CHARACTERISATION OF PHOSPHATIDATE PHOSPHOHYDROLASE ACTIVITY IN HUMAN COLORECTAL CANCER

By

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ABSTRACT

Established chemotherapeutic agents often produce dose limiting toxicities in normal tissues due to lack of selectivity for tumour cells. Identification of abnormalities in the control of signal transduction in tumour cells may suggest more specific targets. Phosphatidate phosphohydrolase (PAP) is involved in the generation of diradylglycerol (DRG) from phosphatidylcholine (PC) in response to a mitogenic signal. The aims of the present studies were firstly to determine whether the activity of PAP is altered in transformed cell lines and in human tumours and secondly to determine whether PAP is a target for anticancer drug development.

An assay was established and characterised to measure PAP activity. Two activities are present in rat liver. PAP1 is mainly cytosolic and is involved in glycerolipid synthesis. It requires Mg^{2+} and is inhibited by the thiol-blocking reagent n-ethylmaleimide (NEM). PAP2 is associated with the plasma membrane and is thought to be involved in mitogenic signal transduction. PAP activity can be measured in tissue homogenates and the two activities distinguished by their differential sensitivity to NEM. PAP activity was shown to be present in a range of mouse tissues The highest activities were present in the brain and the lowest in muscle tissue.

Transformation of mouse fibroblast cell lines by a mutant *ras* oncogene results in an increase in DRG mass. This DRG could be derived from the breakdown of either phosphatidylinositol (PI) by a phospholipase C activity or PC by the sequential activity of phospholipase D and PAP. PAP2 activity was shown to be decreased in the transformed cells and clearly could not account for the increased DRG mass. However, the level of phosphatidate was also increased and this was consistent with the decreased activity of PAP2. The association of a mutant *ras* oncogene and decreased activity of PAP2 was also observed in human colon tumour cell lines. Cell lines DLD-1 and LoVo contain a mutant Ki-*ras* oncogene and were shown to have lower PAP2 activities than HT29 and Colo320DM which express wild type Ki-*ras*. The phosphatidate and DRG mass were also higher in the cell lines with a mutant *ras* oncogene. Thus, in both cell models a decrease in PAP2 activity is associated with increased levels of phosphatidate, a second messenger with potent mitogenic activity.

PAP activity was measured in human colon tumours and in adjacent pathologically normal colonic mucosa. Two activities, with properties representative of PAP1 and PAP2, were identified. A wide range of activities were observed in the normal colon tissue. Both activities were significantly increased in the majority of tumours. Furthermore, the phosphatidate and DRG mass were decreased in the tumour tissue. An increase in PAP activity is consistent with the decreased phosphatidate mass but does not account for the decreased DRG mass. These changes

are in contrast to those observed in the *ras* transformed cell lines and it is probable that they are unrelated to *ras* since only 50% of the tumours contained a mutation in the Ki-*ras* oncogene. However, the tumours with a *ras* mutation tended to have the highest activities of PAP2.

In an attempt to account for the decreased levels of DRG in the colon tumours two enzymes that are responsible for the metabolism of DRG were measured. A wide range of activities of both DRG kinase and DRG lipase were observed in the normal colonic mucosa. A DRG kinase activity was increased in the tumour tissue but the substrate specificity of this activity suggests that it metabolises DRG derived from PI rather than PC. In contrast, a DRG lipase activity was also increased in the tumour tissue and the substrate specificity of this activity indicated a preference for PC derived DRG. Thus there is evidence for a general increase in DRG turnover in colon tumours.

Studies of PAP activity in intact cell would be aided by a specific inhibitor. A number of cationic amphiphilic agents, including propranalol and sphingosine, were shown to inhibit PAP activity in rat liver homogenates and to inhibit proliferation of normal and *ras* transformed mouse fibroblast cell lines. These agents are known to inhibit other enzymes such as protein kinase C and thus lack specificity for PAP. Sphingosine was identified as the lead compound for future development of a more specific inhibitor of PAP.

The role of PAP in the control of proliferation in transformed cells remains unclear. However, the consistent increase in activity observed in colon tumours suggests that this may be a target for anticancer drug development.

CONTENTS

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List of Figures

CHAPTER 5

CHAPTER 6

CHAPTER 7

List of Tables

CHAPTER 6

CHAPTER 7

List of Abbreviations

BCNU	bis-chloroethylnitrosourea
BSA	bovine serum albumin
CAAs	cationic amphiphilic agents
DAG	*sn*-1,2-diacylglycerol
DMEM	Dulbecco's modified Eagle's Medium
DOG	*sn*-1,2-di-oleoylglycerol
DRG	diradylglycerol
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NEM	N-ethylmaleide
OBG	n-octyl-β,D-glucopyronaside
PAP	phosphatidate phosphohydrolase
PC	phosphatidylcholine
PCR	polymerase chain reaction
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
POG	1-palmitoyl-2-oleoyl-*sn*-glycerol
SAG	1-stearoyl-2-arachidonyl-*sn*-glycerol
t.l.c.	thin layer chromatography
TMAC	tetramethylammonium chloride

CHAPTER 1

GENERAL INTRODUCTION

In Britain, approximately 20% of deaths in any year are due to malignant disease. This is the second main cause of mortality in this country with heart disease being the biggest killer. Amongst the major forms of cancer arising include lung, colon and prostate in males and breast, lung and colon in females. Cancer patients generally die as a result of metastatic spread and therefore systemic therapy is essential to treat the disseminated disease.

Chemotherapy has an important role in cancer treatment. It can be used as a major curative modality for some malignancies, as adjuvant treatment before and after surgery or in combination with other modalities such as radiotherapy. Chemotherapy has had relatively major success in the treatment of a small number of malignancies including Hodgkin's disease, testicular cancer and lymphocytic leukaemia (DeVita, 1989). However, its impact on advanced solid tumours such as lung, colon and breast, is relatively limited. Since the main characteristic of tumours is their uncontrolled proliferation, many conventional chemotherapeutic agents were selected for their ability to inhibit DNA synthesis. However, many normal tissues such as the bone marrow, intestinal epithelium, skin and hair follicles demonstrate significant rates of cell renewal. As a result, these tissues are damaged by cytotoxic drugs and dose-limiting toxicities such as neutropenia and diarrhoea are associated with chemotherapy. A further complication is the development of resistance to cytotoxic drugs. Patients who show an initial response to treatment often relapse with a tumour that is resistant not only to the initial chemotherapeutic regimen but also to many other agents to which the tumour has not yet been exposed. In addition, some tumour types such as non-small cell lung and colon cancers frequently demonstrate 'intrinsic' resistance such that the patient fails to respond to treatment with cytotoxic drugs. Clearly, there is a need for more effective chemotherapeutic agents.

1.1 Colorectal Cancer

Colorectal cancer is the second most prevalent cancer in the United States and Europe preceeded only by lung cancer. Its etiology, like that of most human neoplasms, is probably multifactorial. Hereditory factors certainly play a predominant role in the development of familial polyposis coli and familial nonpolyposis colon cancer (Bussey, 1970). However, in the majority of colon cancer patients, dietary factors probably provide the overriding cause for tumour development and there is a positive association among dietary lipid intake, faecal bile acid secretion and increased risk of colon cancer (Doll & Peto, 1981; Nair, 1988; Reddy *et al.*, 1992).

The cure rate for colorectal cancer has remained relatively unchanged over the last 10-20 years. The most effective treatment is surgical resection of the tumour and the 5 year survival is high for patients who present early with a localised tumour (Cohen *et al.*, 1989). However, once the tumour has metastasised, surgery has limited success and additional chemotherapy is required. The most effective agent is 5-fluorouracil but 5 year survival rates are very low at only 10% (Vaughn & Haller, 1992). New chemotherapeutic drugs are required for treatment of this cancer. A better understanding of the biochemical changes involved in colon carcinogenesis may identify potential therapeutic targets for development of more selective agents.

