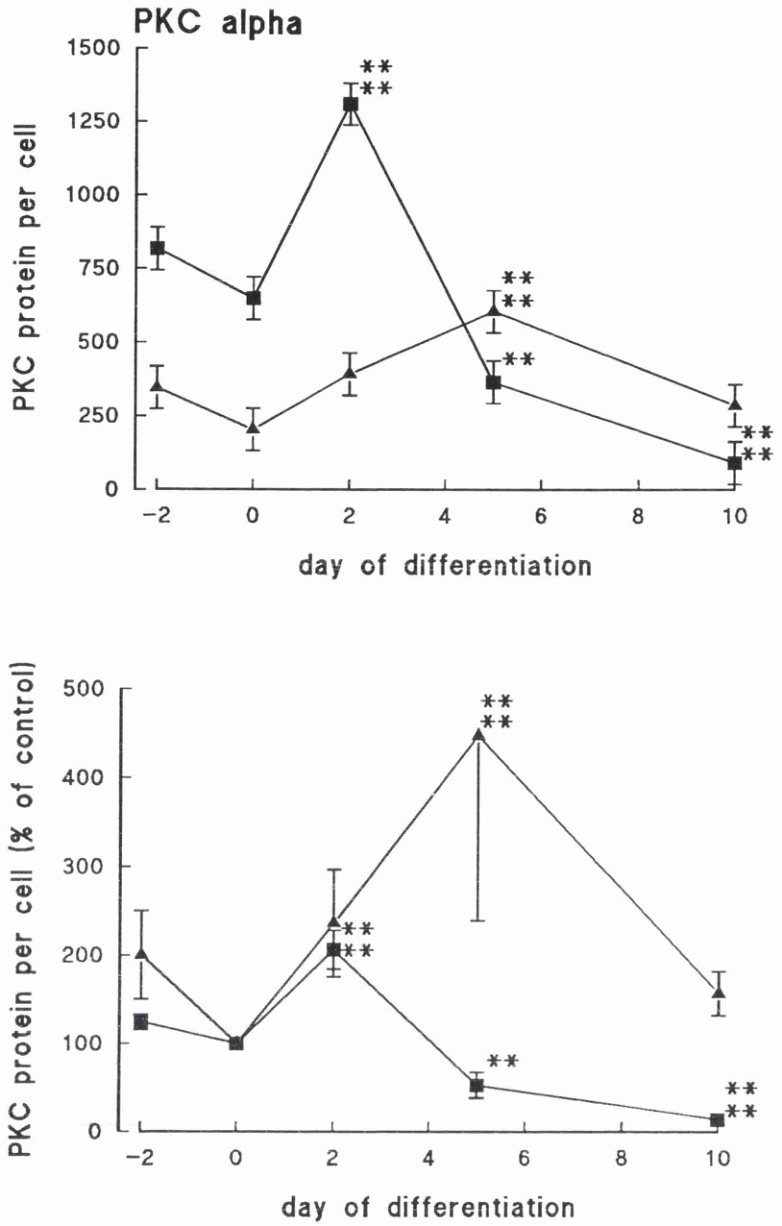


Figure 4.5A Changes in protein kinase C α expression during differentiation of 3T3-F442A preadipocytes and control (non-differentiating) 3T3-C2 cells

Cellular lysates were prepared from 3T3-F442A (■) and 3T3-C2 (▲) cells at confluence (day -2), just prior to treatment with differentiation medium (day 0) and at days 2, 5 and 10 of treatment with differentiation-inducing agents for assessment of PKC α levels by immunoblotting (Figure 4.4). Expression of PKC α was quantified by densitometric analysis of immunoblots and expressed on a per cell basis (Table 4.3). The changes in expression of PKC α during treatment with differentiation medium are shown both on a per cell basis and as a percentage of expression in day 0 preadipocytes (control = 100 %). Data represent means ± S.E.M. from six separate experiments carried out on different cell preparations. *, **, *** and **** indicate the value differs significantly from that for day 0 cells, $p < 0.05$, 0.02, 0.01, 0.001 respectively. Statistical analysis was by ANOVA.

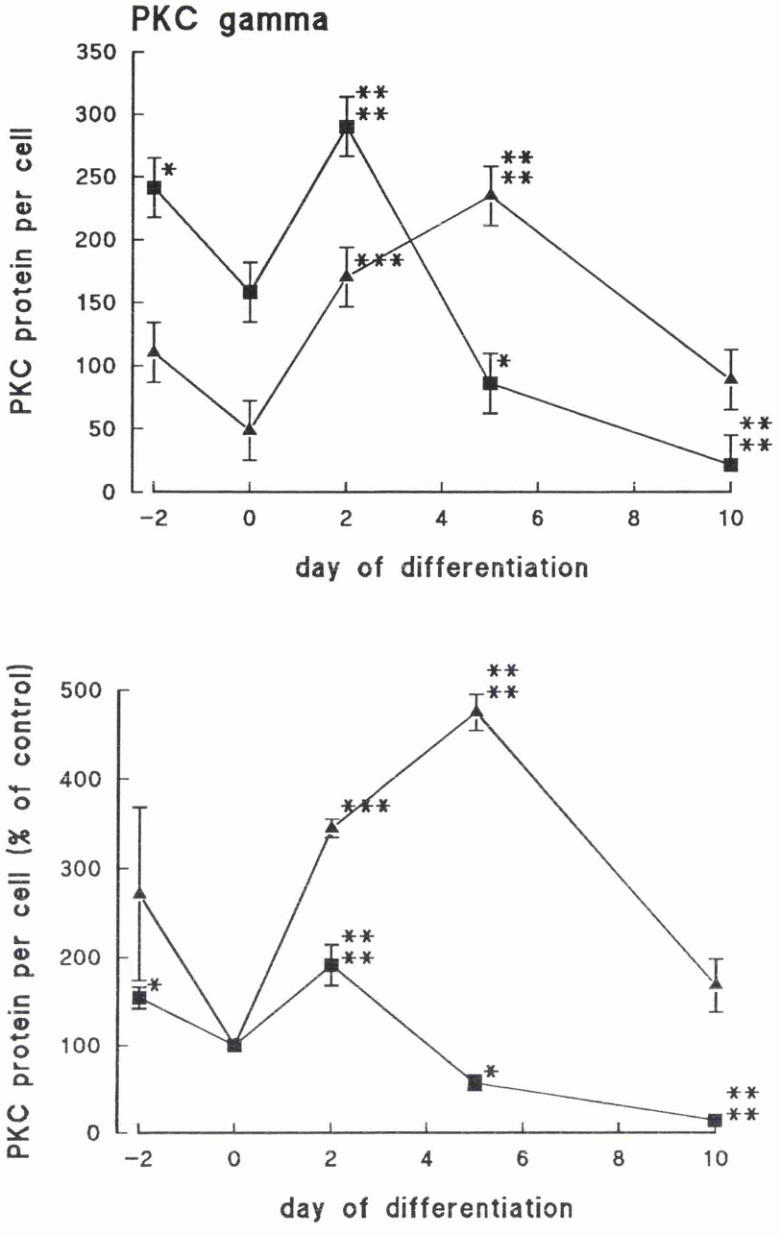


Figure 4.5B Changes in protein kinase C γ expression during differentiation of 3T3-F442A preadipocytes and control (non-differentiating) 3T3-C2 cells

Cellular lysates were prepared from 3T3-F442A (■) and 3T3-C2 (▲) cells at confluence (day -2), just prior to treatment with differentiation medium (day 0) and at days 2, 5 and 10 of treatment with differentiation-inducing agents for assessment of PKC γ levels by immunoblotting (Figure 4.4). Expression of PKC γ was quantified by densitometric analysis of immunoblots and expressed on a per cell basis (Table 4.3). The changes in expression of PKC γ during treatment with differentiation medium are shown both on a per cell basis and as a percentage of expression in day 0 preadipocytes (control = 100 %). Data represent means ± S.E.M. from six separate experiments carried out on different cell preparations. *, **, *** and **** indicate the value differs significantly from that for day 0 cells, $p < 0.05$, 0.02, 0.01, 0.001 respectively. Statistical analysis was by ANOVA.

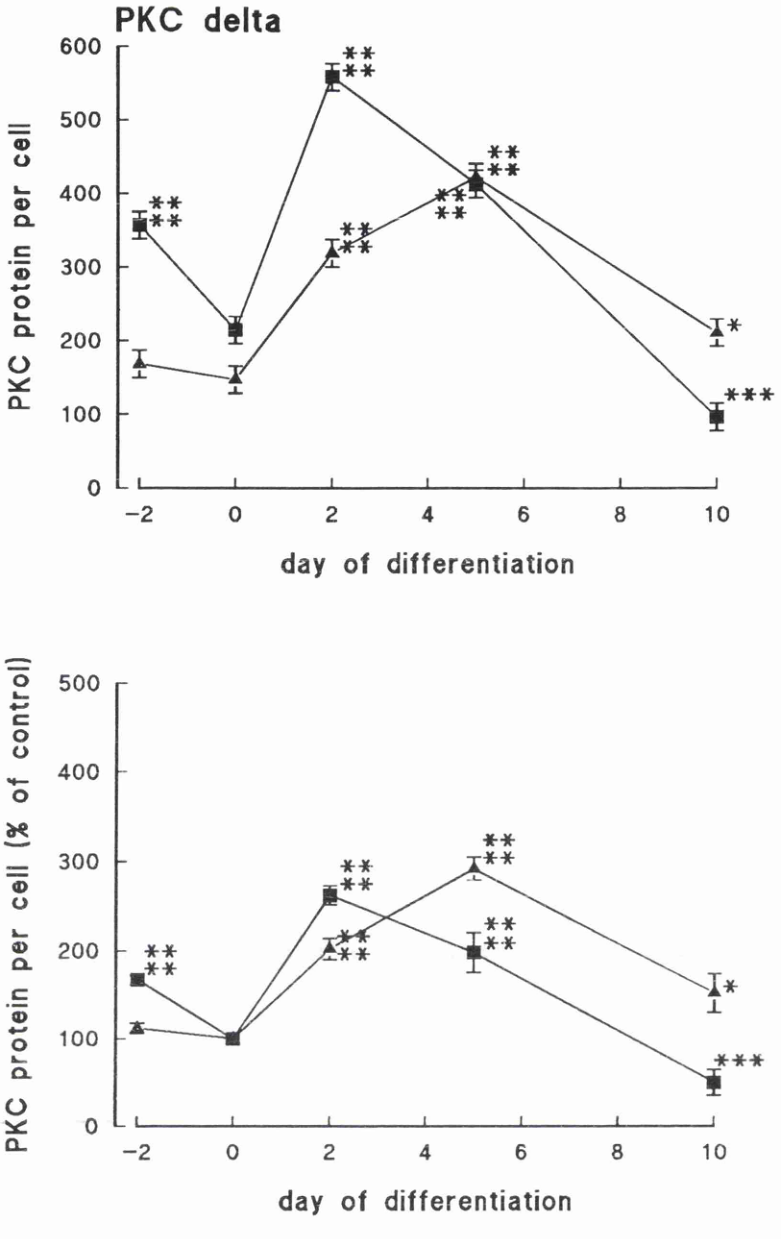


Figure 4.5C Changes in protein kinase C δ expression during differentiation of 3T3-F442A preadipocytes and control (non-differentiating) 3T3-C2 cells

Cellular lysates were prepared from 3T3-F442A (■) and 3T3-C2 (▲) cells at confluence (day -2), just prior to treatment with differentiation medium (day 0) and at days 2, 5 and 10 of treatment with differentiation-inducing agents for assessment of PKC δ levels by immunoblotting (Figure 4.4). Expression of PKC δ was quantified by densitometric analysis of immunoblots and expressed on a per cell basis (Table 4.3). The changes in expression of PKC δ during treatment with differentiation medium are shown both on a per cell basis and as a percentage of expression in day 0 preadipocytes (control = 100 %). Data represent means ± S.E.M. from six separate experiments carried out on different cell preparations. *, **, *** and **** indicate the value differs significantly from that for day 0 cells, $p < 0.05$, 0.02, 0.01, 0.001 respectively. Statistical analysis was by ANOVA.

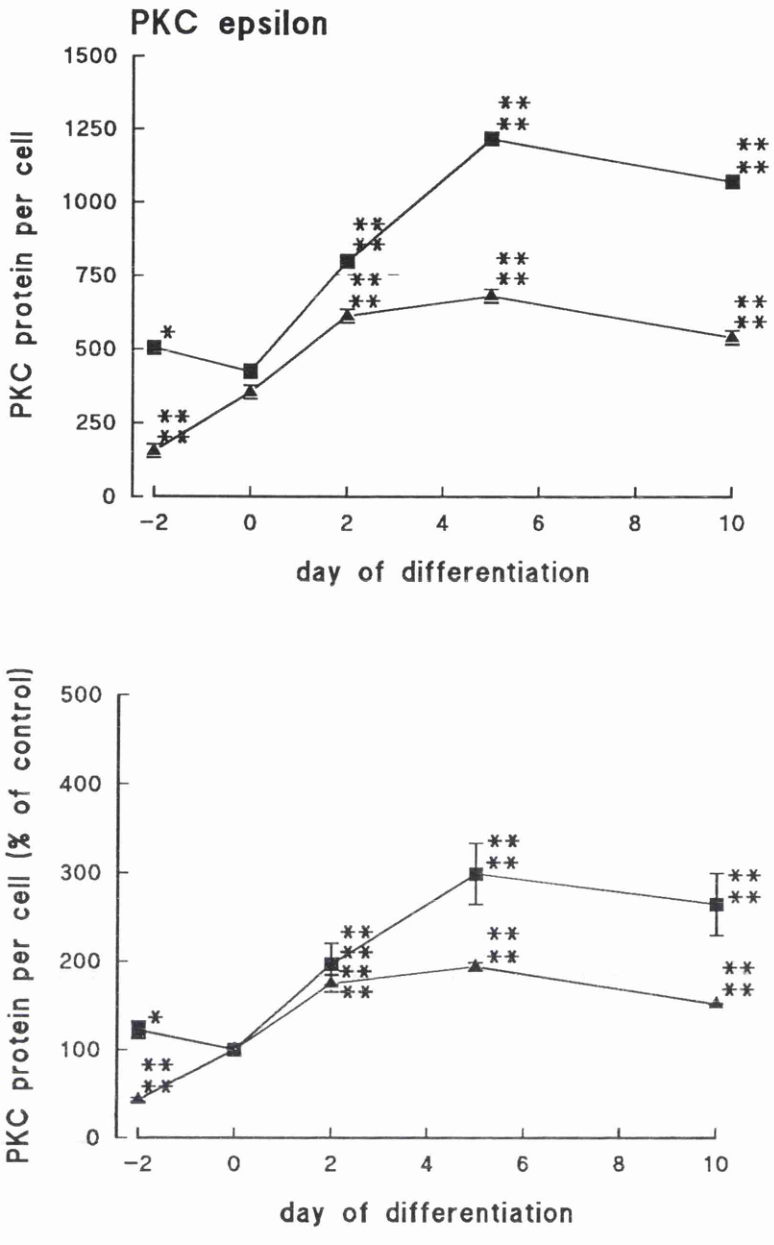


Figure 4.5D Changes in protein kinase C ε expression during differentiation of 3T3-F442A preadipocytes and control (non-differentiating) 3T3-C2 cells

Cellular lysates were prepared from 3T3-F442A (■) and 3T3-C2 (▲) cells at confluence (day -2), just prior to treatment with differentiation medium (day 0) and at days 2, 5 and 10 of treatment with differentiation-inducing agents for assessment of PKC ε levels by immunoblotting (Figure 4.4). Expression of PKC ε was quantified by densitometric analysis of immunoblots and expressed on a per cell basis (Table 4.3). The changes in expression of PKC ε during treatment with differentiation medium are shown both on a per cell basis and as a percentage of expression in day 0 preadipocytes (control = 100 %). Data represent means ± S.E.M. from six separate experiments carried out on different cell preparations. *, **, *** and **** indicate the value differs significantly from that for day 0 cells, $p < 0.05$, 0.02, 0.01, 0.001 respectively. Statistical analysis was by ANOVA.

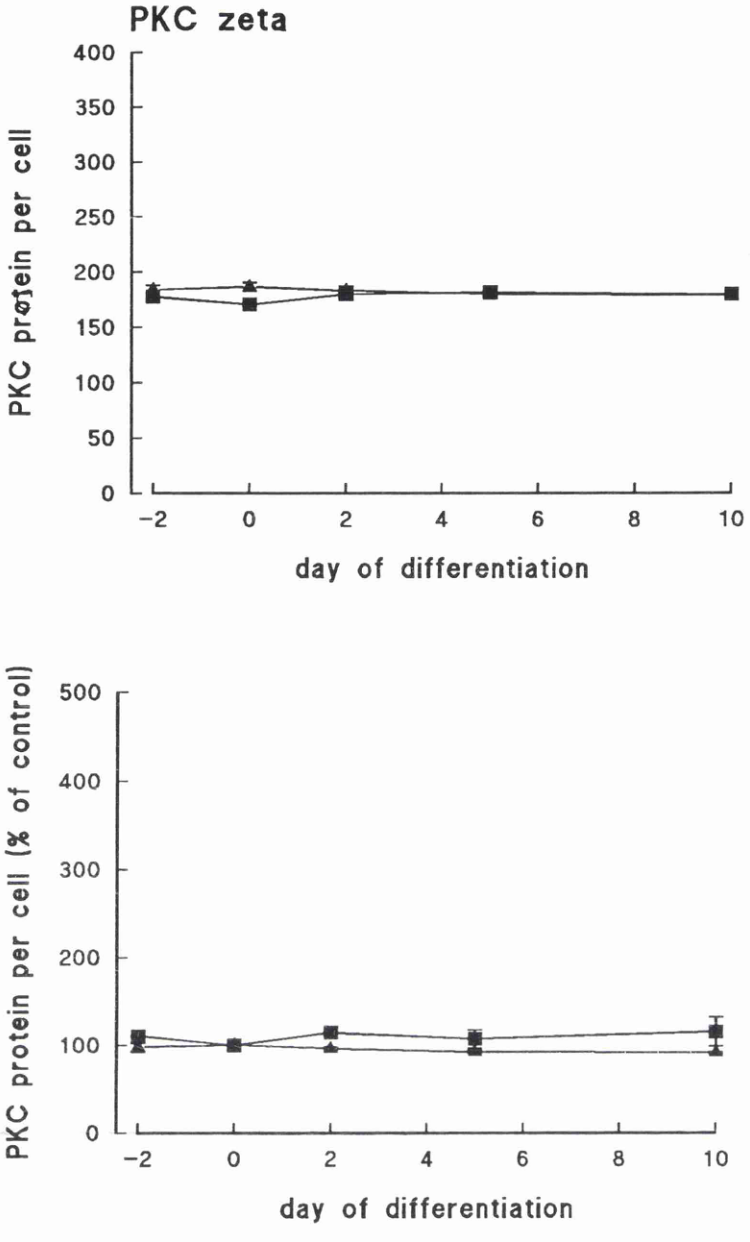


Figure 4.5E Changes in protein kinase C ζ expression during differentiation of 3T3-F442A preadipocytes and control (non-differentiating) 3T3-C2 cells

Cellular lysates were prepared from 3T3-F442A (■) and 3T3-C2 (▲) cells at confluence (day -2), just prior to treatment with differentiation medium (day 0) and at days 2, 5 and 10 of treatment with differentiation-inducing agents for assessment of PKC ζ levels by immunoblotting (Figure 4.4). Expression of PKC ζ was quantified by densitometric analysis of immunoblots and expressed on a per cell basis (Table 4.3). The changes in expression of PKC ζ during treatment with differentiation medium are shown both on a per cell basis and as a percentage of expression in day 0 preadipocytes (control = 100 %). Data represent means ± S.E.M. from six separate experiments carried out on different cell preparations. *, **, *** and **** indicate the value differs significantly from that for day 0 cells, $p < 0.05$, 0.02, 0.01, 0.001 respectively. Statistical analysis was by ANOVA.

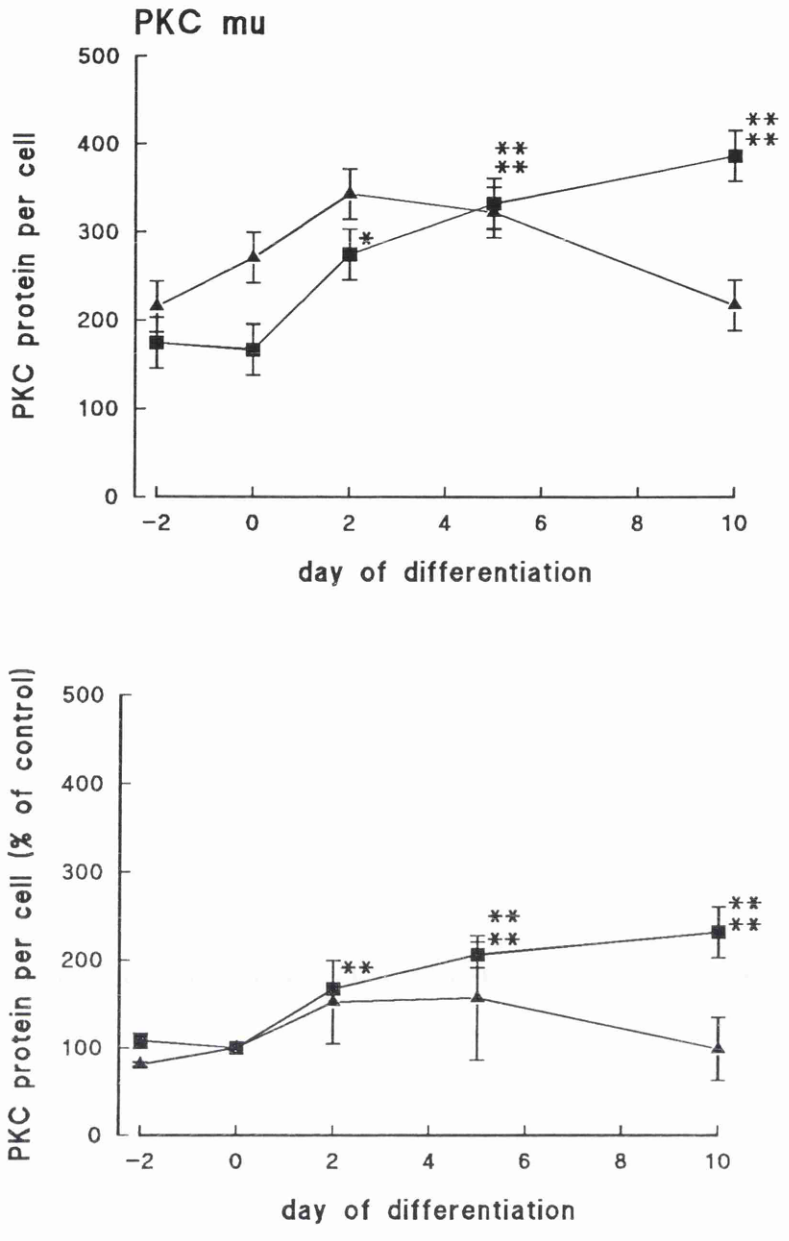
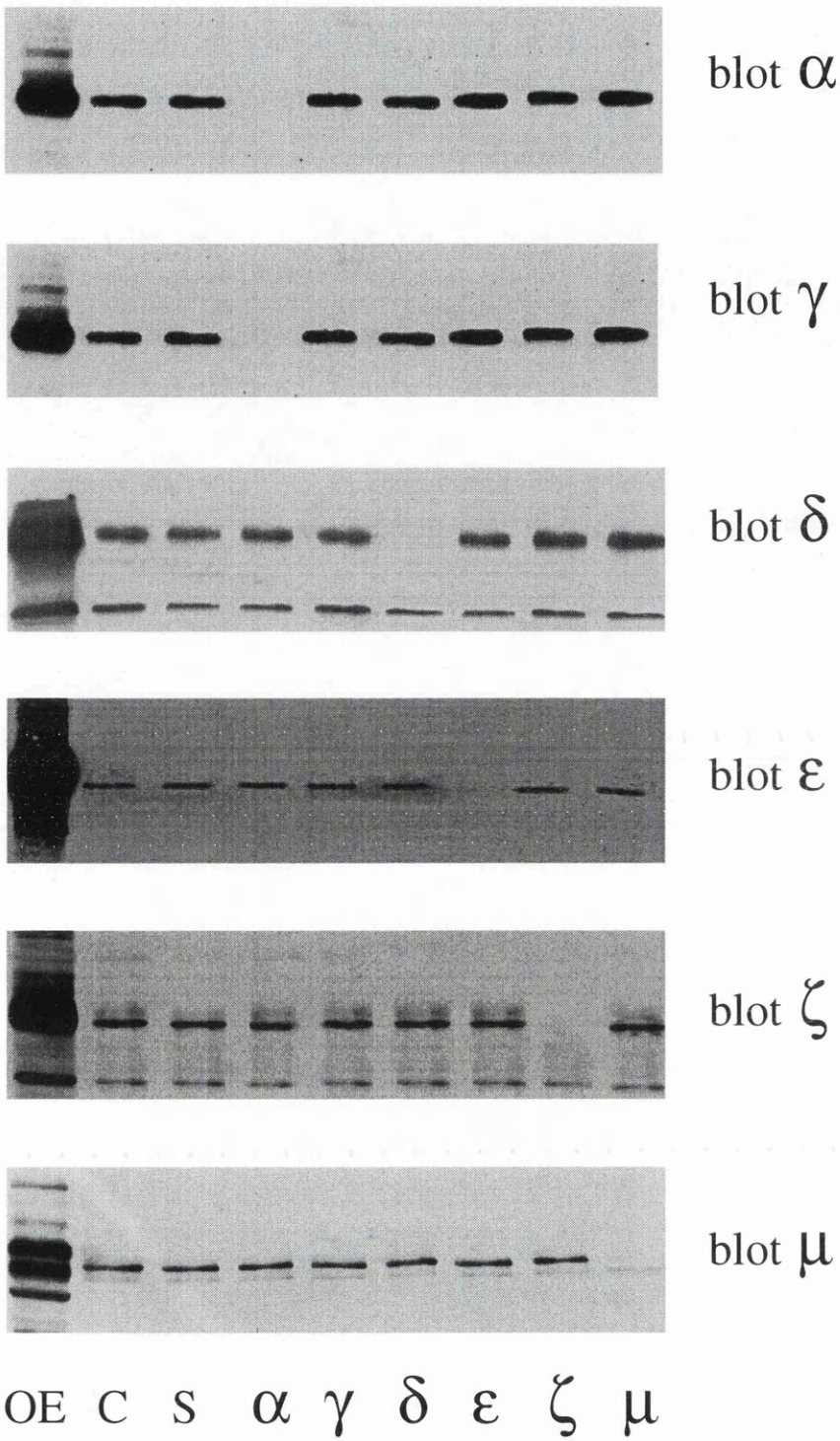


Figure 4.5F Changes in protein kinase C μ expression during differentiation of 3T3-F442A preadipocytes and control (non-differentiating) 3T3-C2 cells

Cellular lysates were prepared from 3T3-F442A (■) and 3T3-C2 (▲) cells at confluence (day -2), just prior to treatment with differentiation medium (day 0) and at days 2, 5 and 10 of treatment with differentiation-inducing agents for assessment of PKC μ levels by immunoblotting (Figure 4.4). Expression of PKC μ was quantified by densitometric analysis of immunoblots and expressed on a per cell basis (Table 4.3). The changes in expression of PKC μ during treatment with differentiation medium are shown both on a per cell basis and as a percentage of expression in day 0 preadipocytes (control = 100 %). Data represent means ± S.E.M. from six separate experiments carried out on different cell preparations. *, **, *** and **** indicate the value differs significantly from that for day 0 cells, $p < 0.05$, 0.02, 0.01, 0.001 respectively. Statistical analysis was by ANOVA.

Figure 4.6 Specific depletion of protein kinase C isoforms in 3T3-F442A preadipocytes by antisense oligodeoxynucleotide treatment

3T3-F442A preadipocytes were preincubated with ODNs for 48 h prior to the preparation of lysates for assessment of PKC levels by immunoblotting. Immunoblots show the amount of each indicated isoform in 25 μg of lysate prepared from NIH-3T3 cells over-expressing the appropriate PKC subtype (OE), control 3T3-F442A preadipocytes treated with lipofectin alone (C), 3T3-F442A preadipocytes treated with the appropriate sense ODN (S) and 3T3-F442A preadipocytes treated with antisense ODN to the PKC isoform indicated. Immunoblots are representative of experiments performed on at least four occasions for each PKC isoform.


blot α
blot γ
blot δ
blot ε
blot ζ
blot μ
OE C S α γ δ ε ζ μ

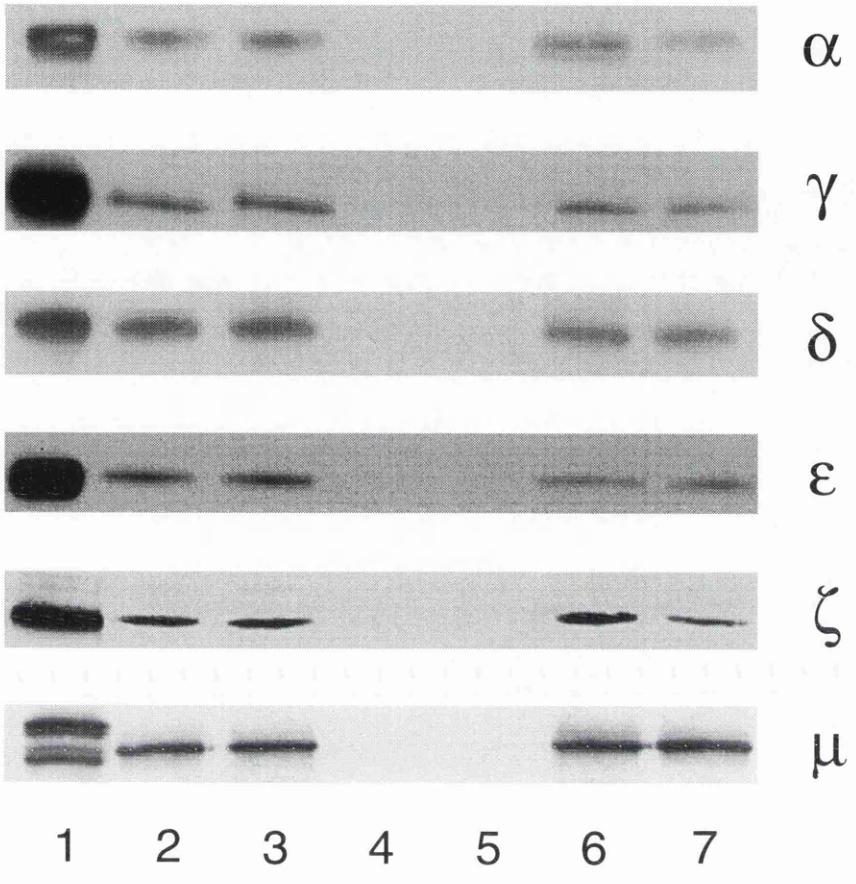


Figure 4.7 The effect of time on suppression of protein kinase C isoforms by antisense oligodeoxynucloetides

3T3-F442A preadipocytes were preincubated with antisense ODNs to the PKC isoforms indicated for 48 h prior to induction of differentiation. Equal quantities (25 μg) of lysate protein prepared from cells at days 0 (lane 4), 3 (lane 5), 4 (lane 6) and 7 (lane 7) of differentiation were subjected to immunoblotting with isoform-specific monoclonal antibodies to PKC. Immunoblots also show the amounts of each indicated PKC isoform in 25 μg of lysate protein prepared from NIH-3T3 cells over-expressing the appropriate PKC isoform (lane 1), control 3T3-F442A preadipocytes treated with lipofectin alone (lane 2) and 3T3-F442A preadipocytes treated with the appropriate sense ODN (lane 3). Immunoblots are representative of results obtained on at least four occasions for each PKC isoform.

Figure 4.8 A The effect of protein kinase C α oligodeoxynucleotides on changes in 3T3-F442A cell morphology during differentiation

3T3-F442A preadipocytes were preincubated with sense or antisense ODNs to PKC α, or lipofectin alone, for 48 h prior to the induction of differentiation. Changes in cell morphology following induction of differentiation were examined by photographing cells daily over the next seven days. Results are representative of experiments performed on at least six occasions.

PKC Alpha

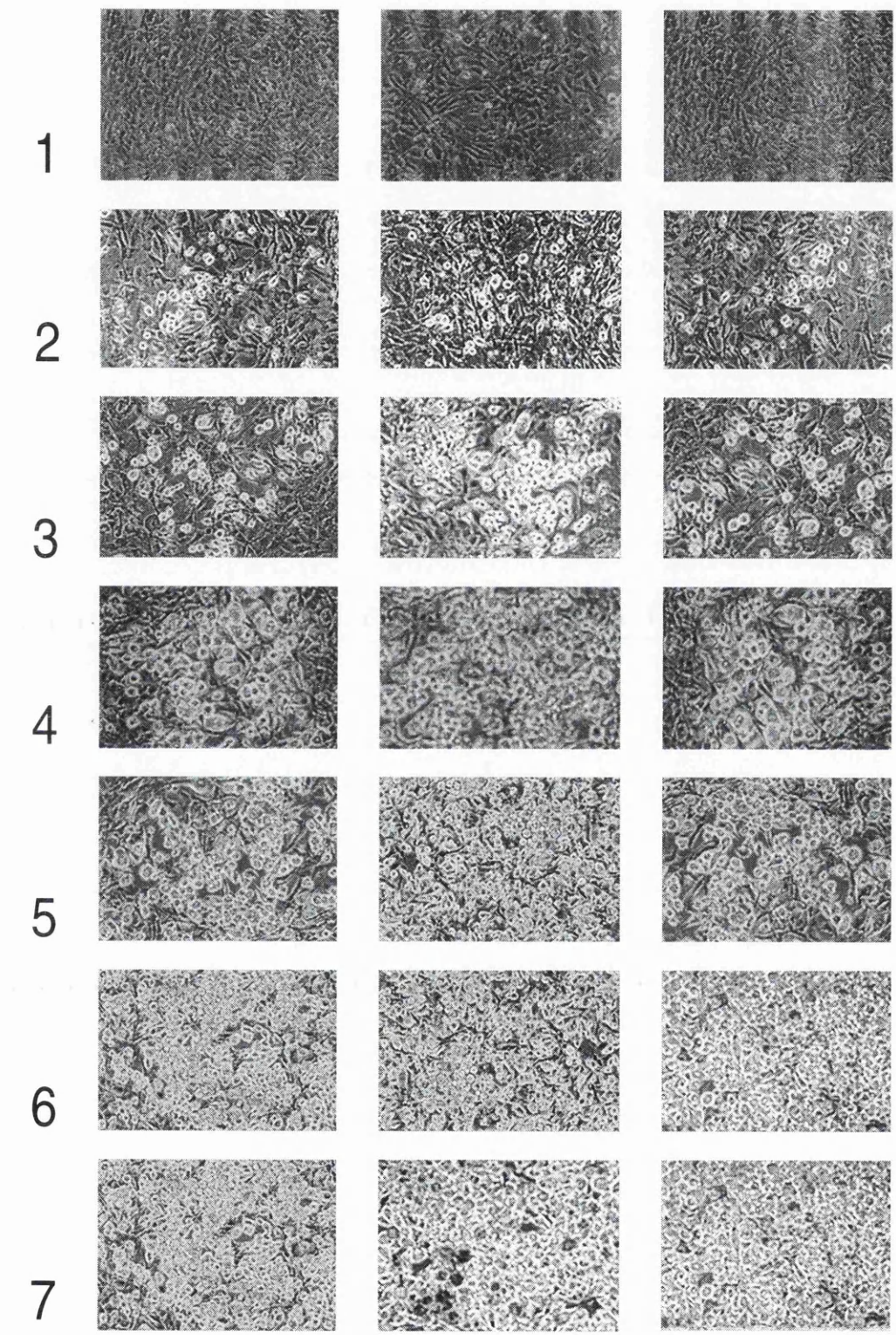


Figure 4.8 B The effect of protein kinase C γ oligodeoxynucleotides on changes in 3T3-F442A cell morphology during differentiation

3T3-F442A preadipocytes were preincubated with sense or antisense ODNs to PKC γ, or lipofectin alone, for 48 h prior to the induction of differentiation. Changes in cell morphology following induction of differentiation were examined by photographing cells daily over the next seven days. Results are representative of experiments performed on at least six occasions.

PKC Gamma

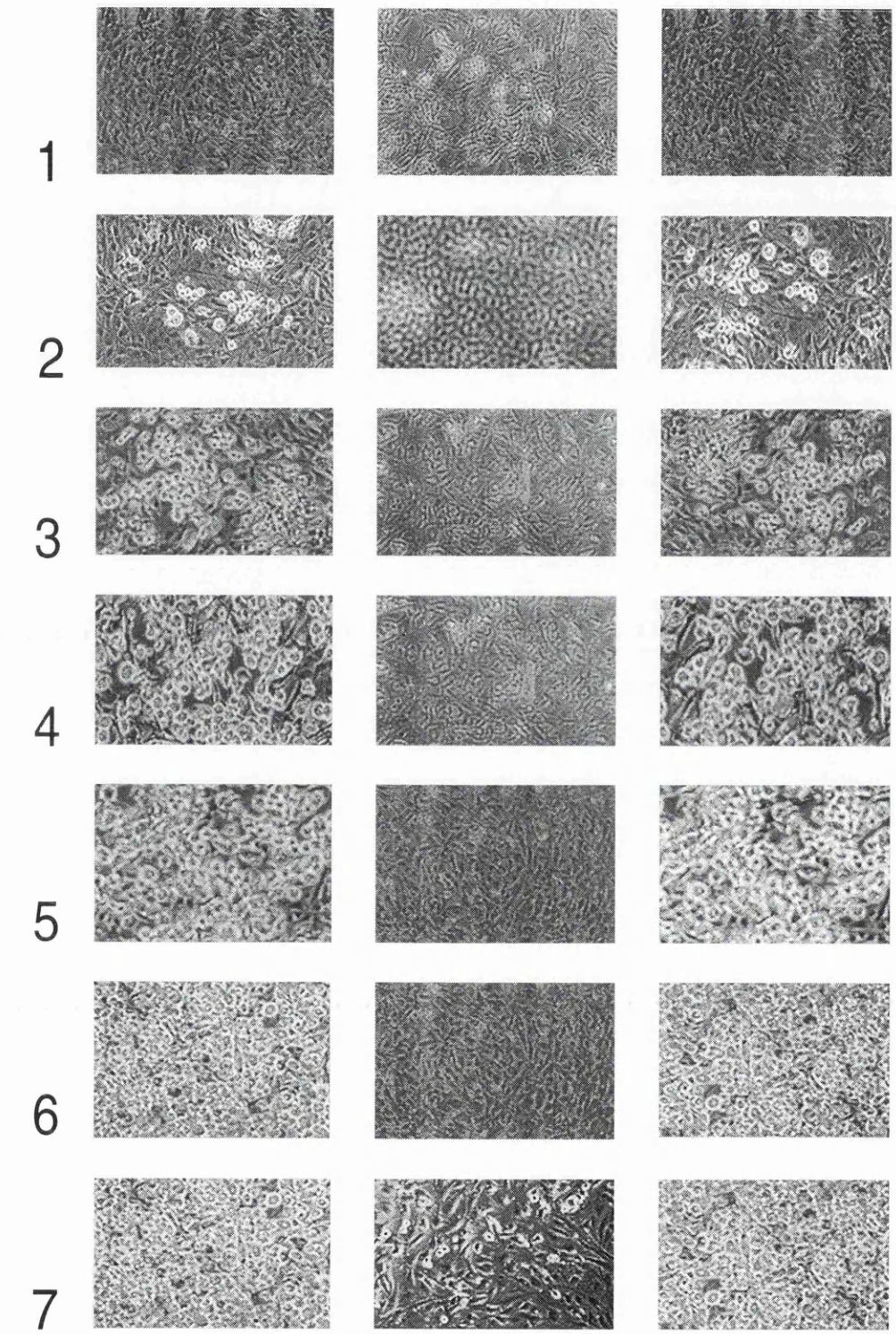


Figure 4.8 C The effect of protein kinase C δ oligodeoxynucleotides on changes in 3T3-F442A cell morphology during differentiation

3T3-F442A preadipocytes were preincubated with sense or antisense ODNs to PKC δ, or lipofectin alone, for 48 h prior to the induction of differentiation. Changes in cell morphology following induction of differentiation were examined by photographing cells daily over the next seven days. Results are representative of experiments performed on at least six occasions.

PKC Delta

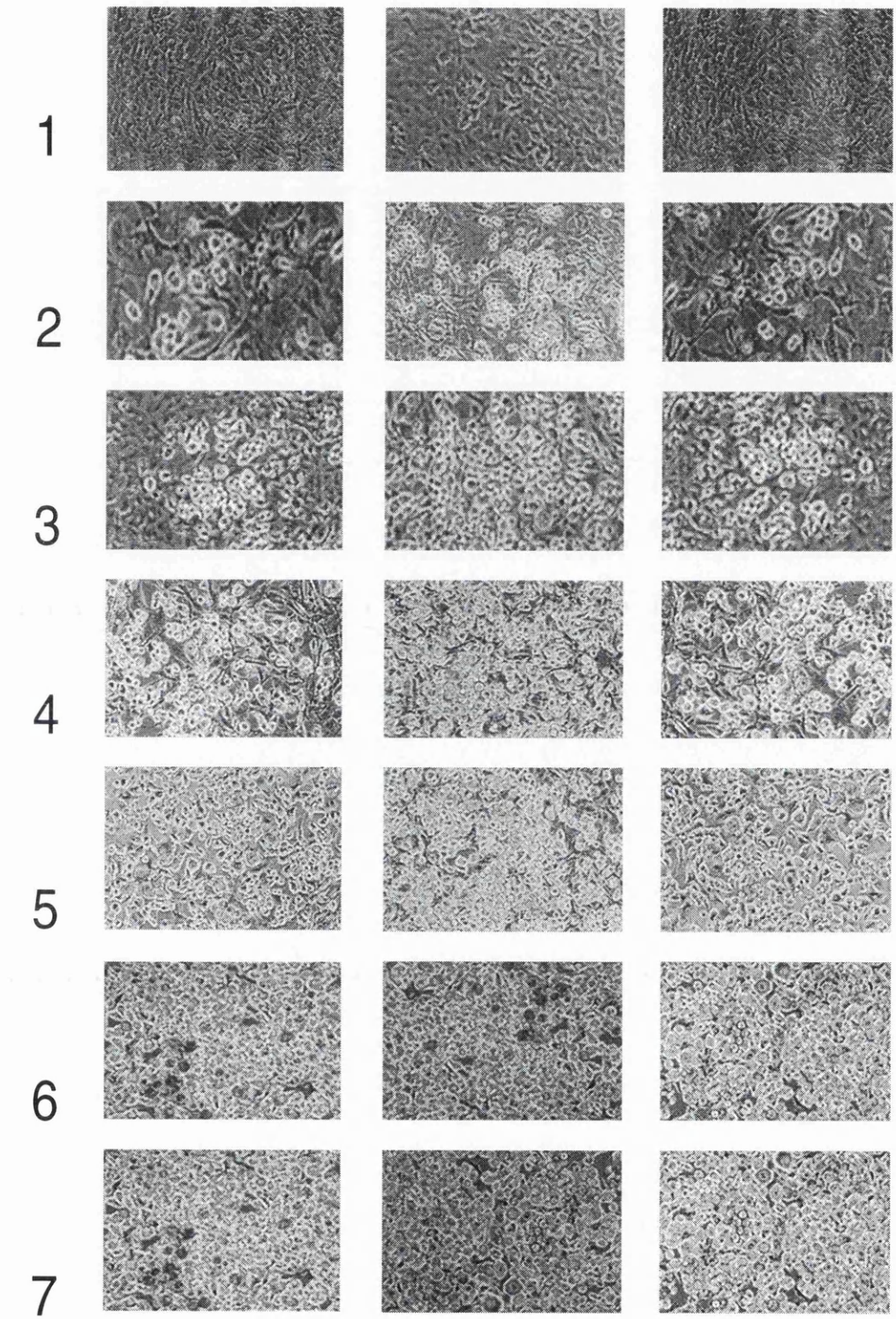


Figure 4.8 D The effect of protein kinase C ε oligodeoxynucleotides on changes in 3T3-F442A cell morphology during differentiation

3T3-F442A preadipocytes were preincubated with sense or antisense ODNs to PKC ε, or lipofectin alone, for 48 h prior to the induction of differentiation. Changes in cell morphology following induction of differentiation were examined by photographing cells daily over the next seven days. Results are representative of experiments performed on at least six occasions.

PKC Epsilon

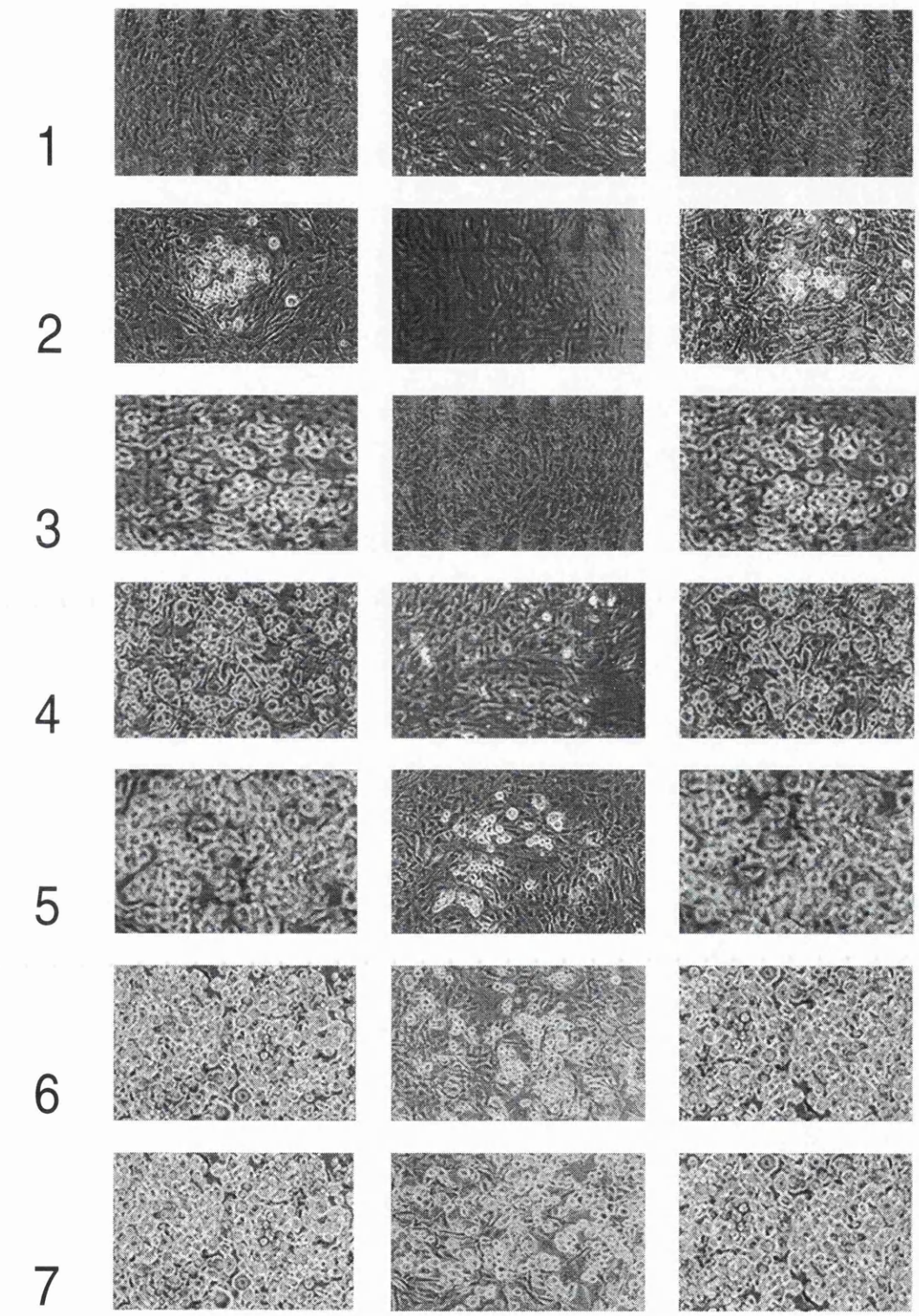


Figure 4.8 E The effect of protein kinase C ζ oligodeoxynucleotides on changes in 3T3-F442A cell morphology during differentiation

3T3-F442A preadipocytes were preincubated with sense or antisense ODNs to PKC ζ, or lipofectin alone, for 48 h prior to the induction of differentiation. Changes in cell morphology following induction of differentiation were examined by photographing cells daily over the next seven days. Results are representative of experiments performed on at least six occasions.

PKC Zeta

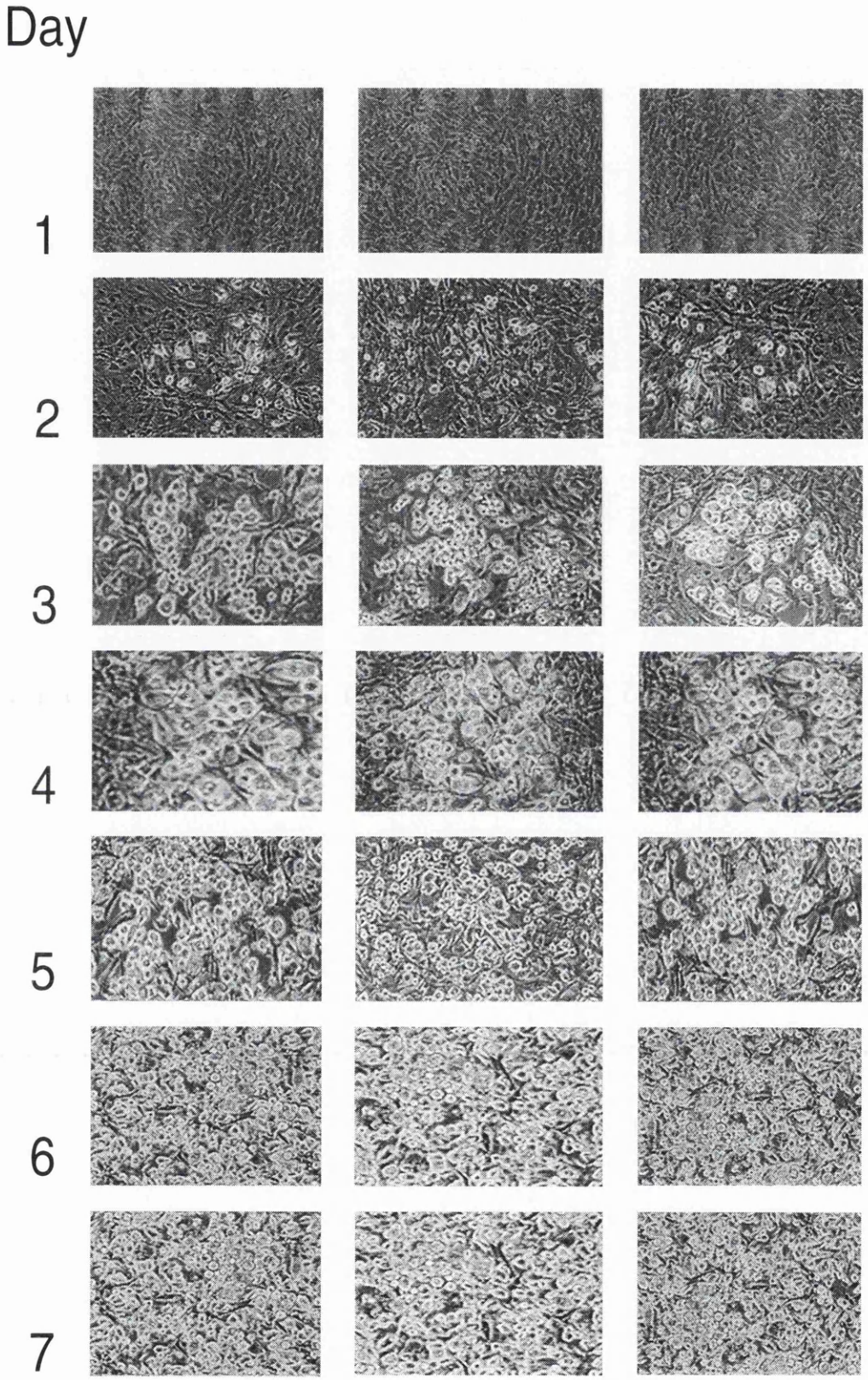
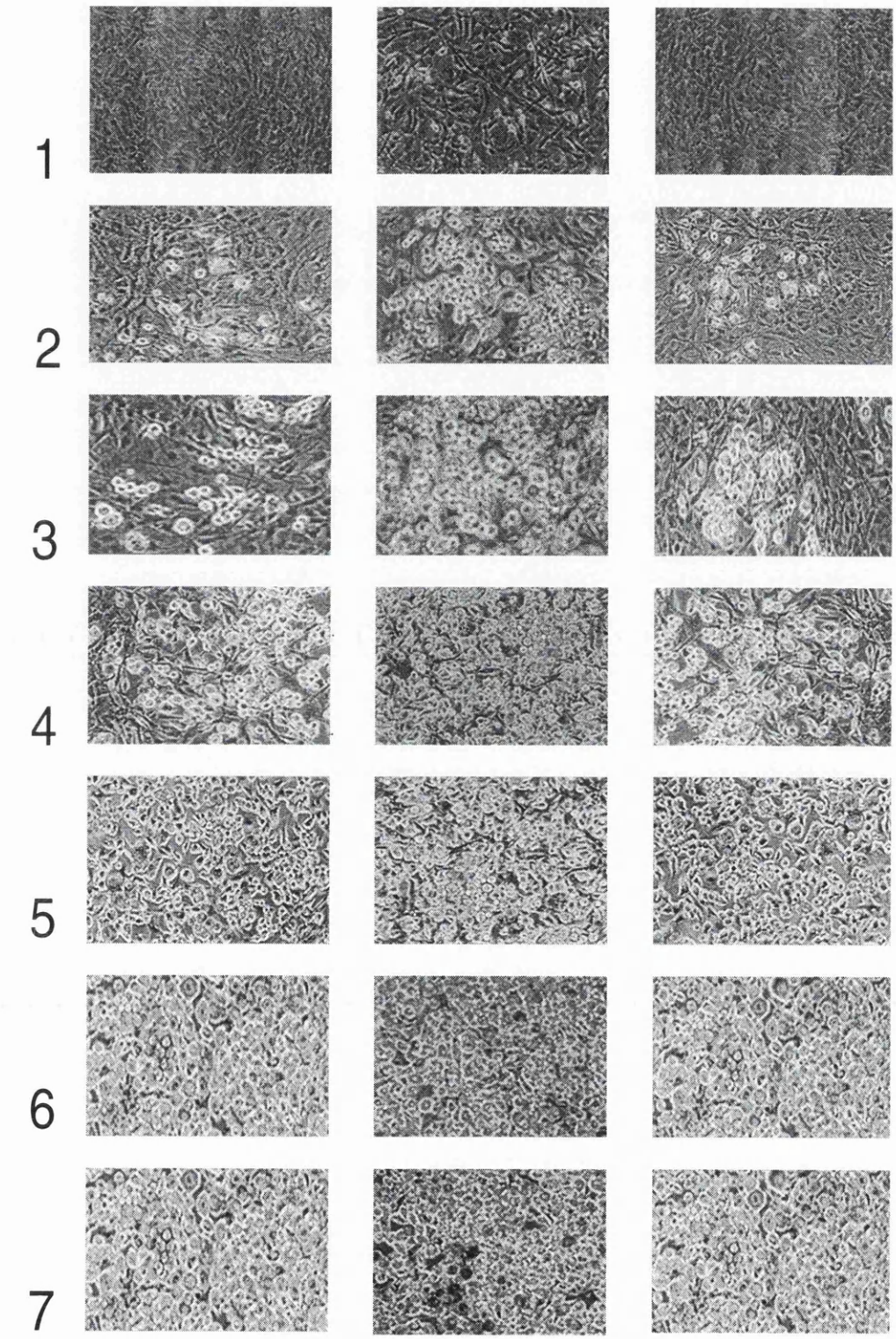


Figure 4.8 F The effect of protein kinase C μ oligodeoxynucleotides on changes in 3T3-F442A cell morphology during differentiation

3T3-F442A preadipocytes were preincubated with sense or antisense ODNs to PKC μ, or lipofectin alone, for 48 h prior to the induction of differentiation. Changes in cell morphology following induction of differentiation were examined by photographing cells daily over the next seven days. Results are representative of experiments performed on at least six occasions.

PKC Mu



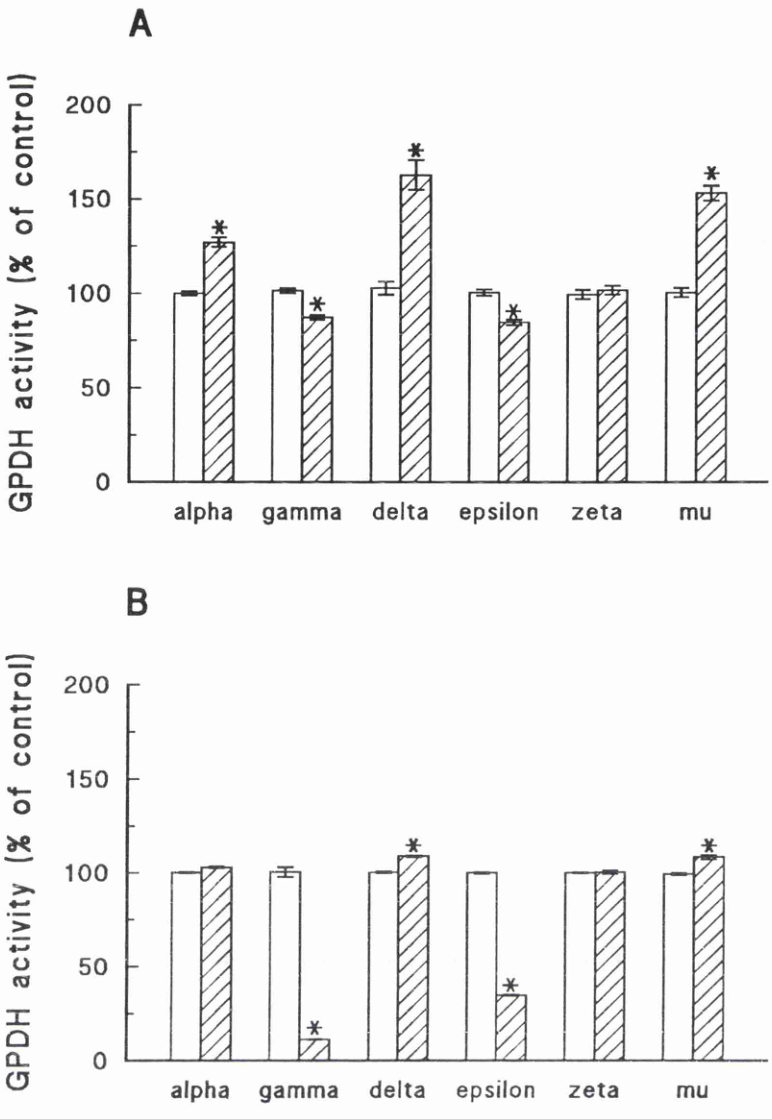
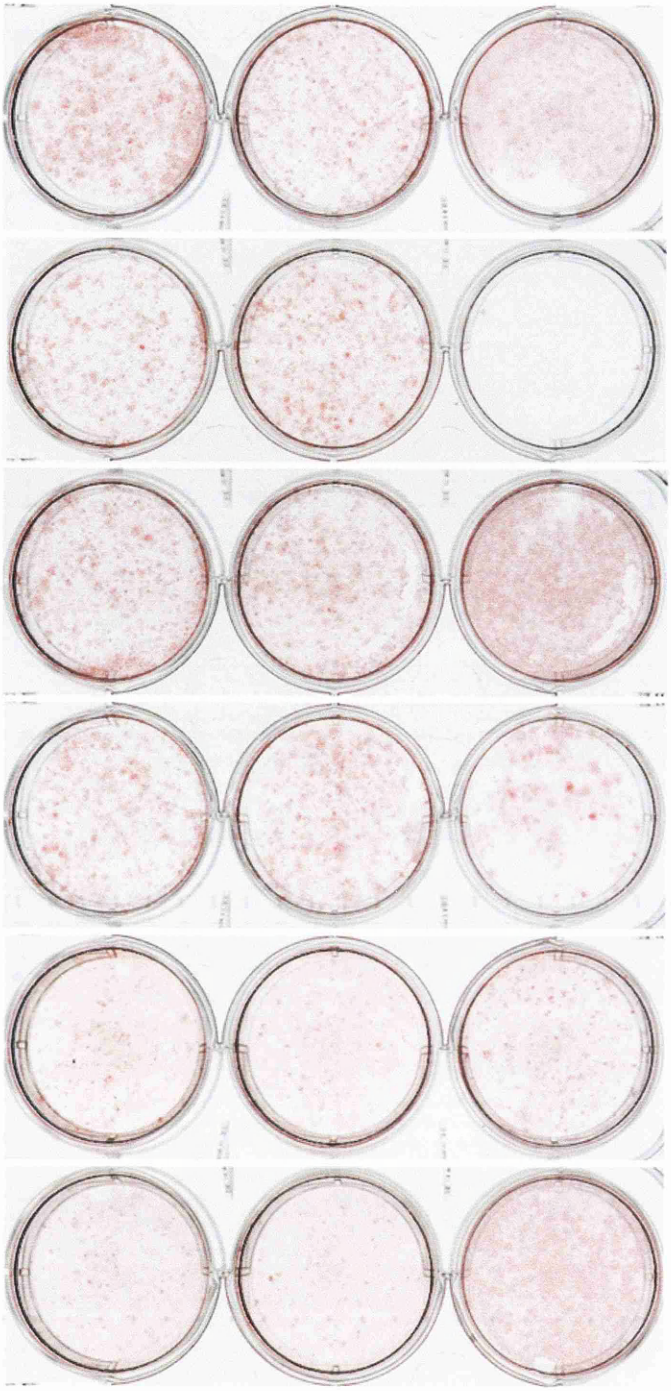


Figure 4.9 The effect of protein kinase C isoform-specific oligodeoxynucleotides on induction of glycerol-3-phosphate dehydrogenase activity during 3T3-F442A preadipocyte differentiation

3T3-F442A cells were preincubated with sense or antisense ODNs to the PKC isoform indicated, or lipofectin alone, prior to the induction of differentiation. Homogenates were prepared from cells at day 4 (A) and day 7 (B) of differentiation for determination of GPDH activity as described in section 2.2.2.8. GPDH activity of homogenates pretreated with sense and antisense ODNs is expressed as a percentage of the activity in homogenates prepared from control cultures differentiated following pretreatment with lipofectin alone (control = 100 %). Data are means ± S.E.M. of six separate experiments carried out on different cell preparations. * indicates the value differs significantly from that for cells treated with lipofectin alone, $p < 0.001$. Statistical analysis was by Student's t-test for paired samples.

Figure 4.10 The effect of protein kinase C isoform-specific oligodeoxynucleotides on lipid accumulation during 3T3-F442A preadipocyte differentiation

3T3-F442A preadipocytes were preincubated with sense or antisense ODNs to the PKC isoform indicated, or lipofectin alone, for 48 h prior to the induction of differentiation. At day 7 of differentiation, cells were fixed with formalin and stained for lipid with Oil red O as described in section 2.2.2.5.



Lipofectin	+	+	+
Sense	-	+	-
Antisense	-	-	+

Table 4.5 The effect of protein kinase C isoform-specific oligodeoxynucleotides on total homogenate DNA content during 3T3-F442A preadipocyte differentiation

3T3-F442A preadipocytes were pretreated with sense or antisense ODNs to the PKC subtype indicated, or lipofectin alone (control), prior to the induction of differentiation. Cellular homogenates were prepared either just prior to (day 0) or 2, 4 or 7 days after the induction of differentiation for determination of DNA content as described in section 2.2.2.10. Data are means ± S.E.M. of six separate experiments carried out on different cell preparations. * indicates the value differs significantly from that for cells treated with lipofectin alone, $p < 0.001$. Statistical analysis was by Student's t-test for paired samples.

	Total DNA per plate (μg/ml of extract)			
	Day of differentiation			
ODN Treatment	0	2	4	7
None	7.4 ± 0.41	14.9 ± 1.44	14.9 ±1.35	15.0 ± 1.51
Sense α	7.4 ± 0.39	15.0 ± 1.90	15.1 ± 1.47	15.1 ± 1.62
Antisense α	7.6 ± 0.50	15.1 ± 1.72	15.1 ± 1.44	15.2 ± 1.65
Sense γ	7.5 ± 0.49	14.9 ± 1.61	15.2 ± 1.56	15.1 ± 1.61
Antisense γ	7.3 ± 0.29	7.4 ± 0.52*	7.2 ± 0.49*	14.7 ± 1.43
Sense δ	7.3 ± 0.35	14.8 ± 1.83	15.5 ± 1.82	15.5 ± 1.62
Antisense δ	7.7 ± 0.69	15.2 ± 2.11	16.0 ± 1.98	16.1 ± 2.10
Sense ε	7.4 ± 0.42	14.7 ± 1.20	15.0 ± 1.39	15.3 ± 1.71
Antisense ε	7.4 ± 0.30	14.4 ± 1.71	14.7 ± 1.52	15.0 ± 1.11
Sense ζ	7.5 ± 0.61	14.9 ± 1.51	15.4 ± 1.61	15.6 ± 1.66
Antisense ζ	7.3 ± 0.40	15.1 ± 1.79	15.5 ± 1.64	15.5 ± 1.56
Sense μ	7.0 ± 0.22	15.0 ± 1.61	15.3 ± 1.71	15.3 ± 1.64
Antisense μ	7.4 ± 0.51	15.4 ± 1.73	15.5 ± 1.81	16.0 ± 2.01

4.4 Discussion

There is now mounting evidence to show that the multiple members of the PKC family assume distinct functional roles in the regulation of cellular growth and differentiation (see Section 1.6.3). PKC is inhibitory to differentiation in a number of cell-lines (Minana *et al*, 1989; Felipo *et al*, 1990; Taoka *et al*, 1990; Sahai *et al*, 1994). However, in contrast, selective over-expression or up-regulation of PKC isoforms has been shown to promote differentiation (Catalioto *et al*, 1992; Mischak *et al*, 1993; Livneh *et al*, 1996; McGowan *et al*, 1996). These studies suggest differential roles for individual PKC isoforms at distinct phases of the differentiation process. In this study, the 3T3-F442A preadipocyte cell-model was used to investigate the roles of individual PKC isoforms in the regulation of adipocyte development.

Initially, in order to gain an indication of the functional roles of PKC isoforms in 3T3-F442A preadipocyte differentiation, changes in the expression of individual isoforms during the differentiation of 3T3-F442A cells were examined. Two control systems were employed to distinguish between changes in cellular levels of PKC isoforms induced by the differentiation process *per se* and changes attributable to cell growth or treatment. Initially, cellular levels of PKC isoforms in 3T3-F442A adipocytes were compared to those of both just-confluent 3T3-F442A preadipocytes and cells maintained at confluence in growth medium for twelve days which did not undergo adipose conversion. This gave an indication of whether changes in cellular levels of PKC isoforms in adipocytes were specific to differentiation. Secondly, temporal patterns of PKC isoform expression during the differentiation of 3T3-F442A cells were compared to those observed in 3T3-C2 cells treated identically. 3T3-C2 cells are related to 3T3-F442A cells but do not differentiate in response to adipogenic stimuli (Green and Kehinde, 1976). Immunoblot analysis of 3T3-C2 cells revealed that they expressed the same PKC isoforms as 3T3-F442A cells. That both cell lines had a similar PKC complement suggested that differences in the patterns of change of cellular levels of PKC isoforms following treatment with differentiation-inducing agents may be important in regulating the differentiation process. Comparison of the changes in cellular levels of PKC isoforms during the differentiation of 3T3-F442A cells with those of 3T3-C2 cells treated identically gave an indication of changes in PKC isoforms that were important for the initiation and progression of differentiation.

Cellular levels of PKC ζ were unchanged both during the differentiation of 3T3-F442A cells and during the identical treatment of 3T3-C2 cells. This suggested that PKC ζ assumed no obligatory role in the regulation of the differentiation process.

Cellular levels of PKC μ increased throughout the differentiation of 3T3-F442A cells but were unaltered during the identical treatment of 3T3-C2 cells. This suggested that increases in the expression of PKC μ were important for the differentiation of 3T3-F442A cells. The fact that cellular levels of PKC μ were also elevated in 3T3-F442A preadipocytes maintained at confluence for twelve days, however, suggested that elevated PKC μ expression was not specific to the process of differentiation. Thus, following temporal studies, the role of PKC μ in 3T3-F442A preadipocyte differentiation remained unclear.

Following the induction of differentiation in 3T3-F442A preadipocytes, cellular levels of PKC α, γ and δ increased rapidly, peaking at around day 2, correlating with the time-point at which clonal expansion of the differentiating cells was observed. Subsequently cellular levels of the α, γ and δ isoforms decreased, such that a significant reduction in their expression was observed as cells attained the adipocyte phenotype. Although expression of PKC α, γ and δ followed a similar pattern of expression in 3T3-C2 cells treated with differentiation medium, the observation that levels of PKC α, γ and δ were reduced during 3T3-F442A preadipocyte differentiation prior to the reduction in 3T3-C2 cells supported a possible role for the reduction of these isoforms in the differentiation process. This hypothesis was strengthened by the fact that cellular levels of PKC α, γ and δ were not reduced below the levels observed in untreated 3T3-C2 cells at any point during treatment with differentiation medium. Thus, following the clonal expansion stage, these isoforms may exert an inhibitory influence on the differentiation process.

Cellular levels of PKC ε increased throughout the first five days of differentiation and were elevated significantly in 3T3-F442A adipocytes compared to preadipocytes. The expression profile of PKC was similar in 3T3-C2 cells treated identically. However, the proportional increase in expression was much larger in 3T3-F442A cells

suggesting that the level of PKC expression in 3T3-F442A cells may be important for differentiation to progress.

Consistent with the present observations in 3T3-F442A cells, preadipocyte differentiation was found to have no effect on cellular levels of PKC ζ in the related 3T3-L1 cell-line, but was accompanied by an increase in expression of PKC ε and a reduction in expression of PKC α (Frevert and Kahn, 1996; McGowan *et al*, 1996). It was also reported that, although PKC β is not expressed in 3T3-L1 preadipocytes or adipocytes, brief, transient expression of this isoform occurs during differentiation of the cells (McGowan *et al*, 1996). However, in the present study, expression of PKC β could not be detected at any point during the differentiation of 3T3-F442A cells.

3T3-F442A and 3T3-C2 cells also displayed differences in PKC isoform expression prior to the induction of differentiation (between day -2 and day 0), during the period in which confluent cells become quiescent. Cellular levels of PKC α, γ, δ, ζ and μ were unaltered during this period in 3T3-C2 cells whereas cellular levels of PKC γ, δ and ε decreased as 3T3-F442A cells entered growth arrest. In contrast to the decrease in cellular levels of PKC ε observed in growth-arrested 3T3-F442A cells, cellular levels of PKC ε in 3T3-C2 cells were elevated compared to those in just-confluent cells. The differences in PKC expression profiles of quiescent 3T3-F442A and 3T3-C2 cells may have important implications for the differentiation process. For example, growth-arrested 3T3-F442A cells can be induced to enter a unique state in which they become insensitive to mitogens that can induce cell cycle re-entry and sensitive to the differentiating activity of hormones that induce expression of the adipose phenotype (Guller et al, 1988; Corin et al, 1990). Growth-arrested 3T3-C2 cells cannot be induced to reach this anti-mitogenic state (Corin et al, 1990), suggesting that it is an integral part of the differentiation process. A number of studies indicate that PKCs can contribute to the induction of growth arrest, cell cycle exit and, hence, differentiation by a number of mechanisms, including the suppression of cyclins and the induction of cyclin-dependent kinase inhibitors (reviewed in Livneh and Fishman, 1997; see also Section 1.6.3.2). Thus, the differential expression of PKC isoforms in 3T3-F442A cells and 3T3-C2 cells at growth arrest may be important for the induction of the differentiation process.

Thus, temporal studies suggested that several isoforms of PKC might have roles at different stages in the differentiation of 3T3-F442A preadipocytes. To define the dependence of 3T3-F442A preadipocyte differentiation on PKC isoforms, antisense ODNs were used to achieve the selective depletion of individual isoforms prior to differentiation. Immunoblotiing studies revealed that complete depletion of PKCs could be achieved by using the sequence-specific antisense ODNs designed to hybridise with the isoform of interest. Importantly, none of the antisense treatments significantly affected the expression of other PKC subtypes. In addition, the effects of antisense treatment were fully reversible, indicating that the ODNs were not toxic to the cells. Maximal depletion of PKC isoforms also persisted for at least the first three days of differentiation. Thus, the role of individual PKC isoforms during the early stages of differentiation could be assessed.

Using the antisense approach it was demonstrated that, in agreement with the expression data, depletion of PKC ζ had no detectable effect upon adipocyte differentiation assessed either qualitatively, by examining changes in cell morphology and accumulation of cell lipid, or quantitatively, by assay of the adipocyte marker enzyme GPDH. Thus, it appears that PKC ζ plays no obligatory role in the process of 3T3-F442A preadipocyte differentiation.

In contrast, depletion of either PKC α, δ or μ significantly accelerated the differentiation of preadipocytes into adipocytes. Hence, these three isoforms each appear to exert an inhibitory influence upon adipocyte differentiation. Therefore, the decrease in expression of the α and δ isoforms, which occurs following the clonal expansion phase of differentiation, may be required for the attainment and maintenance of the adipocyte phenotype.

As suppression of PKC α or PKC δ accelerated the differentiation of 3T3-F442A preadipocytes, the reason for the initial increase in these isoforms at the onset of differentiation is unclear. Although levels of PKC α and δ are elevated around the time of clonal expansion, antisense studies indicate that neither of these isoforms is

essential for this increase in cell number to occur. It is therefore possible that PKC α and δ play a modulatory role in the clonal expansion phase of differentiation. It is also possible that these isoforms can functionally substitute for each other during the differentiation of 3T3-F442A cells and that clonal expansion would not occur in the simultaneous absence of both isoforms.

Cellular levels of PKC μ increase throughout the process of adipocyte development, although antisense studies demonstrated that this isoform is not required for differentiation to occur. This may point to a functional role for PKC μ in the adipocyte. That preadipocyte differentiation is actually accelerated in the absence of PKC μ may indicate that, like PKC α and δ, PKC μ plays a modulatory role during the early stages of the differentiation process. Antisense depletion of PKC α, δ and μ also appeared to increase the growth rate of 3T3-F442A preadipocytes (as assessed by morphological examination of cells; I. Fleming, S. MacKenzie and E. Kilgour, unpublished observation). Thus, PKC α, δ and μ may play a modulatory role in the regulation of growth and the early stages of differentiation in 3T3-F442A cells.

Depletion of PKC γ, which displayed a similar temporal pattern of expression to the α and δ PKC isoforms, severely inhibited adipocyte differentiation. Further antisense studies revealed that PKC γ was essential for the process of clonal expansion, which occurs early during differentiation. Antisense experiments also demonstrated that neither PKC α nor PKC δ, whose expression levels follow a similar temporal pattern to those of PKC γ and thus are elevated during the clonal expansion phase, are able to functionally substitute for PKC γ. From the expression studies, it appeared that, following clonal expansion, decreases in PKC γ, like PKC α and δ, may be important for the progression of differentiation.

Depletion of PKC ε also severely attenuated adipocyte differentiation, thus demonstrating that other PKC isoforms are unable to assume the essential functional role that this isoform plays in adipocyte development. Further studies revealed that PKC ε is not required for clonal expansion of differentiating cells, but is likely to be necessary in the later stages of differentiation, when its expression is markedly elevated, for attainment and maintenance of the adipocyte phenotype.

Preadipocyte differentiation is a complex process, requiring the strict ordering of a cascade of multiple and interacting molecular events, leading to the expression of multiple genes required for maintenance of the adipocyte phenotype. PKCs are involved in a number of the signalling pathways that are known to modulate the process of preadipocyte differentiation (Anderson, 1993; Nakanishi *et al*, 1993; Toker *et al*, 1994; Cobb and Goldsmith, 1995; Sale *et al*, 1995; Uehara *et al*, 1995; Yeh *et al*, 1995) and there is considerable evidence that the PKC family of isoforms play an important part in the "cross talk" which occurs between different signalling pathways (Houslay, 1991; Hata *et al*, 1993; Stabel and Parker, 1993; Chin, *et al*, 1994; Houslay, 1995). Thus, the fluctuations in cellular levels of PKC isoforms that occur throughout adipocyte development are likely to be important for the modulation and integration of the variety of signals that regulate the differentiation process.

At present, the mechanisms by which the PKC family regulate adipocyte development are uncertain. However, the results of this study indicate that PKC isoforms assume distinct functional roles during 3T3-F442A preadipocyte differentiation and are, thus, likely to target different cellular substrates. Members of the C/EBP and the PPAR family of transcription factors, which are especially prominent in the control of adipogenesis (see Yeh and McKnight, 1995), display similar temporal expression profiles to individual PKC isoforms during preadipocyte differentiation (see Figure 1.3). In particular, C/EBP β and C/EBP δ, which are induced during the early stages of differentiation, peak during the clonal expansion phase and subsequently decline as cells attain the adipocyte phenotype, follow a similar temporal pattern of expression to PKC α, γ and δ. In addition, PPAR γ, which is induced during the clonal expansion phase of differentiation and continues to increase as cells attain the adipocyte phenotype, follows a similar temporal pattern of expression to PKC ε and μ. This implies that the C/EBP and PPAR proteins may be plausible candidiates as the ultimate down-stream targets of individual PKC isoforms during preadipocyte differentiation. Indeed, a role for PKCs in the regulation of PPAR γ has already been implied by studies demonstrating that suppression of adipogenesis by TPA involves the MAP kinase-mediated phosphorylation of PPAR γ and attenuation of its

transcriptional activity (Hu *et al*, 1996).

In summary, selective changes in PKC isoform expression accompany the differentiation process in 3T3-F442A cells, implying distinct functional roles for PKC isoforms in the regulation of adipocyte development and function. Antisense depletion of PKC isoforms revealed that PKC ζ is not essential for any stage of the differentiation process. PKC α, δ and μ each exert an inhibitory influence upon the early stages of adipocyte development and are therefore likely to modulate this stage of the differentiation process. PKC ε is required for the attainment of the adipocyte phenotype and PKC γ is essential for the clonal expansion of differentiating preadipocytes. These results clearly demonstrate that PKC subtypes assume distinct functional roles in 3T3-F442A cells and are therefore likely to target different cellular substrates.