There is already a lot known about colon cancer in terms of the genetic alterations which occur in its progression from adenoma to carcinoma. It is known that colorectal tumours arise as a result of mutational activation of oncogenes coupled with the mutational inactivation of tumour suppressor genes; the latter stages of which predominate (Fearon & Jones, 1992) (Figure 1.1). Mutations in at least four or five genes are required before development of the full malignant phenotype. In patients with familial adenomatous polyposis coli (FAP), a mutation on chromosome 5q is inherited. This may be responsible for the hyperproliferative epithelium present in these patients (Groden *et al.*, 1991). In tumours arising in patients without

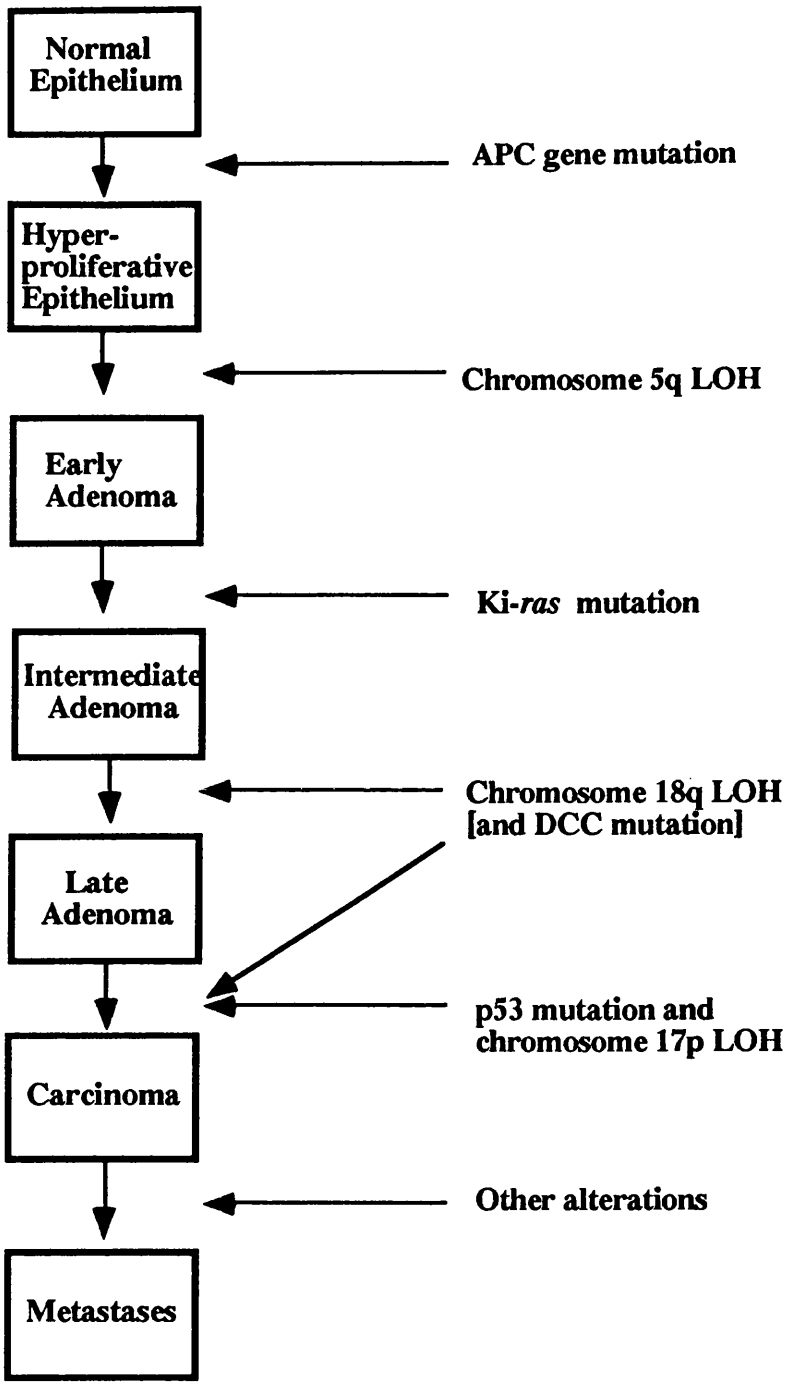


Figure 1.1 A genetic model for colorectal tumourigenesis

Abbreviations used: APC, adenomatous polyposis coli; DCC, deleted in colorectal cancer; LOH, loss of heterozygosity.

polyposis, the same region has also been shown to be altered at an early stage of tumourigenesis in a number of cases (Groden *et al.*, 1991). The early, intermediate and late stages of adenoma, represent stages of increasing size, dysplasia and villous content (Fearon & Vogelstein, 1990). It is thought that the *ras* gene mutations may be an initiating event in a subset of colorectal tumours and adenomas with the *ras* mutation are more likely to progress than adenomas without the *ras* gene mutation (Vogelstein *et al.,* 1988). The chromosomes most frequently deleted include 17p and 18q and these contain p53, a common tumour suppressor found in human cancers (Vogelstein & Kinzler, 1992), and deletion in colorectal cancer (DCC) genes, respectively (Thomas, 1991). These occur in later stages of tumourigenesis than deletions of chromosome 5q or *ras* mutations. Although these genetic alterations often occur according to a preferred sequence, the total accumulation of changes, rather than their order of occurrence, appear to be responsible for the tumour arising (Fearon & Vogelstein, 1990). Although the exact function of all these genetic alterations is not known, transformation of some genes, such as p53, is associated with development of more invasive and metastatic tumours. Another group of genes appear to promote the expansion of the adenoma, for example, DCC encodes a cell surface molecule that is homologous to N-CAM, a transmembrane cell-cell adhesion protein. A defect in this gene could produce the irregular cell packing that is characteristic of adenoma growth (Chuong *et al.*, 1994).

1.2 Signal Transduction

As a result of an increase in understanding the control of cell proliferation and differentiation, a number of new approaches to cancer treatment have been proposed. The stimulation of cell proliferation by growth factors involves a series of biochemical events whose regulation is controlled by the generation of intracellular messages in response to the binding of the mitogen to its cell surface receptor. Signals implicated in the onset of mitogenesis are tyrosine kinase activity and changes in the

levels of second messengers cyclic AMP, *sn*-1,2-diradylglycerol (DRG) and inositol 1,4,5-trisphosphate. Most oncogene products are components of signal transduction pathways (Figure 1.2) and transfection of cells with a number of oncogenes has been shown to subvert the regulation of pathways controlling second messenger concentration. If the key sites of regulation of these pathways can be identified, they might serve as targets for novel anticancer agents. For example, the *ras* genes appear to play an intermediary role in signal transduction pathways and this may provide a range of potential therapeutic targets upstream or downstream of *ras* (Burns & Balmain, 1992).

1.3 Protein Kinase C

Activation of protein kinase C (PKC) appears to be critical for mitosis. Chronic treatment of cells with the tumour promoting phorbol esters leads to down-regulation of PKC activity (Nishizuka, 1989). As a result cells are unable to enter mitosis in response to growth factor treatment (Gescher, 1985). PKC is therefore an obvious target for anticancer drug development. However, this has proved to be a very complex target. At least twelve subspecies of PKC exist which can be divided into three main groups (Dekker & Parker, 1994). The conventional PKCs, PKCα, β_I, β_{II} and γ, are Ca^{2+}-dependent, the novel PKCs, PKCδ, ε, θ, η and μ are Ca^{2+}-independent whilst the atypical PKCs, PKCζ, λ and ι, are not activated by DRG or phorbol esters. These subspecies have distinct tissue distributions implying different roles in these cell types. For example, some isoforms such as PKCα, δ and ζ are widespread, whereas others are restricted to one of a few tissues, such as PKCγ which is found only in a neuronal cell line (Hug & Sarre, 1993). Furthermore, PKC activity is also associated with expression of the differentiated phenotype in some cell lines *in vitro*. For example, differentiation of the HL-60 promyelocytic leukaemia cell line towards a macrophage-like phenotype is induced in response to phorbol ester (Vandenbark *et al.*, 1984; Vandenbark & Niedel, 1984).

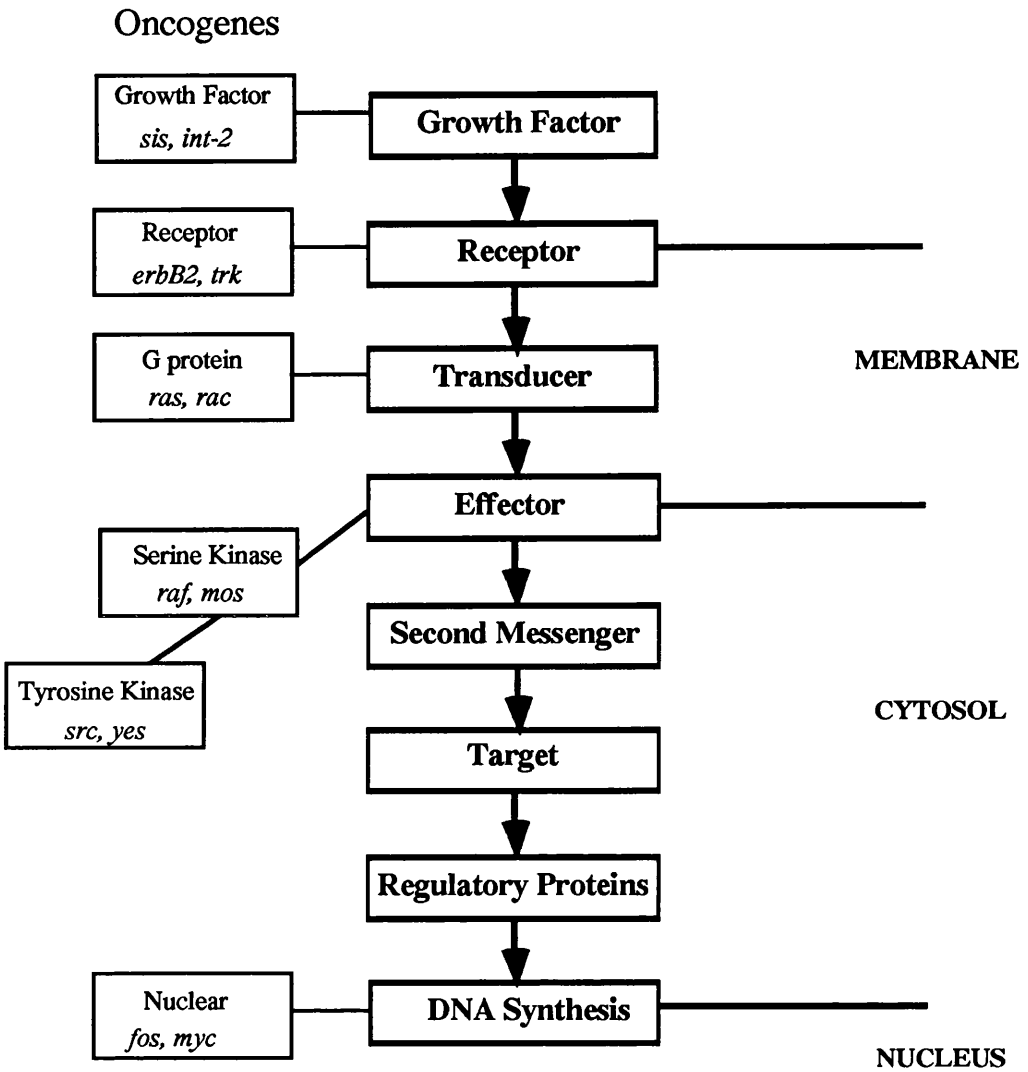


Figure 1.2 **An overview of signal transduction and examples of oncogenes involved in the process**

A number of inhibitors of PKC have been developed. Staurosporine is often used as the lead compound and the majority of inhibitors act at the ATP binding site of PKC (Grunicke & Uberall, 1992). This site is common to all PKC isoforms and shares sequence homology with the ATP binding site of other cellular kinases. This is clearly not the ideal site for development of selective inhibitors but attempts to target other sites on the protein have been largely unsuccessful. Bryostatins are a group of natural compounds which activate PKC but still show antitumour activity both*in vitro* and *in vivo* (Hornung *et al.* , 1992). They activate PKC at the same site on the regulatory domain as phorbol esters, however, these compounds can antagonise certain effects of the phorbol esters including tumour promotion in mouse skin (Hennings *et al.*, 1987). Bryostatin 1 exhibits activity against a number of tumours *in vivo* and is currently undergoing clinical trial as a chemotherapeutic drug (Prendiville *et al.*, 1993) .

An alternative approach to direct targetting of PKC is to prevent formation or to increase degradation of the stimulatory regulator of PKC.

1.4 Generation of DRG

Protein kinase C is activated physiologically by DRG. Stimulation of cells by several hormones and neurotransmitters (Berridge & Irvine, 1989) as well as mitogenic growth factors such as platelet-derived growth factor (PDGF) and bombesin (Berridge, 1987; Exton, 1990), result in the generation of DRG (Figure 1.3). Originally, it was thought that the sole source of agonist-stimulated DRG was the phospholipase C catalysed breakdown of phosphatidylinositol 4,5-bisphosphate (PI-PLC) (Wakelam *et al.*, 1986). PI-PLC is stimulated through either a G protein coupled reaction (PLCβ) (Plevin *et al.* , 1990) or phosphorylation by an activated receptor tyrosine kinase (PLCγ) (Nishibe *et al.*, 1990) (Figure 1.3). Hydrolysis of phosphatidylinositol 4,5-bisphosphate yields two second messengers, inositol trisphosphate and DRG (Berridge & Irvine, 1989). Inositol trisphosphate translocates

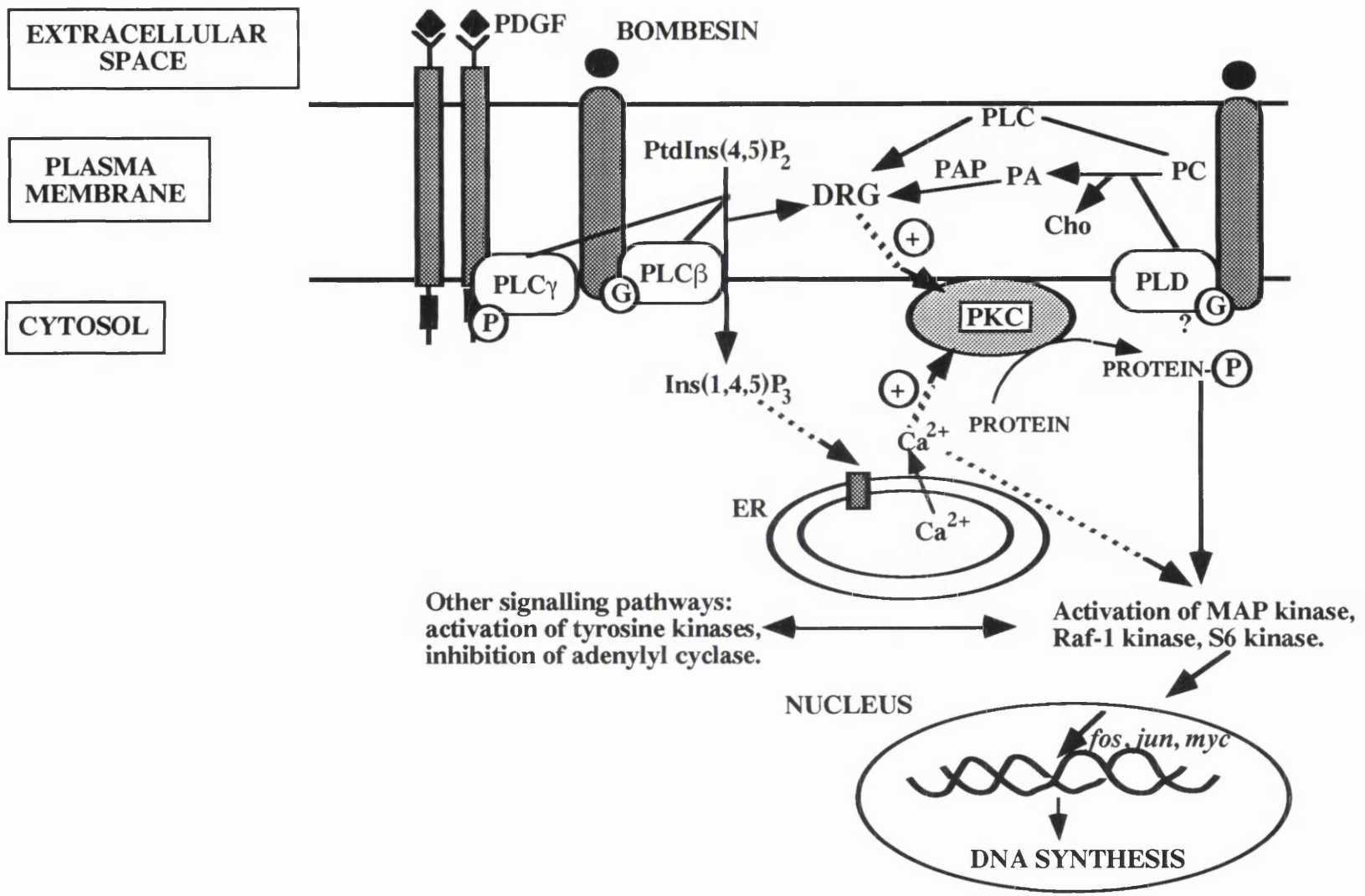

EXTRACELLULAR SPACE
PLASMA MEMBRANE
CYTOSOL
PDGF
BOMBESIN
PtdIns(4,5)P2
PLC
PAP
PA
PC
DRG
Cho
PLCγ
PLCβ
PLD
PKC
PROTEIN-P
Ins(1,4,5)P3
PROTEIN
Ca2+
ER
Ca2+
Other signalling pathways:
activation of tyrosine kinases,
inhibition of adenylyl cyclase.
Activation of MAP kinase,
Raf-1 kinase, S6 kinase.
NUCLEUS
fos, jun, myc
DNA SYNTHESIS

Figure 1.3 Potential pathways for receptor-mediated generation of diradylglycerols and downstream effects of PKC activation
Growth factors such as PDGF bind to specific receptors with intrinsic tyrosine kinase domains. PLCγ associates with the activated receptor and this results in hydrolysis of PtdIns(4,5)P_2 to form IP_3 and DRG. IP3 binds to specific receptors on endoplasmic reticulum which results in the release of Ca^{2+}. Mitogens such as bombesin can also activate this pathway, however, this receptor is G protein linked and associates with PLCβ. Activation of this receptor also leads to hydrolysis PC either by PLC, generating DRG directly, or by a PLD/PAP coupled pathway, generating phosphatidate as an intermediate. DRG activates PKC whose activity is also regulated by Ca^{2+} levels. PKC phosphorylates many proteins leading to activation of kinase cascades which have not yet been fully characterised. Following activation of regulatory proteins, transcription factors such as c-*fos*, c-*jun* and c-*myc* are expressed in the nucleus. *Abbreviations used:* cho, choline; DRG, diradylglycerol; ER, endoplasmic reticulum; G, G-protein; Ins(1,4,5)P_3, inositol (1,4,5)-trisphosphate; PA, phosphatidate; PAP, phosphatidate phosphohydrolase; PC, phosphatidylcholine; PDGF, platelet-derived growth factor; PKC, protein kinase C; PLC, phospholipase C; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate.

from the plasma membrane to specific receptors on the endoplasmic reticulum, resulting in the release of Ca^{2+}. An increase in intracellular Ca^{2+} modulates the activity of certain regulatory proteins such as PKC (Berridge, 1993). The PI-PLC pathway, however, is rapidly desensitised, within 90s, following stimulation and it is clear that it represents only a minor component of the elevation of DRG observed in response to mitogens (Cook *et al.*, 1990). A more sustained source of DRG is thought to arise from phosphatidylcholine.