Chapter 5

Investigation of the roles of individual protein kinase C isoforms in growth hormone- and insulin- mediated effects on lipogenesis in 3T3-F442A adipocytes

5.1 Introduction

The key features of adipose tissue metabolism are the synthesis and subsequent hydrolysis of fat. The enzymes involved in lipid metabolism in the adipocyte are subject to strict regulation by a number of hormones. Two important hormonal regulators of adipocyte metabolism are insulin, which promotes fat deposition, and growth hormone (GH), which reduces adiposity (see Sections 1.2.1 and 1.2.1.1).

Several studies have produced evidence of a role for PKC in some of the signalling events mediated by GH (see Section 1.6.3.4). GH increases levels of DAG, a natural activator of PKC, in various cells (Doglio *et al*, 1989; Catalioto *et al*, 1992). In addition, studies with phorbol esters suggest that, in preadipocytes, activation of MAP kinases, and s6 kinases (Anderson 1992 and 1993) and promotion of terminal differentiation (Gurland *et al*, 1990; Campbell *et al*, 1992) by GH occur by PKC-dependent mechanisms. Furthermore, at least in the rat adipocyte, data are consistent with GH exerting inhibitory effects on lipogenesis (Smal and De Meyts, 1987) and stimulatory effects on lipolysis (Gorin *et al*, 1990) *via* signal transduction which is, at least in part, mediated by the activation of PKC. Thus, one or more isoforms of PKC are likely to be involved in the signalling pathway(s) by which GH regulates adipocyte metabolism.

The role of PKC in mediating the effects of insulin remains more controversial (see Section 1.6.3.5). PKC has been associated with attenuation of the insulin response and the induction of insulin resistance (see Houslay, 1991; Chin *et al*, 1993). However, insulin has been noted to activate a number of PKC isoforms in adipocytes (Farese *et al*, 1992; Bandyopadhyay *et al*, 1997). In addition, PKC appears to be, at least in part, required to mediate insulin-stimulated lipogenesis (Smal and De Meyts, 1987) and glucose transport (Standaert *et al*, 1990; Grunberger, 1991; Yano *et al*, 1993; Chalfant *et al*, 1995) in the adipocyte. Therefore, the role of PKC isoforms in mediating the metabolic effects of insulin appears to be complex. This may be due, in part, to interactions of a range of PKC isoforms.

In this study, the involvement of specific PKC isoforms in the signalling mechanisms by which GH and insulin alter the rate of lipogenesis in the adipocyte was

investigated. Initially, the effects of GH *in vivo* on the expression of PKC isoforms in the rat adipocyte were examined. The effects of GH and insulin on the expression of PKC isoforms *in vitro* were then examined in 3T3-F442A adipocytes. Finally, the selective depletion of individual PKC isoforms from 3T3-F442A adipocytes was carried out using specific antisense ODNs, in an attempt to define the roles of individual PKC isoforms in the GH- and insulin-mediated effects on lipid synthesis in the adipocyte.

5.2 Methods

5.2.1 Assessment of the effects of growth hormone and insulin on protein kinase C isoform expression in adipocytes

5.2.1.1 Assessment of protein kinase C isoform expression in rats treated with antiserum to rat growth hormone

5.2.1.1.1 Immunoneutralisation of circulating rat growth hormone

Female Wistar rats of 150-180g were treated twice daily with antiserum to rat GH, either alone, or in combination with ovine GH, for four days as described in section 2.2.3.1. Control animals received equivalent injections of carrier solutions. Rat body weights were taken prior to the commencement of treatments and following their completion to assess the effectiveness of treatments in changing serum GH concentrations. On day 5, rats were anaesthetised as described in section 2.2.3.1 prior to the extraction of parametrial fat pads and blood samples. Upon removal, the parametrial fat pads were weighed rapidly and placed in saline at 37° C prior to the isolation of adipocytes.

5.2.1.1.2 Isolation of rat adipocytes and homogenate preparation

Parametrial fat pads, removed from rats treated as described above in section 5.2.1.1.1, were washed in Krebs-Ringer-Hepes buffer and minced finely as described in section 2.2.3.2. Adipocytes were subsequently extracted by collagenase digestion as described in section 2.2.3.2. Undigested material was removed by filtration and adipocyte homogenates were prepared at room temperature as described in section 2.2.3.2.

5.2.1.1.3. Assessment of fat pad composition

Prior to homogenisation, aliquots of adipocyte suspension were retained and placed at 37° C until transfer to a warm haemocytometer where the diameter of one hundred cells was measured to allow calculation of the mean cell volume as described in section 2.2.3.3. Further aliquots of adipocyte suspension were oven dried at 100° C. An estimate of the number of cells per gram tissue was obtained by dividing the total

lipid content of the tissue by the average lipid content of the fat cells as described by Di Girolamo *et al* (1971). Lipid content of the adipose tissue was taken to be equal to its dry weight and the average lipid content of the fat cells derived from the mean cell volume x density of lipid (= 0.9).

5.2.1.1.4 Preparation of serum from whole blood and assay of IGF-1

Blood samples, taken from rats treated as described above in section 5.2.1.1.1, were allowed to clot on ice prior to the extraction of serum by centrifugation as described in section 2.2.3.6. Endogenous serum IGF-1 levels were determined by radioimmunoassay as described in section 2.2.3.7.

5.2.1.1.5 Immunoblotting

Cellular homogenates were prepared from the isolated adipocytes of rats treated as described above in section 5.2.1.1.1. The protein content of cell homogenates was determined as described in section 2.2.4.1 and confirmed by Coomassie staining of mini-gels as described in section 2.2.4.3. Equal quantities of homogenate protein were subjected to SDS-PAGE alongside a homogenate prepared from rat brain that served as a positive control for the PKC antibodies. PKC isoform expression was examined by immunoblotting with isoform-specific monoclonal antibodies to PKC as described in section 2.2.4.4. PKC isoform expression was quantified by densitometric analysis of immunoblots.

5.2.1.2 Assessment of protein kinase C isoform expression in 3T3-F442A adipocytes treated with growth hormone and insulin

5.2.1.2.1 Cell culture

3T3-F442A preadipocytes were grown to confluence in 100 mm diameter dishes. Two day confluent cultures were induced to differentiate with foetal calf serum and insulin as described in section 2.2.1.2. Insulin was removed from the medium on day 8 of differentiation and adipocytes were treated as required at day 10 of differentiation following a 16-18 h incubation in the absence of serum.

5.2.1.2.2 Hormone treatment of 3T3-F442A adipocytes

Fully differentiated 3T3-F442A adipocytes were incubated for 16-18 h in the absence

of exogenous hormones and then incubated for 24 h in the presence or absence of 4.5 nM GH or 8.7 nM insulin, either alone, or in combination. Subsequently, cellular lysates were prepared as described in section 2.2.2.6. The protein content of cell lysates was determined as described in section 2.2.4.1 and confirmed by Coomassie staining of mini-gels as described in section 2.2.4.3. PKC isoform expression in adipocyte lysates was examined and quantified as descibed above in section 5.2.1.1.5.

5.2.2 Characterisation of the effects of growth hormone and insulin on the rate of lipid synthesis in 3T3-F442A adipocytes

3T3-F442A preadipocytes were grown to confluence in 30 mm diameter wells. Two-day confluent cultures were induced to differentiate as described above in section 5.2.1.2.1. Fully differentiated adipocytes were incubated with hormones as described above in section 5.2.1.2.2, except that incubations were carried out for 24 and 48 h. Hormones were replenished every 24 h and the rate of lipogenesis subsequently determined as described in section 2.2.2.9.

5.2.2.1 Treatment of cells with kinase inhibitors

For some experiments, the effects of GH and insulin on the rate of lipid synthesis in 3T3-F442A adipocytes were determined in the presence of wortmannin, an inhibitor of PI 3-kinase (Arcaro and Wymann, 1993; Powis *et al*, 1994), and PD98059, an inhibitor of MAP kinase kinase and, hence, MAP kinase (Alessi *et al*, 1995). Stock solutions of wortmannin and PD98059 were prepared in DMSO and stored at -20° C until use. Solutions of wortmannin were kept dark until required.

3T3-F442A adipocytes were incubated with hormones as described above in section 5.2.1.2.2. Wortmannin (100 nM) and PD98059 (50 μM) were added immediately following hormones and wortmannin was replenished every 8 h. The rate of lipogenesis was subsequently determined as described in section 2.2.2.9.

5.2.3 Assessment of the role of protein kinase C isoforms in the growth hormone- and insulin-mediated effects on lipid synthesis in 3T3-F442A adipocytes

3T3-F442A preadipocytes were grown and differentiated in 30 mm diameter wells. Adipocytes were treated with sense or antisense ODNs (20 μM) specific for

individual isoforms of PKC or PKD at day 8 of differentiation as described in section 2.2.2.4.2. (Insulin was effectively removed from the culture medium at this stage). For the first 6 h of treatment, adipocytes were incubated with appropriate ODN in combination with lipofectin reagent (40 μg/ml). The lipofectin was subsequently removed from the culture medium and incubations continued in the presence of ODN alone. After a further 42 h, cells were either used immediately for the preparation of lysates for assessment of immunoreactive PKC levels by immunoblotting or, alternatively, cells were incubated with hormones as desribed above in section 5.2.1.2.2 prior to the assessment of lipogenesis as described in section 2.2.2.9.

5.2.4 Statistical analysis

Results are presented as means ± S.E.M. and, unless stated otherwise, statistical analysis was by Student's t-test for paired samples.

5.3 Results

5.3.1 The effects of growth hormone and insulin on protein kinase C isoform expression in adipocytes

In order to gain an indication of the possible functional roles of PKC isoforms in fat cells, the involvement of individual isoforms in the signalling pathways utilised by GH and insulin in adipocytes was investigated. Initially, it was established whether GH and insulin affected the expression of adipocyte PKC isoforms.

To establish whether GH affected the expression of PKC isoforms *in vivo*, rats were treated with antiserum to rat GH to neutralise circulating levels of the hormone. In addition to the antiserum, some animals also received ovine GH. The purpose of these animals was to serve as a control against the non-specific effects of the antiserum. The effectiveness of treatments in changing serum GH concentrations was assessed by measuring changes in rat growth rate (Figure 5.1) and serum IGF-I concentration (Table 5.1). In addition, the effect of treatments on fat pad composition was examined (Table 5.1).

The total changes in body weight following treatments indicated that significant changes in serum GH levels were being achieved (Figure 5.1). Antiserum treated animals exhibited significant weight loss ($p < 0.001$) compared to control animals indicating that neutralisation of circulating GH was being achieved (Figure 5.1). This was confirmed by determination of serum IGF-I levels (Table 5.1). Antiserum-treated animals displayed significantly reduced ($p < 0.01$) levels of IGF-I compared to control animals and those treated with antiserum to rat GH in combination with oGH (Table 5.1). In accordance with others (Kilgour *et al*, 1995), it was found that neutralisation of circulating GH had no significant effect on the size or number of parametrial adipocytes (Table 5.1).

Simultaneous administration of oGH reversed the effect of antiserum treatment on rat growth rate (Figure 5.1), resulting in a weight gain greater that that of control animals ($p < 0.001$). This suggested that the oGH replacement regime achieved serum GH levels greater than those in control rats. Thus, PKC isoform expression could be

examined both in the absence of GH and in the presence of excess GH. Isolated adipocytes prepared from treated rats were subjected to immunoblotting with isoform-specific antibodies to PKC (Figure 5.2) and PKC isoform expression was quantified by densitometric analysis of immunoblots (Table 5.2).

Removal of circulating GH had no effect on the level of expression of PKC α, γ or δ in homogenates prepared from isolated rat adipocytes (Table 5.2). However, addition of excess GH suppressed significantly ($p < 0.05$) the levels of expression of these isoforms (Table 5.2), suggesting a possible threshold serum GH concentration, above which GH suppresses levels of PKC α, γ and δ in rat adipocytes.

On removal of circulating GH, levels of PKC ζ and μ were suppressed significantly ($p < 0.05$; Table 5.2), suggesting that GH enhances the expression of these PKC isoforms. In agreement with this, administration of excess GH reversed the effect of antiserum treatment on cellular levels of PKC ζ and μ (Table 5.2).

The situation with PKC ε was more complicated in that levels of expression were enhanced significantly ($p < 0.002$) in both the absence of GH and upon addition of excess GH (Table 5.2). Collectively, these results suggested that GH differentially regulates the expression of PKC isoforms in rat adipocytes.

The effects of GH on PKC isoform expression were further assessed *in vitro*. In addition, the effects of insulin on PKC isoform expression were examined *in vitro*. 3T3-F442A adipocytes were incubated in the absence of exogenous hormones for 16-18 h and then incubated in the presence or absence of 4.5 nM GH or 8.7 nM insulin for a period of 24 h. PKC isoform expression in adipocyte lysates was subsequently examined by immunoblotting (Figure 5.3) and quantified by densitometric analysis of immunoblots (Table 5.3). PKC isoform expression in adipocytes treated with GH or insulin did not differ significantly from that observed in control adipocytes incubated in the absence of hormones (Table 5.3). Thus, direct *in vitro* addition of GH or insulin to 3T3-F442A adipocytes did not effect significantly the expression of PKC isoforms. This suggested that GH and insulin did not exert their chronic effects in adipocytes *via* changes in the expression of PKC isoforms.

5.3.2 Characterisation of the effects of growth hormone and insulin on the rate of lipid synthesis in 3T3-F442A adipocytes

It is well established that chronic incubation with insulin increases the rate of lipid synthesis in the adipocyte whereas chronic incubation with GH both suppresses the basal rate of lipid synthesis and antagonises the ability of insulin to maintain or increase the basal rate (Vernon, 1982; Vernon and Flint, 1989; see Section 1.2.1.1.2). The involvement of PKC isoforms in the signalling pathway(s) utilised by GH and insulin to exert these effects on the rate of lipogenesis was investigated in 3T3-F442A adipocytes.

The effects of treatment with hormones on the rate of lipid synthesis were initially characterised. 3T3-F442A adipocytes were incubated with GH and insulin, either alone, or in combination, for 24 or 48 h and the rate of lipid synthesis determined by incorporation of radiolabelled acetate into cell lipid. Incubation with GH for 24 h reduced significantly ($p < 0.001$) the rate of lipogenesis to approximately 40 % of that observed in cells incubated in the absence of hormones (Figure 5.4). Incubation with insulin for 24 h increased significantly ($p < 0.001$) the rate of lipogenesis to approximately 160 % of control (Figure 5.4). Following incubation with GH and insulin in combination, no significant alteration in the rate of lipogenesis was observed (Figure 5.4), indicating that GH was antagonising the ability of insulin to stimulate lipogenesis. The effects of both GH and insulin on the rate of lipid synthesis were not enhanced further by treatment with hormones for 48 h (Figure 5.4). Hence, in all subsequent experiments, hormone incubations were carried out for a period of 24 h.

GH and insulin are known to stimulate the signalling pathways of MAP kinase and PI 3-kinase (Anderson, 1992; Kilgour *et al*, 1996; Denton and Tavare, 1995; Moule *et al*, 1995), both of which have been shown to involve isoforms of PKC (Cobb and Goldsmith, 1995; Nakanishi *et al*, 1993; Toker *et al*, 1994). It is, however, unclear whether these pathways are involved in the signalling mechanisms by which GH and insulin alter the rate of lipid synthesis in 3T3-F442A adipocytes. The involvement of the MAP kinase and PI 3-kinase pathways in the GH- and insulin-mediated effects on lipid synthesis in 3T3-F442A adipocytes was, therefore, investigated by making use

of the kinase inhibitors wortmannin and PD98059. When used at appropriate concentrations, wortmannin is a potent and relatively specific inhibitor of PI 3-kinase (Arcaro and Wymann, 1993; Powis *et al*, 1994). PD98059 has been shown to prevent the activation of MAP kinase by its activating kinase, MEK (Alessi *et al*, 1995). 3T3-F442A adipocytes were incubated in the absence of exogenous hormones for 16-18 h and then incubated with GH or insulin for 24 h in the presence of 100 nM wortmannin or 50 μM PD98059.

Following treatment with GH, the rate of lipogenesis in control adipocytes was reduced significantly ($p < 0.001$) to 37.1 ± 6.2 % of that observed in cells incubated in the absence of hormones (Table 5.4). Following treatment with insulin, the rate of lipogenesis was enhanced significantly ($p < 0.001$) to 165.5 ± 9.8 % of that observed in cells incubated in the absence of hormones (Table 5.4).

In the presence of wortmannin, the basal rate of lipogenesis was reduced significantly ($p < 0.001$) to 41.3 ± 4.5 % of that observed in control adipocytes (Table 5.4). Hence, the rate of lipogenesis following treatment with GH was reduced significantly ($p < 0.001$) in wortmannin-treated cells compared to control adipocytes (Table 5.4). However, treatment with wortmannin had no significant effect on the ability of GH to suppress the rate of lipogenesis. GH reduced the rate of lipogenesis to 52.7 ± 6.7 % and 41.3 ± 4.5 % of that observed in adipocytes incubated in the absence of hormones in wortmannin-treated cells and control adipocytes respectively (Table 5.4). This suggested that, although PI 3-kinase was required to maintain the basal rate of lipogenesis, this kinase was not required for the suppression of lipogenesis by GH.

Similarly, the rate of lipogenesis following treatment of adipocytes with insulin was reduced significantly ($p < 0.001$) in wortmannin-treated cells compared to control adipocytes incubated in the absence of inhibitors (Table 5.4). Insulin stimulated significantly ($p < 0.01$) the rate of lipogenesis in wortmannin-treated cells (Table 5.4). However, the stimulation by insulin was reduced slightly, but significantly ($p < 0.05$), to 80.4 ± 5.1 % of that observed in control adipocytes (Table 5.3). Thus, it would appear that insulin affects lipogenesis through multiple routes, one of which involves the PI 3-kinase pathway.

Incubation of cells with the MEK inhibitor PD98059 was found to have no effect on the basal rate of lipogenesis or on the ability of GH and insulin to suppress or stimulate the rate of lipogenesis respectively (Table 5.4). This suggested that MAP kinases are not involved in the signalling pathways utilised by GH or insulin to exert their effects on the rate of lipid synthesis in 3T3-F442A adipocytes.

5.3.3 The effects of depletion of individual protein kinase C isoforms with antisense oligodeoxynucleotides on growth hormone- and insulin-induced changes in the rate of lipid synthesis in 3T3-F442A adipocytes

To define whether PKC isoforms were required for the GH and insulin-mediated effects on the rate of lipid synthesis in 3T3-F442A adipocytes, antisense ODNs were used to deplete individual isoforms of PKC selectively from 3T3-F442A adipocytes prior to stimulation with hormones. 3T3-F442A adipocytes were treated with sense or antisense ODNs in combination with lipofectin reagent, or lipofectin was used alone as a control, for 6 h at day 8 of differentiation. Immunoblot analysis of adipocytes after 48 h revealed that over 90 % of the appropriate PKC isoform was depleted following treatment with the appropriate antisense ODN (Figure 5.5). PKC-depleted adipocytes were treated with GH or insulin, either alone, or in combination, for 24 h and the rate of lipid synthesis determined.

Treatment of cells with lipofectin alone had no significant effect on the stimulation of lipogenesis by insulin, the direct suppression of lipogenesis by GH or the ability of GH to antagonise the insulin-mediated stimulation of lipogenesis (Table 5.5). Similarly, incubation of 3T3-F442A adipocytes with the appropriate sense ODNs had no significant effect on the GH- and insulin-mediated effects on the rate of lipogenesis (Tables 5.6 A-F).

Following depletion of PKC α, δ or ζ, there was no significant effect on the suppression of lipogenesis by GH, the stimulation of lipogenesis by insulin or the ability of GH to antagonise the insulin-stimulated increase in the rate of lipogenesis (Tables 5.6 A, C and E). This suggested that these isoforms of PKC were not essential for GH or insulin to exert their effects on lipogenesis in 3T3-F442A adipocytes.

Following depletion of PKC γ, the rate of lipogenesis following treatment with either GH or insulin did not differ significantly from that observed in control adipocytes incubated with lipofectin alone (Table 5.6 B). In the absence of PKC γ, however, the rate of lipogenesis in cells incubated with GH and insulin in combination did not differ significantly from that observed when cells were incubated with insulin alone (Table 5.6 B). Thus, in the absence of PKC γ, GH did not antagonise the insulin-mediated increase in lipid synthesis. Therefore, in 3T3-F442A adipocytes, PKC γ does not appear to be required for the insulin-stimulated increase in lipogenesis or the inhibition of basal lipogenesis by GH. PKC γ appears to be essential, however, for the inhibition of insulin-stimulated lipogenesis by GH

Following depletion of PKC ε, the rate of lipogenesis following treatment with GH, either alone, or in combination with insulin, did not differ significantly from that observed in control adipocytes incubated with lipofectin alone (Table 5.6 D). This suggested that PKC ε is not essential for the inhibition of basal or insulin-stimulated lipogenesis by GH in 3T3-F442A adipocytes. In the absence of PKC ε, treatment with insulin stimulated significantly ($p < 0.05$) the rate of lipogenesis (Table 5.6 D). However, the rate of lipogenesis in insulin-stimulated cells depleted of PKC ε did not differ significantly from the basal rate of lipogenesis in cells treated with lipofectin alone (Table 5.6 D). This was due to a small, but significant ($p < 0.05$) reduction in the insulin response; the insulin-induced stimulation of lipogenesis in adipocytes depleted of PKC ε was reduced to 81.3 ± 3.4 % of that observed in control adipocytes treated with lipofectin alone (Table 5.6 D). Thus, in 3T3-F442A adipocytes, PKC ε appears to be one of the multiple components required for the stimulation of lipogenesis by insulin.

Following depletion of PKC μ, treatment with GH reduced significantly ($p < 0.05$) the rate of lipogenesis compared to that observed in adipocytes incubated in the absence of hormones (Table 5.6 F). However, the rate of lipogenesis in PKC μ-depleted cells following treatment with GH did not differ significantly from the basal rate of lipogenesis observed in control cells treated with lipofectin alone (Table 5.6 F). The suppression of lipogenesis by GH was, thus, reduced significantly to 51.1 ± 9.7 % of

that observed in control cells incubated with lipofectin alone (Table 5.6 F). That the suppression of lipogenesis by GH was reduced significantly in adipocytes depleted of PKC μ indicated that PKC μ is a component of the signalling mechanism by which GH suppresses the basal rate of lipogenesis in 3T3-F442A adipocytes and that other, PKC μ-independent pathways are also required.

More strikingly, the rate of lipogenesis in PKC μ-depleted adipocytes treated with insulin did not differ from that observed in cells incubated in the absence of hormones (Table 5.6 F). Thus, in the absence of PKC μ, insulin did not stimulate lipogenesis. This suggested that PKC μ is an essential component of the signalling mechanism by which insulin increases the rate of lipogenesis in 3T3-F442A adipocytes.

When PKC μ-depleted adipocytes were incubated with GH and insulin in combination, the rate of lipogenesis did not differ significantly from that observed following incubation of cells with insulin alone or following incubation in the absence of hormones (Table 5.6 F). Thus, GH did not alter the rate of lipogenesis in the presence of insulin. Since insulin does not alter the rate of lipogenesis in the absence of PKC μ, there is no insulin response for GH to antagonise. Thus, these results are likely to reflect the fact that the suppression of basal lipogenesis by GH requires PKC μ.

Antisense depletion of PKC μ reduced the direct suppression of lipogenesis by GH to approximately 50 % of that observed in control adipocytes (Table 5.6 F). In the absence of PKC γ, the direct suppression of lipogenesis by GH was reduced to approximately 60 % of that observed in control adipocytes. However, the effect of depletion of PKC γ on the suppression of lipogenesis by GH was found not to be significant. To investigate further the involvement of PKC γ in the signalling mechanism by which GH suppresses the basal rate of lipid synthesis, lipogenesis was examined in the absence of both PKC γ and PKC μ. 3T3-F442A adipocytes were incubated with antisense ODNs to PKC γ and PKC μ in combination and the rate of lipogenesis following hormone stimulation subsequently determined. Immunoblotting studies revealed that incubation of cells with PKC γ and PKC μ antisense ODNs in

combination resulted in complete depletion of the appropriate PKC isoforms (Figure 5.6). The rate of lipogenesis in PKC γ/μ-depleted adipocytes following GH-treatment, however, did not differ significantly from that observed in adipocytes depleted of PKC μ alone (Table 5.7 and Table 5.6 F). That the GH-response was not reduced further following depletion of PKC γ in addition to PKC μ indicated that PKC μ was important for the suppression of basal lipogenesis by GH and that PKC γ was not essential, as earlier studies had suggested

Thus, in 3T3-F442A adipocytes, PKC μ appears to be, at least in part required for the suppression of basal lipogenesis by GH and essential for the stimulation of lipogenesis by insulin. Given the importance of the results obtained using the antisense ODN to PKC μ, 3T3-F442A adipocytes were also treated with an antisense ODN to PKD, which is thought to be the murine homologue of this protein (Van Lint *et al*, 1995). PKC μ was originally identified in cells of human origin (Johannes *et al*, 1994). Thus, the ODN used to deplete PKC μ from murine 3T3-F442A adipocytes was derived from the human gene sequence. The PKD ODN was designed to hybridise specifically with bases within the murine (PKD) coding sequence which were not present within the human (PKC μ) coding sequence (see Section 2.1.4). Immunoblotting studies revealed that treatment of adipocytes with the PKD antisense ODN resulted in the complete depletion of the 112 kDa protein detected with the monoclonal antibody to PKC μ (Figure 5.7). Thus, treatment with either the PKC μ antisense ODN or the PKD antisense ODN resulted in depletion of the same 112 kDa protein from 3T3-F442A adipocytes. PKD-depleted cells were incubated with GH and insulin either alone, or in combination, and the rate of lipogenesis determined.

The rates of lipogenesis following treatment of PKD-depleted adipocytes with GH and insulin were found to be essentially identical to those observed following hormone treatment of PKC μ-depleted adipocytes (Table 5.8 and Table 5.6 F). Thus, the suppression of lipogenesis by GH was reduced to 48 ± 10.9 % of that observed in control cells incubated with lipofectin alone (Table 5.8), suggesting a role for PKD in the GH-mediated suppression of lipogenesis in 3T3-F442A adipocytes. In the absence of PKD, insulin was unable to stimulate lipogenesis (Table 5.8) suggesting that PKD is essential for the insulin-mediated stimulation of lipogenesis in 3T3-F442A

adipocytes. That the rate of lipogenesis in cells incubated with GH and insulin in combination did not differ significantly from that observed in cells incubated with insulin alone (Table 5.8), is likely to reflect the reduced GH response in these cells since insulin has no effect on lipogenesis in the absence of PKD.

Thus, the PKD antisense studies confirmed that the murine PKC μ/PKD protein was, at least in part, required for the direct suppression of lipogenesis by GH and essential for the stimulation of lipogenesis by insulin. In addition, the results of these studies suggest that PKC μ and PKD are indeed homologues of the same protein.

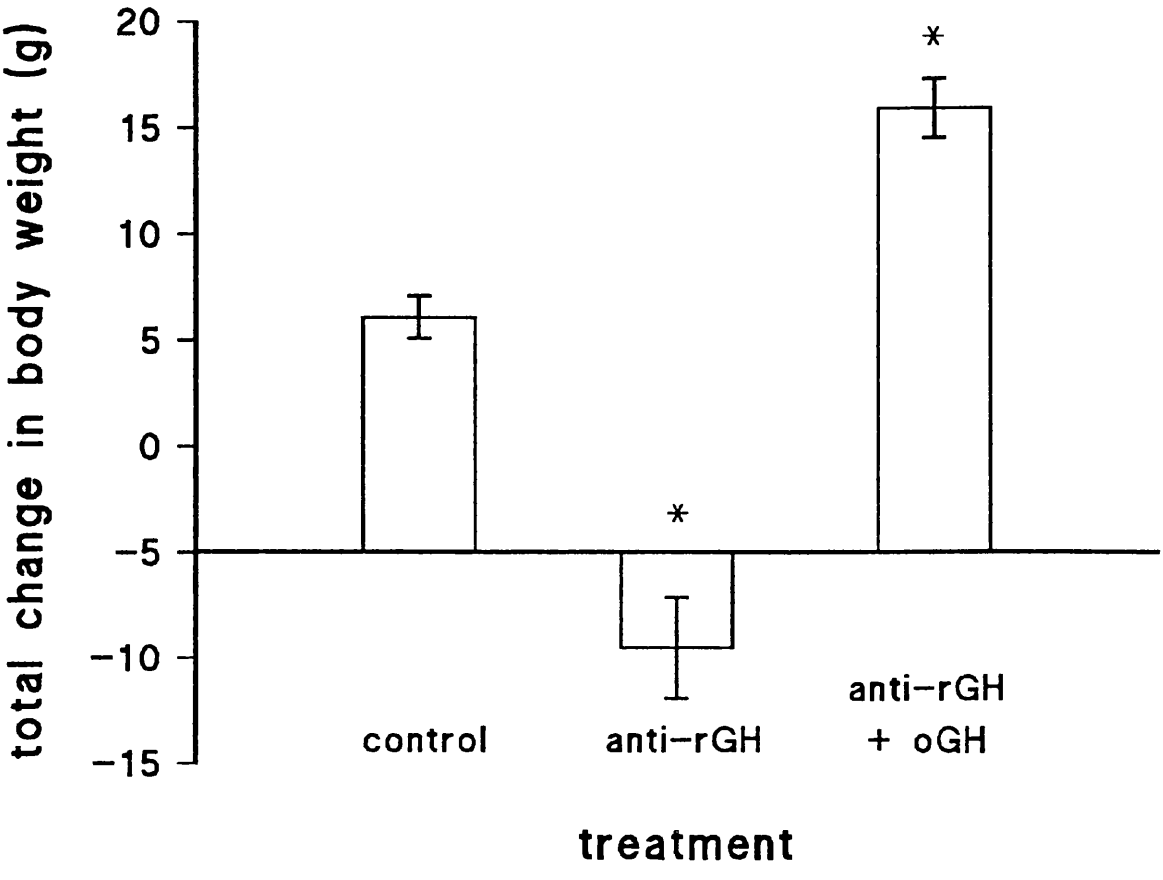


Figure 5.1 Body weight changes in rats treated with antiserum to rat growth hormone
Rats were treated with antiserum to rat GH (anti-rGH), either alone, or in combination with ovine GH (oGH) twice daily for four days as described in section 5.2.1.1.1. Control animals received carrier solutions. Body weights were taken prior to the commencement of treatments and following their completion and the difference calculated. Results are means ± SEM from eight rats. * indicates the value is significantly different from that for control animals, $p < 0.001$. Statistical analysis was by Student's t-test for unpaired samples.

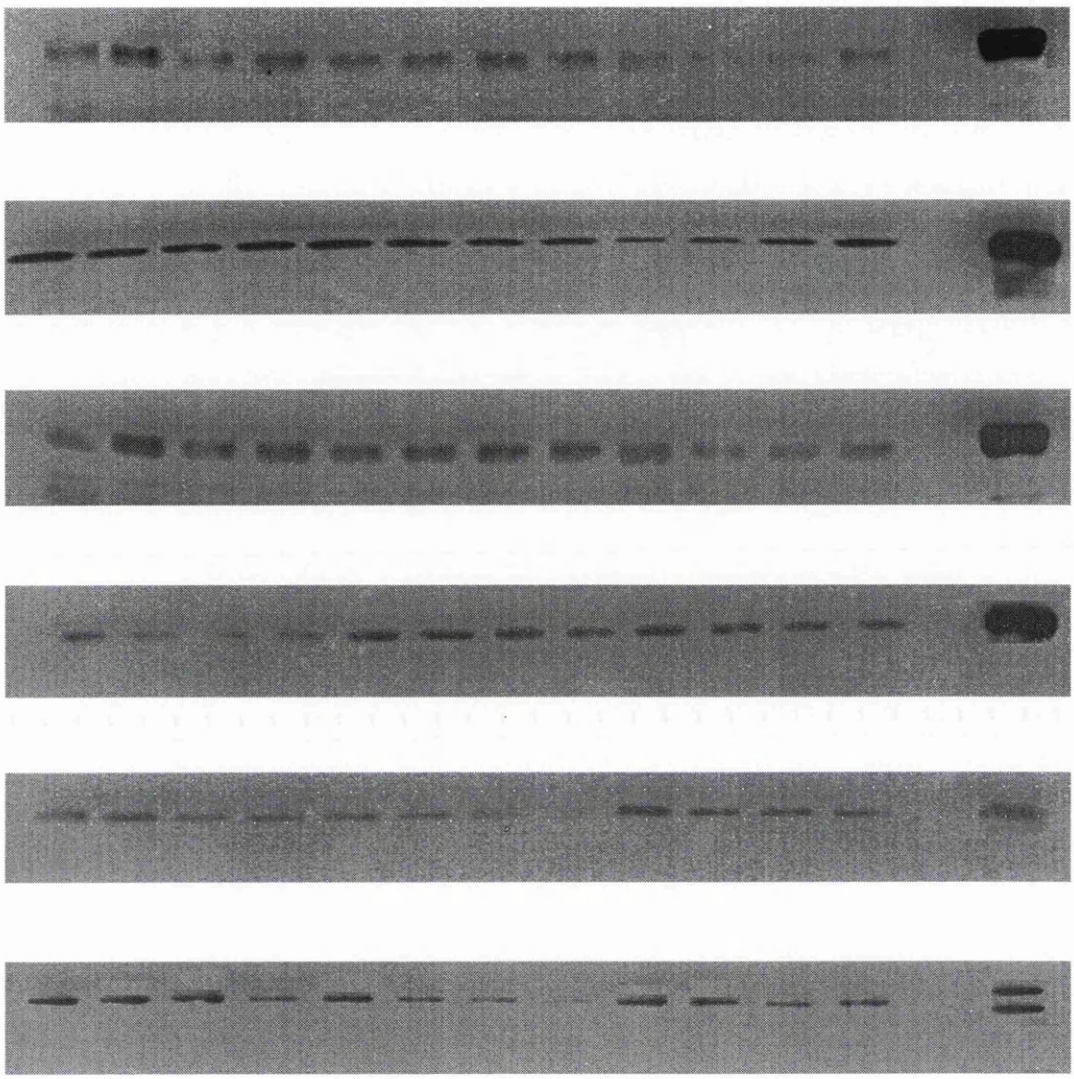
Table 5.1 Fat pad composition and serum IGF-1 levels in rats treated with antiserum to rat growth hormone

Rats were treated with antiserum to rat GH (Anti-rGH), either alone, or in combination with ovine GH (+ oGH) twice daily for four days as described in section 5.2.1.1.1. Control animals received carrier solutions. Subsequently, parametrial fat pads were removed and the fat pad composition determined as described in section 5.2.1.1.3. Samples of blood were also taken for the determination of serum IGF-1 levels as described in section 5.2.1.1.4. Results are means ± S.E.M from four rats. * indicates the value differs significantly from that observed for control animals, $p < 0.001$. Statistical analysis was by Student's t-test for unpaired samples.

		Fat pad composition		
Treatment	Serum IGF-1 (ng/ml)	Mean cell volume (pl)	Fat pad weight (g)	Adipocytes per fat pad (x 10^6)
Control	167.8 ± 14.9	165.4 ± 9	2.01 ± 0.2	4.40 ± 0.34
Anti-rGH	54.4 ± 4.7*	222.1 ± 30	1.97 ± 0.1	2.70 ± 0.64
Anti-rGH + oGH	198.9 ± 17.1	171.6 ± 20	2.33 ± 0.2	4.95 ± 1.43

Figure 5.2 Protein kinase C isoform expression in rats treated with antiserum to rat growth hormone

Homogenates were prepared from the isolated adipocytes of rats that had been treated with antiserum to rat GH alone (Anti-rGH; lanes 5-8), or antiserum to rat GH in combination with ovine GH (+ oGH; lanes 9-12) and control rats which had been treated with carrier solutions (lanes 1-4). Equal quantities (50 μg) of homogenate protein were subjected to immunoblotting with isoform-specific monoclonal antibodies to PKC alongside 50 μg of homogenate protein prepared from rat brain (RB; lane 13) that served as a positive antibody control. Results are representative of four separate experiments carried out on different cell preparations.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δ
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μ
1 2 3 4 5 6 7 8 9 10 11 12 13

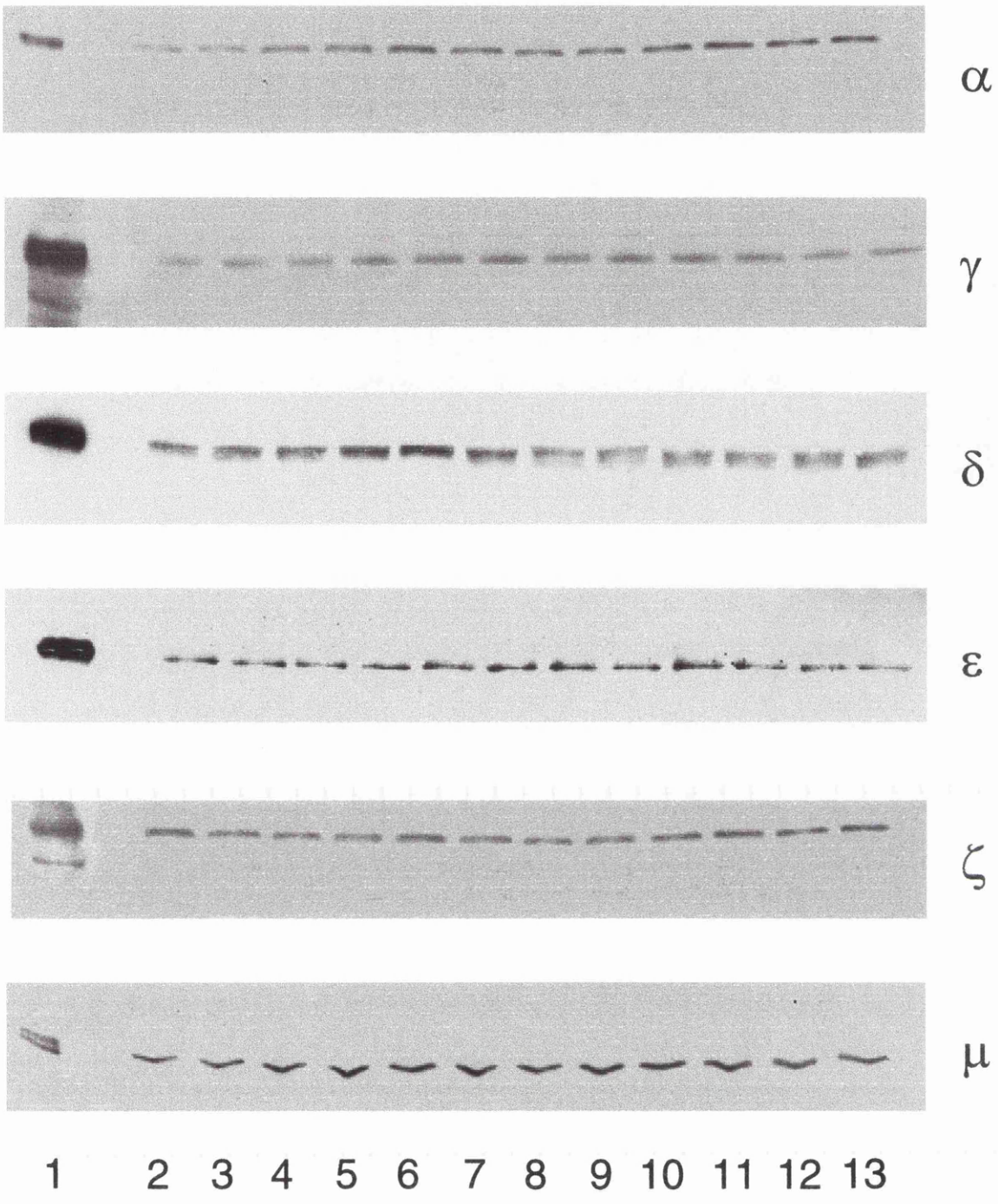
Table 5.2 Cellular levels of protein kinase C isoforms in rats treated with antiserum to rat growth hormone

Rats were treated with antiserum to rat GH (Anti-rat GH), either alone, or in combination with ovine GH (+ oGH) twice daily for four days as described in section 5.2.1.1.1. Control animals received carrier solutions. Homogenates were prepared from isolated adipocytes and subjected to immunoblotting with isoform-specific monoclonal antibodies to PKC. Data are expressed as arbitrary units following densitometric analysis of immunoblots. Results are means ± S.E.M. of four observations. * and ** indicate the value is significantly different from that for control animals, $p < 0.05$ and $p < 0.002$ respectively. Statistical analysis was by analysis of variance.