Phosphatidylcholine (PC) can be hydrolysed directly by a PC-specific PLC to yield DRG and phosphocholine (Pcho) or by a coupled pathway where phospholipase D (PLD; EC 3.1.4.4) activity gives rise to phosphatidate and choline (cho). These pathways were identified from studies of the rates of formation of [^{3}H]cho and [^{3}H]Pcho in stimulated cells, representative of PLC and PLD activity, respectively (Figure 1.4) (Cook & Wakelam, 1991a). However, activation of PLD was also demonstrated in whole cells in a transphosphatidylationreaction (Yang, 1967). In this reaction the PC pool is labelled specifically with [^{3}H]palmitate and a primary alcohol is used to substitute for water in the phosphatidyltransferase reaction of PLD. PLD activity is determined from the rate of production of radiolabelled phosphatidylalcohol which can be measured.

Phosphatidate, produced by the action of PLD, is dephosphorylated by phosphatidate phosphohydrolase (PAP; EC 3.1.3.4) to generate DRG. This DRG can activate PKC but is also metabolised by both kinase and lipase activities to yield phosphatidate and monoacylglycerols respectively (Bishop & Bell, 1988) (Figure 1.4). Exogenous phosphatidate and particularly its lyso-derivative, lyso-phosphatidate, are mitogenic in a number of cell systems (Moolenaar, 1994; van Corven *et al.* , 1991; van Corven *et al.* , 1992). This lipid has been shown to stimulate phosphatidylinositol hydrolysis (van Corven *et al.*, 1989), to activate PLD in a PKC dependent manner (van der Bend *et al.*, 1992) and to modulate the amount of GTPase activating protein (GAP).and hence increase the amount of activated Ras protein (Tsai *et al.*, 1989; Van Corven *et al.*, 1993).

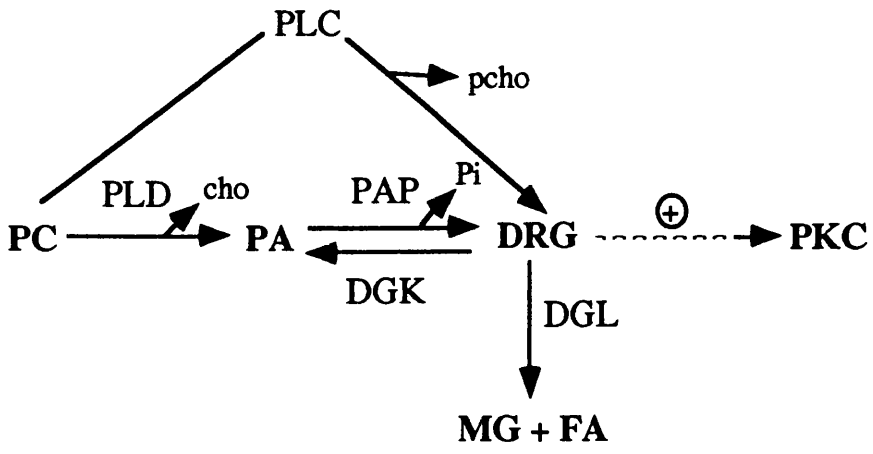


Figure 1.4 Generation of DRG from phosphatidylcholine

PC can be converted directly to DRG by the action of PLC. Alternatively, DRG can be generated from PC by the activity of PLD and PAP. DRG can then activate PKC and can be metabolised by kinase or lipase action which generate phosphatidate and free fatty acids, respectively. *Abbreviations used:* cho, choline; DRG, diradylglycerol; DGK, diacylglycerol kinase; DGL, diacylglycerol lipase; FA, fatty acid; MG, monoacylglycerol; PA, phosphatidate; PAP, phosphatidate phosphohydrolase; PC, phosphatidylcholine; pcho, phosphocholine; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D.

The two candidate enzymes for PC hydrolysis, PLC and PLD, have been reported in a number of cell systems but it is difficult to assess which activity predominates (Billah *et al.*, 1989; Cook & Wakelam, 1992; Cuadrado *et al.*, 1993). Pcho levels are increased in growth factor stimulated cells (Exton, 1990) and as a result of transformation by oncogenes (Lacal *et al.*, 1987), an indication of PC-PLC action. This hypothesis, however, has been questioned (Lacal, 1990; Plevin *et al.*, 1991; Song *et al.*, 1991) since Pcho can also be generated by phosphorylation of choline by choline kinase, the choline resulting from PLD activation. The fact that choline kinase activity is increased in *ras* transformed cells (Teegarden *et al.*, 1990) provides support for this explanation. In addition, Cuadrado and coworkers have also demonstrated that both Pcho and DRG are generated by sequential action of PLD, choline kinase and PAP in serum-stimulated and oncogene transformed cells (Cuadrado *et al.*, 1993). It may be that sustained DRG levels are a composite of PLC and PLD/PAP pathways, however, DRG synthesis *de novo* can also not be ruled out (Chiarugi *et al.*, 1989).

The role of PLD and PAP in the generation of DRG from PC was identified mainly from studies with 3T3 fibroblasts, both Swiss and NIH, and neutrophils. These two systems have proved invaluable in studies of cell signalling. In particular, the effects of oncogenes on signalling have been studied in fibroblast cells. For example, one of the responses to transformation by the *ras* oncogene is the generation of several lipids such as phosphatidate and DRG and these were found to be derived from PC (Lacal *et al.*, 1987). However, it is far from clear if results from such systems can be extrapolated to other cell types such as epithelial cells and, in particular, to tumour cells. Although bombesin stimulates breakdown of PC by PLD in Swiss 3T3 fibroblasts it is itself only a weak mitogen in these cells (Rozengurt & Sinnett-Smith, 1983). A much greater mitogenic response is obtained with bombesin and insulin as co-mitogens. This may be explained by the stimulation of a second signalling pathway by insulin which acts in a synergistic manner with the products of the breakdown of PC by PLD/PAP. In the neutrophil, the chemotactic peptide formyl-

methionyl-leucyl-phenylalanine (fmet-leu-phe) stimulates the breakdown of PC by PLD and the resultant production of phosphatidate is essential for the response to the peptide (Bonser *et al.*, 1989; Perry *et al.*, 1992). However, the response is secretion not mitogenesis suggesting that PLD could be involved in control of the secretory pathway. This suggestion is supported by the observation that PLD activation in neutrophils requires a small molecular weight cytosolic protein ADP ribosylation factor (ARF). ARF is a GTP binding protein involved in the control of vesicle formation in the golgi. It has been proposed that PLD is involved in transmission of the secretory signal from the cell surface receptor to the golgi through association with ARF (Donaldson & Klausner, 1994; Kahn *et al.*, 1993). Thus, there is evidence for the involvement of PLD mediated breakdown of PC in both mitogenic signalling as well as in membrane trafficking. The relative importance of the pathways of PLD activation is not clear and may be cell type specific. Furthermore, mitogenesis requires synthesis and modification of new proteins such that effects on trafficking through the golgi may not be distinct from mitogenic signalling.

There is little information available on the metabolism of PC in tumour cells. In view of the generation of two important lipid second messengers, phosphatidate and DRG, by the sequential action of PLD and PAP on PC, both these enzymes could be regarded as potential targets for anticancer drug development. Whilst inhibition of PLD would prevent production of both mitogenic lipids, it is also apparent that this will result in effects on membrane trafficking in normal cells such as neutrophils.

1.5 Phosphatidate Phosphohydrolase

It is only in recent years that PAP has been shown to play a role in mitogenic signal transduction (Billah & Anthes, 1990; Billah *et al.*, 1989; Bocckino *et al.*, 1987) and, in comparison to some other signalling enzymes, very little information is available concerning its regulation and function. The established function of this enzyme is as an intermediate in glycerolipid synthesis and it has been studied in great

detail particularly in the liver (Brindley, 1987). Subsequent studies have revealed two distinct activities based on differential sensitivity to N-ethylmaleimide (NEM) (Jamal *et al.*, 1992; Jamal *et al.*, 1991). PAP1 is stimulated by Mg^{2+} and is inhibited by NEM (Brindley, 1987). The other form of PAP, PAP2, is an integral plasma membrane protein which does not require Mg^{2+} for its activity and is not inhibited by thiol-blocking reagents (Jamal *et al.*, 1991).