	PKC isoform expression (arbitrary units)			
	Treatment			
Isoform	Control	Anti-rat GH	Anti-rat GH + oGH	S.E.M
α	203.51	139.75	57.31*	26.86
γ	723.90	583.00	354.20*	112.4
δ	702.50	516.60	277.60*	126.8
ε	89.22	232.98**	195.38**	16.15
ζ	104.26	65.97*	128.61	12.00
μ	418.80	159.10*	305.90	69.82

Figure 5.3 The effect of growth hormone and insulin on protein kinase C isoform expression in 3T3-F442A adipocytes

3T3-F442A adipocytes were incubated for 16-18 h in the absence of exogenous hormones and for a further 24 h in the absence of hormones (No Additions; lanes 2-5) or in the presence of 4.5 nM GH (lanes 6-9) or 8.7 nM insulin (INS; lanes 10-13). Cellular lysates were subsequently prepared and equal quantites (50 μg) of lysate protein were subjected to immunoblotting with isoform-specific monoclonal antibodies to PKC alongside 50 μg of homogenate protein prepared from rat brain (RB; lane 1) that served as a positive antibody control. Immunoblots are representative of results obtained on at least six occasions with different cell preparations.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1 2 3 4 5 6 7 8 9 10 11 12 13

Table 5.3 The effect of growth hormone and insulin on cellular levels of protein kinase C isoforms in 3T3-F442A adipocytes

3T3-F442A adipocytes were incubated for 16-18 h in the absence of exogenous hormones and for a further 24 h in the absence of hormones or in the presence of 4.5 nM GH or 8.7 nM insulin. Cellular lysates were subsequently prepared and subjected to immunoblotting with isoform-specific monoclonal antibodies to PKC. Following densitometric analysis of immunoblots, changes in expression of PKC isoforms are shown as a percentage of the expression level in control adipocytes incubated in the absence of hormones (100 %). Results are means ± S.E.M. of six separate experiments carried out on different cell preparations.

	PKC isoform expression [% of control (= untreated adipocytes; 100 %)]	
	Hormone treatment	
Isoform	Growth hormone	Insulin
α	109.7 ± 4.8	129.6 ± 14.2
γ	149.8 ± 28.8	116.1 ± 23.85
δ	98.9 ± 2.2	74.5± 12.1
ε	179.4 ± 65.9	132.3 ± 21.4
ζ	91.1 ± 7.8	77.4 ± 19.6
μ	125.3 ± 14.7	103.9 ± 5.6

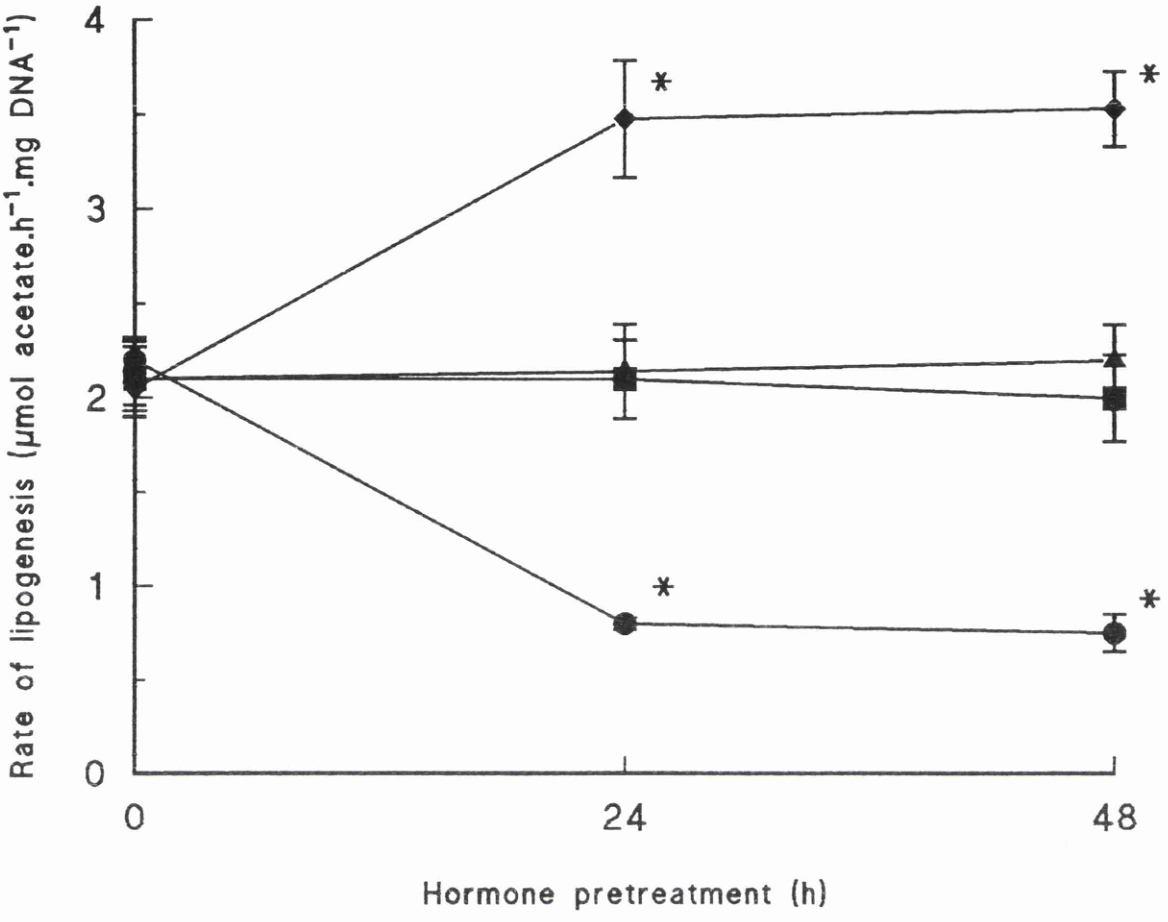

Figure 5.4 The effect of growth hormone and insulin on the rate of lipogenesis in 3T3-F442A adipocytes

3T3-F442A adipocytes were incubated for 16-18 h in the absence of exogenous hormones and for 24 or 48 h in the absence of hormones (■) or in the presence of 4.5 nM GH (●) or 8.7 nM insulin (◆), either alone, or in combination (▲). Subsequently the rate of lipogenesis was determined as described in section 2.2.2.9. Results are means ± S.E.M. of six separate observations carried out on different cell preparations. * indicates the value differs significantly from that for control adipocytes incubated in the absence of hormones, $p < 0.001$. Statistical analysis was by Student's t-test for paired samples.

Table 5.4 The effect of wortmannin and PD98059 on growth hormone- and insulin-induced changes in the rate of lipogenesis in 3T3-F442A adipocytes

3T3-F442A adipocytes were incubated for 16-18 h in the absence of exogenous hormones and for 24 h in the absence of hormones (No additions) or in the presence of 4.5 nM GH or 8.7 nM insulin (INS). Incubations were carried out in the absence of inhibitors (control), or in the presence of 100 nM wortmannin or 50 μM PD98059. Subsequently, the rate of lipogenesis was determined as described in section 2.2.2.9. Results are means ± S.E.M. of six separate observations carried out on different cell preparations. The rate of lipogenesis following hormone treatment as a percentage of the rate of lipogenesis in control adipocytes incubated in the absence of hormones (100 %) is indicated in parenthesis. * indicates the rate of lipogenesis differs significantly from that for control adipocytes incubated in the absence of inhibitors, $p < 0.001$. • indicates the effect of hormone treatment differs significantly from that observed for control adipocytes incubated in the absence of inhibitors, $p < 0.05$. Statistical analysis was by Student's t-test for paired samples.

	Rate of Lipogenesis (μmol acetate.h^{-1}.mg DNA^{-1})		
	Inhibitor		
Hormone treatment	Control	Wortmannin	PD98059
No Additions	2.22 ± 0.29 (100 %)	0.91 ± 0.23* (100 %)	2.01 ± 0.34 (100 %)
GH	0.78 ± 0.05 (41.3 ± 4.5 %)	0.48 ± 0.12* (52.7 ± 6.7 %)	0.74 ± 0.11 (39.2 ± 5.2 %)
INS	3.76 ± 0.33 (169.1 ± 10.2 %)	1.27 ± 0.25* (139.4 ± 3.4 %•)	3.52 ± 0.81 (175.5 ± 12.4 %)

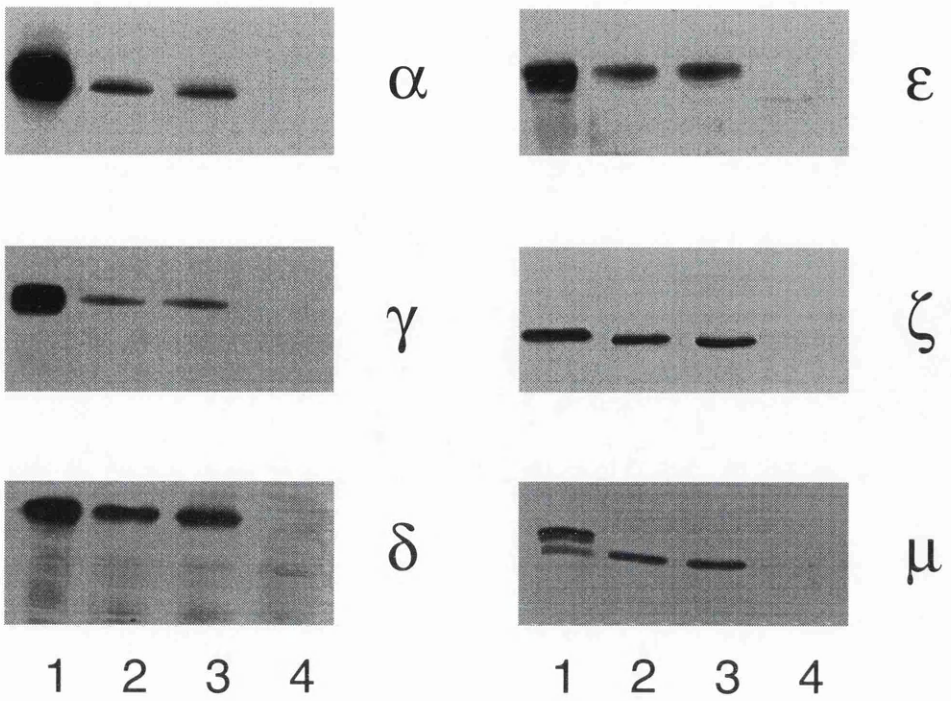

Figure 5.5 Specific depletion of protein kinase C isoforms in 3T3-F442A adipocytes by antisense oligodeoxynucleotide treatment

3T3-F442A adipocytes were preincubated with ODNs for 48 h prior to the preparation of lysates for the assessment of immunoreactive PKC isoforms by immunoblotting. Immunoblots show the amount of each indicated isoform in 25 μg of homogenate prepared from rat brain (lane 1), control adipocytes incubated with lipofectin alone (lane 2), 3T3-F442A adipocytes treated with the appropriate sense ODN (lane 3) and 3T3-F442A adipocytes treated with the appropriate antisense ODN (lane 4). Immunoblots are representative of results obtained on at least four occasions for each PKC isoform.

Table 5.5 The effect of lipofectin on growth hormone- and insulin-induced changes in the rate of lipogenesis in 3T3-F442A adipocytes

3T3-F442A adipocytes were preincubated in the presence or absence (control) of lipofectin reagent. Subsequently, adipocytes were incubated for 24 h in the absence of hormones (No additions) or in the presence of 4.5 nM GH or 8.7 nM insulin (INS), either alone, or in combination and the rate of lipogenesis determined as described in section 2.2.2.9. Results are means ± S.E.M. of six separate observations carried out on different cell preparations. The rate of lipogenesis following hormone treatment as a percentage of the rate of lipogenesis in control adipocytes incubated in the absence of hormones (100 %) is indicated in parenthesis.

	Rate of Lipogenesis (μmol acetate.h^{-1}.mg DNA^{-1})	
	Pretreatment	
Hormone	None	Lipofectin
No Additions	2.14 ± 0.20 (100 %)	2.21 ± 0.28 (100%)
GH	0.76 ± 0.06 (36.7 ± 5.9 %)	0.80 ±0.09 (37.1 ± 6.1 %)
INS	3.51 ± 0.41 (166.5 ± 18.4 %)	3.60 ± 0.48 (163.7 ± 17.6 %)
GH + INS	2.29 ± 0.19 (108.4 ± 9.1 %)	2.31 ± 0.21 (106.2 ± 10.7 %)

Table 5.6 A The effect of protein kinase C α-specific oligodeoxynucleotides on growth hormone- and insulin-induced changes in the rate of lipogenesis in 3T3-F442A adipocytes

3T3-F442A adipocytes were preincubated with sense or antisense ODNs to PKC α, or lipofectin alone (Control), prior to stimulation with hormones. During the final 16-18 h of the ODN treatment period, exogenous hormones were removed from the culture medium. Subsequently, adipocytes were incubated for 24 h in the absence of hormones (No Additions) or in the presence of 4.5 nM GH or 8.7 nM insulin (INS), either alone, or in combination and the rate of lipogenesis determined as described in section 2.2.2.9. Results are means ± SEM of four separate observations carried out on different cell preparations. The rate of lipogenesis following hormone treatment as a percentage of the rate of lipogenesis in control adipocytes incubated in the absence of hormones (100 %) is indicated in parenthesis. The values for individual PKC isoforms that differ significantly ($p < 0.05$) carry different superscripts. Statistical analysis was by analysis of variance.

	Rate of Lipogenesis (μmol acetate.h^{-1}.mg DNA^{-1})			
	Oligodeoxynucleotide pretreatment			
Hormone treatment	Control	Sense	Antisense	S.E.M
No Additions	2.10^{a} (100 %)	2.12^{a} (100 %)	2.22^{a} (100 %)	0.16
GH	0.80^{b} (39.1 ± 5.2 %)	0.91^{b} (41.2 ± 6.4 %)	0.91^{b} (40.7 ± 3.7)	0.16
INS	3.48^{c} (168.3 ± 17.5 %)	3.35^{c} (155.8 ± 15.6 %)	3.27^{c} (147.4 ± 11.8 %)	0.16
GH + INS	2.40^{a} (115.3 ± 11.2 %)	2.51^{a} (117.1 ± 12.1 %)	2.71^{a} (102.0 ± 1.3 %)	0.16

Table 5.6 B The effect of protein kinase C γ-specific oligodeoxynucleotides on growth hormone- and insulin-induced changes in the rate of lipogenesis in 3T3-F442A adipocytes

3T3-F442A adipocytes were preincubated with sense or antisense ODNs to PKC γ, or lipofectin alone (Control), prior to stimulation with hormones. During the final 16-18 h of the ODN treatment period, exogenous hormones were removed from the culture medium. Subsequently, adipocytes were incubated for 24 h in the absence of hormones (No Additions) or in the presence of 4.5 nM GH or 8.7 nM insulin (INS), either alone, or in combination and the rate of lipogenesis determined as described in section 2.2.2.9. Results are means ± SEM of four separate observations carried out on different cell preparations. The rate of lipogenesis following hormone treatment as a percentage of the rate of lipogenesis in control adipocytes incubated in the absence of hormones (100 %) is indicated in parenthesis. The values for individual PKC isoforms that differ significantly ($p < 0.05$) carry different superscripts. ** indicates the effect of hormone treatment differs significantly from that observed for control adipocytes incubated with lipofectin alone, $p < 0.01$. Statistical analysis was by analysis of variance.

	Rate of Lipogenesis (μmol acetate.h^{-1}.mg DNA^{-1})			
	Oligodeoxynucleotide pretreatment			
Hormone treatment	Control	Sense	Antisense	S.E.M
No Additions	2.00^{a} (100 %)	1.94^{a} (100 %)	1.70^{a} (100 %)	0.18
GH	0.75^{b} (35.4 ± 6.6 %)	0.82^{b} (39.8 ± 7.5 %)	1.02^{b} (60.5 ± 8.5 %)	0.18
INS	3.51^{c} (170.4 ± 16.1 %)	3.54^{c} (180.4 ± 18.9 %)	3.38^{c} (201.7 ± 24.7 %)	0.18
GH + INS	2.20^{a} (123.4 ± 7.1 %)	2.17^{a} (114.8 ± 8.7 %)	3.03^{c} (181.1 ± 17.1 %**)	0.18

Table 5.6 C The effect of protein kinase C δ-specific oligodeoxynucleotides on growth hormone- and insulin-induced changes in the rate of lipogenesis in 3T3-F442A adipocytes

3T3-F442A adipocytes were preincubated with sense or antisense ODNs to PKC δ, or lipofectin alone (Control), prior to stimulation with hormones. During the final 16-18 h of the ODN treatment period, exogenous hormones were removed from the culture medium. Subsequently, adipocytes were incubated for 24 h in the absence of hormones (No Additions) or in the presence of 4.5 nM GH or 8.7 nM insulin (INS), either alone, or in combination and the rate of lipogenesis determined as described in section 2.2.2.9. Results are means ± SEM of four separate observations carried out on different cell preparations. The rate of lipogenesis following hormone treatment as a percentage of the rate of lipogenesis in control adipocytes incubated in the absence of hormones (100 %) is indicated in parenthesis. The values for individual PKC isoforms that differ significantly ($p < 0.05$) carry different superscripts. Statistical analysis was by analysis of variance.

	Rate of Lipogenesis (μmol acetate.h^{-1}.mg DNA^{-1})			
	Oligodeoxynucleotide pretreatment			
Hormone treatment	Control	Sense	Antisense	S.E.M
No Additions	2.02^{a} (100 %)	2.21^{a} (100 %)	2.44^{a} (100 %)	0.17
GH	0.76^{b} (38.7 ± 5.3 %)	0.93^{b} (41.9 ± 4.8 %)	0.97^{b} (40.8 ± 5.0 %)	0.17
INS	3.29^{c} (167.3 ± 17.1 %)	3.51^{c} (160.2 ± 15.4 %)	3.53^{c} (147.2 ± 11.2 %)	0.17
GH + INS	2.05^{a} (103.2 ± 10.8 %)	2.32^{a} (105.3 ± 11.2 %)	2.44^{a} (100.0 ± 2.2 %*)	0.17

Table 5.6 D The effect of protein kinase C ε-specific oligodeoxynucleotides on growth hormone- and insulin-induced changes in the rate of lipogenesis in 3T3-F442A adipocytes

3T3-F442A adipocytes were preincubated with sense or antisense ODNs to PKC ε, or lipofectin alone (Control), prior to stimulation with hormones. During the final 16-18 h of the ODN treatment period, exogenous hormones were removed from the culture medium. Subsequently, adipocytes were incubated for 24 h in the absence of hormones (No Additions) or in the presence of 4.5 nM GH or 8.7 nM insulin (INS), either alone, or in combination and the rate of lipogenesis determined as described in section 2.2.2.9. Results are means ± SEM of four separate observations carried out on different cell preparations. The rate of lipogenesis following hormone treatment as a percentage of the rate of lipogenesis in control adipocytes incubated in the absence of hormones (100 %) is indicated in parenthesis. The values for individual PKC isoforms that differ significantly ($p < 0.05$) carry different superscripts. * indicates the effect of hormone treatment differs significantly from that observed for control adipocytes incubated with lipofectin alone, $p < 0.05$. Statistical analysis was by analysis of variance.

	Rate of Lipogenesis (μmol acetate.h^{-1}.mg DNA^{-1})			
	Oligodeoxynucleotide pretreatment			
Hormone treatment	Control	Sense	Antisense	S.E.M
No Additions	2.48^{ad} (100 %)	2.45^{ad} (100 %)	1.96^{a} (100 %)	0.28
GH	0.74^{b} (30.8 ± 3.0 %)	0.92^{b} (35.7 ± 4.6 %)	0.86^{b} (45.2 ± 9.2 %)	0.28
INS	3.93^{c} (163.5 ± 7.2 %)	3.75^{c} (160.1 ± 5.8 %)	2.77^{d} (138.1 ± 1.3 %*)	0.28
GH + INS	2.75^{ad} (108.0 ± 12.0 %)	2.77^{ad} (114.2 ± 11.2 %)	2.03^{a} (103.5 ± 4.4 %)	0.28

Table 5.6 E The effect of protein kinase C ζ-specific oligodeoxynucleotides on growth hormone- and insulin-induced changes in the rate of lipogenesis in 3T3-F442A adipocytes

3T3-F442A adipocytes were preincubated with sense or antisense ODNs to PKC ζ, or lipofectin alone (Control), prior to stimulation with hormones. During the final 16-18 h of the ODN treatment period, exogenous hormones were removed from the culture medium. Subsequently, adipocytes were incubated for 24 h in the absence of hormones (No Additions) or in the presence of 4.5 nM GH or 8.7 nM insulin (INS), either alone, or in combination and the rate of lipogenesis determined as described in section 2.2.2.9. Results are means ± SEM of four separate observations carried out on different cell preparations. The rate of lipogenesis following hormone treatment as a percentage of the rate of lipogenesis in control adipocytes incubated in the absence of hormones (100 %) is indicated in parenthesis. The values for individual PKC isoforms that differ significantly ($p < 0.05$) carry different superscripts. Statistical analysis was by analysis of variance.

	Rate of Lipogenesis (μmol acetate.h^{-1}.mg DNA^{-1})			
	Oligodeoxynucleotide pretreatment			
Hormone treatment	Control	Sense	Antisense	S.E.M
No Additions	2.40^{a} (100 %)	2.44^{a} (100 %)	2.46^{a} (100 %)	0.26
GH	0.80^{b} (36.7 ± 0.6 %)	0.93^{b} (39.1 ± 5.1 %)	0.92^{b} (39.5 ± 9.6 %)	0.26
INS	3.98^{c} (166.5 ± 18.3 %)	3.87^{c} (161.3 ± 17.2 %)	3.61^{c} (147.5 ± 12.1 %)	0.26
GH + INS	2.86^{a} (119.8 ± 10.5 %)	2.63^{a} (109.3 ± 11.1 %)	2.37^{a} (95.6 ± 2.5 %)	0.26

Table 5.6 F The effect of protein kinase C μ-specific oligodeoxynucleotides on growth hormone- and insulin-induced changes in the rate of lipogenesis in 3T3-F442A adipocytes

3T3-F442A adipocytes were preincubated with sense or antisense ODNs to PKC μ, or lipofectin alone (Control), prior to stimulation with hormones. During the final 16-18 h of the ODN treatment period, exogenous hormones were removed from the culture medium. Subsequently, adipocytes were incubated for 24 h in the absence of hormones (No Additions) or in the presence of 4.5 nM GH or 8.7 nM insulin (INS), either alone, or in combination and the rate of lipogenesis determined as described in section 2.2.2.9. Results are means ± SEM of four separate observations carried out on different cell preparations. The rate of lipogenesis following hormone treatment as a percentage of the rate of lipogenesis in control adipocytes incubated in the absence of hormones (100 %) is indicated in parenthesis. The values for individual PKC isoforms that differ significantly ($p < 0.05$) carry different superscripts. ** and *** indicate the effect of hormone treatment differs significantly from that observed for control adipocytes incubated with lipofectin alone, $p < 0.01$ and $p < 0.001$ respectively. Statistical analysis was by analysis of variance.

	Rate of Lipogenesis (μmol acetate.h^{-1}.mg DNA^{-1})			
	Oligodeoxynucleotide pretreatment			
Hormone treatment	Control	Sense	Antisense	S.E.M
No Additions	2.59^{ad} (100 %)	2.61^{ad} (100 %)	3.13^{ac} (100 %)	0.29
GH	0.78^{b} (31.3 ± 3.1 %)	0.93^{b} (36.3 ± 5.7 %)	2.05^{d} (65.2 ± 6.1 %**)	0.29
INS	3.90^{c} (151.9 ± 13.2 %)	3.92^{c} (150.9 ± 11.3 %)	3.00^{a} (96.0 ± 8.6 %***)	0.29
GH + INS	2.95^{a} (112.4 ± 9.9 %)	2.77^{a} (108.8 ± 7.6 %)	2.62^{ad} (83.9 ± 1.9 %)	0.29

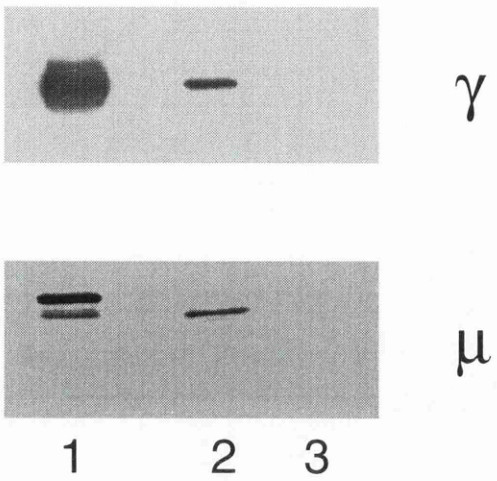

Figure 5.6 Specific depletion of protein kinase C γ and protein kinase C μ in 3T3-F442A adipocytes by combined antisense oligodeoxynucleotide treatment

3T3-F442A adipocytes were preincubated with sense or antisense ODNs to PKC γ and PKC μ in combination. Subsequently, cellular lysates were prepared and subjected to immunoblotting with isoform-specific monoclonal antibodies to PKC γ and PKC μ. Immunoblots show the amount of PKC γ and PKC μ in 25 μg of homogenate prepared from rat brain (lane 1), 3T3-F442A adipocytes treated with sense ODNs (lane 2) and 3T3-F442A adipocytes treated with antisense ODNs (lane 3). Immunoblots are representative of results obtained on at least four occasions using different cell preparations.

Table 5.7 The effect of treatment with protein kinase C γ- and μ-specific oligodeoxynucleotides in combination on growth hormone- and insulin-induced changes in the rate of lipogenesis in 3T3-F442A adipocytes

3T3-F442A adipocytes were preincubated with sense or antisense ODNs to PKC γ and PKC μ in combination, or lipofectin alone (Control), prior to stimulation with hormones. During the final 16-18 h of the ODN treatment period, exogenous hormones were removed from the culture medium. Subsequently, adipocytes were incubated for 24 h in the absence of hormones (No Additions) or in the presence of 4.5 nM GH or 8.7 nM insulin (INS), either alone, or in combination and the rate of lipogenesis determined as described in section 2.2.2.9. Results are means ± SEM of four separate observations carried out on different cell preparations. The rate of lipogenesis following hormone treatment as a percentage of the rate of lipogenesis in control adipocytes incubated in the absence of hormones (100 %) is indicated in parenthesis. The values for individual PKC isoforms that differ significantly ($p < 0.05$) carry different superscripts. ** and *** indicate the effect of hormone treatment differs significantly from that observed for control adipocytes incubated with lipofectin alone, $p < 0.01$ and $p < 0.001$ respectively. Statistical analysis was by analysis of variance.

	Rate of Lipogenesis (μmol acetate.h^{-1}.mg DNA^{-1})			
	Oligodeoxynucleotide pretreatment			
Hormone treatment	Control	Sense	Antisense	S.E.M
No Additions	2.53^{ad} (100 %)	2.49^{ad} (100 %)	2.63^{a} (100 %)	0.19
GH	0.73^{b} (29.5 ± 2.3 %)	0.80^{b} (32.2 ± 4.6 %)	1.99^{d} (75.3 ± 9.5 %**)	0.19
INS	4.08^{c} (165.6 ± 13.1 %)	4.10^{c} (166.9 ± 12.1 %)	2.66^{a} (104.7 ± 5.0 %***)	0.19
GH + INS	2.87^{a} (111.6 ± 10.6 %)	2.81^{a} (114.1 ± 11.2 %)	2.59^{a} (98.6 ± 1.5 %)	0.19

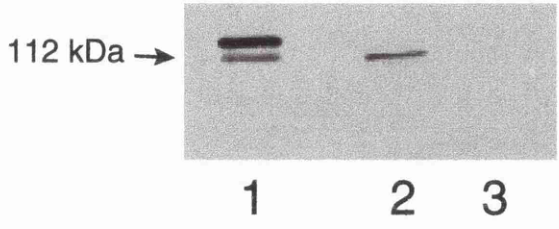

Figure 5.7 The effect of protein kinase D-specific oligodeoxynucleotides on expression of the 112 kDa protein detected with the monoclonal antibody specific for protein kinase C μ

3T3-F442A adipocytes were preincubated with sense or antisense ODNs to PKD. Subsequently, cellular lysates were prepared and subjected to immunoblotting with a PKC μ-specific monoclonal antibody. Immunoblots show the amount of the 112 kDa protein detected with the PKC μ antibody in 25 μg of homogenate prepared from rat brain (lane 1), 3T3-F442A adipocytes treated with the sense ODN to PKD (lane 2) and 3T3-F442A adipocytes treated with the antisense ODN to PKD (lane 3). The immunoblot is representative of results obtained on at least four occasions using different cell preparations.

Table 5.8 The effect of protein kinase D-specific oligodeoxynucleotides on growth hormone- and insulin-induced changes in the rate of lipogenesis in 3T3-F442A adipocytes

3T3-F442A adipocytes were preincubated with sense or antisense ODNs to PKD, or lipofectin alone (control), prior to stimulation with hormones. During the final 16-18 h of the ODN treatment period, exogenous hormones were removed from the culture medium. Subsequently, adipocytes were incubated for 24 h in the absence of hormones (No additions) or in the presence of 4.5 nM GH or 8.7 nM insulin (INS), either alone, or in combination and the rate of lipogenesis determined as described in section 2.2.2.9. Results are means ± SEM of four separate observations carried out on different cell preparations. The rate of lipogenesis following hormone treatment as a percentage of the rate of lipogenesis in control adipocytes incubated in the absence of hormones (100 %) is indicated in parenthesis. Values that differ significantly ($p < 0.05$) carry different superscripts. ** and *** indicate the effect of hormone treatment differs significantly from that observed for control adipocytes incubated with lipofectin alone, $p < 0.01$ and $p < 0.001$ respectively. Statistical analysis was by analysis of variance.

	Rate of Lipogenesis (μmol acetate.h^{-1}.mg DNA^{-1})			
	Oligodeoxynucleotide pretreatment			
Hormone treatment	Control	Sense	Antisense	S.E.M
No Additions	2.73^{ad} (100 %)	2.69^{ad} (100 %)	3.21^{ac} (100 %)	0.30
GH	0.81^{b} (31.4 ± 4.5 %)	0.79^{b} (30.9 ± 3.2 %)	2.22^{d} (65.5 ± 5.2 %**)	0.30
INS	4.12^{c} (153.2 ± 14.0 %)	4.10^{c} (152.8 ± 13.7 %)	3.14^{a} (95.8 ± 7.8 %***)	0.30
GH + INS	3.14^{a} (113.5 ± 10.4 %)	3.12^{a} (116.1 ± 11.1 %)	2.85^{ad} (85.8 ± 1.7 %)	0.30

5.4 Discussion

A number of studies suggest that PKC isoforms may assume distinct functional roles in adipocyte regulation. For example, studies with preadipocyte cell lines have revealed that PKC isoform expression is altered as a result of the differentiation of preadipocytes into adipocytes (Frevert and Khan, 1996; McGowan *et al*, 1996; data presented in Chapter four). In addition, selective changes in the expression of adipocyte PKC isoforms have been associated with diseased states such as obesity (Frevert and Khan, 1996). The metabolic pathways in the adipocyte are strictly regulated by a number of hormones. In order to investigate the possible functional roles of PKC isoforms in adipocytes, their involvement in the signalling pathways by which two important hormonal regulators of adipocyte metabolism, GH and insulin, alter the rate of lipogenesis was investigated using the 3T3-F442A adipocyte cell model.

Several studies suggest that the mechanisms by which GH exerts its metabolic effects in the adipocyte are, at least in part, mediated by one or more isoforms of PKC (see Section 1.6.3.4). In order to investigate the involvement of PKC isoforms in the GH-regulated signalling pathways in the adipocyte, the effects of GH on the expression of adipocyte PKC isoforms were examined. PKC isoform expression was examined initially *in vivo* in rat adipocytes in the absence of GH or in the presence of excess GH.

It was found that GH differentially regulated the expression of PKC isoforms *in vivo* in rat adipocytes. Levels of conventional PKC α and γ and novel PKC δ were suppressed by GH whereas levels of atypical PKC ζ and μ were enhanced. The effects of GH on PKC ε expression were complex and, at present, difficult to explain, since GH both enhanced and suppressed expression of PKC ε depending on the hormone serum concentration. Collectively, these observations implied the individual or combinational involvement of PKC isoforms in GH-mediated responses in rat adipocytes.

When the effects of GH on PKC isoform expression were examined *in vitro* in 3T3-F442A adipocytes, however, GH had no direct effect on PKC isoform levels. This

discrepancy may have been attributable to differences in the lengths of the treatment periods. 3T3-F442A adipocytes were incubated with GH for 24 h, whereas rat adipocytes were treated with anti-rat GH (± oGH) for a total of 96 h. Alternatively, the data obtained *in vitro* may indicate that the effects of GH on PKC isoform expression observed *in vivo* are not direct and are attributable to secondary changes in circulating levels of other hormones involved in adipocyte regulation.

It is less clear whether PKC is involved in the chronic metabolic effects of insulin in the adipocyte (see Section 1.6.5). This appears to be, at least in part, due to the existence of multiple isoforms. The effects of chronic exposure to insulin on the expression of PKC isoforms in 3T3-F442A adipocytes were therefore examined. However, as was the case with GH, treatment with insulin had no direct effect on the expression of PKC isoforms in 3T3-F442A adipocytes.

Thus, direct *in vitro* addition of GH or insulin to 3T3-F442A adipocytes was found to have no chronic effect on the expression of PKC isoforms. The fact that GH differentially regulated PKC isoforms in rat adipocytes, either directly or indirectly, however, suggests they may individually or combinationally regulate different GH-induced signal transduction events. At least in 3T3-F442A preadipocytes, GH acutely activates PKC γ, δ and ε, as determined by translocation from the cytosol to the membrane (S. MacKenzie, E. Kilgour, N. Anderson and I. Fleming, unpublished observation), whereas no such effect was observed upon PKC α and ζ. No such studies have been done on PKC μ to date. Insulin has been shown to acutely translocate/activate PKC α, β and ζ in 3T3-L1 adipocytes (Bandyopadhyay *et al*, 1997) and PKC α, β, γ, ε and ζ in rat adipocytes (Farese *et al*, 1992). The subsequent insulin-induced down-regulation of PKC isoforms was found to be attended by selective increases in their mRNAs in rat adipocytes (Avignon *et al*, 1995). Thus, although GH and insulin had no chronic effect on the expression of PKC isoforms in 3T3-F442A adipocytes, a role for PKC isoforms in the signalling pathways utilised by these hormones in the adipocyte could not be ruled out. The studies presented here were therefore aimed at evaluating directly whether the individual isoforms expressed in 3T3-F442A adipocytes contribute to the effects of GH and insulin on a single major metabolic process which characterises adipocyte metabolism, namely lipogenesis.

Studies with PKC activators suggest that both GH and insulin alter the rate of lipogenesis in the adipocyte by mechanisms that are, at least in part, PKC-dependent (Smal and De Meyts, 1987). The compounds used during these studies, however, cannot be construed as being totally specific. The involvement of PKC isoforms in the signalling pathway(s) utilised by GH and insulin to exert their effects on the rate of lipid synthesis was therefore investigated in 3T3-F442A adipocytes using an antisense procedure to deplete selectively specific PKC isoforms. Initial characterisation of the effects of GH and insulin on the rate of lipid synthesis in 3T3-F442A adipocytes revealed that insulin increased significantly the rate of lipogenesis (to approximately 160 % of that observed in control adipocytes), whereas GH both decreased significantly the rate of lipogenesis (to approximately 40 % of that observed in control adipocytes) and completely prevented the increase in lipogenesis in response to insulin. Studies here with the MEK inhibitor, PD 98059 (Arcaro and Wymann, 1993; Powis *et al*, 1994) revealed that the MAP kinase pathway was not required by either GH or insulin to exert these effects. Studies here with the PI 3-kinase inhibitor, wortmannin (Alessi *et al*, 1995) revealed that, whereas PI 3-kinase was found to be required for maintaining the basal rate of lipogenesis, the inhibitory effects of GH on lipid synthesis did not involve this pathway. In contrast, PI 3-kinase was found to be one of a number of components required for the stimulation of lipogenesis by insulin.

These observations, at least for insulin, are consistent with the results of others. It has been demonstrated that stimulation of MAP kinases by insulin is not required for its effects on glucose transport, glycogen synthesis or on the activity of the lipogenic enzymes, acetyl CoA carboxylase and pyruvate dehydrogenase (Lazar *et al*, 1985; Denton and Tavare, 1995). Similarly, activation of PI 3-kinase alone is not sufficient for mediating the metabolic effects of insulin (Krook et al, 1997; Wiese *et al*, 1995; Moule *et al*, 1995). PI 3-kinase appears to be, at least in part, required for the stimulation of glucose utilisation, glycogen synthesis and acetyl CoA carboxylase by insulin (Moule *et al*, 1995). However, stimulation by insulin of other lipogenic enzymes, such as pyruvate dehydrogenase and fatty acid synthase, appears not to involve the PI 3-kinase pathway (Moule *et al*, 1995). Thus, multiple signalling pathways appear to be involved in the stimulation of lipogenic enzymes and lipogenesis by insulin. The PI 3-kinase pathway, but not the MAP kinase pathway,

appears to be involved.

In order to establish whether PKC isoforms were required for the GH- and insulin-mediated effects on lipid synthesis in 3T3-F442A adipocytes, antisense ODNs were used to deplete selectively individual isoforms from adipocytes prior to stimulation with hormones. Immunoblotting studies revealed that incubation with antisense ODNs resulted in the complete depletion of the appropriate PKC isoform whereas incubation with the appropriate sense ODN (or lipofectin alone) had no effect on expression of PKC isoforms in 3T3-F442A adipocytes.

Depletion of PKC α, δ and ζ from adipocytes was found to have no effect on the suppression of lipogenesis by GH or the stimulation of lipogenesis by insulin. This suggested that these PKC isoforms were not essential for the GH- and insulin-mediated effects on lipid synthesis in 3T3-F442A adipocytes. It is possible that these isoforms may functionally substitute for each other in the signalling pathways utilised by GH and insulin to regulate the rate of lipid synthesis. However, PKC δ has been shown to be required for MAP kinase activation by GH in 3T3-F442A preadipocytes (MacKenzie *et al*, 1997). Therefore, the lack of involvement of PKC δ in the signalling pathway utilised by GH to reduce the rate of lipid synthesis in adipocytes would appear consistent with the observation that the MAP kinase pathway is not involved.

Depletion of PKC ε from 3T3-F442A adipocytes had no effect on the ability of GH to suppress the basal or insulin-stimulated rate of lipogenesis. However, following depletion of PKC ε, the stimulation of lipogenesis by insulin was reduced slightly, but significantly. This indicated that PKC ε is one of the multiple components utilised by insulin to increase the rate of lipogenesis in 3T3-F442A adipocytes. These results also indicated that other, PKC ε-independent signalling pathway(s), are involved. Calcium-independent PKC isoforms such as PKC ε can be activated by the products of PI 3-kinase (Nakanishi *et al*, 1993; Toker *et al*, 1994). The insulin response was reduced by approximately 20 % both following the inhibition of PI 3-kinase and in the absence of PKC ε. This suggests that PKC ε may be activated in a PI 3-kinase-dependent manner during the stimulation of lipogenesis by insulin in 3T3-F442A

adipocytes.

Depletion of PKC μ totally abolished the ability of insulin to stimulate the rate of lipid synthesis in 3T3-F442A adipocytes. Thus, PKC μ appears to be an essential component of the signalling mechanism by which insulin increases the rate of lipogenesis in 3T3-F442A adipocytes. The antisense sequence used to deplete PKC μ was derived from the human protein coding sequence. The results obtained with the PKC μ ODN were, therefore, confirmed by making use of an antisense ODN directed against PKD, which is thought to be the murine homologue of this protein (Van Lint *et al*, 1995). That identical results were obtained using the PKD ODN suggested that PKC μ and PKD were indeed human/murine homologues and that this protein was essential for the insulin-mediated effects on lipid synthesis in 3T3-F442A adipocytes.

Insulin alters the rate of lipid synthesis in 3T3-F442A adipocytes by a mechanism that is, at least in part, mediated by PKC ε and apparently totally dependent on PKC μ. Interestingly, PKC μ (PKD) can be activated *via* PKC ε in intact cells (Zugaza *et al*, 1996). This suggests the simultaneous activation by insulin of a signalling pathway involving PKC ε, possibly that of PI 3-kinase, and the activation of other PKC ε-independent pathway(s) which diverge at the level of PKC μ in stimulating the rate of lipid synthesis.

Thus, at least with regard to the stimulation of lipogenesis, insulin exerts its metabolic effects in the adipocyte *via* signalling pathways that are mediated by the activation of PKC. The phorbol ester-sensitive PKC ε isoform is, at least in part, required for the stimulation of lipogenesis by insulin. Therefore, the results of Smal and De Meyts (1987), who, using phorbol esters, observed that PKC was partially required for the lipogenic effects of insulin, are likely to reflect down-regulation of the PKC ε isoform. However, seemingly contradictory reports suggest that insulin responses are unimpaired in cells in which PKC isoforms have been down-regulated by chronic exposure to phorbol esters (reviewed in Blackshear, 1994). The data presented in Chapter three and the results of others (Olivier and Parker, 1992) indicate that PKC ε is relatively resistant to down-regulation by such treatment. This fact, coupled to the observation that the insulin response is only partially dependent on the presence of

PKC ε, could account for an intact insulin response following prolonged exposure of cells to phorbol esters. In addition, PKC μ, which is essential for the insulin-stimulated increase in the rate of lipogenesis, is not down-regulated by chronic phorbol ester treatment (Johannes *et al*, 1995; Rennecke *et al*, 1996).

PKC μ was also found to be an important component of the signalling mechanism by which GH exerts its effects on the rate of lipid synthesis. Following the depletion of PKC μ from 3T3-F442A adipocytes, the ability of GH to suppress basal lipogenesis was reduced significantly. This indicated that PKC μ was an important component of the signalling mechanism by which GH suppresses the basal rate of lipogenesis in 3T3-F442A adipocytes and that other PKC μ-independent signalling pathways were also required. When PKC μ/PKD-depleted adipocytes were incubated with GH and insulin in combination, the rate of lipogenesis did not differ significantly from that observed when cells were incubated with insulin alone. This is most likely due to the fact that PKC μ is, at least in part, required for the suppression of basal lipogenesis by GH in 3T3-F442A adipocytes, although interpretation of these results is, of course, complicated by the fact that insulin exerts no response in the absence of PKC μ and there is therefore no response for GH to antagonise.

Depletion of PKC γ from 3T3-F442A adipocytes had no effect on the ability of insulin to enhance the rate of lipogenesis and did not effect significantly the ability of GH to suppress the basal rate of lipid synthesis. However, PKC γ was found to be essential for the ability of GH to antagonise the insulin-mediated increase in the rate of lipid synthesis in 3T3-F442A adipocytes. Given that PKC μ is essential for the stimulation of lipogenesis by insulin, it is possible that activation of PKC γ by GH prevents the insulin-induced activation of PKC μ, or alters its subsequent down-stream effects.

Thus, in 3T3-F442A adipocytes, GH alters the basal rate of lipid synthesis by a mechanism that is, at least in part, mediated by PKC μ, although other PKC-independent signalling pathways appear to be involved. PKC γ is not essential for the suppression of basal lipogenesis by GH but is an essential component of the signal transduction mechanism by which GH antagonises the insulin-stimulated increase in

the rate of lipid synthesis. Thus, both the direct anti-lipogenic and the insulin-antagonistic effects of GH involve isoforms of PKC. The results of these studies also suggest that the mechanisms by which GH exerts its direct anti-lipogenic effects and its insulin-antagonisitc effects on the rate of lipid synthesis in 3T3-F442A adipocytes are distinct, at least with respect to the activation of PKC. From these studies, it appears that PI 3-kinase is the basal regulator of lipogenesis in 3T3-F442A adipocyes that is inhibited by wortmannin and by GH and this does not involve PKC γ. A PI 3-kinase-independent process also affects the lipogenic parhway that is stimulated by insulin and which may be blocked by GH through PKC γ.

PKC μ was found to be an important component of the signalling mechanism by which both GH and insulin exert their metabolic effects in the adipoctye, at least with regard to altering the rate of lipid synthesis. Support of a role for PKC μ (PKD) in mediating insulin responses also comes from studies of the desensitisation of hormone receptors. PKCμ (PKD) has been shown to be involved in mediating glucagon desensitisation of adenylate cyclase in cos cells (Tobias *et al*, 1997). This could be considered as an insulin-like effect in that it is a process that antagonises glucagon action and hence is anti-gluconeogenic. Since PKC μ is also important for the effects of GH on lipid synthesis, at least in 3T3-F442A adipocytes, it is tempting to speculate that this PKC isoform could have an involvement in GH-induced insulin resistance in adipocytes.

PKC μ, like a number of other signalling molecules (see Sections 1.4 and 1.5), is utilised by both GH and insulin in order to exert opposite effects. PKC μ has now been shown to be activated by a number of hormones and growth factors (Zugaza *et al*, 1997) and may therefore be an important target for hormones in mediating down-stream events. This suggests that either the timing or duration of activation, or the down-stream substrates, are likely to be crucial in determining the final response following hormonal stimulation. Given that PKCs are regulated by phosphorylation (see Section 1.6.2.3), it is possible that GH and insulin (and other hormones) induce the phosphorylation of PKC μ at distinct sites, or in different sub-cellular compartments, which alters its specificity/affinity for, or regulation of, down-stream substrates. Studies to date suggest that PKC μ is activated by hormones in a PKC-

dependent manner and that PKC isoforms such as PKC ε and η are involved (Zugaza *et al*, 1996 and 1997) Indeed, the results of the studies presented here suggest that PKC μ may be activated down-stream of PKC ε in response to insulin stimulation. However, other signalling mechanisms appear to be required for the stimulation of lipogenesis by insulin as PKC ε is only one of a number of components required for the response to insulin and PKC μ appears to be essential for the insulin response. Furthermore, PKC μ appears to be required for the suppression of basal lipogenesis by GH and no other PKC isoform was found to be essential for this response. Interestingly, PKC γ was found to be required for the antagonism of insulin-stimulated lipogenesis by GH, suggesting that PKC γ may be involved in the regulation of PKC μ. From these studies it appears that, either PKC-independent mechanisms are involved in the activation of PKC μ by both GH and insulin in 3T3-F442A adipocytes, or that other PKC isoforms can functionally substitute for each other in the activation of PKC μ.

The results of these studies clearly indicate that both GH and insulin exert their metabolic effects in the adipocyte by signalling pathways involving PKC. At present the mechanisms by which individual PKC isoforms regulate the GH- and insulin-mediated responses on the rate of lipogenesis in adipocytes are unclear. The lipogenic pathway comprises a number of strictly regulated enzymes (see Figure 1.1). There are, therefore, multiple potential points at which PKC isoforms could regulate the pathway, including the rate of enzyme substrate synthesis and degredation as well as the expression and activity of lipogenic enymes. To date, the effects of GH and insulin on the rate of lipogenesis have been largely attributed to alterations in the expression and activity of key lipogenic enzymes such as acetyl CoA carboxylase, fatty acid synthase and pyruvate dehydrogenase (Dietz and Schwartz, 1991; Bauman and Vernon, 1993; Harris *et al*, 1993; Vernon *et al*, 1993; Denton and Tavare, 1995; Moule *et al*, 1995). GH has been shown to suppress both the activation status and expession of acetyl CoA carboxylase and fatty acid synthase (Dietz and Schwartz, 1991; Bauman and Vernon, 1993; Harris *et al*, 1993; Vernon *et al*, 1993) and prevent the activation of acetyl CoA carboxylase by insulin (Vernon *et al*, 1991). It would therefore be of interest to determine the effects of antisense depletion of PKC

isoforms, particularly that of PKC μ and PKC γ, on the expression and activation status of such lipogenic enzymes following stimulation with GH and insulin.

It is also unclear at present which, if any, PKC isoforms are involved in the signalling pathways utilised by GH and insulin to regulate other aspects of adipocyte metabolism. However, studies with PKC activators and inhibitors imply a role for PKC isoforms in the regulation of lipolysis by GH (Gorin *et al*, 1990; Doris *et al*, 1998) and glucose transport by insulin (Standaert *et al*, 1990; Grunberger, 1991; Yano *et al*, 1993; Chalfant *et al*, 1995). Further support of a role for PKC in the regulation of lipolysis comes from the observation that PKC appears to phosphorylate the inhibitory G protein, G_i-2, at least in rat hepatocytes (Morris *et al*, 1994), and G_i-2 appears to play a pivotal role in the functioning of adenylate cyclase (Bushfield *et al*, 1991).

In summary, both GH and insulin alter the rate of lipid synthesis in 3T3-F442A adipocytes *via* signalling pathways involving isoforms of PKC. PKC α, δ and ζ are not essential for the stimulation of lipogenesis by insulin or the suppression of basal or insulin-stimulated lipogenesis by GH. PKC ε is one of a number of components required by insulin to increase the rate of lipogenesis. PKC γ is an essential component of the signalling mechanism utilised by GH to antagonise the stimulation of lipogenesis by insulin. PKC μ is, at least in part, required for the direct anti-lipogenic effect of GH. This PKC isoform is also an essential component of the signal transduction mechanism by which insulin stimulates lipogenesis in 3T3-F442A adipocytes. These results indicate that PKC isoforms assume differential functional roles in 3T3-F442A adipocytes.

Chapter 6

General Discussion

Initial interest in the role of PKC in the regulation of cellular function arose from the discovery that the enzyme was activated by tumour promoting phorbol esters (Gschwendt *et al*, 1991; Bell and Burns, 1991). Phorbol esters have, therefore, been used widely as a pharmacological tool to investigate the biological functions of PKC in cells. By this approach, PKC has been implicated in a wide range of cellular processes, including growth and differentiation, tumourigenesis, apoptosis and a variety of cell functions (Hug and Sarre , 1993; Kindregan *et al*, 1994; Li *et al*, 1994; Glazer, 1994; Borner *et al*, 1995; Blobe *et al*, 1996; Acs *et al*, 1997; Deacon *et al*, 1997). In addition, it has been established that PKC activity is provided for by a family of closely related kinases, perhaps suggesting an explanation for the range of processes in which PKC has been implicated. The marked difference in tissue distributions of PKC isoforms indicates that their functions are both isoform- and tissue-specific (Borner *et al*, 1992; Hata *et al*, 1993; Hug and Sarre, 1993; Stabel and Parker, 1993; Selbie *et al*, 1993; Akimoto *et al*, 1994; Dekker and Parker, 1994; Hirai *et al*, 1994; Dieterich *et al*, 1996;). However, as of yet, little is known about the functions of individual PKC isoforms in any system. The purpose of this study was to investigate the functional roles of individual PKC isoforms in the regulation of adipocyte development and function using the 3T3-F442A preadipocyte cell model.

Knowledge of the PKC isoforms in 3T3-F442A cells was required initially to enable the subsequent evaluation of the roles of individual isoforms in adipocyte regulation. The PKC complement of 3T3-F442A cells was characterised by immunoblotting using a panel of antibodies with application of strict criteria to ensure the correct identification of PKC isoforms. These criteria were employed due to the high degree of sequence homology shared between PKC sub-species and exploited the known structural and regulatory properties of the PKC sub-types in order to assess the specificty of the antibodies used for characterisation of the cells. Using this approach, both 3T3-F442A preadipocyte and adipocytes were found to express PKC α, γ, δ, ε, ζ and μ. The PKC complement of 3T3-F442A adipocytes was found to be identical to that of rat adipocytes. This supported the suitability of using 3T3-F442A cells as a model system of adipocyte development and function.

A number of potential problems exist in assigning functional roles to individual PKC

isoforms. Importantly, most PKC activators and inhibitors cannot be construed as being isoform-specific (Castagna *et al*, 1982; Blobe *et al*, 1996; Jaken, 1996; Quest, 1996; Hofmann, 1997). In this study, an alternative approach was, therefore, taken to investigate the roles of individual PKC isoforms in 3T3-F442A cells. Changes in expression of PKC isoforms were examined throughout adipocyte development in order to gain initial insight into the roles of PKC isoforms in 3T3-F442A cell regulation. Subsequently, antisense oligonucleotides were employed to deplete individual PKC isoforms selectively from 3T3-F442A cells in an attempt to define the dependence of specific cellular processes on individual PKC isoforms.

PKCs are involved in a number of the signalling pathways that are known to modulate the preadipocyte differentiation process (Anderson, 1993; Nakanishi *et al*, 1993; Toker *et al*, 1994; Cobb and Goldsmith, 1995; Sale *et al*, 1995; Uehara *et al*, 1995, Yeh *et al*, 1995). Thus, fluctuations in cellular levels of PKC isoforms throughout adipocyte development are likely to be important for the modulation and integration of the variety of signals that regulate the differentiation process. Indeed, differential changes in the expression of individual PKC isoforms were observed to accompany the differentiation process in 3T3-F442A cells, implying distinct functional roles for PKC isoforms in the regulation of adipocyte development and function. Comparison of the cellular levels of PKC isoforms during 3T3-F442A preadipocyte differentiation with cellular levels of PKC isoforms in two separate control systems, gave an indication of the functional roles of individual isoforms in adipogenesis. Cellular levels of PKC ζ were unchanged during the differentiation process suggesting that this isoform played no obligatory role in preadipocyte differentiation. Although cellular levels of PKC μ were elevated in adipocytes, this was not attributable to the differentiation process *per se*. Following the induction of differentiation, cellular levels of PKC α, γ and δ increased rapidly, peaking at around day 2, correlating with the time-point at which clonal expansion of the differentiating cells was observed. Subsequently, cellular levels of these isoforms decreased such that a significant reduction in their expression was observed as cells attained the adipocyte phenotype. Thus, following the clonal expansion stage, these isoforms may exert an inhibitory influence on the differentiation process. Cellular levels of PKC ε increased during adipocyte development implying a requirement for this isoform for the induction and

maintenance of the adipocyte phenotype.

To define the dependence of 3T3-F442A preadipocyte differentiation on PKC isoforms, antisense ODNs were used to achieve the selective depletion of individual isoforms prior to the induction of differentiation. By this approach it was confirmed that PKC ζ is not essential for any stage of the differentiation process in 3T3-F442A preadipocytes. PKC α, δ and μ each exert an inhibitory influence upon the early stages of adipocyte development and are therefore likely to modulate this stage of the differentiation process. PKC ε and γ are both essential for 3T3-F442A preadipocyte differentiation. Further antisense studies revealed that PKC γ and PKC ε are required for distinct stages of the differentiation process. PKC γ is essential for the clonal expansion of differentiating 3T3-F442A cells that is necessary for subsequent differentiation to occur (Smas and Sul, 1995; Mandrup and Lane, 1997). PKC ε is not required for clonal expansion but is essential for later stages of the differentiation process, when its expression is markedly elevated, for the attainment of the adipocyte phenotype.

Thus it was shown that PKC γ and PKC ε were essential for preadipocyte differentiation and that no other PKC isoforms could functionally substitute for these isoforms during the differentiation process. Both PKC γ and PKC ε were found to be required for stages of the differentiation process when their expression levels were markedly elevated. Although, like PKC γ, expression of PKC α and δ was elevated during the clonal expansion phase of preadipocyte differentiation, neither isoform was found to be essential for the process to occur. The increase in expression of these isoforms upon induction of differentiation is, therefore, difficult to explain. It is possible that PKC α and δ can functionally substitute for each other during the clonal expansion stage of the preadipocyte differentiation process. This could be established by depletion of both PKC α and δ by combined antisense ODN treatment prior to the initiation of differentiation.

PKC α, δ and μ are expressed in a wide variety of tissues (see Quest, 1996). Such ubiquitous expression implies a general role in the regulation of cell growth, differentiation or function. That the rate of adipocyte development was enhanced in

the absence of PKC α, δ and μ suggested that these isoforms modulate the early stages of the differentiation process. In addition, antisense depletion of these PKC isoforms increased the growth rate of 3T3-F442A preadipocytes (as assessed by morphological examination of cells; I. Fleming, S. MacKenzie and E. Kilgour, unpublished observation). Thus, loss of PKC α, δ or μ from 3T3-F442A preadipocytes led to enhanced cell growth and differentiation. These isoforms could, therefore, be part of a mechanism acting as a brake on signal transduction, perhaps by inducing the suppression of a down-stream kinase or the activation of a down-stream phosphatase. Thus, loss of these isoforms could ultimately result in the disorganised cell growth associated with tumourigenesis.

PKC ζ is also a ubiquitously expressed PKC isoform (see Quest, 1996). Although depletion of PKC ζ from 3T3-F442A cells had no detectable effects on cell growth or differentiation, it is possible that other ubiquitously expressed PKC isoforms can functionally substitute for PKC ζ in the regulation of proliferation and differentiation. It would be relatively simple to investigate whether, for example, the PKC α, δ and ζ isoforms functionally substitute for each other in the regulation of cell growth and differentiation by performing multiple depletion of isoforms using a combination of antisense ODNs.

At present the mechanisms by which the PKC family regulate preadipocyte differentiation are uncertain. However, the results of these studies indicate that PKC isoforms assume distinct functional roles during preadipocyte differentiation and may therefore target different cellular substrates. Members of the C/EBP and the PPAR family of transcription factors are especially prominent in the control of adipogenesis (see Yeh and McKnight, 1995). A role for PKC isoforms in the regulation of such adipogenic treanscription factors has been implied by studies demonstrating that suppression of adipogenesis by phorbol esters involves the MAP kinase-mediated phosphorylation of PPAR γ and attenuation of its transcriptional activity (Hu *et al*, 1996). In addition, these transcriptional regulatory proteins display similar temporal expression profiles to individual PKC isoforms during preadipocyte differentiation (see Yeh and McKnight; Figure 1.3 and Figures 4.5 A-F). In particular C/EBP β and C/EBP δ, which are induced during the early stages of differentiation, peak during the

clonal expansion phase and subsequently decline as cells attain the adipocyte phenotype, follow a similar temporal pattern of expression to PKC α, γ and δ. In addition, PPAR γ, which is induced during the clonal expansion phase of differentiation and continues to increase as cells attain the adipocyte phenotype, follows a similar temporal pattern of expression to PKC ε and μ. C/EBP β and δ and PPAR γ are thought to induce the expression and channel the adipogenic potential of CEBP α, the ultimate regulator of terminal adipogenesis (see Yeh and McKnight, 1996). It would, therefore, be extremely interesting to determine the effects of antisense depletion of individual PKC isoforms on the expression and transcriptional activity of members of the C/EBP and PPAR family of transcription factors and establish whether these proteins are the ultimate down-stream targets of individual PKC isoforms during preadipocyte differentiation.

In order to investigate the functional roles of PKC isoforms in adipocytes, their involvement in the signalling pathways of two important hormonal regulators of adipocyte metabolism, GH and insulin, was examined in 3T3-F442A adipocytes. As studies with PKC activators suggested that both GH and insulin alter the rate of lipogenesis in the adipocyte by PKC-dependent mechanisms (Smal and DeMeyts, 1987), the involvement of PKC isoforms in the signalling mechanisms by which GH and insulin alter the rate of lipid synthesis was investigated.

Using the antisense approach it was established that PKC α, δ and ζ are not essential components of the signalling mechanisms by which either GH or insulin alter the rate of lipid synthesis in 3T3-F442A adipocytes. PKC ε is not required for the suppression of basal or insulin-stimulated lipogenesis by GH but is one of the multiple components required by insulin for the stimulation of lipogenesis. Studies with the PI 3-kinase inhibitor, wortmannin (Alessi *et al*, 1995) suggested that PKC ε may be activated *via* PI 3-kinase during the stimulation of lipogenesis by insulin. PKC μ is an important component of the signalling mechanism by which GH suppresses basal lipogenesis in 3T3-F442A adipocytes. The results of these studies indicated that PKC μ-independent mechanisms were also required for the suppression of lipogenesis by GH. PKC μ also appears to be an essential component of the signal transduction

mechanism by which insulin stimulates lipogenesis in 3T3-F442A adipocytes. The stimulation of lipogenesis by insulin was reduced in the absence of PKC ε but no stimulation of lipogenesis by insulin was observed in the absence of PKC μ. This suggests the simultaneous activation by insulin of a signalling pathway involving PKC ε, and other PKC ε-independent signalling pathway(s), which diverge at the level of PKC μ in the stimulation of lipogenesis. PKC γ is an essential component of the signalling mechanism by which GH antagonises the stimulation of lipogenesis by insulin. This suggests that activation of PKC γ by GH may prevent the activation of PKC μ by insulin, or its subsequent down-stream events.

Thus, PKC γ, ε and μ were found to be involved in the signalling mechanisms by which GH and insulin alter the rate of lipid synthesis in 3T3-F442A adipocytes. Cellular levels of PKC ε and μ are markedly elevated in 3T3-adipocytes as compared to preadipocytes. This implied a functional role for these PKC isoforms in adipocyte regulation. Indeed, PKC μ appears to be an important component of the signalling mechanisms by which both GH and insulin exert their effects on the lipogenic pathway. In addition, PKC ε appears to be a component of the signalling mechanism by which insulin alters the rate of lipid synthesis in the adipocyte. In contrast, cellular levels of PKC γ were dramatically reduced following differentiation of 3T3-F442A cells, implying that PKC γ played a minimal role in adipocyte regulation. However, this PKC isoform was found to be essential for the antagonism of insulin action by GH, at least with regard to lipogenesis. Thus, although 3T3-F442A adipocytes exhibit reduced levels of PKC γ at the protein level, adipocyte PKC γ may possess increased sensitivity or affinity for substrates following hormonal stimulation.

The mechanisms by which individual PKC isoforms regulate the lipogenic pathway are unclear. That PKC μ is targeted by both GH and insulin in exerting opposing effects on the rate of lipogenesis, suggests that this isoform may be differentially regulated by these hormones. For example, phosphorylation at distinct sites in response to hormones may mediate distinct down-stream events such as the activation or suppression of down-stream substrates. In addition, that PKC γ is essential for the antagonism of insulin-stimulated lipogenesis by GH suggests that this PKC isoform may be involved in the regulation of PKC μ or subsequent down-stream events

mediated by PKC μ.

Given that PKC μ was found to be an important component of the signal transduction mechanism by which both GH and insulin exert their effects on lipid synthesis in the adipocyte, it would be interesting to determine how PKC μ is regulated by these hormones. PKC μ has been shown to be activated by a number of hormones and growth factors (Zugaza *et al*, 1997) and studies to date suggest PKC μ activation is dependent on other PKC isoforms such as PKC ε and η (Zugaza *et al*, 1996 and 1997). Indeed, the results of the studies presented here suggest that PKC μ may be activated down-stream of PKC ε in response to insulin stimulation. However, other signalling components, in addition to PKC ε, appear to be required for the stimulation of lipogenesis by insulin. PKC μ is also required for the suppression of basal lipogenesis by GH and no other PKC isoform was found to be essential for this response. From these studies it appears that, either, PKC-independent processes are involved in the activation of PKC μ by both GH and insulin in 3T3-F442A adipocytes, or, that other PKC isoforms can functionally substitute for each other in the activation of PKC μ. This could again be established by performing multiple depletion of PKC isoforms using a combination of antisense ODNs.

At present the mechanisms by which individual PKC isoforms regulate the GH- and insulin- mediated responses on the rate of lipogenesis in adipocytes are unclear. The lipogenic pathway comprises a number of strictly regulated enzymes (see Figure 1.1). To date, the effects of GH and insulin on the rate of lipogenesis have been largely attributed to hormone-induced alterations in the expression and activity of key lipogenic enzymes such as acetyl CoA carboxylase, fatty acid synthase and pyruvate dehydrogenase (Dietz and Schwartz, 1991; Bauman and Vernon, 1993; Harris *et al*, 1993; Vernon *et al*, 1993; Denton and Tavare, 1995; Moule *et al*, 1995). It would, therefore, be of interest to determine the effects of antisense depletion of PKC isoforms from 3T3-F442A adipocytes on the expression and activation status of such enzymes following hormonal stimulation in order to establish if these key lipogenic enzymes are the ultimate down-stream targets of PKC isoforms in the lipogenic pathway.

It is also unclear at present which, if any PKC isoforms, are involved in the signalling pathways utilised by GH and insulin to regulate other aspects of adipocyte metabolism in 3T3-F442A adipocytes. However, in theory, the antisense approach could be used to provide further insight into the roles of individual PKC isoforms in the signalling mechanisms used by GH and insulin or, indeed, other hormones involved in adipocyte regulation, to exert their effects on other aspects of adipocyte metabolism. The selective depletion of PKC isoforms from adipocytes could be used to analyse the involvement of specific isoforms in hormone-controlled processes such as changes in the rate of glucose transport, glucose transporter translocation, alterations in the rate of lipolysis, changes in the activity of key lipolytic enzymes and altered regulation of cyclic AMP phosphodiesterases. Given that the functional roles of PKC ζ in 3T3-F442A cells remain undefined following the present study, it would be of particular interest to determine if this isoform is required for GH- and insulin-induced alterations in the rate of lipolysis and glucose transport, particularly as a role for PKC ζ in the stimulation of glucose transport by insulin has been implied in 3T3-L1 adipocytes (Bandypadhyay *et al*, 1997).

In summary, PKC isoforms were found to assume distinct functional roles in 3T3-F442A cell regulation. Cellular levels of PKC ζ were maintained throughout the differentiation of preadipocytes, suggesting that this isoform plays an as yet unidentified role in 3T3-F442A cell regulation. PKC α and δ play a modulatory role in the early stages of the differentiation process whereas PKC γ and PKC ε are both essential for preadipocyte differentiation to occur. In fully differentiated adipocytes, PKC ε is one of a number of components required for the stimulation of lipogenesis by insulin and PKC γ appears to be an essential component of the signal transduction mechanism by which GH antagonises the stimulation of lipogenesis by insulin. PKC μ, like PKC α and δ, appears to modulate the early stages of the preadipocyte differentiation process. In addition, this PKC isoforms appears to play an important role in the regulation of adipocyte metabolism by hormones, at least with regard to lipogenesis. PKC μ is an important component of the signalling mechanism by which GH suppresses basal lipogenesis in adipocytes and this isoform appears to be essential for the stimulation of lipogenesis by insulin.

Although, the precise mechanisms by which individual PKC isoforms regulate adipocyte development and function remain to be determined, the results of these studies clearly indicate that individual PKC isoforms assume distinct functional roles in 3T3-F442A cells. These findings may, therefore, be relevant to our understanding of the diseased state of obesity which involves both changes in the turnover of adipocyte cells in adipose tissue (Prins and O'Rahilly, 1997) and selective changes in the expression of adipocyte PKC isoforms (Frevert and Kahn, 1996).

References

ABUMRAD, N.A., Elmaghrabi, M.R., Amri, E.Z., Lopez, E. and Grimaldi, P.A. (1993) Cloning of a rat adipocyte membrane-protein implicated in binding or transport of long-chain fatty-acids that is induced during preadipocyte differentiation - Homology with human CD36. *J. Biol. Chem.*, **268.** 17665-17668.

ACS, P., Wang, Q.M.J., Bogi, K., Marquez, A.M., Lorenzo, P.S., Biro, T., Szallasi, Z., Mushinski, J.F. and Blumberg, P.M. (1997) Both the catalytic and regulatory domains of protein kinase C chimaeras modulate the proliferative properties of NIH-3T3 cells. *J. Biol. Chem.*, **272.** 28793-28799.

AHIMA, R.S., Prabakaran, D., Mantzoros, C., Qu, D.Q., Lowell, B., Maratosflier, E. and Flier, J.S. (1996) Role of leptin in the neuroendocrine response to fasting. *Nature,* **382.** 250-252.

AILHAUD, G. (1982) Adipose cell-differentiation in culture. *Mol. Cell. Biochem.*, **49.** 17-31

AILHAUD, G., Dani, C., Amri, E.Z., Djian, P., Vannier, C., Doglio, A., Forest, C., Gaillard, D., Negrel, R. and Grimaldi, P. (1989) Coupling growth arrest and adipocyte differentiation. *Environ. Health Perspec.*, **80.** 17-23

AILHAUD, G., Grimaldi, P. and Negrel, R. (1992) Cellular and molecular aspects of adipose-tissue development. *Ann. Rev. Nutrit.*, **12.** 207-233.

AILHAUD, G., Grimaldi, P. and Negrel, R. (1994) Hormonal regulation of adipose differentiation. *Trends Endocrinol. Metab.*, **5.** 132-136.

AKIMOTO, K., Mizuno, K., Osada, S-I., Hirai, S., Tanuma, S., Suzuki, K. and Ohno, S. (1994) A new member of the third class in the protein kinase C family, PKCλ, expressed dominantly in an undifferentiated mouse embryonal cell line and also in many tissues and cells. *J. Biol. Chem.*, **269.** 12677-12683.

AKIMOTO, K., Takahashi, R., Moriya, S., Nishioka, N., Takayanagi, J., Kimura, K., Fukui, Y., Osada, S., Mizuno, K., Hirai, S., Kazlauskas, A. and Ohno S. (1996) EGF or PDGF receptors activate atypical PKCλ through phosphatidylinositol 3-kinase. *EMBO J.*, **15**. 788-798.

ALESSI, D.R., Cuenda, A., Cohen, P., Dudley, D.T. and Saltiel, A.R. (1995) PD-098059 is a specific inhibitor of the activation of mitogen-activated protein-kinase kinase in vitro and in vivo. *J. Biol. Chem.*, **270.** 27489-27494.

ALESSI, D.R., James, S.R., Downes, C.P., Holmes, A.B., Gaffney, P.R.J., Reese, C.B. and Cohen, P. (1997) Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase B alpha. *Current Biol.*, **7.** 261-269.

ALLEN, D.O. (1985) Rate-limiting steps in isoproteranol and forskolin stimulated lipolysis. *Biochem. Pharmacol.*, **34.** 843-846

ALTZULER, N. (1974) Growth hormone and carbohydrate metabolism. In: *Handbook of Physiology,* Section 7, Volume 4. p233 (Knobie, E. and Sawyer, W., eds.), Am. Physiol. Soc., Washington D.C.

AMRI, E.Z., Dani, C., Doglio, A., Grimaldi, P. and Ailhaud, G. (1986) Coupling of growth arrest and early markers during adipose conversion of preadipocyte cell-lines. *Biochem. Biophys. Res. Commun.*, **137.** 903-910.

AMRI, E.Z., Ailhaud, G. and Grimaldi, P. (1991a) Regulation of adipose cell-differentiation. 2. Kinetics of induction of the AP2 gene by fatty-acids and modulation by dexamethasone. *J. Lipid Res.*, **32.** 1457-1463.

AMRI, E.Z., Bertrand, B., Ailhaud, G. and Grimaldi, P. (1991b) Regulation of adipose cell-differentiation. 1. Fatty-acids are inducers of the AP2 gene-expression. *J. Lipid Res.*, **32.** 1449-1456.

AMRI, E.Z., Bonino, F., Ailhaud, G., Abumrad, N.A. and Grimaldi, P.A. (1995) Cloning of a protein that mediates transcriptional effects of fatty-acids in preadipocytes - Homology to peroxisome proliferator-activated receptors. *J. Biol. Chem.*, **270.** 2367-2371.

ANDERSON, N.G. (1992) Growth hormone activates mitogen-activated protein kinase and S6 kinase and promotes intracellular tyrosine phosphorylation in 3T3-F442A preadipocytes. *Biochem. J.*, **284**. 649-652.

ANDERSON, N. G. (1993) Simultaneous activation of $p90^{rsk}$ and $p70^{s6k}$ S6 kinases by growth hormone in 3T3-F442A preadipocytes. *Biochem. Biophys. Res. Commun.*, **193.** 284-290.

ANDERSON, W.B., Estival, A., Tapiovaara, H and Gopalakrishna, R. (1985) Altered subcellular-distribution of protein kinase-C (A phorbol ester receptor) - Possible role in tumour promotion and the regulation of cell growth - Relationship to changes in adenylate cyclase activity. *Adv. Cyclic Nucleotide Prot. Phosphoryl. Res.*, **19.** 287-306.

ANTRAS, J., Lasnier, F. and Pairault, J. (1991) The beta-adrenergic-cyclic AMP signalling pathway modulates cell-function at the transcriptional level in 3T3-F442A adipocytes. *Mol. Cell. Endocrinol.*, **82.** 183-190.

ARAKI, E., Lipes, M.A., Patti, M.E., Bruning, J.C., Haag, B., Johnson, R.S. and Kahn, C.R. (1994) Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. *Nature*, **372.** 186-190.

ARCARO, A. and Wymann, M.P. (1993) Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor - The role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. *Biochem. J.*, **296.** 297-301.

ARGETSINGER, L.S., Campbell, G.S., Yang, X., Witthuhn, B.A., Silvennoinen, O., Ihle, J.N. and Carter-Su, C. (1993) Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase. *Cell*, **74**. 237-244.

ARGETSINGER, L.S., Hsu, G.W., Myers, Jr, M.G., Billestrup, N., White, M.F. and Carter-Su, C. (1995) Growth hormone, interferon-γ, and leukemia inhibitory factor promoted tyrosyl phosphorylation of insulin receptor substrate-1. *J. Biol. Chem.*, **270**. 14685-14692.

ARGETSINGER, L.S. and Carter-Su, C. (1996) Mechanism of signalling by growth hormone receptor. *Physiol. Rev.*, **76**. 1089-1107.

ARGETSINGER, L.S., Norstedt, G., Billestrup, N., White, M.F. and Carter-Su, C. (1996) Growth hormone, interferon-γ, and leukemia inhibitory factor utilize insulin receptor substrate-2 in intracellular signalling. *J. Biol. Chem.*, **271**. 29415-29421.

ARNOLD, T.P., Standaert, M.L., Hernandez, H., Watson, J., Mischak, H., Kazanietz, M.G., Zhao, L., Cooper, D.R. and Farese, R.V. (1993) Effect of insulin and phorbol esters on MARCKS (myristoylated alanine-rich C-kinase substrate) phosphorylation (and other parameters of protein kinase C activation) in rat adipocytes, rat soleus muscle and BC3H-1 myocytes. *Biochem. J.*, **295**. 155-164.

ASAOKA, Y., Yoshida, K. and Nishizuka, Y. (1993) Potential role of phospholipase A_2 in HL-60 cell differentiation to macrophages induced by protein kinase C activation. *Proc. Natl. Acad. Sci. USA*, **90.** 4917-4921.

AUGER, K.R., Serunian, L.A., Soltoff, S.P., Libby, P. and Cantley, L.C. (1989) PDGF-dependent tyrosine phosphorylation of stimulates production of novel phosphoinositides in intact-cells. *Cell*, **57.** 167-175.

AVIGNON, A., Standaert, M.L., Yamada, K., Mischak, H., Spencer, B. and Farese, R.V. (1995) Insulin increases mRNA of protein kinase C-α and -β in rat adipocytes and protein kinase C-α, -β and -θ in rat skeletal muscle. *Biochem. J.*, **308.** 181-187.

BABAZONO, T., Kapor-Drezgic, J., Dlugosz, J.A. and Whiteside, C. (1998) Altered expression of and subcellular localization of diacylglycerol-sensitive protein kinase C isoforms in diabetic rat glomerular cells. *Diabetes*, **47.** 666-676.

BACHER, N., Zisman, Y., Berent, E. and Livneh, E. (1991) Isolation and characterization of PKC-L, a new member of the protein kinase C-related gene family specifically expressed in lung, skin and heart. *Mol. Cell Biol.* **11.** 126-133.

BACKER, J. M., Myers, M. G., Shoelson, S. E., Chin, D. J., Sun, X-J., Miralpeix, M., Hu, P., Margolis, B., Skolinik, E. Y., Schlessinger, J. and White, M. F. (1992) Phosphatidylinositol 3'-kinase is activated by association with IRS-1 during insulin stimulation. *EMBO J.*, **11.** 3469-3479.

BAI, Y.L., Zhang, S.Y., Kim, KS, Lee, J.K. and Kim, K.H. (1996) Obese gene-expression alters the ability of 30A5 preadipocytes to respond to lipolytic hormones. *J. Biol. Chem.*, **271.** 13939-13942.

BANDYOPADHYAY, G., Standaert, M.L., Zhao, L., Yu, B., Avignon, A., Galloway, L., Karnam, P., Moscat, J. and Farese, R. (1997) Activation of protein kinase C (α, β, and ζ) by insulin in 3T3/L1 cells. Transfection studies suggest a role for PKC-ζ in glucose transport. *J. Biol. Chem.*, **272**. 2551-2558.

BARCELLINI-COUGET, S., Pradines-Figueres, A., Rous, P., Dani, C. and Ailhaud, G. (1993) The regulation by growth-hormone of lipoprotein-lipase gene-expression is mediated by c-fos protooncogene. *Endocrinol. (Baltimore)*, **132.** 53-60.

BARTHEL, A., Nakatani, K., Dandekar, A.A. and Roth, R.A. (1998) Protein kinase C modulates the insulin-stimulated increase in Akt1 and Akt3 activity in 3T3-L1 adipocytes. *Biochem. Biophys. Res. Commun.*, **243.** 509-513.

BAUMAN, D. E. and Vernon, R. G. (1993) Effects of exogenous bovine somatotrophin on lactation. *Annu. Rev. Nutr.*, **13.** 437-461.

BELFRAGE, P. (1984) Hormonal regulation of lipid degradation. In: *New Perspectives in Adipose Tissue*. p121-145. (Cryer, A and Van, R. L. R., eds.) Butterworths, London.

BELL, R. M. and Burns, D. J. (1991) Lipid activation of protein kinase C. *J. Biol. Chem.*, **266.** 4661-4664.

BERLANGA, J. J., Gualillo, O., Buteau, H., Applanat, M., Kelly, P. A. and Edery, M. (1997) Prolactin activates tyrosyl phosphorylation of insulin receptor substrate 1 and phosphatidylinositol-3-OH kinase. *J. Biol. Chem.*, **272.** 2050-2052.

BERNLOHR, D.A., Bolanowski, M.A., Kelly, T.J. and Lane, M.D. (1985) Evidence for an increase in transcription of specific messenger-RNAs during differentiation of 3T3-L1 preadipocytes. *J. Biol. Chem.*, **260.** 5563-5567.

BERRA, E., Diaz-Meco, M.T., Lozano, J., Frutos, S., Municio, M.M., Sánchez, P., Sanz, L. and Moscat, J. (1995) Evidence for a role of MEK and MAPK during signal transduction by protein kinase C ζ. *EMBO J.*, **14**. 6157-6163.