1.5.1 PAP1

PAP1 plays a role in glycerolipid metabolism, controlling the direction of *de novo* glycerolipid biosynthesis from phosphatidate either to diglycerides and triglycerides, or to the zwitterionic phospholipids, PC and phosphatidylethanolamine. Phosphatidate is derived from stepwise acylation via dihydroxyacetone phosphate and glycerol 3-phosphate in the endoplasmic reticulum. The cytosol acts as a reservoir of PAP1 activity which associates with the endoplasmic reticulum when active (Martin *et al.*, 1986). Translocation occurs as a result of accumulation of fatty acids, acyl CoA esters or phosphatidate within the membranes (Butterwith *et al.*, 1984; Martin *et al.*, 1987). This promotes the production of triacylglycerol and increased secretion of very low density lipoproteins which occurs during gluconeogenesis (Brindley, 1987). In addition, PAP1 activity is increased in the long term by glucagon, cyclic AMP, glucacorticoids and growth hormone (Brindley, 1987; Pittner *et al.*, 1985), whereas insulin antagonises these effects (Pittner *et al.*, 1986). Thus, PAP1 activity is increased, for example, in diabetes and the increase in enzyme activity is reversed by insulin (Woods *et al.*, 1981). It has also been suggested that this form of PAP could play a role in signal transduction (Gomez-Munoz *et al.*, 1992) either by an association with the internal surface of the plasma membrane or by regulation of the balance between phosphatidate and DRG formed *de novo* in the cell (Farese *et al.*, 1987; Rossi *et al.* , 1991). This may be particularly important in cells where PAP2 activity

is absent but agonist-stimulated PLD activation results in DRG generation (Truett *et al.*, 1992).

1.5.2 PAP2

An NEM-insensitive PAP enzyme has been described (Brindley, 1987; Cascales *et al.* , 1984; Martin *et al.*, 1987) but the role of this enzyme has only recently been elucidated. Billah and Anthes first proposed that phosphatidate, formed in response to agonist stimulation, is converted to DRG by PAP activity (Billah and Anthes, 1990). This form of the enzyme, PAP2, is located in the plasma membrane, an ideal position for carrying out a signalling role. The degradation of PC by PLD coupled to the activation of PAP is now recognised as an important route of DRG formation (Billah & Anthes, 1990; Exton, 1990; Exton, 1992). The action of PAP is, therefore, closely linked to the release and control of lipid second messengers as has been shown in the fibroblast system (Martin *et al.*, 1993) and in neutrophils (Taylor *et al.*, 1993).

Unlike PAP1 which is known to be under complex, long term regulation, the factors which control PAP2 activity are unknown. Cultured vascular smooth muscle cells incubated with phorbol ester to deplete the cells of PKC, have been shown to generate more phosphatidate and less DRG than untreated cells in response to stimulation with angiotensin II (Lassegue *et al.* , 1993). This observation suggests that PAP may be regulated by PKC. Like PAP1, PAP2 activity may also be associated with physiological and pathological processes. An increase in an NEM-insensitive, plasma membrane PAP activity was associated with the inflammatory response in cyrrotic liver (Day *et al.*, 1993).

1.5.3 Purification of Phosphatidate Phosphohydrolase

To date, PAP has not been purified to homogeneity from mammalian tissue although a number of partially purified preparations have been described (Butterwith *et al.*, 1984; Day & Yeaman, 1992; Hosaka *et al.*, 1975; Ide & Nakazawa, 1989). A membrane-associated PAP, however, has been purified from the yeast *Saccharomyces cerevisiae* (Lin & Carman, 1989; Morlock *et al.*, 1991).

In yeast, two forms of PAP have been identified with molecular weights of 45- and 104-kDa, located in mitochondrial and microsomal yeast fractions, respectively. These enzymes are regulated by certain nutritional requirements and growth conditions of the cell (Morlock *et al.*, 1991). Levels of both forms increase as *S.cerevisae* progresses from exponential to stationary phase of growth. The expression of the two forms is regulated differentially by inositol and phosphorylation (Morlock *et al.*, 1991; Quinlan *et al.*, 1992). The levels of the 45 kDa form are induced in cells supplemented with inositol, whereas the other form is uneffected. Also, only the 45kDa form is phosphorylated by cAMP-dependent protein kinase *in vitro* and *in vivo*, resulting in activation of PAP activity (Quinlan *et al.*, 1992). The effects of growth phase, inositol and phosphorylation state on PAP activity correlates with changes in lipid synthesis (Morlock *et al.*, 1988; Quinlan *et al.*, 1992), implying a close involvement in their regulation of lipid synthesis.

Whilst investigating the molecular properties of diacylglycerol kinase, an enzyme which catalyses the conversion of DRG to phosphatidate, Kanoh and co-workers purified not only this enzyme, but also PAP from the plasma membrane of porcine thymus (Kanoh *et al.*, 1992). The properties of the purified 83kDa enzyme were clearly distinct from the yeast forms but were similar to those described for the PAP2 enzyme in hepatocytes (Jamal *et al.*, 1991). Unfortunately, subsequent analysis of the 'pure' protein using silver staining techniques revealed that there were four bands on the gel. It is not clear which band is responsible for PAP2 activity. Purification studies by Professor D.N.Brindley's group have also identified a 83kDa

protein that co-purifies with PAP2, but this protein has no activity under the conditions used to measure the PAP2 enzyme (Brindley, D.N., personal communication).

1.6 Aims of the Thesis

The main aims of these studies can be summarised as follows:

(i) to establish an assay for measuring PAP1 and PAP2 enzyme activities and determine their tissue distribution.

(ii) to determine whether PAP activity is altered in *ras* transformed fibroblasts.

(iii) to measure PAP activity in colorectal cancer and in adjacent normal tissue and to relate changes in PAP to levels of the second messengers phosphatidate and DRG.

(iv) to determine the effects of known PAP inhibitors on enzyme activity and cell proliferation.

(v) to establish whether PAP is a potential target for anticancer drug development.

1.7 Lay-out of Thesis

Each chapter begins with a self-contained introduction and hence full details of the rational behind the various individual stages of the work are not discussed here.

Chapter 2 describes the establishment and characterisation of an assay for the measurement of PAP activity in tissue samples. This was an essential requirement for the later studies and a well characterised assay in routine use in the laboratory of Professor D.N.Brindley was chosen. PAP1 and PAP2 differ in their sensitivity to the thiol-blocking reagent, NEM, and this property is used as the basis of an assay that allows estimation of the two activities without the need for subcellular fractionation to separate the two forms of PAP.

Transformation of fibroblasts by a mutant *ras* oncogene has been shown to increase cellular levels of DRG and to increase PKC activity (Price *et al.*, 1989). This implies an increase in the activity of the enzymes involved in generation of DRG. If the breakdown of PC by PLD/PAP is an important source of DRG for PKC activation, then it would be expected that PLD and/or PAP activity might be increased in *ras* transformed fibroblasts. This possibility was examined in a study described in Chapter 3. Although levels of DRG were indeed higher in the *ras* transformed fibroblasts, the activity of PAP2 was decreased.

Surgical resection is the major form of treatment of colon cancer. Since large sections of the colon are removed, it is possible to obtain samples of both tumour and adjacent pathologically normal colon. As a result, this disease lends itself to comparative studies. In view of the poor prognosis of this form of cancer and of the importance of *ras* mutations in tumour progression, colon cancer was chosen as a model to study PAP activity in human tumours. Chapter 4 describes a study of PAP activity in human colon cancer samples and in the adjacent normal colon. It has already been reported that PKC activity and levels of DRG are decreased in colon tumours and it was suggested that the pathways for generation of DRG is down-regulated in this tumour type (Guillem *et al.*, 1987a; Phan *et al.*, 1991). In contrast, PAP1 and PAP2 activities were both shown to be elevated in the tumour tissue.

Not all colon cancers contain *ras* mutations and it is possible that the signalling pathways in the tumours will show different changes depending on the *ras* status of the tumour. Chapter 5 describes a study of Ki-*ras* mutations present in the human colon cancer samples. Mutations were observed in a proportion of the tumour samples and this tended to be the samples with the greatest increase in PAP activity.

An increase in PAP activity in the tumour tissue is apparently inconsistent with the observed decreased levels of DRG. Whilst this may be explained by a decrease in the generation of DRG from other sources, it is possible that the enzymes responsible for DRG metabolism are also affected by transformation. Chapter 6

describes a study of the activity of two enzymes involved in DRG metabolism, DRG kinase and DRG lipase, in the colon tissue samples.

The observation that PAP activity is increased in tumour tissue lends support to the suggestion that this may be a target for anticancer drug development. In the absence of a purified form of PAP, direct design of inhibitors is not easy. A group of compounds, the cationic amphiphilic agents (CAAs), have been used in the past as inhibitors of PAP activity in whole cells (Mullmann *et al.*, 1991). However, these agents are not specific for this enzyme and can effect other signalling enzymes such as PKC (Sozzani *et al.*, 1992). These compounds may be a suitable basis for the development of specific inhibitors of PAP. Chapter 7 describes a study of the potential cytotoxicity of the CAAs as well as their effects on partially purified preparations of PAP1 and PAP2.

Finally, Chapter 8 is a general summary and discussion of the experimental evidence presented in this thesis. That PAP activity is altered in transformed cells suggests that it may play a role in the altered control of proliferation in these cells. Furthermore, the observation that PAP activity is increased in colon tumours lends support to the identification of this enzymes as a target for novel chemotherapies.

CHAPTER 2

ESTABLISHMENT AND CHARACTERISATION OF AN ASSAY TO MEASURE PHOSPHATIDATE PHOSPHOHYDROLASE ACTIVITY IN TISSUE EXTRACTS

2.1 Introduction

Much of the information available on the properties of PAP comes from the work of Professor D.N.Brindley (University of Alberta, Canada). An assay for measurement of PAP activity, routinely used in his laboratory (Jamal *et al.,* 1991), was thus chosen for use in these studies. This chapter focuses on the characterisation of enzyme activites in plasma membrane and cytosolic fractions from animal tissues. Differential assays based on inhibition by N-ethylmaleimide (NEM) are used to distinguish between the plasma membrane PAP activity, which is not inhibited by NEM, and a cytosolic form which is sensitive to this compound. A well-established and reliable assay will enable further studies on the characterisation and functional properties of the PAP enzymes in cells lines and cancer tissues.

PAP has been shown to occur as two distinct activities within rat liver (Jamal *et al.*, 1992; Jamal *et al.*, 1991). The form of the enzyme involved in glycerolipid synthesis, PAP1, is present in the cytosol but can translocate to the endoplasmic reticulum to become metabolically functional (Brindley, 1984; Brindley, 1987). In contrast, PAP2 is an integral plasma membrane protein. To date, the two forms of PAP have not been purified and thus their properties are not yet clearly defined. Since the activities have been identified as present in distinct cellular compartments, preparations of subcellular fractions can be used as a source of each activity. Until the two activities are purified, it is not certain that they represent two separate isoforms of the enzyme. However, PAP1 is known to be highly sensitive to the thiol-blocking reagent, NEM, whilst the activity of PAP2 is unaffected by this agent. This is strong

supporting evidence that the activities represent two separate enzymes and this property forms the basis of a differential assay. The assay allows estimation of PAP1 and PAP2 in tissues without the need for subcellular fractionation (Jamal *et al.*, 1991). A disadvantage of enzyme assays based on pure or partially pure preparations is that they estimate total activity and do not reflect enzyme activity under physiological conditions where substrate and co-factor availability and feedback inhibition or activation controls are present. This problem can sometimes be overcome in studies with cell lines *in vitro* by estimation of activities in intact cells. An example of such an assay is used for measurement of PLD. This enzyme catalyses the hydrolysis of phosphatidylcholine (PC) to form phosphatidate and choline. If cells are incubated with primary alcohols, these can substitute for water in the phosphatidyltransferase reaction giving rise to the corresponding phosphatidylalcohol (Yang *et al.* , 1967). Moreover, since the phosphatidylalcohols are poor substrates for PAP (Metz & Dunlop, 1991), they accumulate in the cell and allow estimation of product formation. No such assay is yet available for measurement of PAP activity. Furthermore, an assay was required that could be used both for cell lines in culture and for biopsies of human colon tumours.

There are other methods available for measurement of PAP activity and these are based on the release of inorganic phosphate from a phosphatidate emulsion. Such methods are valid if there is no degradation of phosphatidate by phospholipase A (Figure 2.1). Release of inorganic phosphate in subsequent reactions would result in an overestimation of PAP activity (Sturton & Brindley, 1978). When rat liver was used as a source of PAP, activity measured by release of inorganic phosphate was shown to be 32% higher than that measured by other methods. This discrepancy was found to be due to deacylation of phosphatidate leading to the production of glycerol phosphate and glycerol (Sturton & Brindley, 1978). These problems can be overcome by the estimation of the product of PAP activity, DRG, as in the method chosen for these studies. In this method the substrate is radiolabelled and the DRG formed in the reaction is separated by addition of aluminium oxide which absorbs unreacted

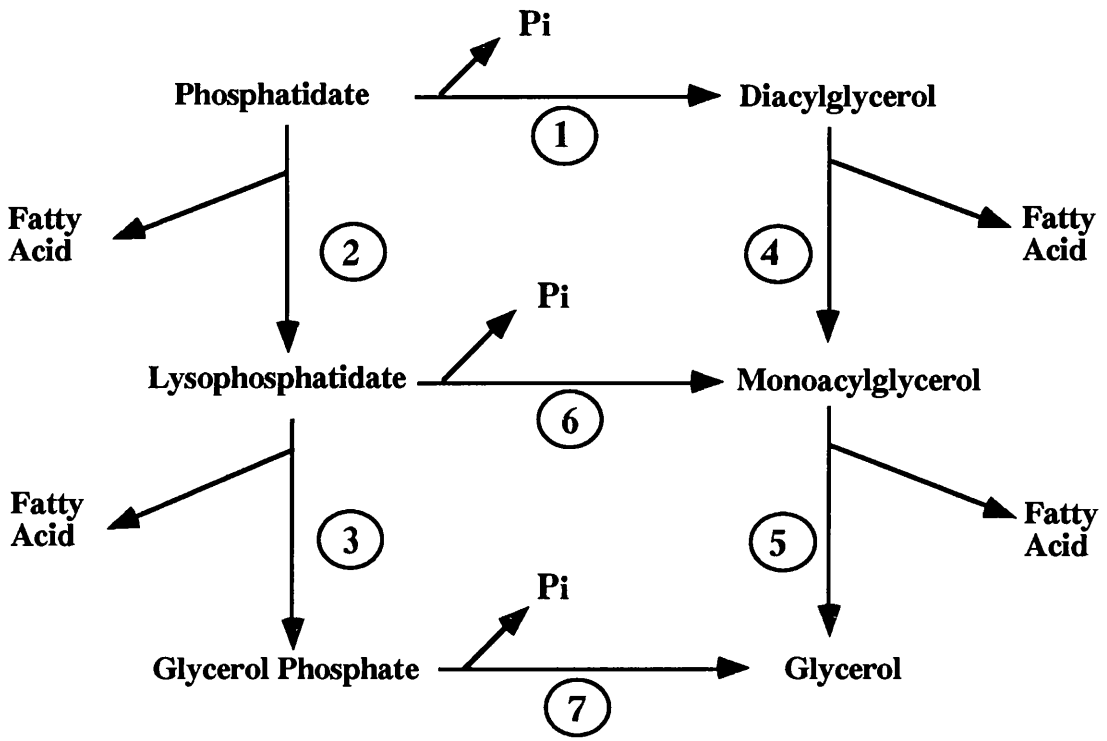


Figure 2.1 Possible routes for the degradation of phosphatidate phosphohydrolase assay

The reactions are catalysed as follows: (1) phosphatidate phosphohydrolase, (2) phospholipase A activity, (3) lysophospholipase, (4) diacylglycerol lipase, (5) monoacylglycerol lipase, (6) probably phophatidate phosphohydrolase, (7) acid or alkaline phosphatase.

phosphatidate (Martin *et al.*, 1987). Significant hydrolysis of DRG by lipase action would interfere with this assay and this is particularly relevant in adipose tissue where lipase activity is high. This problem is avoided by addition of a lipase inhibitor (Ide & Nakazawa, 1989).

Phosphatidate is regarded as a relatively minor membrane constituent with less than 1% of total phospholipids being accounted for by this lipid in eukaryotic cells (Zachowski, 1993). This water-insoluble lipid is found only in membranes where it can act as a substrate for PAP activity. Since PAP2 is an integral plasma membrane protein the substrate is readily available for its action. However, PAP1, found mainly in the cytosol, has to translocate to endoplasmic reticulum to come in contact with the substrate and, hence, carry out its role in glycerolipid synthesis. A major problem in determining PAP activity is to present this substrate in a suitable form for measuring enzyme activity in aqueous solution. In the past, a membrane-bound substrate was used but this has the disadvantage that the substrate is relatively undefined in terms of lipid composition and phosphatidate concentration (Mangiapane *et al.*, 1973). Incorporation of phosphatidate into a mixed micelle with PC produces a substrate which closely resembles the natural one. This substrate has been used to measure PAP1 activity (Martin *et al.*, 1987). PAP2 has highest activity when a phosphatidate/Triton X-100 micelle is used as substrate (Jamal *et al.*, 1991).

This chapter describes the assay used to estimate PAP activity. Distinct activities were characterised in rat liver in terms of their dependence on Mg^{2+} and Triton X-100 and sensitivity to NEM. Once established, the assay was used to determine PAP activities in a variety of tissues in mice.

2.2 Materials and Methods

2.2.1 Chemicals and Reagents

All general chemicals were of Analar grade and supplied by Merck (Thornliebank, Glasgow) or Sigma (Fancy Road, Poole, Dorset) unless otherwise stated. Tetrahydralipstatin was a kind gift from Dr.M.K.Meier (Hoffman-La-Roche Ltd., Basel, Switzerland). [^{3}H]Phosphatidate was provided by Professor Brindley and co-workers (University of Alberta, Canada).