BERRIDGE, M. J. (1993) Inositol triphosphate and calcium signalling. *Nature*, **361.** 315-325.

BHANDARI, B., Saini, K.S. and Muller, R.E. (1991) Glycerol 3-phosphate dehydrogenase gene-expression in cultured 3T3-L1 adipocytes - Regulation by insulin, dexamethasone and dibutyryl cyclic AMP at the level of messenger-RNA abundance, transcription and messenger-RNA stability. *Mol. Cell. Endocrinol.*, **76.** 71-77.

BIRKENMEIR, E., Gwynn, B., Howard, S., Jerry, J., Gordon, J., Landschluz, W. H. and McKnight, S. L. (1989) Tissue-specific expression, developmental regulation, and genetic mapping of the gene encoding CCAAT/enhancer binding protein. *Genes Dev*, **3.** 1146-1156.

BLACKSHEAR, P. J. (1994) The role (or lack thereof) of protein kinase C in insulin action. In *Molecular Biology of Diabetes, Part II.* p229-244. (Draznin, B. and LeRoth, D., eds.), Humana Press Inc, Totowa, N.J.

BLASCKSHEAR, P. J., Witters, L. A., Girard, P. R., Kuo, J. F. and Quamo, S. N. (1985) Growth factor-stimulated protein phosphorylation in 3T3-L1 cells. Evidence for protein kinase C-dependant and -independent pathways. *J. Biol. Chem.*, **260**. 13304-13315.

BLOBE, C. G., Sachs, C. W., Khan, W. A., Fabbro, D., Stabel, S., Wetsel, W.C., Obeid, L.M., Fine, R.L. and Hannun, Y.A. (1993a) Selective regulation of expression of protein kinase C (PKC) isoenzymes in multidrug-resistant MCF-7 cells: functional significance of enhanced expression pf PKC α. *J. Biol. Chem.*, **268.** 658-664.

BLOBE, G. C., Khan, W. A., Halpern, A. E., Obeid, L. M. and Hannun, Y. A. (1993b) Selective regulation of expression of protein kinase C β isoenzymes occurs via alternative splicing. *J. Biol. Chem.*, **268.** 10627-10635.

BLOBE, C. G., Khan, W. A. and Hannun, Y. H. (1995) Protein kinase C: cellular target of the second messanger arachadonic acid? *Prostoglandins Leukotrienes Essential Fatty Acids*, **52.** 129-135.

BLOBE, G. C., Stribling, S., Obeid, L. M. and Hannan, Y. A. (1996) Protein kinase C isoenzymes: regulation and function. *Cancer Surveys: Cell Signalling*, **27.** 213-248.

BONI, L. T. and Rando, R. R. (1985) The mature of protein kinase C activation by physically defined phospholipid vesocles and diacylglycerols. *J. Biol. Chem.*, **260.** 10819-10825.

BONNET, F., Vanderschueren-Lodeweyckx, M., Eeckels, R. and Malvaux, P. (1974) Subcutaneous adipose tissue and lipids in blood in growth deficiency before and after treatment with human growth hormone. *Pediat. Res.*, **8.** 800-806.

BORNER, C., Guadagno, S. N., Fabbro, D. and Weinstein, I. B. (1992) Expression of four protein kinase C isoforms in rat fibroblasts. Distinct subcellular distribution and regulation by calcium and phorbol esters. *J. Biol. Chem.*, **267**. 12892-12899.

BORNER, C., Eeffiny, M., Jaken, S., Parker, P.J. and Weinstein, I.B. (1995) Two closely related isoforms of protein-kinase-C produce reciprocal effects on the growth of rat fibroblasts - Possible molecular mechanisms. *J. Bio. Chem.*, **270.** 78-86.

BOURGEOIS, F., Alexin, A. and Lemmonier, D. (1983) Diet-induced obesity. - Effect of dietary fats on adipose-tissue cellularity in mice. B*ritish J. Nutrit.*, **49.** 17-26.

BOWERS, B.J., Parham, C.L., Sikela, J.M. and Wehner, J.M. (1993) Isolation of a sequence of mouse-brain cDNA coding for protein kinase-C-gamma. *Gene*, **123**, 263-265.

BOYD, R. D. and Bauman, D. E. (1989) In: *Current Concepts of Animal Growth Regulation*: p257-293 (Campion, D. R., Hausman, G. J. and Martin, R. J., eds.), Plenum Press, New York

BRADFORD, M. M. (1976) A rapid and sensitive method for quantification of microgram quantities of protein utilising the principle of protein dye binding. *Analyt. Biochem.*, **72**. 248-254.

BUDAY, L. and Downward, J. (1993) Epidermal growth-factor regulares p21 (Ras) through the formation of a complex of receptor, Grb2 adaptor protein and Sos nucleotide exchange factor. *Cell,* **73.** 611-620.

BUDD, T.J., Atkinson, J.L., Buttery, P.J., Salter, A.M. and Wiesman, J. (1994) Effeccts of insulin and isoproterenol on lipid-metabolism in porcine adipose-tissue from different depots. *Comparative. Biochem. Physiol. C - Pharmacol., Toxicol. Endocrinol.*, **108.** 137-143.

BURGERING, B.M.T. and Coffer, P.J. (1995) Protein-kinase-B (c-Akt) in phosphatidylinositol-3-OH kinase signal-transduction. *Nature,* **376.** 599-602.

BUSHFIELD, M., Griffiths, S.C., Straussheim, D., Tang, E., Shakur, Y., Lavan, B. and Houslay, M.D. (1991) Guanine-nucleotide-binding proteins in diabetes and insulin-resistant states. *Biochem. Soc. Sympos.*, **56.** 137-154.

CAI, H., Smola, U., Wixler, V., Eisenmann-Tappe, I., Diaz-Meco, M. T., Moscat, J., Rapp, U. and Cooper, G. M. (1997) Role of diacylglycerol-regulated protein kinase C iosforms on growth factor activation of the Raf-1 protein kinase. *Mol. & Cell. Biol.* **17.** 732-741.

CAMPBELL, G.S., Pang, L., Miyasaka, G,m Saltiel, A.R. and Cater-Su, C. (1992) Stimulation by growth-hormone of MAP kinase-activation in 3T3-F442A fibroblasts. *J. Biol. Chem.*, **267.** 6974-6080.

CAMPBELL, G. S., Christian, L. J. and Carter-Su, C. (1993) Evidence for involvement of the growth hormone receptor-associated tyrosine kinase in actions of growth hormone. *J. Biol. Chem.* **268.** 7427-7434.

CAMPBELL, G.S., Meyer, D.J., Raz, R., Levy, D.E., Schwartz, J. and Carter-Su, C. (1995) Activation of acute-phase response factor (APRF)/STAT3 transcription factor by growth-hormone. *J. Biol., Chem.*, **270.** 3974-3979.

CAMPOS, S.P., Wang, Y. and Baumann, H. (1996) Insulin modulates STAT3 protein activation and gene transcription in hepatic cells. *J. Biol. Chem.*, **271**. 24418-24424.

CAO, Z., Umek, R. and McKnight, S. L. (1991) Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes Dev.*, **5.** 1538-1552.

CASTAGNA, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.*, **257.** 7847-7851.

CATALIOTO, R-M., Gaillard, D., Ailhaud, G. and Negrel, R. (1992) Terminal differentiation of mouse preadipocyte cells: the mitogenic-adipogenic role of growth hormone is mediated by the protein kinase C signalling pathway. *Growth Factors*, **6**. 255-264.

CAZAUBON, S., Bornancin, F. and Parker, P. J. (1994) Threonine-497 is a critical site for permissive activation of protein kinase C α. *Biochem. J.*, **301.** 443-448.

CERESA, B.P. and Pessin, J.E. (1996) Insulin stimulates the serine phosphorylation of the signal transducer and activator of transcription (STAT3) isoform. *J. Biol. Chem.*, **271**. 12121-12124.

CHALFANT, C.E., Mischak, H., Watson, J.E., Winkler, B.C., Goodnight, J., Farese, R.V. and Cooper, D.R. (1995) Regulation of alternative splicing of protein kinase Cβ by insulin. *J. Biol. Chem.*, **270.** 13326-13332.

CHANG, Y.W.E. and Traugh, J.A. (1997) Phosphorylation of elongation factor 1 and ribosomal protein S6 by multipotential S6 kinase and insulin stimulation of translational elongation. *J. Biol. Chem.*, **272.** 28252-28257.

CHAPMAN, A.B., Knight, D.M., Dieckman, B.S. and Ringold, G.M. (1984) Analysis of gene-expression during differentiation of adipogenic cells in culture and hormonal control of the developmental program. *J. Biol. Chem.*, **259.** 15548-15555.

CHAUHAN, V. P. S., Chauhan, A., Deshumkh, D. S. and Brokerhoff, H. (1990) Lipid activators of protein kinase C. *Life Sci.*, **47.** 981-986.

CHAWLA, A. and Lazar, M.A. (1994) Peroxisome proliferator and retinoid signalling pathways coregulate preadipocyte phenotype and survival. *Proc. Natl. Acad. Sci. USA*, **91.** 1786-1790.

CHAWLA, A., Schwartz, E. J., Dimaculangan, D. D. and Lzar, M. A. (1994) Peroxisome proliferator-activated receptor (PPAR) γ: adipocyte-predominant expression and induction early in adipocyte differentiation. *Endocrinology*, **135.** 798-800.

CHEATHAM, B., Vlahos, C.J., Cheatham, L., Wang, L., Blenis, J. and Kahn, C.R. (1994) Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose-transporter translocation. *Mol. Cell. Biol.*, **14.** 4902-4011.

CHEN, J., Sadowski, H.B., Kohanski, R.A. and Wang, L.H. (1997a) Stat 5 is a physiological substrate for the insulin receptor. *Proc. Natl. Acad. Sci. USA*, **94.** 2295-2300.

CHEN, P. L., Scully, P., Shew, J. Y., Wang, J. Y. Y. and Lee, W. H. (1989) Phosporylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. *Cell*, **58.** 1193-1198.

CHEN, R.-H., Corbalan-Garcia, S. and Bar-Sagi, D. (1997b) The role of the PH domain in the signal-dependant membrane targeting of Sos. *EMBO J.*, **16**. 1351-1359.

CHIDA, K., Kato, N. and Kuroki, T. (1986) Downregulation of phorbol diester receptors by proteolytic degradation of protein kinase C in a cultured cell line of fetal rat skin keratinocytes. *J. Biol. Chem.*, **261.** 13013-13018.

CHIN, J.E., Dickens, M., Tavare, J.M. and Roth, R.A. (1993) Overexpression of protein kinase-C isoenzymes-alpha, beta-I, gamma, and epsilon in cells overexpressing the insulin receptor - Effects on receptor phosphorylation and signalling. *J. Biol. Chem.*, **268.** 6338-6347.

CHIN, J.E., Liu, F. and Roth, R.A. (1994) Activation of protein kinase Cα inhibits insulin-stimulated tyrosine phosphorylation of insulin receptor substrate-1. *Mol. Endocr.*, **8**. 51-58.

CHRISTY, R. J., Yang, V., Ntambi, J., Geiman, D., Landschulz, W. H., Friedman, A., Nakabeppu, Y., Kelly, T. and Lane, M. D. (1989) Differentiation-induced gene expression in 3T3-L1 preadipocytes: CCAAT/enhancer binding protein interacts with and activates the promoters of two adipocyte-specific genes. *Genes Dev.*, **3.** 1323-1335.

CHRISTY, R. J., Kaestner, K. H., Geiman, D. E. and Lane, M. D. (1991) CCAAT/enhancer binding protein gene promoter: binding of nuclear factors during differentiation of 3T3-L1 preadipocytes. *Proc. Natl. Acad. Sci. USA,* **88.** 2593-2597.

CHUNG, J.K., Uchida, E., Grammer, T.C. and Blenis, J. (1997) STAT3 serine phosphorylation by ERK-dependent and -independent pathways negatively modulates its tyrosine phosphorylation. *Mol. Cell. Biol.*, **17.** 6508-6516.

CLARKSON, R.W.E., Chen, C.M., Harrison, S., Wells, C., Muscat, G.E.O. and Waters, M.J. (1995) Early responses of transactivating factors to growth-hormone in preadipocytes - Differential regulation of CCAAT enhancer-binding protein beta (C/EBP beta) and C/EBP delta. *Mol. Endocrinol.*, **9.** 108-120.

COBB, M.H. and Goldsmith, E.J. (1995) How MAP kinases are regulated. *J. Biol. Chem.*, **270**. 14843-14846.

CONSIDINE, R.V., Nyce, M.R., Allen, L.E., Morales, L.M., Triester, S., Serrano, J., Colberg, J., Lanza-Jacoby, S. and Caro, J.F. (1995) Protein kinase C is increased in the liver of humans and rats with non-insulin-dependent diabetes mellitus: an alteration not due to hyperglycemia. *J. Clin. Invest.*, **95.** 2938-2944.

COOK, J.S., Lucas, J.J., Sibley, E., Bolanowski, M.A., Christy, R.J., Kelly, T.J. and Lane, M.D. (1988) Expression of the differentiation-induced gene for fatty acid-binding protein is activated by glucocorticoid and cAMP. *Proc. Natl. Acad. Sci. USA,* **85.** 2949-2953.

CORIN, R.E., Guller, S., Wu, K.-Y. and Sonenberg, M. (1990) Growth hormone and adipose differentiation: growth hormone-induced antimitogenic state in 3T3-F442A preadipose cells (terminal differentiation/hormonal pathway). *Proc. Natl. Acad. Sci.USA,* **87**. 7507-7511.

CORNELIUS, P., MacDougald, O. A. and Lane, M. D. (1994) Regulation of adipocyte development. *Annu. Rev. Nutr.*, **14.** 99-129.

COUSSENS, L., Parker, P. J., Rhee, L., Yangfeng, T.L., Chen, E., Waterfield, M.D., Francke, U. and Ullrich, A. (1986) Multiple, distinct forms of bovine and human protein kinase C suggests diversity in cellular signalling pathways. *Science,* **233.** 859-866.

COUSSENS, L., Rhee, L., Parker, P. and Ullrich, A. (1987) Alternative splicing increases the diversity of the human protein kinase family. *DNA,* **6.** 389-394.

CROSS, D.A.E., Alessi, D.R., Cohen, P., Andjelkovich, M., Hemmings, B.A. (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature,* **378**. 785-789.

DANI, C., Doglio, A., Grimaldi, P. amd Ailhaud, G. (1986) Expression of phosphoenolpyruvate carboxykinase gene and its insulin regulation during differentiation of preadipose cell-lines. *Biochem. Biophys. Res. Comm.*, **138.** 468-475.

DANI, C., Amri, E., Bertrand, B., Enerback, S., Bjursell, G., Grimaldi, P. and Ailhaud, G. (1990) Expression and regulation of pob24 and lipoprotein-lipase genesduring adipose conversion. *J. Cell. Biochem.*, **43.** 103-110.

DANIELSEN, A.G., Liu, F., Hosomi, Y., Shii, K. and Roth, R.A. (1995) Activation of protein kinase Cα inhibits signalling by members of the insulin receptor family. *J. Biol. Chem.*, **270**. 21600-21605.

DAVID, M., Petricoin, E., Benjamin, C., Pine, R., Weber, M.J. and Larner, A.C. (1995) Requirement for MAP kinase (ERK2) activity in interferon-alpha-stimulated and interferon-beta-stimulated gene expression through STAT proteins. *Science,* **269.** 1721-1723.

DAVIS, R.J. (1993) The mitogen-activated-protein-kinase signal-transduction pathway. *J. Biol. Chem.*, **268.** 14553-14556.

DE COY, D.L., Snapper, J.R. and Breyer, M.D. (1995) Anti sense DNA down-regulates protein-kinase C-epsilon and enhances vasopressin-stimulated Na^+ absorption in rabbit cortical collecting duct. *J. Clin. Invest.*, **95**, 2749-2756.

DE FEA, K. and Roth, R.A. (1997) Protein kinase C modulation of insulin receptor substrate-1 tyrosine phosphorylation requires serine 612. *Biochem.*, **36.** 12939-12947.

DE FRONZO, R.A., Bonadonna, R.C. and Ferrannini, E. (1992) Pathogenesis of NIDDM. - A balanced overview. *Diabetes Care*, **15.** 318-368.

DE HERREROS, A.G. and Birnbaum, M.J. (1989) The acquisition of increased insulin-responsive hexose-transport in 3T3-L1 adipocytes correlates with expression of a novel transporter gene. *J. Biol. Chem.*, **264.** 19994-19999.

DE VENTE, J., Kukoly, C. A., Bryant, W. O., Posekany, K. J., Chen, J., Fletcher, D. J., Parker, P. J., Pettit, G. J., Lozano, G., Cook, P. P. and Ways, D. K. (1995) Phorbol esters induce death in MCF-7 breat cancer cells with altered expression of protein kinase C isoforms. *J. Clin. Invest.*, **96.** 1874-1886.

DE VENTE, J., Bryant, W., Posekany, K., Garris, T., Chen, J., Carey, J., Cook, P. and Ways, K. (1996a) Phorbol ester stimulated expression in p53-negative leukemic cells: death in the absence of a G1 arrest of differentiation. *Oncol. Rep.*, **3.** 213-217.

DE VENTE, J.E., Carey, J.O., Bryant, W.O., Pettit, G.J. and Ways, D.K. (1996b) Transcriptional regulation of insulin receptor substrate 1 by protein kinase C. *J. Biol. Chem.*, **271**. 32276-32280.

DEACON, E.M., Pongracz, J., Griffiths, G. and Lord, J.M. (1997) Isoenzymes of protein kinase C: differential involvement in apoptosis and pathogenesis. *J. Clin. Pathol. Mol. Pathol.*, **50**. 124-131.

DENNING, M.F., Dlugosz, A.A., Howett, M.K. and Yuspa, S.H. (1993) Expression of an oncogenic ras^{Ha} gene in murine keratinocytes induces tyrosine phosphorylation and reduced activity of protein kinase C δ. *J. Biol. Chem.*, **268.** 26079-26081.

DENNING, M.F., Dlugosz, A.A., Threadgill, D.W. Magnuson, T. and Yuspa, S.H. (1996) Activation of the epidermal growth factor receptor signal transduction pathway stimulates tyrosine phosphorylation of protein kinase C δ. *J. Biol. Chem.*, **271.** 5325-5331.

DENTON, R.M. and Tavare, J.M. (1995) Does mitogen-activated protein-kinase have a role in insulin action? - The cases for and against. *Eur. J. Biochem.*, **227.** 597-691.

DI GIROLAMO, M. Mendliger, S. and Fertig, J. W. (1971) A simple method to determine fat cell size and number in four mammalian species. *Am. J. Physiol.*, **221**. 850-858.

DIAZ-MECO, M. T., Lozabo, J., Muncinio, M. M., Berra, E., Futos, S., Sauz, L. and Moscat, J. (1994) Evidence of the in vitro and in vivo interaction of Ras with protein kinase C. *J. Biol. Chem.*, **269.** 31706-31710.

DIAZ-MECO, M.T., Municio, M.M., Fruts, S., Sanchez, P., Lozano, J., Sanz, L. and Moscat, J. (1996) The product of *par*-4, a gene induced during apoptosis, interacts selectively with the atypical isoforms of protein kinase C. *Cell,* **86.** 777-786.

DIETERICH, S., Herget, T., Link, G., Bottinger, H. Pfizenmaier, K. and Johannes, F. J. (1996) In vitro activation and substrates of recombinant, baculovirus expressed human protein kinase C mu. *FEBS Lett.*, **381**. 183-187.

DIETZ, J. and Schwartz, J. (1991) Growth hormone alters lipolysis and hormone-sensitive lipase activity in 3T3-F442A adipocytes. *Metabolism*, **40**. 800-806.

DOBROWSKY, R. T. and Hannun, Y. A. (1992) Ceramide stimulates a cytosolic protein phosphatase. *J. Biol. Chem.*, **267.** 5048-5051.

DOGLIO, A., Dani, C., Grimaldi, P. and Ailhaud, G. (1986) Growth-hormone regulateion of the expression of differentiation-dependent genes in preadipocyte OB1771 cells. *Biochem. J.*, **238.** 123-129.

DOGLIO, A., Dani, C., Fredrikson, E., Grimaldi, P. and Ailhaud, G. (1987) Acute regulation of IGF-I gene-expression by growth-hormone in OB1771 cells. *Diabete Metabolisme,* **5.** 560-564.

DOGLIO, A., Dani, C., Grimaldi, P. and Ailhaud, G. (1989) Growth hormone stimulates c-*fos* gene expression by means of protein kinase C without increasing inositol lipid turnover. *Proc. Natl. Acad. Sci. USA*, **86**. 1148-1152.

DONG, L.Q., Du, H.Y., Porter, S.G., Kolakowski, L.F., Lee, A.V., Mandarino, J., Fan, J.B., Yee, D. and Liu, F. (1997) Cloning, chromosome localization, expression, and characterization of an Src homology 2 and pleckstrin homology domain-containing insulin receptor binding protein hGrb10 gamma. *J. Biol. Chem.*, **272.** 29104-29112.

DORIS, R., Vernon, R. G., Houslay, M. D. and Kilgour, E. K. (1994) Growth hormone decreases the response to anti-lipolytic agonists and decreases the levels of G_i2 in rat adipocytes. *Biochem. J.*, **297.** 41-45.

DORIS, R.A., Kilgour, E., Houslay, M.D. and Vernon, R.G. (1998) Regulation of the GTP-binding protein-based antilipolytic system of sheep adipocytes by growth hormone. *Endocrinol.*, **158.** 295-303.

DREVER, C., Krey, G., Keller, H., Givel, F., Helftenbein, G. and Wahli, W. (1992) Control of the peroxisomal β-oxidation pathway by a novel family of nuclear hormone receptors. *Cell* **68.** 879-887.

DUCHER, L., Croquet, F., Gil, S., Davy, J., Féger, J. and Bréhier, A. (1995) Differential expression of five protein kinase C isoenzymes in FAO and HEPG2 hepatoma cell lines compared with normal rat hepatocytes. *Biochem. Biophys. Res. Commun.*, **217**. 546-553.

EDMONDSON, D.G. and Olson, E.N. (1993) Helix-loop-helix proeins as regulators of muscle-specific transcription. *J. Biol. Chem.*, **268.** 755-758.

ERIKSSON, H., Ridderstrale, M., Degerman, E., Ekholm, D., Smith, C.J., Manganiello, V.C., Belfrage, P. and Tornqvist, H. (1995) Evidence for the key role of the adipocyte cGMP-inhibited cAMP phosphodiesterase in the antilipolytic action of insulin. *Biochim. Biophys. Acta,* **1266.** 101-107.

ERIKSSON, R.S.M., Thunberg, L. and Eriksson, U.J. (1989) Effects of interrupted insulin-treatment on fetal-outcome of pregnant diabetic rats. *Diabetes*, **38.** 764-772.

EVANS, R.M. (1988) The steroid and thyroid-hormone receptor superfamily. *Science,* **240.** 889-895.

FAIN, J.N. (1985) Activation of plasma-membrane phosphatidylinositol turnover by hormones. *Vitamins Hormones - Advances Res. Applic.,* **41.** 117-160.

FARAH, S., Agazie, Y., Ohan, N., Ngsee, J.K. and Liu, X.J. (1998) A rho-associated protein kinase, ROK alpha, binds insulin receptor substrate-1 and modulates insulin signalling. *J. Biol. Chem.,* **273.** 4740-4746.

FARESE, R. V., Standaert, M. L., Francois, A. J., Ways, K., Arnold, T. P., Hernandez, H. and Cooper, D. R. (1992) Effects of insulin and phorbol esters on subcellular distribution of protein kinase C isoforms in rat adipocytes. *Biochem. J.,* **288**. 319-323.

FELGNER, P.L. Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M. and Danielson, M. (1987) Lipofectin - A highly efficient, lipid-mediated DNA-transfection process. *Proc. Natl. Acad. Sci. USA,* **84.** 7413-7417.

FELIPO, V., Minana, M.D. and Grisolia, S. (1990) A specific inhibitor of protein kinase-C induces differentiation of neuro-blastoma cells. *J. Biol. Chem.,* **265.** 9599-9601.

FERVERT, E. J. and Kahn, B. B. (1996) Protein kinase C isoforms ε, η, δ and ζ in murine adipocytes: expression, subcellular localization and tissue-specific regulation in insulin-resistant states. *Biochem. J.,* **316**. 865-871.

FLEISCHMAN, L. F., Chahwala, S. B. and Cabtley, L. (1986) *Ras*-transformed cells: altered levels of phosphatidylinositol-4,5-biphosphate and catabolites. *Science,* **231.** 407-410.

FLORES-DELGADO, G., Marsch-Moreno, M. and Kuri-Harcuch, W. (1987) Thyroid-hormone stimulates adipocyte differentiation of 3T3 cells. *Mol. Cell. Biochem.,* **76.** 35-43.

FOLGUEIRA, L., McElhinny, J.A., Bren, G.D., MacMorran, W.S., Diazmeco, M.T., Moscat, J. and Paya, C.V. (1996) Protein kinase C-zeta mediates NF-kappa B activation in human immunodeficiency virus-infected monocytes. *J. Virol.*, **70**. 223-231.

FOSTER, C.M., Hale, P.M., Jing, H.W. and Schwartz, J. (1988) Effects of human growth-hormone on insulin-stimulated glucose-metabolism in 3T3-F442A adipocytes. *Endocrinol.*, **123.** 1082-1088.

FOWELIN, J., Attvall, S., von Schenck, H., Smith, U. and Lager, I. (1991) Characterization of the insulin-antagonistic effect of growth hormone in man. *Diabetologia.*, **34**. 500-506.

FRAKER, P.J. and Speck, J.C.Jnr. (1978) Protein and cell membrane iodinations with a sparingly soluble chloroamide 1,3,4,6-tetra-chloro-3α,6α-diphenylglycoluril. *Biochem. Biophys. Res. Commun.*, **80.** 849-857.

FREDERICH, R.C., Lollmann, B., Hamann, A., Napolitanorosen, A., Kahn, B.B., Lowell, B.B. and Flier, J.S. (1995) Expression of ob messenger-RNA and its encoded protein in rodents. (Impact of nutrition and obesity). *J. Clin. Invest.*, **96.** 1658-1663.

FREVERT, E.U. and Kahn, B.B. (1997) Differential effects of constitutively phosphatidylinositol 3-kinase on glucose transport, glycogen synthase activity, and DNA synthesis in 3T3-L1 adipocytes. *Mol. & Cell. Biol.*, **17**. 190-198.

FREYTAG, S. and Geddes, T. (1992) Reciprocal regulation of adipogenesis by Myc and C/EBPα. *Science*, **256**. 279-282.

FREYTAG, S., Paielli, D. L. and Gilbert, J. D. (1994) Ectopic expression of the CCAAT/enhancer-binding protein α promotes the adipogenic program in a variety of mouse fibroblastic cells. *Genes Dev.*, **8**. 1654-1663.

FRIEDMAN, J. M. and Lehel, R. L. (1992) Tackling a weighty problem. *Cell*, **69.** 217-220.

FRUHBECK, G., Aguado, M. and Martinez, J.A. (1997) In vitro lipolytic effect of leptin on mouse adipocytes. -Evidence for a possible autocrine/paracrine role. *Biochem. Biophys. Res. Comm.*, **240.** 590-594.

GAMOU, S. and Shimizu, N. (1986) Adipocyte differentiation of 3T3-L1 cells in serum-free hormone-supplemented media. - Effects of insulin and dihydroteleocidin B. *Cell Struc. Funct.*, **11.** 21-30.

GHARBI-CHIHI, J., Bismuth, J., Lissitzky, S. and Torresani, J. (1983) The effect of triiodothyronine on fatty-acid synthase - Activity and control in differentiating OB17 preadipocytes. *Biochim. Biophys. Acta*, **750.** 282-290.

GLAZER, R. I. (1994) Protien kinase C in multidrug resistance, neoplastic trasnformation and differentiation. In: *Protein Kinase C.* (Kuo, J. F., ed.) Oxford University Press.

GLENN, K.C., Shieh, J.J. and Laird, D.M. (1992) Characterization of 3T3-L1 storage lipid metabolism: effect of stomatotrophin and insulin on specific pathways. *Endocrinology*, **131**. 1115-1124.

GONZALEZ, G.A. and Montminy, M.R. (1989) Cyclic-AMP stimulates somatostatin gene-transcription by phosphorylating CREB at Ser-133. *Cell*, **59.** 675-680.

GOODMAN, H. M. (1993) In: *The Endocrinology of Growth, Development and Metabolism in Vertebrates*: p93-115 (Schreibman, M. P., Scanes, C. G. And Pang, P. K. T., eds), Academic Press, San Diego.

GOODMAN, H.M., Coiro, G.P., Gorin, E., Grichting, G., Honeyman, T.W. and Szecowka, J. (1987) Effects of growth-hormone on the rat adipocyte - A model for studying direct actions of growth-hormone. *Endocrinologia Japonica*, **34.** 59-72.

GOODMAN, H. M., Schwartz, Y., Tai, L. R. and Gorin, E. (1990) Actions of growth hormone on adipose tissue: possible involvement of autocrine or paracrine factors. *Acta Paediatr. Scand.*, **367.** 132-136.

GOODNIGHT, J., Mischak, H., Kolch, W. and Mushinski, J.F. (1995) Immunocytochemical localisation of 8 protein-kinase-C isozymes overexpressed in NIH 3T3 fibroblasts - Isozyme-specific association with microfilaments, golgi, endoplasmic-reticulum, and nuclear and cell-membranes. *J. Biol. Chem.*, **270.** 9991-10001.

GORIN, E., Tai, L.-R., Honeyman, T. W. and Maurice, H. (1990) Evidence for a role of protein kinase C in the stimulation of lipolysis by growth hormone and +isoproterenol. *Endocrinology*, **126**. 2973-2982.

GOTTLICHER, M., Widmark, E., Li, Q. and Gustafsson, J. A. (1992) Fatty acids activate a chimera of the clofibric acid-activated receptor and the glucocorticoid receptor. *Proc. Natl. Acad. USA*, **89.** 4653-4657.

GOUILLEUX, F., Pallard, C., Dusanterfourt, I., Wakao, H., Haldosen, L.A., Norstedt, G., Levy, D. and Groner, B. (1995) Polactin, growth-hormone, erythropoietin and granulocyte-macrophage colony-stimulating factor induce MGF-STAT 5 DNA-binding activity. *EMBO J.*, **14.** 2005-2013.

GOULD, G.W. and Bell, G.I. (1990) Facilitative glucose transporters - An expanding family. *Trends Biol. Sci.*, **15.** 18-23.

GREEN, H. and Kehinde, O. (1974) Sublines of mouse 3T3 cells that accumulate lipid. *Cell*, **1.** 113-116.

GREEN, H. and Kehinde, O. (1975) An established preadipose cell line and its differentiation in culture. *Cell*, **5.** 19-27.

GREEN, H. and Kehinde, O. (1976) Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells. *Cell*, **7.** 105-113.

GREEN, H. and Kehinde, O. (1979) Formation of normally differentiated subcutaneous fat pads by an established preadipose cell line. *J. Cell. Physiol.*, **101.** 169-171.

GREEN, H., Morikawa, M. and Nixon, T. (1985) A dual effector theory of growth-hormone action. *Differentiation*, **29**. 195-198.

GREEN, S. and Chambon, P. (1988) Nuclear receptors enhance our understanding of transcription regulation. T*rends Genet.*, **4.** 309-314.

GREENWOOD, M.R.C. and Hirsch, J. (1974) Postnatal development of adipose cellularity in the normal rat. *J. Lipid Res.*, **15.** 474-483.

GRIGORIADIS, A.E., Heersche, J.N.M. and Aubin, J.E. (1988) Differentiation of muscle, fat, cartillage, and bone from progenitor cells present in a bone-derived clonal cell-population - Effects of dexamethasone. *J. Cell Biol.*, **106.** 2139-2151.

GRIMALDI, P., Djian, P., Negrel, R. and Ailhaud, G. (1982) Differentiation of OB17 preadipocytes to adipocytes - Requirement of adipose conversion factor(s) for fat-cell cluster formation. *EMBO J.*, **1.** 687-692.

GRONOWSKI, A.M. and Rotwein, P. (1994) Rapid changes in nuclear-protein tyrosine phophorylation after growth-hormone treatment *in vivo* - Identification of phosphorylated mitogen-activated protein-kinase and STAT91. *J. Biol. Chem.*, **269.** 7874-7878.

GRUNBERGER, G. (1991) Interplay between insulin signalling and protein kinase C. *Cell. Signalling*, **3.** 171-177.

GSCHWENDT, M., Kittstein, W. and Marks, F. (1991) Protein-kinase-C activation by phorbol esters - Do cysteine-rich regions and pseudosubstrate motifs play a role? *Trends Biol. Sci.*, **16.** 167-169.

GSCHWENDT, M., Leibersperger, H., Kittstein, W. and Marks, F. (1992) Protein kinase C ζ and η in murine epidermis TPA induces down-regulation of PKCη but not PKCζ. *FEBS Lett.*. **307.** 151-155.

GSCHWENDT, M., Kielbassa, K., Kittstein, W. and Marks, F. (1994) Tyrosine phosphorylation and stimulation of protein kinase Cδ from porcine spleen by scr in vitro: dependence on the activated state of protein kinase Cδ. *FEBS Lett.*, **347.** 85-89.

GU, W., Schneider, J. W., Conorell, G., Kaushal, S., Mahdavi, V. and Nadal-Ginard, B. (1993) Interaction of mygenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. *Cell*, **72.** 309-324.

GUEST, S.J., Hadcock, J.R., Watkins, D.C. and Malbon, C.C. (1990) Beta-1-adrenergic and beta-2-adrenergic receptor expression in differentiating 3T3-L1 cells - Independent regulation at the level of messenger-RNA. *J. Biol. Chem.*, **265.** 5370-5375.

GULLER, S., Corin, R.E., Mynarick, D.C., London, B.M. and Sonenberg, M. (1988) Role of insulin in growth hormone-stimulated 3T3 cell adipogenesis. *Endocrinology*, **122**. 2084-2089.

GURLAND, G., Ashcom, E., Cochran, B.H. and Schwartz, J. (1990) Rapid events in growth-hormone action - Induction of c-fos and c-jun transcription in 3T3-F442A preadipocytes. *Endocrinol.*, **127.** 3187-3195.

GYSIN, S. and Imber, R. (1997) Phorbol-ester-activated protein kinase C-alpha lacking phosphorylation at Ser657 is down-regulated by a mechanism involving dephosphorylation. *Europ. J. Biochem.*, **249.** 156-160.

HAGER, A. (1981) Adipose-tissue cellularity in childhood in relation to the development of obesity. *British Med. Bulletin*, **1981.** 287-290.

HAGIWARA, M., Brindle, P., Harostunian, A., Armstrong, R., Rivier, J., Vale, W., Tsien, R. and Montminy, M.R. (1993) Coupling of hormonal-stimulation and transcription via the cyclic AMP-responsive factor CREB is rate-limited by nuclear entry of protein kinase-A. *Mol. Cell. Biol.*, **13.** 4852-4859.

HALAAS, J.L., Gajiwala, K.S., Maffei, M., Cohen, S.L., Chait, B.T., Rabinowitz, D., Lallone, R.L., Burley, S.K. and Friedman, J.M. (1995) Weight-reducing effects of the plasma-protein encoded by the obese gene. *Science*, **269.** 543-546.

HALEVY, O., Novitch, B. G., Spicer, D. B., Spapek, S. X., Rhee, J., Hannon, G. J., Beach, D. and Lassar, A. B. (1995) Correlation of terminal cell cycle arrest of sceletal muscle with induction of p21 by myoD. *Science*, **267.** 1018-1021.

HAMADA, K., Takuwa, N., Zhou, W., Kumada, M. and Takuwa, Y. (1996) Protein kinase C inhibits the CAK-CDK2 cyclin-dependent kinase cascade and G1/S cell cycle progression in human diploid fibroblasts. *Biochim. Biophys. Acta*, **1310.** 149-156.

HANKS, S. K., Quinn, A. M. and Hunter, T. (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science*, **241.** 45-52.

HANNUN, Y. A. and Bell, R. M. (1986) Phorbol ester binding and activation of protein kinase C on triton X-100 mixed micelles containing phosphatidylserine. *J. Biol. Chem.*, **261.** 9341-9347.

HANNUN, Y. A., Loomis, C. R. and Bell, R. M. (1986) Protein kinase C activation in mixed micelles: mechanistic implications of phospholipid, diacylglycerol and calcium interdependencies. *J. Biol. Chem.*, **261.** 7184-7190.

HARA, K., Yonezawa, K., Sakaue, H., Kotani, K., Kotani, K., Kojima, A., Waterfield, M. D. and Kasuga, M. (1995) Normal activation of p70 S6 kinase by insulin in cells overexpressing dominat negative 85kD subunit of phophoinositide 3-kinase. *Biochem. Biophys. Res. Commun.*, **208.** 735-741.

HARRIS, D.M., Dunshea, F.R., Bauman, D.E., Boyd, R.D., Wang, S.Y., Johnson, P.A. and Clarke, S.D. (1993) Effect of in vivo somatotropin treatment of growing pigs on adipose-tissue lipogenesis. *J. Anim. Sci.*, **71.** 3293-3200.

HATA, A., Akita, Y., Suzuki, K. and Ohno, S. (1993) Functional divergence of protein-kinase-C (PKC) family members - PKC-gamma differs from PKC-alpha and PKC-beta II and nPKC-epsilon in its competence to mediate 12-O-tetradecanoyl phorbol 13-acetate (TPA)-responsive transcriptional activation through a TPA-response element. *J. Biol. Chem.*, **268.** 9122-9129

HAUNER, H. (1990) Complete adipocyte differentiation of 3T3-L1 cells in a chemically defined medium - compared to serum-containing culture conditions. *Endocrinol.*, **127.** 865-872.

HAUNER, H., Entemann, G., Wabitsch, M., Gaillard, D., Ailhaud, G., Negrel, R. and Pfeiffer, E.F. (1989) Promotional effect of glucocorticoids on the differentiation of human adipocyte precursor cells cultured in a chemically defined medium. *J. Cli. Invest.*, **84.** 1663-1670.

HAUNER, H., Rotrig, F. and Petruschke, T. (1995) Effects of epidermal growth-factor (EGF), platelet-derived growth-factor (PDGF) and fibroblast growth-factor (FGF) on human adipocyte development and function. *J. Clin. Invest.*, **25.** 90-96.

HAUSDORF, S., Clement, J. and Loos, U. (1988) Expression of SI4 gene during differentiation of 3T3-L1 cells. *Hormone Metabolic Res.*, **20.** 723-724.

HAUSDORFF, S. F., Bennet, A. M., Neel, B. G. and Birnbaum, M. J. (1995) Different signalling roles of SHPTP2 in insulin-induced GLUT1 expression and GLUT4 translocation. *J. Biol. Chem.*, **270.** 12965-12968.

HAUSMAN, G.J. and Martin, R.J. (1989) The influence of human growth hormone on preadipocyte development in serum-supplemented and serum-free cultures of stromal-vascular cells from pig adipose-tissue. *Dom. Ani. Endocrinol.*, **6.** 331-337.

HAYSTEAD, T. A. J., Weiel, J. E., Litchfield, D. W., Tsukitani, Y., Fischer, E. H. and Krebs, E. G. (1990) Okadaic acid mimics the action of insulin in stimulating protein kinase activity in isolated adipocytes. *J. Biol. Chem.*, **265.** 16571-16580.

HEICHMAN, K. A. and Roberts, J. M. (1994) Rules to replicate by. *Cell*, **79.** 557-562.