2.2.2 Preparation of Phosphatidate as Enzyme Substrate

[^{3}H]Phosphatidate was synthesised enzymatically from [^{3}H]palmitate and glycerol phosphate. Stock solutions of cold phosphatidate and PC were prepared in chloroform. Two substrates were required for this assay. For PAP1, a mixed micelle was prepared from [^{3}H]phosphatidate, cold phosphatidate and PC to give a molar ratio of phosphatidate:PC of 3:2 and specific activity of 0.4Ci/mol (Sturton*et al.* , 1978). For PAP2, the plasma membrane form, a mix of [^{3}H]phosphatidate and cold phosphatidate was used. The solvent was removed in a stream of N_2 and the chelating reagents EDTA (5.56mM) and sodiumEGTA (5.56mM, adjusted to pH 7 with KOH) were added to give a concentration of phosphatidate in both cases of 3.33mM. The mixture was sonicated at 22kHz with an amplitude of 8μm peak for 30s at 22^{o}C using an MSE probe sonicator (Fisons Instruments, Loughborough, Leicestershire). To every 9 volumes of this mixture, 1 volume of 100mg/ml fatty acid free bovine serum albumin (BSA) was added to give a final concentration of phosphatidate of 3mM. The substrate for PAP2 was prepared in the same way except that PC was omitted. Both substrates were stored at -20^{o}C.

2.2.3 Crude Enzyme Preparations

Subcellular fractions from rat liver were used as a source of the enzymes for characterisation of the assay and enzyme kinetic analysis. Enzyme activity was also measured in a variety of homogenate preparations from tissues of nude mice.

Plasma membrane and cytosolic fractions were prepared as in Jamal *et al* (Jamal *et al.*, 1991). At all stages the tissues and fractions were kept cold at 4°C or on ice. Livers from male Wistar rats (120-200g) (Harlam Olac, Glasgow) were perfused with saline (NaCl; 0.15M) through the inferior vena cava, dissected out and minced with scissors in 4 volumes of ice-cold Tris/HCl (5mM, pH7.4) buffer containing sucrose (0.25M), $MgCl_2$ (0.5mM) and dithiothreitol (DTT, 0.5mM) (buffered sucrose). Mouse tissues were dissected out and washed in saline. All tissues were homogenised with 3 strokes up and down in a homogeniser (Citeco Varilab, Citenco Ltd., Borhamwood, Herts.) with a loose fitting pestle. The homogenate was filtered through four layers of gauze presoaked in buffered sucrose and the homogenate was centrifuged in a Centaur 1 benchtop centrifuge (Fisons Instruments) at 280 x g for 5min at 4°C. Mouse tissues were split into aliquots at this stage and stored at -70°C.

The supernatant was centrifuged at 1,500 x g for 10min in a benchtop centrifuge. The supernatant was transferred to a clean tube and centrifuged at 18,000 x g in a J2-21 Beckman centrifuge (Beckman Instruments, High Wycombe, Bucks.). These steps removed mitochondria, lysosomes and golgi from the preparation. The supernatant was then centrifuged at 100,000 x g in a Beckman L-60 ultracentrifuge using an SW 28 rotor for 60min to yield a cytosolic supernatant fraction. This was stored frozen in aliquots at -70°C.

The 280 x g pellet from rat liver was used to prepare plasma membrane fractions (for PAP2). The pellet was resuspended in buffered sucrose (1-2ml/g liver). The sucrose density of this homogenate was adjusted to 1.186g/ml using buffered sucrose solution prepared with 2M sucrose. Samples (35ml) were overlaid with 2 to 4 ml 0.25M buffered sucrose and, after centrifuging for 60min at 82,000 x g in the

Beckman L-60 ultracentrifuge using an SW 28 rotor and no brake, the interfacial layer was collected and resuspended with sufficient 0.25M buffered sucrose to obtain a sucrose density of 1.05g/ml. This was centrifuged at 280 x g for 5min as before and the supernatant discarded. The crude plasma membrane preparation was resuspended in a minimum volume of 0.25M buffered sucrose and layered over a self-forming gradient consisting of 3.5ml Percoll (Sigma), 16ml buffer A (Tris/HCl 10mM, pH7.4, sucrose 0.25M and sodiumEGTA 2mM) and 0.5ml buffer B (Tris/HCl 80mM, pH7.4, sucrose 2M and sodiumEGTA 8mM) for which the final density of sucrose was 1.05g/ml. The mixture was centrifuged at 10,000 x g for 15min in the J2-21 Beckman centrifuge with a JA-20 fixed angle rotor to yield a plasma membrane fraction just below the surface. This was removed and washed in 10 volumes of sucrose (0.25M, adjusted to pH7.4 with $KHCO_3$) containing DTT (0.5mM) and centrifuged at 10,000 x g for 5 min as before. The pellet was resuspended in the same sucrose solution and stored frozen in aliquots at -70°C.

Protein concentration in the fractions was determined using the Bio-rad protein assay (Bio-rad Laboratories Ltd., Homesdale Road, Bromley, Kent) which is based on the method of Bradford (Bradford, 1976).

2.2.4 Estimation of Phosphatidate Phosphohydrolase Activity

Solutions:

PAP1 Incubation Solution:	Tris/maleate	500mM, pH6.5
	DTT	5mM
	$MgCl_2$	15mM
	Tetrahydralipstatin	1mM
PAP2 Incubation Solution:	Tris/maleate	500mM, pH6.5
	Tetrahydralipstatin	1mM
	NEM	21mM
	Triton X-100	2.5%

PAP activity was determined by measuring the amount of [^{3}H]DRG formed from a [^{3}H]phosphatidate substrate prepared as described. Assays for two distinct PAP activities were based on the differential effect of NEM; PAP1 is sensitive to this reagent, PAP2 is insensitive. Tetrahydralipstatin was added to prevent the degradation of DRG by lipase action (Jamal *et al.*, 1991). PAP1 and PAP2 activities were measured using 20μg protein unless otherwise stated.

The reaction was carried out in polypropylene test tubes. The enzyme preparation was added in a volume of 10μl and was either rat liver cytosolic fraction for PAP1, plasma membrane fraction for PAP2 or crude tissue homogenate in which both enzymes were present. The incubation solution for PAP1 or PAP2 was added to to each tube in a volume of 20μl. The reaction volume in each tube was then made up to 80μl with water. The tubes were preincubated for 10min at 37°C and the reaction started by addition of 20μl substrate, either a phosphatidate/PC micelle for PAP1 or phosphatidate alone for PAP2 activity. This gives a final concentration of reaction mixture in the tubes of Tris/maleate buffer 100mM, DTT 1mM, $MgCl_2$ 3mM, tetrahydralipstatin 200μM, NEM 4.2mM and Triton X-100 0.5%. The final concentration of substrate was 0.6mM [^{3}H]phosphatidate for PAP1 and PAP2 and 0.4mM PC for PAP1. Also, albumin (0.2mg), EDTA (1mM) and sodiumEGTA (1mM) were added along with the substrate. In the case where both forms of the enzyme were present, parallel incubations were performed for PAP1 with NEM (4.2mM) in the incubation solution. Control reactions contained no enzyme preparation.

The reaction was terminated after incubation for 60min at 37°C by addition of 2.2ml chloroform/methanol (19:1, v/v) containing 0.08% olive oil as carrier. The [^{3}H]DRG formed in the reaction was purified by adding 1g dry aluminium oxide (80-200 mesh from Merck). The tubes were capped, vortexed and then centrifuged for 10min at 250 x g at 4°C. This pelleted the aluminium oxide to which unreacted phosphatidate was absorbed. The [^{3}H]DRG formed in the reaction was in the top chloroform phase. One ml of this was removed and placed in scintillation vials. The

chloroform was evaporated by placing the tubes in a boiling waterbath and 3ml scintillation fluid (Ecoscint A; B.S&S. Scotland Ltd., Edinburgh) was then added to each vial and the radioactivity determined by scintillation counting using a 1600TR scintillation counter (Canberra Packard, Berkshire, UK). Samples were counted after 24h to attain maximum counting efficiency. For both PAP1 and PAP2 enzymes, samples were assayed in triplicate and activities expressed as nmoles DRG formed / min / mg protein.

Kinetics of the PAP1 and PAP2 reactions were analysed by studying the reaction at increasing concentrations of substrate. In this case, a 9mM stock of [^{3}H]phosphatidate was prepared and diluted to a range of substrate concentrations between 0.1 and 1.8 mM phosphatidate for PAP1 and between 0.01 and 1.8mM for PAP2. For the former enzyme, the ratio of the [^{3}H]phosphatidate to PC was kept constant (3:2) as was the ratio of phosphatidate to Mg^{2+} (1:5). Enzyme kinetic data was analysed by use of the "Enzyme Kinetics" package of European Scientific Software (Britannia Centre, Point Pleasant, Wallsend).

2.3 Results

2.3.1 Effect of Protein Concentration and Time on Reaction Rate

For both forms of the enzyme, DRG production was proportional to protein concentration at least up to 100μg and to time up to 60min (Figure 2.2). For this reason, the majority of experiments were carried out for 60min using concentrations of protein on the linear portion of the graph, usually 20μg.