HIRAGUN, A., Sato, M. and Mitsui, H. (1980) Establishment of a clonal cell-line that differentiates into adipose-cells in vitro. *In vitro J. Tiss. Cult. Assoc.*, **16.** 685-693.

HIRAI, S., Izumi, Y., Higa, K., Kaibuchi, K., Mizuno, K., Osada, S., Suzuki, K. and Ohno, S. (1994) Ras-dependant signal transduction is indispensable but not sufficient for the activation of AP1/Jun by PKCδ. *EMBO J.*, **13**. 2331-2340.

HOFMANN, J. (1997) The potential for isoenzyme-selective modulation of protein kinase C. *FASEB J.*, **11**. 649-669.

HOUSE, C. and Kemp, B. E. (1987) Protein kinase contains a pseudosubstrate prototype in its regulatory domain. *Science*, **238**. 1726-1728.

HOUSLAY, M.D. (1991) 'Crosstalk': a pivotal role for protein kinase C in modulating relationships between signal transduction pathways. *Eur. J. Biochem.*, **195**. 9-27.

HOUSLAY, M.D. (1995) Compartmentalisation of cyclic AMP phosphodiesterases: signalling crosstalk, desensitisation and the phosphorylation of G(i)-2 add cell-specific personalisation to the control of the levels of the 2nd messenger cyclic AMP. *Adv. Enz. Reg.*, **35.** 303-338.

HU, E., Kim, J.B., Sarraf, P. and Spiegelman, B.M. (1996) Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARγ. *Science*, **274**. 2101-2103.

HUANG, C. L. and Ives, H. E. (1987) Growth inhibition by protein kinase C late in mitogenesis. *Nature*, **329.** 849-850.

HUANG, K.P. and Huang, F.L. (1986) Conversion of protein kinase C from a Ca^{2+}-dependent to and independent form of phobol ester-binding protein by digestion with trypsin. *Biochem. Biophys. Res. Comm.*, **139.** 320-326

HUBEL, D.H. and Wiesel, T.N. (1979) The Brain, A Scientific American Book. W.H. Freeman, San Francisco.

HUG, H. and Sarre, T. F. (1993) Protein kinase C isozymes: divergence in signal transduction? *Biochem. J.*, **291.** 329-343.

HUNDLE, B., McMahon, T., Dadgar, J. and Messing, R.O (1995) Overexpression of epsilon-protein-kinase-C enhances nerve growth factor-induced phosphorylation of mitogen-activated protein-kinases and neurite outgrowth. *J. Biol. Chem.*, **270.** 30134-30140.

HUNTER, T. (1993) Signal-transduction - Cytokine connections. *Nature*, **366.** 114-116.

HUWILER, A., Fabbro, D. and Pfeilschifter, J. (1991) Differential recovery of protein kinase C-α and -ε isozymes after long term phorbol ester treatment in rat renal mesangial cells. *Biochem. Biophys. Res. Commun.*, **180.** 1422-1428.

INOUE, G., Kuzuya, H., Hayashi, T.,Okamoto, M., Yoshimasa, Y., Kosaki, A., Kono, S., Okamoto, M., Maeda, I., Kubota, M. and Imura, H. (1993) Effects of ML-9 on insulin stimulation of glucose transport in 3T3-L1 adipocytes. *J. Biol. Chem.*, **268**, 5272-5278.

INOUE, M., Kishimoto, A., Takai, Y. and Nishizuka, Y. (1977) Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissue: proenzyme and its activation by calcium-dependent protease from rat brain. *J. Biol. Chem.*, **252.** 7610-7616.

INUKAI, K., Funaki, M., Ogihara, T., Katagiri, H., Kanda, A., Anai, M., Fukushima, Y., Hosaka, T., Suzuki, M., Shin, B.-C., Takata, K., Yazaki, Y., Kikuchi, M., Oka, Y. and Asano, T. (1997) p85α gene generates three isoforms of regulatory subunit for phosphatidylinositol 3-kinase (PI 3-kinase), p50α. P55α and p85α, with different PI 3-kinase activity elevating responses to insulin. *J. Biol. Chem.*, **272**. 7873-7882.

ISAKOV, N., McMahon, P. and Altman, A. (1990) Selective post-translational down-regulation of protein kinase C isozymes in leukemic T cells chronically treated with phorbol ester. *J. Biol. Chem.*, **265.** 2091-2097.

ISSEMAN, I. and Green, S. (1990) Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature* **347.** 645-649.

ITO, M., Tanabe, F., Sato, A., Ishida, E., Takami, Y. and Shigeta, S. (1989) Possible involvment of microfilaments in protein kinase C translocation. *Biochem. Biophys. Res. Commun.*, **160.** 1344-1349.

JAKEN, S. (1996) Protein kinase C isozymes and substrates. *Curr. Opin. Cell Biol.*, **8.** 168-173.

JOHANNES, F. J., Prestle, J., Eis, S., Oberhagemann, P. and Pfizenmaier, K. (1994) PKCu is a novel, a typical member of the protein kinase C family. *J. Biol. Chem.*, **269**. 6140-6148.

JOHANNES, F. J., Prestle., J., Dieterich, S., Oberhagemann, P., Link, G. and Pfizenmaier, K. (1995) Characterisation of activators and inhibitors of protein-kinase C-mu. *Eur. J. Biochem.*, **227**. 303-307.

KAESTNER, K., Christy, R. and Lane, M. D. (1990) Mouse insulin-responsive glucose transporter gene: characterization of the gene and trans-activation by the CCAAT/enhancer binding protein. *Proc. Natl. Acad. Sci. USA*, **87.** 251-255.

KAIBUCHI, K., Takai, Y. and Nishizuka, Y. (1985) Protein kinase C and calcium ion in mitogenic response of macrophage-dependent human peripheral lymphocytes. *J. Biol. Chem.*, **260.** 1366-1369.

KAIBUCHI, K., Fukumoto, Y., Oku, N., Takai, Y., Arai, K. and Muramatsu, M. (1989) Molecular genetic analysis of the regulatory and catalytic domains of protein kinase C. *J. Biol. Chem.*, **264.** 13489-13496.

KARIN, M., Haslinger, A., Holtgreve, H., Richards, R.I., Krauter, P., Westphal, H.M. and Beato, M. (1984) Characterisation of DNA-sequences through which cadmium and glucocorticoid hormones induce human metallothionein-IIA gene. *Nature*, **308.** 513-519.

KAZANIETZ, M.G., Areces, L.B., Bahador, A., Mischak, H., Goodnight, J., Mushinski, J.F. and Blumberg, P.M. (1993) Characterisation of ligand and substrate-specificity for the calcium-dependent and calcium-independent protein-kinase-C isozymes. *Mol. Pharmacol.*, **44.** 298-307

KELLER, H., Dreyer, C., Medin, J., Mahfoudi, A., Ozato, K. and Wahli, W. (1993) Fatty acids and retiniods control lipid metabolism through activation of peroxisome proliferator-activated receptor-retinoid X receptor heterodimers. *Proc. Natl. Acad. USA*, **90.** 2160-2164.

KERANEN, L. M., Dutil, E. M. and Newton, A. C., (1995) Protein kinase C is regulated *in vivo* by three functionally distinct phosphorylations. *Curr. Biol.*, **5.** 1394-1403.

KHAN, W. A., Blobe, G. C., Richards, A. L. and Hannun, Y. A. (1994) Identification, partial purification and characterization of a novel phospholipid-dependent and fatty acid-activated protein kinase from human platelets. *J. Biol. Chem.*, **269.** 9729-9735.

KIESER, A., Goodnight, J., Kölch, W., Mischak, H. and Mushinski J.F. (1995) Identification of the primary growth response gene, ST2/T1, as a gene whose expression is differentially regulated by different protein kinase C isozymes. *FEBS Lett.*, **372**. 189-193.

KIESER, A., Seitz, T., Adler, H.S., Coffer, P., Kremmer, E., Crespo, P., Gutkind, J.S., Henderson, D.W., Mushinski, J.F., Kolch, W. and Mischak, H. (1996) Protein kinase C-ζ reverts v-*raf* transformation of NIH-3T3 cells. *Genes & Dev.*, **10.** 1455-1466.

KILEY, S. C., Parker, P. J., Fabbro, D. and Jaken, S. (1992) Hormone- and phorbol ester-activated protein kinase C isozymes mediate a recognition of the actin cytoskeleton associated with prolactin secretion in GH_4C_1 cells. *Mol. Endocrinology*, **6.** 120-131.

KILEY, S. C., Jaken, S., Whelan, R. and Parker, P. J. (1995) Intracellular targeting of protein kinase C isoenzymes: functional implications. *Biochem. Soc. Trans.*, **23**. 601-605.

KILGOUR, E. and Anderson, N.G. (1993) Changes in the expression of guanine-nucleotide-binding proteins during differentiation of 3T3-F442A cells in a hormonally-defined medium. *FEBS Lett.*, **328.** 271-274.

KILGOUR, E. and Anderson, N.G. (1994) Growth-hormone induces the tyrosine phosphorylation and nuclear accumulation of components of the ISGF3 transcription factor complex. *FEBS Lett.*, **343.** 205-207.

KILGOUR, E., Baldwin, S.A. and Flint, D.J. (1995) Divergent regulation of rat adipocyte GLUT 1 and GLUT 4 glucose transporters by growth hormone. *J. Endocrinol.*, **145.** 27-33.

KILGOUR, E., Gout, I. and Anderson. N. (1996) Requirement for phosphoinositide 3-OH kinase in growth hormone signalling to the mitogen-activated protein kinase and p70 pathways. *Biochem. J.*, **315**. 517-522.

KIM, J.B and Spiegelman, B.M. (1996) ADD1/SREBP1 promotes adipocyte differentiation and gene-expression linked to fatty-acid metabolism. *Genes Devt.*, **9.** 1096-1107.

KIM, J.B., Sarraf, P., Wright, M., Yao, K.M., Mueller, E., Solanes, G., Lowell, B.B. and Spiegelman, B.M. (1998) Nutritional and insulin regulation of fatty acid synthase and leptin gene expression through ADD1/SREBP1. *J. Clin. Invest.*, **101.** 1-9.

KIM, S.O., Jiang, J., Yi, W.S., Feng, G.S. and Frank, S.J. (1998) Involvement of the Src homology 2-containing tyrosine phosphatase SHP-2 in growth hormone signalling. *J. Biol. Chem.*, **273.** 2344-2354.

KINDREGAN, H.C., Rosenbaum, S.E., Ohno, S. and Niles, R.M. (1994) Characterisation of protein-kinase-C (PKC) isotype expression during F9 teratocarcinoma differentiation - Overexpression of PKC-alpha alters the expression of some differentiation-dependent genes. *J. Biol. Chem.*, **269.** 27756-27761.

KISHIMOTO, A., Takai, Y., Mori, T., Kikkawa, U. and Nishizuka, Y. (1980) Activation of calcium and phospholipid-dependent protein kinase by diacylglycerol, its possible relation to phosphatidylinositol turnover. *J. Biol. Chem.*, **255.** 2273-2276.

KISHIMOTO, A., Mikawa, K., Hashimoto, K., Yasuda, I., Tanaka, S., Tominga, M., Kuroda, T. and Nishizuka, Y. (1989) Limited proteolysis of protein kinase C subspesies by calcium-dependent neutral protease (calpain). *J. Biol. Chem.*, **264.** 4088-4092.

KLAUCK, T. M., Faux, M. C., Labudda, K., Langeberg, L. K., Jaken, S. and Scott, J. D. (1996) Coordiation of three signalling enzymes by AKAP 79, a mammalian scaffold proetin. *Science,* **271.** 1589-1592.

KNOPF, J., Lee, M-H., Sultzman, L.A., Kriz, R.W., Loomis, C.R., Henwick, R.M. and Beel, R.M. (1986) Cloning and expression of multiple protein kinase C cDNAs. *Cell,* **46.** 491-502.

KOCHS, G., Hummel, R., Fiebich, B., Sarre, T.F., Marme, D. and Hug, H. (1993) Activation of purified protein kinase C α and βI isoenzymes *in vitro* by Ca^{2+}, phophatidylinositol and phophatidylinositol 4,5-bisphosphate. *Biochem. J.*, **291.** 627-633.

KOLCH, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marme, D. and Rapp, U.R. (1993) Proein kinase-C-alpha activates Raf-1 by direct phosphorylation. *Nature,* **364.** 249-252.

KONIECZNY, S.F. and Emerson, C.P. (1984) 5-azacytidine induction of stable mesodermal stem-cell lineages from 10T1/2 cells - Evidence for regulatory genes-controlling determination. *Cell,* **38.** 791-800.

KONISHI, H., Tanaka, M., Takemura, Y., Matsuzaki, H., Ono, Y., Kikkawa, U. and Nishizuka, Y. (1997) Activation of protein kinase C by tyrosine phosphorylation in response to H_2O_2. *Proc. Natl. Acad. Sci. USA,* **94.** 11233-11237.

KOSAKA, C., Sasaguri, T., Masuda, J., Zen, K., Shimokado, K., Yokota, T. and Ogata, J. (1993) Protein kinase C-mediated inhibition of cyclin A expression in human vascular endothelial cells. *Biochem. Biophys. Res. Commun.,* **193.** 991-998.

KOTANI, K., Carozzi, A. J., Sakaue, H., Hara, K., Robinson, L. J., Clark, S. F., Yonezawa, K., James, D. E. and Kasuga, M. (1995) Requirement for phosphoinositide 3-kinase in insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes. *Biochem. Biophys. Res. Commun.,* **209.** 343-348.

KRAFT, A.S., Anderson, W.B., Cooper, H.L. and Sando, J.J. (1982) Decrease in cytosolic calcium/phospholipid-dependent protein kinase activity following phorbol ester treatment of EL4 thyoma cells. *J. Biol. Chem.,* **257.** 13193-13196.

KRAFT, A.S. and Anderson, W.B. (1983) Phorbol esters increase the amount of Ca^{2+}, phospholipid-dependent protein kinase associated with plasma membrane. *Nature,* **301.** 621-623.

KROOK, A., Whitehead, J.P., Dobson, S.P., Griffiths, M.R., Ouwens, M., Baker, C., Hatward, A.C., Sen, S.K., Maassen, J.A., Siddle, K., Tavare, J.M and O'Rahilly, S. (1997) Two naturally occurring insulin receptor tyrosine kinase mutants provide evidence that phosphoinositide 3-kinase activation alone is not sufficient for the mediation of insulin's metabolic and mitogenic effects. *J. Biol. Chem.,* **272.** 30208-30214.

KU TAI, P-K., Liao, J-F., Chen, E. H., Dietz, J., Schwartz, J. and Carter-Su, C. (1990) Differential regulation of two glucose transporters by chronic growth hormone treatment of cultured 3T3-F442A adipose cells. *J. Biol. Chem.,* **265.** 21828-21834.

KUHNE, M.R., Powson, T., Lienhard, G.E. and Feng, G.S. (1993) The insulin-receptor substrate-1 associates with the SH2-containing protein tyrosine phosphatase Syp. *J. Biol. Chem.*, **268.** 11479-11481.

KULAS, D. T., Zhang, W-R., Goldstein, B. J., Furlanetto, R. W. and Mooney, R. A. (1995) Insulin receptor signalling is augmented by antisence inhibition of the protein tyrosine phosphatase LAR. *J. Biol. Chem.*, **270.** 2435-2438.

KUO J. F., Andersson, R. G. G., Wise, B. C., Mackerlova, L., Salomonsson, I., Brackett, N. L., Katoh, N., Shoji, M. and Wrenn, R. W. (1980) Calcium-dependent protein kinases: Widespread occurrence in various tissues and phyla of the animal kingdom, and comparison of the effects of phospholipid, calmodulin, and trifluoroperazine. *Proc. Natl. Acad. Sci.* USA, 77. 7039-7043.

KURACHI, H., Adachi, H., Ohtsuka, S., Morishige, K., Memuja, K., Keno, Y., Shimonura, I., Tikunaga, K., Miyake, A., Matsuzawa, Y. and Tanizawa, O. (1993) Involvement of epidermal growth-factor in inducing obesity in ovariectomized mice. *Am. J. Physiol.*, **265.** E323-E331.

KURI-HARCUCH, W. and Marsch-Moreno, M. (1983) DNA synthesis and cell division related to adipose differentiation of 3T3 cells. *J. Cell. Physiol.*, **114**. 39-44.

KURTEN, P.C., Navre, M., Gaddy-Kurten, D., Semenkovich, C.F., Ringold, G.M., Chen, L. and Richards, J.S. (1988) Induction of adenosine 3,5'-monophosphate-dependent protein-kinase during adipogenesis in vitro. *Endocrinol.*, **123.** 2408-2418.

KYRIAKIS, J.M., App, H., Zhang, X.F., Banerjee, P., Brautigan, D.L., Rapp, U.R. and Avruch, J. (1992) Raf-1 activates MAP kinase kinase. *Nature*, **358.** 417-421.

LA THANGUE, N. B. (1994) DP and E2F proteins: components of a heterodimeric transcription factor implicated in cell cycle control. *Curr. Opin. Cell Biol.*, **6.** 443-450.

LABARCA, C. and Paigen, K. (1980) A simple, rapid and sensitive DNA assay procedure. *Anal. Biochem.*, **102**, 344-352.

LAGER, I. (1991) The insulin-antagonistic effect of counterregulatory hormones. *J. Internal Med.*, **229.** Suppl. 2: 41-47.

LAMB, P., Seidel, M., Stein, R.B. and Rosen, J. (1996) The role of JAKs and STATs in transcriptional regulation by cytokines. In: *Annual Reports in Medicinal Chemistry*: p269-277 (Wong, ed.), Academic Press Inc.

LAMMERS, R., Moller, N. P. H. and Ullrich, A. (1997) The transmembrane protein tyrosine phosphatase α dephosphorylates the insulin receptor in intact cells. *FEBS Lett.*, **404**. 37-40.

LANE, M.D., Lin, F.T., MacDougald, , O.A. and Vasseur-Cognet, M. (1996) Control of adipocyte differentiation by CCAAT/ enhancer binding protein alpha (C/EBP alpha). *Intl. J. Obesity*, **20.** 591-596.

LASSAR, A.B., Paterson, B.M. and Weintraub, H. (1986) Transfer of a DNA locus that mediates the conversion of 10T1/2 fibroblasts to myoblasts. *Cell*, **47.** 649-646.

LAZAR, D. F., Wiese, R. J., Brady, M. J., Mastick, C. C., Waters, S. B., Yamauchi, K., Pessin, J. E., Cuatrecasas, P and Saltiel, A. R. (1995) Mitogen-activated protein kinase kinase inhibition does not block the stimulation of glucose utilization by insulin. *J. Biol. Chem.*, **270.** 20801-20807.

LEAMAN, D.W., Pisharody, S., Flickinger, T.W., Commane, M.A., Schlessinger, J., Kerr, I.M., Levy, D.E. and Stark, G.R. (1996) Roles of JAKs in activation of STATs and stimulation of c-fos gene expression by epidermal growth-factor. *Mol. Cell. Biol.*, **16.** 369-375.

LECHLEIDER, R.J., Freeman, R.M. and Neel, B.G. (1993) Tyrosyl phosphorylation and growth-factor receptor association of the human corkscrew homolog, SH-PTP2. *J. Biol. Chem.*, **268.** 13434-13438.

LEE, C.H., Li, W., Nishimura, R., Zhou, M., Batzer, A.G., Myers, M.G., White, M.F., Schlessinger, J. and Skolnik, E.Y. (1993) Nck-associates with the SH2 domain-docking protein IRS-1 in insulin-stimulated cells. *Proc. Natl. Acad. Sci. USA*, **90.** 11713-11717.

LEE, J. Y., Hannun, Y. A. and Obeid, L. M. (1996) Ceramide inactivates cellular protein kinase C α. *J. Biol. Chem.*, **271.** 13169-13174.

LEE, M-H., and Bell, R.M. (1991) Mechanism of protein kinase C activation by phosphatidylinositol 4,5-bisphosphate. *Biochemistry,* **30.** 1041-1049.

LEGRAVEREND, C., Antonson, P., Flodby, P. and Xanthopoulos, K.G. (1993) High-level activity of the mouse CCAT enhancer-binding protein alpha (C/EBP alpha) gene promoter involves autoregulation and several ubiquitous transcription factors. *Nuc. Acid Res.*, **21.** 1735-1742.

LEHEL, C., Olah, Z., Jakab, G. and Anderson, W.B. (1995a) The influence of various domains of protein-kinase-C-epsilon on its phorbol ester-induced translocation from the trans-golgi network to the plasma membrane. *Mol. Biol. Cell,* **6.** 400-408.

LEHEL. C., Olah, Z., Jakab, G., Szallasi, Z., Petrovics, G., Harta, G., Blumberg, P.M. and Anderson, W.B. (1995b) Protein-kinase-C-epsilon subcellular localization domains and proteolytic sites - A model for protein-kinase-C conformational changes. *J. Biol. Chem.*, **270.** 19651-19658.

LENORMAND, P., Sardet, C., Pages, G., Lallemain, G., Brunet, A. and Pouyssegur, J. (1993) Growth-factors induce nuclear translocation of MAP kinases (p42 (MAPK) and p44 (MAPK)) but not of their activator MAP kinase kinase (p45 (MAPKK)) in fibroblasts. *J. Cell Biol.*, **122.** 1079-1088.

LEUNG, D.W., Spencer, S.A., Cachianes, G., Hammonds, R.G., Collins, C., Henzel, W.J., Barnard, R., Waters, M.J. and Wood, W.I. (1987) Growth-hormone receptor and serum binding-protein - Purification, cloning and expression. *Nature,* **330.** 537-543.

LEVACHER, C. and Picon, L. (1989) Fatty-acid synthesising enzymes in adipose and stromal vascular fraction of hypothyroid rat adipose tissue. *Horm. Metabol. Res.*, **21.** 537-541.

LI, W., Mischak, H., Yu, J-C., Wang, L.M., Mushinski, J.F., Heidaran, M.A. and Pierce, J.H. (1994) Tyrosine phosphorylation of protein kinase C-δ in response to its activation . *J. Biol. Chem.*, **269.** 2349-2352.

LIAO, L., Hyatt, S.L., Chapline, C. and Jaken, S. (1994) Protein kinase C domains involved in interactions with other proteins. *Biochem.*, **33.** 1229-1233.

LIMATOLA, C., Schaap, D., Moolenaar, W.H. and Van Blitterswijk, W.J. (1994) Phosphatidic acid activation of protein kinase C-zeta overexpressed in COS cells: comparison with other protein kinase C isotypes and other acidic lipids. *Biochem. J.*, **304.** 1001-1008.

LIN, F-T. and Lane, M. D. (1992) Antisence CCAAT/enhancer-binding protein RNA suppresses coordinate gene expression and triglyceride accumulation during differentiation of 3T3-L1 preadipocytes. *Genes Dev.* **6**. 533-544.

LIN, F.T., MacDougald, O.A., Diehl, A.M. and Lane, M.D. (1993) A 30-kDa alternative translational product of the CCAAT enhancer-binding protein alpha messenger-RNA - Translational activation lacking antimitogenic activity. *Proc. Natl. Acad. Sci. USA*, **90.** 9606-9610.

LIN, F.T. and Lane, M.D. (1994) CCAAT/enhancer binding protein α is sufficient to initiate the 3T3-L1 adipocyte differentiation program. *Proc. Natl. Acad. Sci. USA*, **91**. 8757-8761.

LIN, T.A., Kong, T.M., Haystead, T.A.J., Pause, A., Belsham, G., Sonenberg, N. and Lawrence, J.C. (1994) PHAS-1 as a link between mitogen-activated protein-kinase and translation initiation. *Science*, **266.** 653-656.

LIU, F. and Roth, R.A. (1994) Insulin-stimulated tyrosine phosphorylation of protein-kinase C-alpha - Evidence for direct interaction of the insulin-receptor and protein-kinase-C in cells. *Biochem. Biophys. Res. Commun.*, **200.** 1570-1577.

LIVNEH, E., Shimon, T., Bechor, E., Doki, Y., Schieren, I and Weinstein, I. B. (1996) Linking protein kinase C to the cell cycle ectopic expression of PKCη in NIH-3T3 cells alters the expression of cyclins and Cdk inhibitors and induces adipogenosis. *Oncogene*, **12.** 1545-1555.

LIVNEH, E. and Fishman, D.D. (1997) Linking protein kinase C to cell-cycle control. *Eur. J. Biochem.*, **248**. 1-9.

LU, Z.M., Liu, D., Hornia, A., Devonish, W., Pagano, M. and Foster, D.A. (1998) Activation of protein kinase C triggers its ubiquitination and degredation. *Mol. Cell. Biol.*, **18.** 839-8445.

LUO, J-H., Xing, W-Q. and Weinstein, B. I. (1995) The phorbol ester TPA markedly enhances the binding of calcium to the regulatory domain of protein kinase C βI in the absence of phosphatidylserine. *Carcinogenesis,* **16.** 897-905.

MacDOUGALD, O.A., Cornelius, P., Liu, R. and Lane, M.D. (1995) Insulin regulates transcription of the CCAAT/enhancer binding protein (C/EBP) α, β, and δ genes in fully-differentiated 3T3-L1 adipocytes. *J. Biol. Chem.*, **270**. 647-654.

MacKENZIE, S., Fleming, I., Houslay, M.D., Anderson, N.G. and Kilgour, E. (1997) Growth hormone and phorbol esters require specific protein kinase C isoforms to activate mitogen-activated protein kinases in 3T3-F442A cells. *Biochem. J.*, **324**. 159-165.

MADON, R. J., Ensor, D. M., Knight, C. H. and Flint, D. J. (1986) Effects of antiserum to rat growth hormone on lactation in the rat. *J. Endocrinol.*, **111**. 117-123.

MAEGAWA, H., Kashiwagi, A., Fujita, T., Ugi, S., Hesegawa, M., Obata, T., Nishio, Y., Kojima, H., Hidaka, H. and Kikkawa, R. (1996) SHPTP2 serves adapter protein linking between janus kinase 2 and insulin receptor substrates. *Biochem. Biophys. Res. Commun.*, **228**. 122-127.

MALARKEY, K., Belham, C.M., Paul, A., Graham, A., McLees, A., Scott, P.H. and Plevin, R. (1995) The regulation of tyrosine kinase signalling pathways by growth-factor and G-protein-coupled receptors. *Biochem. J.*, **309.** 361-375.

MALOFF, B. L., Levine, J. H. and Lockwood, D. H. (1980) Direct effects of growth hormone on insulin action in rat adipose tissue maintained *in vitro*. *Endocrinology,* **107.** 538-544.

MANDRUP, S. and Lane, M.D. (1997) Regulating Adipogenesis. *J. Biol. Chem.*, **272**. 5367-5370.

MANGANIELLO, V. C., Smith, C. J., Newman, A. H., Rice, K., Degerman, E. and Belfrage, P. (1987) Hormonal regulation of adipocyte particulate "low K_m" cAMP phosphodiesterase. *J. Cyclic Nucleotide Protein Phosphorylation Res.* **11.** 497-511.

MARAIS, R. (1998) Requirement of Ras-GTP-Raf complexes for activation of Raf-1 by protein kinase C. *Science,* **280.** 109-110.

MARAIS, R. M. and Parker, P. J. (1989) Purification and characterisation of bovine brain protein kinase C isotypes α, β and γ. *Eur. J. Biochem.*, **182**. 129-137.

MARSHALL, C. J. (1995) Specificity of receptor tyrosine kinase signalling: transient versus sustained extracellular signal-regulated kinase activation. *Cell,* **80.** 179-185.

McGOWAN, K., De Vente, J., Carey J.O., Ways, D.K. and Pekala, P.H. (1996) Protein kinase C isoform expression during the differentiation of 3T3-L1 preadipocytes: loss of protein kinase C-α isoform correlates with loss of phorbol 12-myristate 13-acetate activation of nuclear factor κB and acquisition of the adipocyte phenotype, *J. Cell. Physiol.*, **167**. 113-120.

McSWINE-KENNICK, R.L., McKeegan, E.M., Johnson, M.D. and Morin, M.J. (1991) Phorbol diester-induced alterations in the expression of protein kinase C isozymes and their mRNAs. Analysis in wild-type and phorbol ester-resistant HL-60 cell clones. *J. Biol. Chem.*, **266.** 15135-15143.

MERRICK, W.C. (1992) Mechanism and regulation of eukaryotic protein-synthesis. *Microbiologic. Rev.*, **56.** 291-315.

MESSINA, J. L., Standaert, M. L., Ishizuka, T., Weinstock, R. S. and Farese, R. V. (1992) Role of protein kinase C in insulin's regulation of c-*fos* transcription. *J. Biol. Chem.*, **267**. 9223-9228.

MEYER, D. J., Campbell, G. S., Cochran, B. H., Argetsinger, L. S., Larner, A. C., Finbloom, D. S., Carter-Su, C. and Schwartz, J. (1994) Growth hormone induces a DNA binding ractor related to the interferon-stimulated 91-kDa transcription factor. *J. Biol. Chem.*, **269.** 4701-4704.

MINANA, M.D., Felipo, V. amd Grisolia, S. (1989) Inhibition of protein kinase-C induces differentiation of neuro-blastoma cells. *Febs Lett.*, **255** 184-186.

MISCHAK, H., Pierce, J.H., Goodnight, J., Kazanietz, M.G., Blumberf, P.M. and Mushinski, J.F. (1993) Phorbol ester-induced myeloid differentiation is mediated by protein-kinase C-alpha and kinase-C-delta and not by protein kinase-C-beta-II, kinase-C-epsilon, kinase-C-zeta and kinase-C-eta. *J. Biol. Chem.*, **268.** 20110-20115.

MISTRY, A.M., Swick, A.G. and Romsos, D.R. (1997) Leptin rapidly lowers food intake and elevates metabolic rates in lean and ob/ob mice. *J. Nutrit.*, **127.** 2065-2072.

MOCHLY-ROSEN, D. (1995) Localization of protein kinases by anchoring proteins: a theme in signal transduction. *Science,* **268.** 247-251.

MOCHLY-ROSEN, D. and Koshland, D.E. (1987) Domain-structure and phosphorylation of protein kinase-C. *J. Biol. Chem.*, **262.** 2291-2297

MOCHLY-ROSEN, D., Henrich, C.J., Cheever, L., Khaner, H. and Simpson, P.C. (1990) A protein kinase C isoenzyme is translocated to the cytoskeletal elements on activation. *Cell Regulation,* **1.** 693-706

MORRIS, N.J., Bushfield, M., Lavan, B.E. and Houslay, M.D. (1994) Multisite phosphorylation of the inhibitory guanine-nucleotide regulatory protein G (I)-2 occurs in intact rat hepatocytes.. *Biochem. J.*, **301.** 693-702.

MOSIOR, M. and McLaughlin, S. (1991) Peptides that mimick the pseudosubstrate region of protein kinase C bind to acidic lipids in membranes. *Biophys. J.*, **60.** 149-159.

MOSIOR, M. and McLaughlin, S. (1992a) Binding of basic peptides to acidic lipids in membranes: effects of inserting alanine(s) between basic residues. *Biochemistry,* **31.** 1767-1773.

MOSIOR, M. and McLaughlin, S. (1992b) Electrostatics and dimentionality can produce apparent cooperativity when protein kinase C and its substrates bind acidic lipids in membranes. In: *Protein Kinase C: Current Concepts and Future Perspectives.* p157-180 (Lester, D. and Epand, R. M., eds), Chichester, Horwood.

MOSIOR, M. and Newton, A.C. (1995) Mechanism of interaction of protein kinase C with phorbol esters. *J. Biol. Chem.*, **270.** 25526-25533.

MOSIOR, M. and Newton, A. C. (1996) Calcium-independent binding to interfacial phorbol esters causes protein kinase C to associate with membranes in the absence of acidic lipids. *Biochemistry,* **35.** 1612-1623.

MOULE, S.K., Edgell, N.J., Welsh, G.I., Diggle, T.A., Foulstone, E.J., Heesom, K.J., Proud, C.G. and Denton, R.M. (1995) Multiple signalling pathways involved in the stimulation of fatty-acid and glcogen-synthesis by insulin in rat epididymal fat-cells. *Biochem. J.*, **311.** 595-601.

MOUSTAID, N. and Sul, H.S. (1991) Regulation of expression of the fatty-acid-synthase gene in 3T3-L1-cells by differentiation and triiodothyrodine. *J. Biol. Chem.*, **266.** 18550-18554.

NAKAIGAWA, N., Hirai, S., Mizuno, K., Shuin, T., Hosaka, M. and Ohno, S. (1996) Differential effects of overexpression of PKCα and PKCδ/ε on cellular E2F activity in late G1 phase. *Biochem. Biophys. Res. Commun.*, **222**. 95-100.

NAKANISHI, H., Brewer, K. A. and Exton, J.H. (1993) Activation of the ζ isoform of protein kinase C by phosphatidylinositol 3,4,5-trispohosphate. *J. Biol. Chem.*, **268.** 13-16.

NALEFSKI, E. A. and Falke, J. J. (1996) The C2 domain calcium-binding motif: structural and functional diversity. *Protein Sci.*, **5.** 2375-2390.

NAVRE, M. and Ringold, G.M. (1989) Differential effects of growth-factor and tumour promoters on the initiation and maintenance of adipocyte differentiation. *J. Cell Biol.*, **109.** 1857-1863.

NELSESTUEN, G.L. and Bazzi, M.D. (1991) Activation and regulation of protein-kinase-C enzymes. *J. Bioenerget. Biomemb.*, **23.** 43-61.

NEWTON, A. C. (1995) Protein kinase C: seeing two domains. *Curr. Biol.*, **5.** 973-976.

NEWTON, A. C. (1997) Regulation of protein kinase C. *Curr. Opin. Cell Biol.*, **9.** 161-167.

NIGG, E. A. (1996) Cyclin-dependent kinase 7: at the cross-roads of transcription, DNA repair and cell cycle control? *Curr. Opin. Cell Biol.*, **8.** 312-317.

NISHIKAWA, K., Yamamoto, S., Nagumo, H., Maruyama, K. and Kato, R. (1995) The presence of phorbol ester responsive and non-responsive forms of the ζ isozyme of protein kinase C in mouse epidermal cells. *Cell. Signal.*, 7. 491-504.

NISHIKAWA, K., Toker, A., Johannes, F.-J., Songyang, Z. and Cantley, L.C. (1997) Determination of the specific substrate sequence motifs of protein kinase C isozymes. *J. Biol. Chem.*, **272.** 952-960.

NISHIZUKA, N. (1988) The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature*, **334**. 661-665.

NISHIZUKA, Y. (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science*, **258.** 607-613.

NIXON, T. and Green, H. (1984a) Contribution of growth-hormone to the adipogenic action of serum. *Endocrinol. (Baltimore)*, **114.** 527-532.

NIXON, T. and Green, H. (1984b) Growth-hormone promotes the differentiation of myoblasts and preadipocytes generated by azacytidine treatment of 10T1/2 cells. *Proc. Natl. Acad. Sci. USA*, **81.** 3429-3432.

OBEID, L.M., Okazaki, T., Karolak, L.A. and Hannun, Y.A. (1990) Transcriptional regulation of protein kinase C by 1,25-dihydroxyvitamin D_3 in HL-60 cells. *J. Biol. Chem.*, **265.** 2370-2374.

OHNO, S., Akita, Y., Konno, Y., Shinobu, I. and Suzuki, K. (1988) A novel phorbol ester-receptor/protein kinase, nPKC, distantly related to the protein kinase C family. *Cell*, **53.** 731-741.

OHNO, S., Mizuno, K., Adachi, Y., Hata, A., Akita, Y., Akimoto, K., Osada, S., Hirai, S. and Suzuki, K. (1994) Activation of novel protein kinase C-δ and ε upon mitogenic stimulation of quienscent rat 3Y1 fibroblasts. *J. Biol. Chem.*, **269.** 17495-17501.

OHNO, S. and Suzuki, K. (1995) In: *The protein kinase C facts book I: Protein-serine kinases.* p80-88 (Hardie, G. and Hanks, S., eds). Academic Press Ltd, London.

OISHI, K., Raynor, R.L., Charp, P.A. and Kuo, J.F. (1988) Regulation of protein kinase-C by lysophospholipids - Potential role in signal transduction. *J. Biol. Chem.*, **263.** 6865-6871.

OKADA, T, Kawano, K., Sakakibara, T., Hazeki, O. and Ui, M. (1994) Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes. Studies with a selective inhibitor wortmannin. *J. Biol. Chem.*, **269.** 3568-3573.

OKAMOTO, M., Hayashi, T., Kono, S., Inoue, G., Kubota, M., Okamoto, M., Kuzuya, H and Imura, H. (1993) Specific activity of phosphatidylinositol 3-kinase is increased by insulin stimulation. *Biochem. J.*, **290.** 327-333.

OLIVIER, A. R. and Parker, P. J. (1992) Identification of multiple PKC isoforms in swiss 3T3 cells: Differential down-regulation by phorbol ester. *J. Cell. Physiol.*, **152.** 240-244.

ONO, Y., Kurokawa, T., Fujii, T., Kawahara, K., Igarishi, K., Kikkawa, U., Ogita, K. and Nishizuka, Y. (1986) Two types of complementary DNAs of rat brain protein kinase C: heterogeneity determined by alternative splicing. *FEBS Lett.*, **206.** 347-352.

ONO, Y, and Nishizuka, Y. (1987) Identification of three additional members of rat PKC family: δ, ε and ζ subspecies. *FEBS Lett.*, **226.** 125-128.

ONO, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarishi, K. and Nishizuka, Y. (1989) Protein kinase C ζ subspecies from rat brain: its structure, expression and properties. *Proc. Natl. Acad. Sci. USA*, **86.** 3099-3103.

ORR, J. W. and Newton, A. C. (1994) Requirement for negative charge on 'activation loop' of protein kinase C. *J. Biol. Chem.*, **269.** 27715-27718.

OSADA, S-I., Mizuno, K., Saido, T.C., Suzuki, K., Kuroki, R. and Ohno, S. (1992) A new member of the protein kinase C Fmily, nPKCθ, predominantly expressed in skeletal muscle. *Mol. Cell/ Biol.*, **12.** 3930-3938.

PAIRAULT, J. and Green H. (1979) A study of the adipose conversion of suspended 3T3 cells by using glycerophosphate dehdrogenase as differentiation marker. *Proc. Natl. Acad. Sci. USA*, **76**. 5138-5142.

PARK, K.R. and Kim, K.H. (1991) Regulation of acetyl-coA carboxylase gene-expression - Insulin induction of acetyl-coA carboxylase and differentiation of 30A5 preadipocytes requires prior cAMP action on the gene. *J. Biol. Chem.*, **19.** 12249-12256

PARKER, P., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel. S., Waterfield, M.D. and Ullrich, A. (1986) The complete primary structure of PKC - the major phorbol ester receptor. *Science*, **233.** 853-859.

PARKER, S. B., Eichele, G., Zhang, P., Rawis, A., Sands, A. T., Bradley, A., Olsen, E., Harper, J. W. and Elledge, S. J. (1995) p53-Independent expression of $p21^{wafl}$ in muscle and other terminally differentiating cells. *Science*, **267.** 1024-1021.

PAULAUSKIS, J.D. and Sul, H.S. (1988) Cloning and expression of mouse fatty-acid synthase and other specific messenger-RNAs - Developmental and hormonal regulation in 3T3-L1 cells. *J. Biol. Chem.*, **263.** 7049-7054.

PAWSON, T. and Gish, G.D. (1992) SH2 and SH3 domains - From structure to function. *Cell*, **71.** 359-362.

PEARSE, A.G.E. (1968) In: *Histochemistry: Theoretical and Applied*, 3rd edition, J & A Churchill, London.

PEARSON, R.B. and Kemp, B.E. (1991) Protein-kinase phosphorylation site sequences and consensus specificity motifs - Tabulations. *Methods Enzymol.*, **200.** 62-81.

PESSINO, A., Passalacqua, M., Sparatore, B., Patrone, M., Melloni, E. and Pontremoli, S. (1995) Antisense oligodeoxynucleotide inhibition of δ protein kinase C expression accelerates induced differentiation of murine erythroleukaemia cells. *Biochem. J.*, **312**. 549-554.

PINES, J. (1994) The cell cycle kinases. *Semin. Cancer Biol.*, **5.** 305-313.

PIRCHER, T.J., Flores-Morales, A., Mui, A.L.F., Saltiel, A.R., Norstedt, G., Gustafsson, J.A. and Haldosen, L.A. (1997) Mitogen-activated protein kinase kinase inhibition decreases growth hormone stimulated transcription mediated by STAT5. *Mol. Cell. Endocrinol.*, **133.** 169-176.

PONTREMOLI, S., Michetti, M., Melloni, E., Sparatore, B., Salamino, F. and Horecker, B.L. (1990) Identification of the proteolytically activated form of protein kinase C in stimulated human neutrophils. *Proc. Natl. Acad. Sci. USA*, **87.** 3705-3707.

POWIS, G., Bonjouklian, R., Berggren, M.M., Gallejos, A., Abraham, R., Ashendel, C., Zalkow, L., Matter, W.F., Dodge, J., Grundey, G. and Vlahos, C.J. (1994) Wortmannin is a potent and selective inhibitor of phophatidylinositol 3-kinase. *Cancer. Res.*, **54.** 2419-2423.

PRINS, J.B. and O'Rahilly, S. (1997) Regulation of adipose cell number in man. *Clin. Sci.*, **92.** 3-11.

PRONK, G.J., McGlade, J., Pelicci, G., Pawson, T. and Bos, J.L. (1993) Insulin-induced phosphorylation of the 46-kDa and 52-kDa Shc proteins. *J. Biol. Chem.*, **268.** 5748-5753.

QUEST, A. F. G. (1996) Regulation of protein kinase C: a tale of lipids and proteins. *Enzyme Protein*, **49.** 231-261.

QUEST, A. F. G., Bardes, E. S. G. and Bell, R. M. (1994) A phorbol ester binding domain of protein kinase Cγ: deletion analysis of the cys2 domain defines a minimal 43-amino acid peptide. *J. Biol. Chem.*, **269.** 2961-2970.

RAHN, T., Ridderstrale, M., Tornqvist, H., Mangaiello, V., Fredrikson, G., Belfrage, P. and Degerman, E. (1994) Essential role of phosphatidylinositol 3-kinase in insulin-induced activation and phosphorylation of the cGMP-inhibited cAMP phosphodiesterase in rat adipocytes. *FEBS Lett.*, **350.** 314-318.

RAY, L.B. and Sturgill, T.W. (1988) Insulin-stimulated microtubule-associated protein-kinase is phosphorylated on tyrosine and threonine *in vivo*. *Proc. Natl. Acad. Sci. USA*, **85.** 3753-3757.

REAVEN, G.M. (1988) Role of insulin resistance in human disease. *Diabetes* **37.** 1595-1607.

REED, B.C. and Lane, M.D. (1980) Insulin receptor synthesis and turnover in differentiating 3T3-L1 preadipocytes. *Proc. Natl. Acad. Sci. USA*, **77.** 285-289.

RENNECKE, J., Johannes, F-J., Richter, K. H., Kittstein, W., Marks, F. and Gschwendt, M. (1996) Immunological demonstration of protein kinase Cμ in murine tissues and various cell lines. Differential recognition of phosphorylated forms and lack of down-regulation upon 10-*O*-tetradecanoylphorphol-13-acetate treatment of cells. *Eur. J. Biochem.*, **242.** 428-432.

RON, D, Luo, J. and Mochly-Rosen, D. (1995) C2 region-derived peptides inhibit translocation and function of β protein kinase C *in vivo*. *J. Biol. Chem.*, **270.** 24180-24187.

RONCARI, D. A. K. (1984) Pre-adipose cell replication and differentiation. *Trends Biochem. Sci.*, **9.** 486-489.

ROZENGURT, E. (1986) Early signals in mitogenic response. *Science*, **234.** 161-166.

RUBIN, C.S., Hirsch, A., Fung, C. amd Rosen, O.M. (1978) Development of hormone receptors and hormonal responsiveness in vitro. Insulin receptors and insulin sensitivity in the preadipocyte and adipocyte forms of 3T3-L1 cells. *J. Biol. Chem.*, **253.** 7570-7578.

RUI, L.Y., Matthews, L.S., Hotta, K., Gustafson, T.A. and Carter-Su, C. (1997) Identification of SH2-B beta as a substrate of the tyrosine kinase JAK2 involved in growth hormone signalling. *Mol. Cell. Biol.*, **17.** 6633-6644.

SAFONOVA, I., Darimont, C., Amri, E.Z., Grimaldi, P. and Ailhaud, G. (1994a) Retinoids are positive effectors of adipose cell-differentiation. *Mol. Cell. Endocrinol.*, **104.** 201-211.

SAFONOVA, I., Reichert, U., Shroot, B., Ailhaud, G. and Grimaldi, P. (1994b) Fatty-acids and retinoids act synergistically on adipose cell-differentiation. *Biochem. Biophys. Res. Commun.*, **204.** 498-504.

SAGER, R. and Kovac, P. (1982) Pre-adipocyte determination either by insulin or by 5-azacytidine. *Proc. Natl. Acad. Sci. USA*, **79.** 480-484.

SAGGERSON, E. D. (1984) Hormonal regulation of biosynthetic activities in white adipose tissue. In: *New Perspectives in Adipose Tissue*. p87-121. (Cryer, A and Van, R. L. R., eds.) Butterworths, London.

SAHAI, A., Patel, M. S., Zavosh, A. S. and Tannen, R. L. (1994) Chronic hypoxia impairs the differentiation of 3T3-L1 fibroblast in culture: Role of sustained protein kinase C activation. *J. Cell. Physiol.*, **160**. 107-112.

SAHARINEN, P., Ekman, N., Sarvas, K., Parker, P., Alitalo, K. and Silvennoinen, O. (1997) The Bmx tyrosine kinase induces activation of the stat signalling pathway, which is specifically inhibited by protein kinase C delta. *Blood*, **90.** 4341-4353.

SALADIN, R., DeVos, P., Gurremillo, M., Leturque, A., Girard, J., Staels, B. and Aniverx, J. (1995) Transient increases in obese gene-expression after food-intake or insulin administration. *Nature*, **377.** 527-529.

SALE, E.M., Atkinson, P.G.P. and Sale, G.J. (1995) Requirement of MAP kinase for differentiation of fibroblasts to adipocytes, for insulin activation of p90 S6 kinase and for insulin or serum stimulation of DNA synthesis. *EMBO J.*, **14**. 674-684.

SAMUELSSON, L., Stromberg, K., Vikman, K., Bjursell, G. and Enerback, S. (1991) The CCAAT/enhancer binding protein and its role in adipocyte differentiation: evidence for direct involvement in terminal adipocyte development. *EMBO J.*, **10.** 3787-3793.

SANCHEZ-MARGALET, V., Goldfine, I. D., Truitt, K., Imboden, J. and Sung, C. K. (1995) Role of p85 subunit of phosphatidylinositol-3-kinase as an adaptor molecule linking the insulin receptor to insulin receptor substrate 1. *Mol. Endocr.*, **9.** 435-442.

SASAGURI, T., Ishida, A., Kosaka, C., Nojima, H. and Ogata, J. (1996) Phorbol ester inhibits the phosphorylation of the retinoblastoma protein without suppressing cyclin D-associated kinase in vascular smooth muscle cells. *J. Biol. Chem.*, **271.** 8345-8351.

SCHIWEK, D.R. and Loffler, G. (1987) Glucocorticoid hormones contribute to the adipogenic activity of human serum. *Endocrinol.*, **120.** 469-474.

SCHMIDT, W., Polljordan, G. and Coffler, G. (1990) Adipose conversion of 3T3-L1 cells in a serum-free culture system depends on epidermal growth-factor, insulin-like growth factor-1, corticosteroids, and cyclic AMP. *J. Biol. Chem.*, **265.** 15489-15495.

SCHMITZ-PEIFFER, C., Browne, C.L., Oakes, N.D., Watkinson, A., Chisholm, D.J., Kraegen, E.W. and Biden, T.J. (1997) Alterations in the expression and cellular localization of protein kinase C isozymes ε and θ are associated with insulin resistance in skeletal muscle of the high-fat-fed rat. *Diabetes,* **46.** 169-178.

SCHOENLE, E., Zapf, J. and Froesch, E.R. (1981) In vivo control of insulin-sensitive phosphodiesterase in rat adipocytes by growth-hormone and its parallel to glucose-transport. *Endocrinol.*, **109.** 561-566.

SCHWARTZ, J. (1984) Growth hormone directly alters glucose utilization in 3T3 adipocytes. *Biochem. Biophys. Res. Commun.*, **125**. 237-243.

SCHWARTZ, J. and Carter-Su, C. (1988) Effects of growth hormone on glucose metabolism and glucose transport in 3T3-F442A cells: dependence on cell differentiation. *Endocrinology,* **122**. 2247-2256.

SCOTT, R.E., Florine, D.L., Willie, J.J. and Yun, K. (1982) Coupling of growth arrest and differentiation at a distinct site in the G1 phase of the cell-cycle - GD. *Proc. Natl. Acad. Sci. USA*, **79.** 845-849.

SELBIE, L. A., Schmitz-Peiffer, C., Sheng, Y. and Bident, T. J. (1993) Molecular-cloning and characterisation of PKC (iota), an atypical protein-kinase-C derived from insulin-secreting cells. *J. Biol. Chem.*, **268**. 24296-24302.

SERRERO, G. (1987) EGF inhibits the differentiation of adipose precursor cells in primary cultures. *Biochem. Biophys. Res. Commun.*, **146.** 194-202.

SERRERO, G. and Mills, D. (1991) Physiological role of epidermal growth-factor in adipose-tissue development in vivo. *Proc. Natl. Acad. Sci. USA*, **88.** 3912-3916.

SHARKEY, N. and Blumberg, P.M. (1985) Kinetic evidence that 1,2-diolein inhibits phorbol ester binding to protein kinase C via a competitive mechanism. *Biochem. Biophys. Res. Commun.*, **133.** 1051-1056.

SHENG, M., Thompson, M.A. and Greenberg, M.E. (1991) CREB - A Ca^{2+}-regulated transcription factor phosphorylated by calmodulin-dependent kinases. *Science*, **252.** 1427-1430.

SHERR, C.J. (1994) G1 phase progression - Cycling on cue. *Cell*, **79.** 551-555.

SHIMIZU, Y., Shimizu, N., Fujuki, H. and Sugimura, T. (1983) Distinct inhibitory effects of dihydroteleocidin-B and the phorbol ester tumour promoters on the adipose differentiation of 3T3-L1 cells. *Cancer Res.*, **10.** 4973-4979.

SIDHU, R.S. (1979) Two-dimensional electrophoretic analyses of proteins synthesised during differentiation of 3T3-L1 adipocytes. *J. Biol. Chem.*, **254.** 11111-11118.

SINGH, S.S., Chauhan, A., Brockerhoff, H. and Chauhan, V.P.S. (1993) Activation of protein kinase C by phosphatidyl inositol 3,4,5-trisphosphate. *Biochem. Biophys. Res. Commun.*, **195.** 104-112.

SKOLNIK, E.Y., Lee, C.H., Batzer, A., Vicentini, L.M., Zhou, M., Daly, R., Myers, M.J., Backer, J.M., Ullrich, A., White, M.F. and Schlessinger, J. (1993) The SH2 SH3 domain-containing protein Grb2 interacts with tyrosine phosphorylated IRS-1 and Shc - Implications for insulin control of Ras signalling. *EMBO J.*, **12.** 1929-1036.

SMAL, J. and De Meyts, P. (1987) Role of kinase C in the insulin-like effects of human growth hormone in rat adipocytes. *Biochem. Biophys. Res. Commun.*, **147.** 1232-1240.

SMAS, C. M. and Sul, H. S. (1995) Control of adipocyte differentiation. *Biochem. J.*, **309.** 697-710.

SMITH, P.J., Wise, L.S., Berkowitz, R., Wan, C. and Rubin, C.S. (1988) Insulin-like growth factor-1 is an essential regulator of the differentiation of 3T3-L1 adipocytes. *J. Biol. Chem.*, **263.** 9402-9408.

SMITH-HALL, J., Pons, S., Patti, M.E., Burks, D.J., Uenush, L., Sun, X.J., Kahn, C.R. and White, M.F. (1997) The 60 kDa insulin receptor substrate functions like an IRS protein ($pp60^{IRS3}$) in adipose cells. *Biochemistry,* **36.** 8304-8310.

SOLANKI, V., Slaga, T.J., Callaham, M. and Huberman, E. (1981) Down regulation of specific binding of [20-3H]phorbol-12,13-dibutyrate and phorbol ester-induced differentiation of human promyelocytic leukaemia cells. *Proc. Natl. Acad. Sci. USA,* **78.** 1722-1725.

SOLOMON, S.S., Sibley, S.D. and Cunningham, T.M. (1990) Growth hormone receptors are up-regulated in diabetic adipocytes. *Endocrinology,* **127.** 1544-1546.

SOLTOFF, S. P. and Toker, A. (1995) Carbachol, substance P, and phorbol ester promote the tyrosine phosphorylation of protein kinase C δ in salivary gland epithelial cells. *J. Biol. Chem.*, **271.** 13490-13495.

SOSSIN, W. S. and Schwartz, J. H. (1993) Ca^{2+}-independent protein kinase Cs contain an amino-terminal domain similar to the C2 consensus sequence. *Trends Biochem. Sci.*, **18.** 207-208.

SOUZA, S. C., Frick, G. P., Yip, R., Lobo, R. B., Tai, L-R. and Goodman, H. M. (1994) Growth hormone stimulates tyrosine phosphorylation of insulin receptor substrate-1. *J. Biol. Chem.*, **269.** 30085-30088.

SPENCE, S., Rena, G., Sweeney, G. and Houslay, M.D. (1995) Induction of Ca^{2+}/calmodulin-stimulated cyclic AMP phosphodiesterase (PDE1) activity in Chinese hamster ovary cells (CHO) by phorbol 12-myristate 13-acetate and by the selective overexpression of protein kinase C isoforms. *Biochem. J.*, **310**. 975-982.

SPIEGELMAM, B.M. and Farmer, S.R. (1982) Decreases in tubulin and actin gene-expression prior to morphological-differentiation of 3T3 adipocytes. *Cell,* **29.** 53-60.

SPIEGELMAN, B.M., Frank, M. and Green, H. (1983) Molecular-cloning of messenger-RNA from 3T3-adipocytes - Regulation of messenger-RNA content for glycerophosphate dehydrogenase and other differentiation-dependent proteins during adipocyte development. *J. Biol. Chem.*, **258.** 10083-10089.

SPIEGELMAN, B.M., Distel, R.J., Ro, H.-S, Rosen, B.S. and Satterberg, B. (1988) *fos* protooncogene and the regulation of gene expression in adipocyte differentiation. *J. Cell Biol.*, **107.** 829-832.

SPOONER, P.M., Chernick, S.S., Garrison, M.M. and Scow, R.O. (1979) Insulin regulation of lipoprotein lipase activity and release in 3T3-L1 adipocytes. *J. Biol. Chem.*, **254.** 10021-10029.

STABEL, S. and Parker, P.J. (1993) Protein kinase C. In: *Intracellular Messengers.* p167-198 (Taylor, C.W. ed), Pergamon Press Ltd, UK.

STANDAERT, M.L., Buckley, D.J., Ishizuka, T., Hoffman, J.M., Cooper, D.R., Pollet, P.J. and Farese, R.V. (1990) Protein-kinase-C inhibitors insulin and PMA-stimulated hexose-transport in isolated rat adipocytes and BC3H-1 myocytes. *Metab. - Clin. Exptal.*, **39.** 1170-1179.

STANDAERT, M.L., Avignon, A., Arnold, T., Saba-Siddique, S.I., Cooper, D.R., Watson, J., Zhou, X., Galloway, L. and Farese, R.V. (1996a) Insulin translocates PKC-ε and phorbol esters induce and persistently translocate PKC-β_2 in BC3H-1 myocytes. *Cell. Signal.*, **8.** 313-316.

STANDAERT, M.L., Avignon, A., Yamada, K., Bandyopadhyay, G. and Farese, R.V. (1996b) The phosphatidylinositol 3-kinase inhibitor, wortmannin, inhibits insulin-induced activation of phosphatidylcholine hydrolysis and associated protein kinase C translocation in rat adipocytes. *Biochem. J.*, **313**. 1039-1046.

STAUDINGER, J., Zhou, J.M., Burgess, R., Elledge, S.J. and Olson, E.N. (1995) PICK1 - A perinuclear binding-protein and substrate for protein-kinase-C isolated by the yeast 2-hybrid system. *J. Cell Biol.*, **128.** 263-271.

STEINBERG, M.M., and Brownstein, B.L. (1982) A clonal analysis of the differentiation of 3T3-L1 preadipose cells - Role of insulin. *J. Cell Physiol.*, **113.** 359-364.

STEPHENS, J.M. and Pekala, P.H. (1992) Transcriptional repression of the C/EBP-alpha and GLUT 4 genes in 3T3-L1 adipocytes by tumour-necrosis-factor-alpha - Regulation is coordinately increased independently of protein synthesis. *J. Biol. Chem.*, **267.** 13580-13584.

STONE, R.L. and Bernlohr, D.A. (1990) The molecular basis for inhibition of adipose conversion of murine 3T3-L1 cells by retinoic acid. *Differentiation,* **45.** 119-127.

STREICHER, R., Kotza, J., Mullerweisland, D., Siemeisler, R., Munck, M., Auci, H. and Krone, W. (1996) SREBP-1 mediates activation of the low-density-lipoprotein receptor promoter by insulin and insulin-like growth-factor-I. *J. Biol. Chem.*, **271.** 7128-7133.

STUMPO, D. J., Haupt, D. M. and Blackshear, P. J. (1994) Protein kinase C isozyme distribution and down-regulation in relation to insulin-stimulated c-*fos* induction. *J. Biol. Chem.*, **269**. 21184-21190.

SUMANTRAN, V. N., Tsai, M and Schwartz, J. (1992) Growth hormone induces c-*fos* and c-*jun* expression in cells with varying requirements for differentiation. *Endocrinology,* **130.** 2016-2024.

SUN, X.J., Rothenberg, P., Kahn, C.R., Backer, J.M., Araki, E., Wilden, P.A., Cahill, D.A., Goldstein, B.J. and White, M.F. (1991) Structure of the insulin-receptor substrate IRS-1 defines a unique signal treansduction protein. *Nature,* **352.** 73-77.

SZALLASI, Z., Bogi, K., Gohari, S., Biro, T., Acs, P. amd Blumberg, P.M. (1996) Nonequivalent roles for the first and second zinc fingers of protein-kinase-C-delta - Effect of their mutation on phorbol ester-induced translocation in NIH-3T3 cells. *J. Biol. Chem.*, **271.** 18299-18301.

TAKAI, Y., Kishimoto, A., Inoue, M. and Nishizuka, Y. (1977) Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues: purification and characterization of an active enzyme from bovine cerebellum. *J. Bio. Chem.*, **252.** 7603-7609.

TANG, E. Y., Parker, P. J., Beattie, J. and Houslay, M. D. (1993) Diabetes induces selective alterations in the expression of protein kinase C isoforms in hepatocytes. *FEBS Lett.*, **326**. 117-123.

TANTI, J-F., Grémeaux, T., Van Obberghen, E. and Le Marchand-Brustel, Y. (1994) Insulin receptor substrate 1 is phosphorylated by the serine kinase activity of phosphatidylinositol 3-kinase. *Biochem. J.*, **304**. 17-21.

TAOKA, T., Tokuda, M., Tasaka, T., Hatase, O., Irino, S. and Norman, A.W. (1990) Induction of differentiation of HL-60 cells by protein-kinase-C inhibitor, K252A. *Biochem. Biophys. Res. Commun.*, **170.** 1151-1156.

TAYLOR, R. (1991) Insulin action 1991. *Clin. Endocrinol.*, **34.** 159-174.

TAYLOR, S.M. and Jones, P.A. (1979) Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. *Cell*, **17.** 771-779.

THOMAS, M. J., Gronowski, A. M., Berry, S. A., Bergad, P. L. and Rotwein, P. (1995) Growth hormone rapidly activates rat serine protease inhibitor 2.1 gene transcription and induces a DNA-binding activity distict from those of stat1, -3, and -4. *Mol. & Cell. Biol.*, **15.** 12-18.

TIMCHENKO, N.A., Wilde, M., Nakanishi, M., Smith, J.R. and Darlington, G.J. (1996) CCAAT/ enhancer-binding protein alpha (C/EBP alpha) inhibits cell proliferation through the p21 (WAF-1/CIP -1/SDI-1) protein. *Genes Devt.*, **10.** 804-815.

TOBE, K., Matuoka, K., Tamemoto, H., Ueki, K., Kaburagi, Y., Asai, S., Noguchi, T., Matsuda, M., Tanaka, S., Hattori, S., Fukui, Y., Akanuma, Y., Yazaki, Y., Takenawa, T., Kadowaki, T. (1993) Insulin stimulates association of insulin-receptor substrate-1 with the protein abundant SRC-homology growth-factor receptor-bound protein-2. *J. Biol. Chem.*, **268.** 11167-11171.

TOBIAS, E.S., Rozengurt, E., Connell, J.M.C. and Houslay, M.D. (1997) Co-transfection with protein kinase C confers phorbol-ester-mediated inhibition on glucagon-stimulated cAMP accumulation in cos cells transfected to overexpress glucagon receptors. *Biochem. J.*, **326.** 545-557.

TOKER, A., Meyer, M., Reddy, K., Falck, J. R., Aneja, R., Aneja, S., Parra, A., Burns, D. J., Ballas, L. M. and Cantley, L. C. (1994) Activation of protein kinase C fammily members by novel phosphoinositides PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3. *J. Biol. Chem.*, **269.** 32358-32367.

TONTONOZ, P., Kim, J. B., Graves, R. A. and Spiegelman, B. M. (1993) ADD1: a novel helix-loop-helix transcription factor associated with adipocyte determination and differentiation. *Mol. Cell Biol.*, **13.** 4753-4759.

TONTONOZ, P., Hu, E. and Spiegelman, B. M. (1994a) Stimulation of adipogenesis in fibroblasts by PPARγ2, a lipid-activated transcription factor. *Cell*, **79.** 1147-1156.

TONTONOZ, P., Hu, E., Graves, R.A., Budavari, A.I and Spiegelman, B.M. (1994b) mPPAR-gamma-2 - Tissue-specific regulator of an adipocyte enhancer. *Genes Develop.*, **8.** 1224-1234.

TSUTAKAWA, S. E., Medzihradszky, K. F., Flint, A. J., Burlingame, A. L. and Koshland D. E. Jnr. (1995) Determination of *in vivo* phospohorylation sites in protein kinase C. *J. Biol. Chem.*, **270.** 26807-26812.

TUOMINEN, R.K., Werner, M.H., Ye, H., McMillan, M.K., Hudson, P.M., Hannun, Y.A. and Hong, T.S. (1993) Biphasic generation of diacylglycerol by angiotensin and phorbol ester in bovine adrenal chromaffin cells. *Biochem. Biophys. Res. Commun.*, **190.** 181-185.

UEDA, Y., Hirai, S-I., Osada, S-I., Suzuki, A., Mizuno, K. and Ohno, S. (1996) Protein kinase C δ activates the MEK-ERK pathway in a manner independent of Ras and dependent on Raf. *J. Biol. Chem.*, **272.** 23512-23519.

UEHARA, T., Tokumitsu, Y. and Normura, Y. (1995) Wortmannin inhibits insulin-induced Ras and mitogen-activated protein kinase activation related to adipocyte differentiation in 3T3-L1 fibroblasts. *Biochem. Biophys. Res. Commun.*, **210.** 574-580.

VALLEJO, M., Ron, D., Miller, C.P. and Habener, J.F. (1993) C/ATF, a member of the activating transcription factor family of DNA-binding proteins, dimerises with CCAAT enhancer-binding proteins and directs their binding to cAMP response elements. *Proc. Natl. Acad. Sci. USA*, **90**. 4679-4683.

VALVERDE, A. M., Sinnett-Smith, J., Van Lint, J. and Rozengurt, E. (1994) Molecular cloning and characterization of protein kinase D: A target for diacylglycerol and phorbol esters with a distinctive catalytic domain. *Biochemistry*, **91**. 8572-8576.

VAN LINT, J., Sinnett-Smith, J. and Rozengurt, E. (1995) Expression and characterization of PKD, a phorbol ester and diacylglycerol-stimulated serine protein kinase. *J. Biol. Chem.*, **270**. 1455-1461.

VAN VLIET, G., Bosson, D., Craen, M., Ducaju, M.V.L., Malvaux, P. and Vanderschuerenlodeweyckx, M. (1987) Comparative-study of the lipolytic potentials of pituitary-derived and biosynthetic human growth-hormone in hypopituitary children. *J. Clin. Endocrinol. Metabol.*, **65.** 876-879.

VANDERKUUR, J., Allevato, G., Billestrup, N., Norstedt, G. and Carter-Su, C. (1995) Growth hormone-promoted tyrosyl phosphorylation of SHC proteins and SHC association with Grb2. *J. Biol. Chem.*, **270.** 7587-7593.

VANNIER, C., Gaillard, D., Grimaldi, P., Amri, E., Djian, P., Cermolacce, C., Forest, C., Etienne, J. Negrel, R. and Ailhaud, G. (1985) Development of lipoprotein-lipase in differentiating OB17-cells. *Int. J. Obesity.*, **9** (Suppl. 1). 41-53.

VASSAUX, G., Gaillard, D., Ailhaud, G. and Negrel, R. (1992) Prostacyclin is a specific effector of adipose cell-differentiation - Its dual role as a cAMP-elevating and Ca^{2+}-elevating agent. *J. Biol. Chem.*, **267.** 11092-11097.

VERNON, R.G. (1982) Effects of growth-hormone on fatty-acid synthesis in sheep adipose-tissue. *Intl. J. Biochem.*, **14.** 255-258.

VERNON, R.G. (1992) In: *Control of fat and lean deposition*, p59-81. (Boorman, K.N., Buttery, P.J. and Lindsay, D.B., eds), Butterworths, London.

VERNON, R.G. and Finley, E. (1988) Roles of insulin and growth hormone in the adaptions of fatty acid synthesis in white adipose tissue during the lactation cycle in sheep. *Biochem. J.*, **256**. 873-878.

VERNON, R. G. and Flint, D. J. (1989) Role of growth hormone in the regulation of adipocyte growth and function. In: *Biotechnology in Growth Regulation*: p57-71 (Heap, R. B., Prosser, C. G. and Lamming, G. E., eds), Butterworths, London.

VERNON, R.G., Barber, M.C. and Finley, E. (1991) Mode of the activation of acetyl-coA carboxylase and other lipogenic enzymes by growth-hormone, insulin and dexamethasone in sheep adipose-tissue and relationship to adaptions to lactation. *Biochem. J.*, **274.** 543-548.

VERNON, R. G., Piperova, L., Watt, P. W. and Lindsay-Watt, S. (1993) Mechanisms involved in the adaptations of the adipocyte adrenergic signal-transduction system and their modulation by growth hormone during the lactation cycle in the rat. *Biochem. J.*, **289.** 845-851.

VIDALPUIG, A., Jimenezlinan, M., Lowell, B. B., Hamann, A., Hu, E., Spiegelman, B., Flier, J. S. and Moller, D. E. (1996) Regulation of PPAR γ gene expression by nutrition and obesity in rodents. *J. Clin. Invest.*, **97.** 2553-2561.

WABITSCH, M., Braun, S., Hauner, H., Heinze, E., Ilonelo, M.M., Shymko, R., DeMeyts, P. amd Teller, W.M. (1996) Mitogenic and antiadipogenic properties of human growth-hormone in differentiating human adipocyte precursor cells in primary culture. *Ped. Res.*, **40.** 450-456

WALDER, K., Filippis, A., Clark, S., Zimmet, P. and Collier, G.R. (1997) Leptin inhibits insulin binding in isolated rat adipocytes. *J. Endocrinol.*, **155.** R5-R7.

WALKER, K.S., Deak, M., Paterson, A., Hudson, K., Cohen, P. and Alessi, D.R. (1998) *Biochem. J.*, **331.** 299-308.

WANG, H.-Y., Goligorsky, M.S. and Malbon, C.C. (1997) Temporal activation of Ca^{2+}-calmodulin-sensitive protein kinase type II is obligate for adipogenesis. *J. Biol. Chem.*, **272.** 1817-1821.

WANG, J., Auger, K.R., Jarvis, L., Shi, Y. And Roberts, T.M. (1995) Direct association of Grb2 with the p85 subunit of phosphatidylinositol 3-kinase. *J. Biol. Chem.*, **270.** 12774-12780.

WANG, J. and Riedel, H. (1998) Insulin-like growth factor-1 receptor and insulin receptor association with a Src homology-2 domain-containing putative adaptor. *J. Biol. Chem.*, **273.** 3136-3139.

WAYS, D.K., Cook, P.P., Webster, C. and Parker, P.J. (1992) Effect of phorbol esters on protein kinase-C-zeta. *J. Biol. Chem.*, **267.** 4799-4805.

WEILAND, M., Bahr, F., Hohne, M., Schurmann, A., Ziehm, D. and Joost, H.G. (1991) The signalling potential of the receptors for insulin and insulin-like growth factor-I (IGF-I) in 3T3-L1 adipocytes - Comparison of glucose-transport activation, induction of oncogene c-fos, glucose transporter messenger-RNA and DNA synthesis. *J. Cell Physiol.*, **149.** 428-435.

WEINBERG, R. A. (1995) The retinoblastoma protein and cell cycle control. *Cell*, **81.** 323-330.

WEINBERG, R. A. (1996) E2F and cell proliferation: a word turned upside down. *Cell*, **85.** 457-459.

WELSH, G. L., Foulstone, E. J., Young, S. W., Tavare, J. M. and Proud, C. G. (1994) Wortmannin inhibits the effects of insulin and serum on the activities of glycogen synthase kinase-3 and mitogen-activated protein kinase. *Biochem. J.*, **303.** 15-20.

WELSH, G.L., Miller, C.M., Loughlin, A.J., Proce, N.T. and Proud, C.G. (1998) Regulation of eukaryotic initiation factor eIF2B: Glycogen synthase kinase-3 phosphorylates a conserved serine which undergoes dephosphorylation in response to insulin. *FEBS Lett.*, **421.** 125-130.

WHITE, M.F., Maron, R. and Kahn, C.R. (1985) Insulin rapidly stimulates tyrosine phosphorylation of a MR-185,000 protein in intact-cells. *Nature*, **318.** 183-186.

WIESE, R. J. Mastick, C. C., Lazar, D. F. and Saltiel, A. R. (1995) Activation of mitogen-activated proteinkinase and phosphatidylinositol 3'-kinase is not sufficient for the hormonal stimulation of glucoseuptake, lipogenesis, or glycogen synthesis in 3T3-L1 adipocytes. *J. Biol. Chem.*, **270.** 3442-3446.

WIJKANDER, J., Landstrom, T.R., Manganiello, V., Belfrage, P. and Degerman, E. (1998) Insulin-induced phosphorylation and activation of phosphodiesterase 3B in rat adipocytes: Possible role for protein kinase B but not mitogen-activated protein kinase or p70 S6 kinase. *Endocrinol.*, **139.** 219-227.

WILKS, A.F., Harpur, A.G., Kurban, R.R., Ralph, S.J., Zurcher, G. and Ziemiecki, A. (1991) Two novel protein-tyrosine kinases, each with a 2nd phophotransferase-related catalytic domain, define a new class of protein-kinase. *Mol. Cell. Biol.*, **11.** 2057-2065.

WINSTON, L. A. and Hunter, T. (1995) KAK2, Ras and Raf are required for activation of extracellular signal-regulated kinase / mitogen-activated protein kinase, by growth hormone. *J. Biol. Chem.*, **270.** 30837-38040.

WISE, L.S. and Green, H. (1979) Participation of one isozyme of cytosolic glycerophosphate dehydrogenase in the adipose conversion of 3T3 cells. *J. Biol. Chem.*, **254**, 273-275.

WITHERS, D.J., Sanchez Gutierrez, J., Towery, H., Burks, D.J., Ren, J-M., Previs, S., Zhang, Y., Bernal, D., Pons, S., Shulman, G.I., Bonner-Weir, S. and White, M.F. (1998) Disruption of IRS-2 causes type 2 diabetes in mice. *Nature*, **391.** 900-904.

WOLFMAN, A. and Macara, I.G. (1987) Elevated levels of diacylglycerol and decreased phorbol ester sensitivity in ras-transformed fibroblasts. *Nature*, **325.** 359-361.

WU, Z., Xie, Y., Bucher, N. and Farmer, S. R. (1995) Conditional ectopic expression of C/EBOβ in NIH-3T3 cells induces PPARγ and stimulates adipogenesis. *Genes Dev.*, **9.** 2350-2363.

WU, Z., Bucher, N. L. R. and Farmer,S. R. (1996) Induction of peroxisome proliferator-activated receptor γ during the conversion of 3T3 fibroblasts into adipocytes is mediated by C/EBP β, and glucocorocoids. *Mol. Cell Biol.*, **16.** 4128-4136.

XU, J.H., Rockow, S., Kim, S., Xiong, W. and Li, W. (1994) Interferons block protein-kinase-C-dependent but not kinase-C-independent activation of Raf-1 and mitogen-activated protein-kinases and mitogenesis in NIH 3T3 cells. *Mol. Cell. Biol.*, **14**, 8018-8027.

XU, X.F. and Bjorntorp, P. (1990) Effects of dexamethasone on multiplication and differentiation of rat adipose precursor cells. *Exptal. Cell Res.*, **189.** 247-252.

YAMAGUCHI, K., Ogita, K., Nakamura, S. and Nishizuka, Y. (1995) The protein kinase C isoforms leading to map-kinase activation in CHO cells. *Biochem. Biophys. Res. Commun.*, **210**. 639-647.

YAMAUCHI, K. and Pessin, J. E. (1994) Insulin receptor substrate-1 (IRS1) and Shc compete for a limited pool of Grb2 in mediating insulin downstream signalling. *J. Biol. Chem.*, **269.** 31107-31114.

YAMAUCHI, T., Kaburagi, Y., Ueki, K., Tsuji, Y., Starks, G. R., Kerr, I. M., Tsushima, T., Akanuma, Y., Komuro, I., Tobe, K., Yazaki, Y. and Kadowaki, T. (1998) Growth hormone and prolactin stimulate tyrosine phophorylation of insulin receptor substrate-1, -2, and -3, their association with p85 phosphatidylinositol 3-kinase (PI3-kinase), and concomitantly PI3-kinase activation via JAK2 kinase. *J. Biol. Chem.*, **273.** 15719-15726.

YANG, V.W., Christy, R.J., Cook, J.S., Kelly, T.J. and Lane, M.D. (1989) Mechanism of regulation of the 422 (AP2) gene by cAMP during preadipocyte differentiation. *Proc. Natl. Acad. Sci. USA.*, **10.** 3629-3633.

YANO, Y., Sumida, Y., Benzing, C.F., Robinson, F.W. and Kono, T. (1993) Primary sites of action of staurospaurine and H7 in the cascade of insulin activation to glucose-transport in rat adipocytes. *Biochim. Biophys. Acta, 1176. 327-332.*

YARWOOD, S.J., Kilgour, E. and Anderson, N.G. (1998) Cyclic AMP potentiates growth hormone-dependent differentiation of 3T3-F442A preadipocytes: Possible involvement of the transcription factor CREB. *Mol. Cell. Endocrinol.*, **138.** 41-50

YEH, W.C. and McKnight, S. L. (1995) Regulation of adipose maturation and energy homeostasis. *Curr. Opin. Cell Biol.*, **7.** 885-890.

YEH, W.C., Cao, Z.D., Classon, M. and McKnight, S.L. (1995) Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine-zipper proteins. *Genes Devt.*, **9.** 168-181.

YI, W., Kim, S.-O., Jiang, J., Park, S.-H., Kraft, A.S., Waxman, D.J. and Frank, S.J. (1996) Growth hormone receptor cytoplasmic domain differentially promotes tyrosine phosphorylation of signal transducers and activators of transcription 5b and 3 by activated JAK2 kinase. *Mol. Endocr.*, **10.** 1425-1443.

YUN, K. and Scott, R.E. (1983) Biological mechanisms of phorbol-myristate acetate-induced inhibition of proadipocyte differentiation. *Cancer Res.*, **43.** 88-96.

ZALEWSKI, P.D., Forbes, I.J., Giannakis, C., Cowled, P.A. and Betts, W.H. (1990) Synergy between zinc and phorbol ester I translocation of protein kinase C to cytoskeleton. *FEBS Lett.*, **273.** 131-134.

ZEZULAK, K. M. and Green, H. (1986) The generation of insulin-like growth factor-1-sensitive cells by growth hormone action. *Science,* **233.** 551-553.

ZHANG, J., Wang, L., Schwartz, J., Boyd, R. W. and Bishop, W. R. (1994a) Phosphorylation of Thr^{642} is an early event in the processing of newly synthesised protein kinase C βI and is essential for its activation. *J. Biol. Chem.* **269.** 19578-19584.

ZHANG, Y.Y., Proenca, R., Maffei, M., Barone, M., Leopold, L. and Friedman, J.M. (1994b) Positional cloning of the mouse obese gene and its human homolog. *Nature,* **372.** 425-432.

ZHOU, G., Wooten, M. W. and Coleman, E. S. (1994a) Regulation of a typical ζ-protein kinase C in cellular signalling. *Exp. Cell Res.*, **214**. 1-11.

ZHOU, W., Takuwa, N., Kumada, M and Takuwa, Y. (1993) Protein kinase C-mediated bidirectional regulation of DNA synthesis, RB protein phosphorylation, and cyclin-dependent kinases in human vascular endothelial cells. *J. Biol. Chem.*, **268.** 23041-23048.

ZHOU, W., Takuwa, N., Kumada, M. and Takuwa, Y. (1994b) E2F1, B-myb and selective members of cyclin / cdk subunits are targets for protein kinase C-mediated bimodal growth regulation in vascular endothelial cells. *Biochem. Biophys. Res. Commun.*, **199.** 191-198.

ZHU, Y.J., Korenberg, J.R., Chen, X.N., Noya, D., Rao, M.S. and Reddy, J.K. (1995) Structural organisation of mouse peroxisome proliferator activator receptor-gamma (mPPAR-gamma) gene - Alternative promoter use and differential splicing yield two mPPAR -gamma isoforms. *Proc. Natl. Acad. Sci. USA,* **17.** 7921-7925.

ZOU, L.L., Menon, R.K. and Sperling, M.A. (1997) Induction of mRNAs for the growth hormone receptor gene during mouse 3T3-L1 preadipocyte differentiation. *Metab. - Clin. Exptal.*, **46.** 114-118.

ZUGAZA, J.L., Sinnett-Smith, J., Van Lint, J. and Rozengurt, E. (1996) Protein kinase D (PKD activation in intact cells through a protein kinase C-dependent signal transduction pathway). *EMBO J.*, **15**. 6220-6230.

ZUGAZA, J.L., Waldrol, R.T., Sinnet-Smith, J. and Rozengurt, E. (1997) Bombesin, vasopressin, endothelin, bradykinin and PDGF repidly activates protein kinase D through a protein kinase C-dependent pathway. *J. Biol. Chem.*, **272.** 23952-23960.