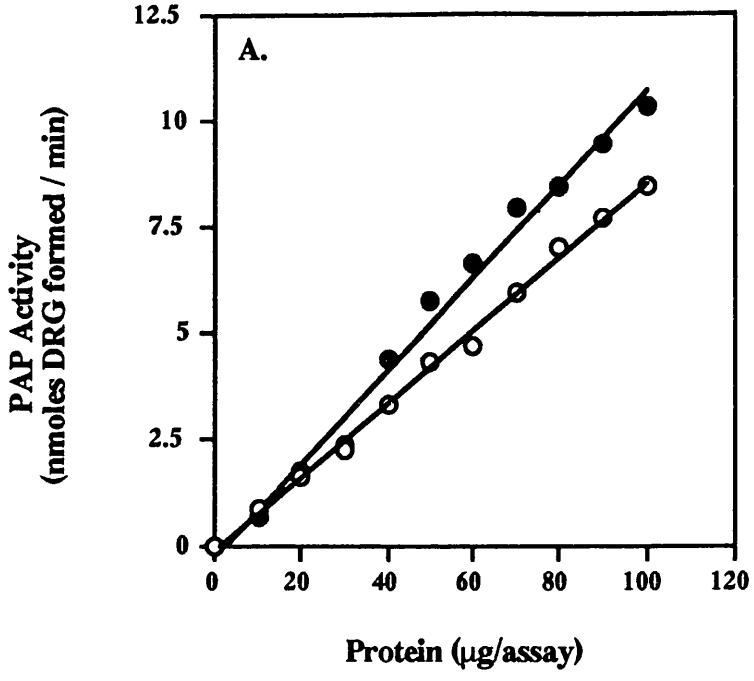
2.3.2 Effect of Mg^{2+}, NEM and Triton X-100 on Enzyme Activity

PAP activity in the rat liver cytosol fractions is dependent on the presence of Mg^{2+} with optimum activity observed between 2 and 3mM (Figure 2.3). Mg^{2+} concentrations above 3mM appear to be inhibitory. All future assays contained 3mM Mg^{2+} for measurement of PAP1 activity. Addition of Mg^{2+} to the assay for PAP2 made no difference to the activity of this form of the enzyme.

One of the main differences between the two forms of PAP is in the sensitivity to NEM as shown in Figure 2.4. In the presence of NEM (4.2mM), the PAP1 enzyme was almost completely inhibited whereas the PAP2 enzyme was insensitive to this reagent. NEM is added to all assays for PAP2 to inhibit any contaminating cytosolic enzyme activity.

When Triton X-100 was present for measurement of PAP2 activity, there was a substantial increase in the observed activity (Figure 2.5). The optimum activity occurred between 7.5 and 8mM which is equivalent to the concentration used for measurement of PAP2 in the assay. Addition of this detergent to the assay for PAP1 severely inhibits this enzyme.

The effect of tetrahydralipstatin, an acylglycerol lipase inhibitor, was also determined. For the PAP preparation from rat liver, there was no significant



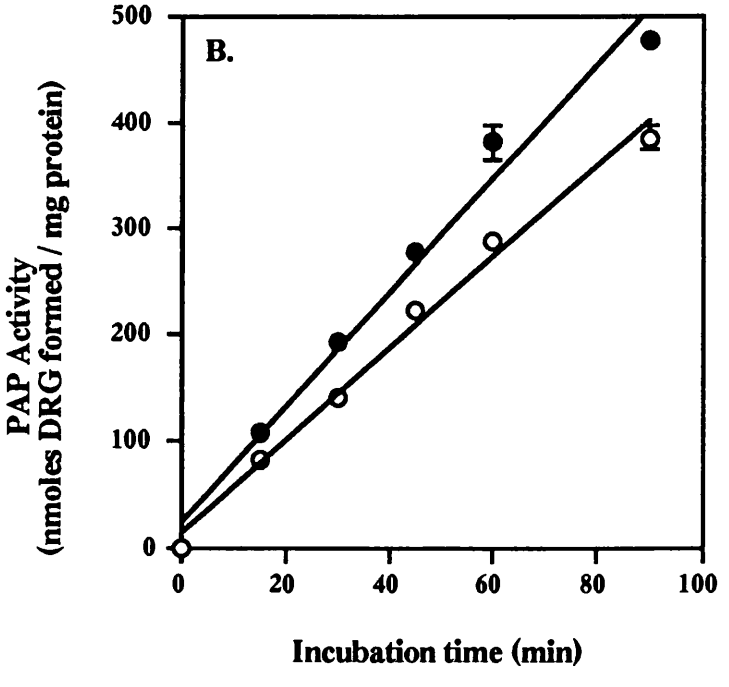


Figure 2.2 Effect of enzyme concentration and incubation time on PAP1 and PAP2 activities.

DRG production was measured with various concentrations of rat liver cytosol (PAP1, O) and plasma membrane (PAP2, ●) as sources of the enzymes (A). DRG production was also determined after incubation of samples (20μg) for various times (B). Each point is a mean ± SE of triplicate determinations and is representative of three observations showing similar results.

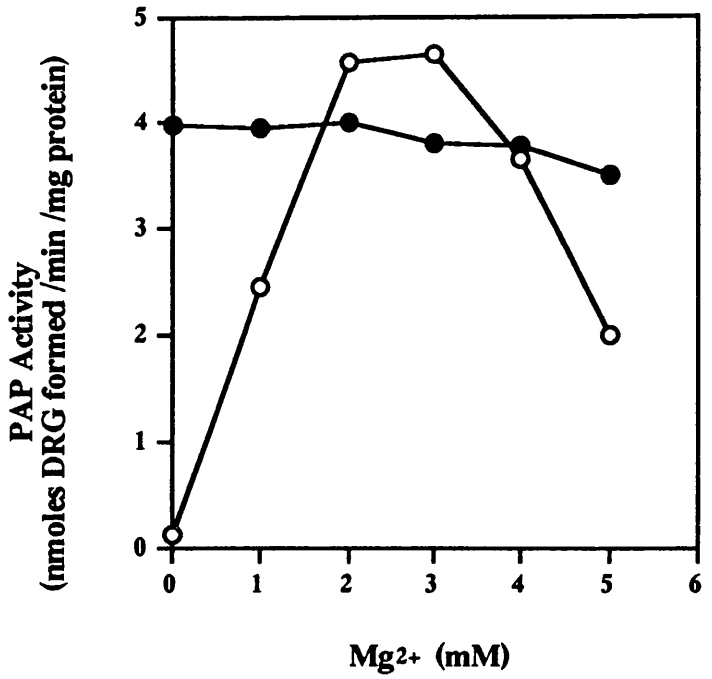


Figure 2.3 Effect of Mg^{2+} on PAP1 and PAP2 enzyme activities

PAP activity was measured in the presence of increasing concentrations of Mg^{2+} as indicated. Rat liver cytosol (PAP1) (○) and plasma membrane (PAP2) (●) fractions were used as sources of the enzymes using 20μg protein in the assay. Values are means ± SE of triplicate determinations and are representative of two observations showing similar results.

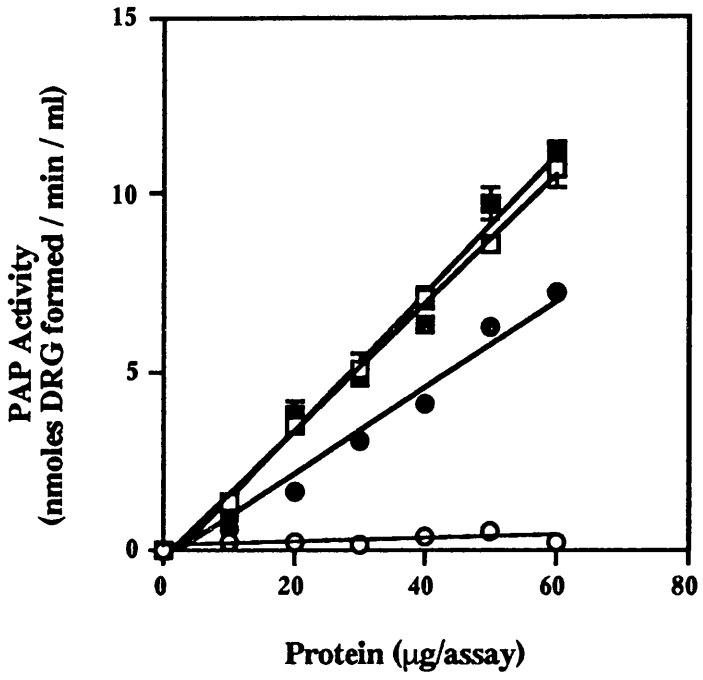


Figure 2.4 Effect of N-ethylmaleimide on PAP1 and PAP2 enzyme activities
PAP activity was measured with various concentrations of protein from rat liver cytosol (PAP1) (○, ●) and plasma membrane (PAP2) (□, ■) fractions. Activity was measured in the presence (open symbols) and absence (filled symbols) of NEM (4.2mM). Values are means ± SE of triplicate determinations and are representative of two independent observations showing similar results.

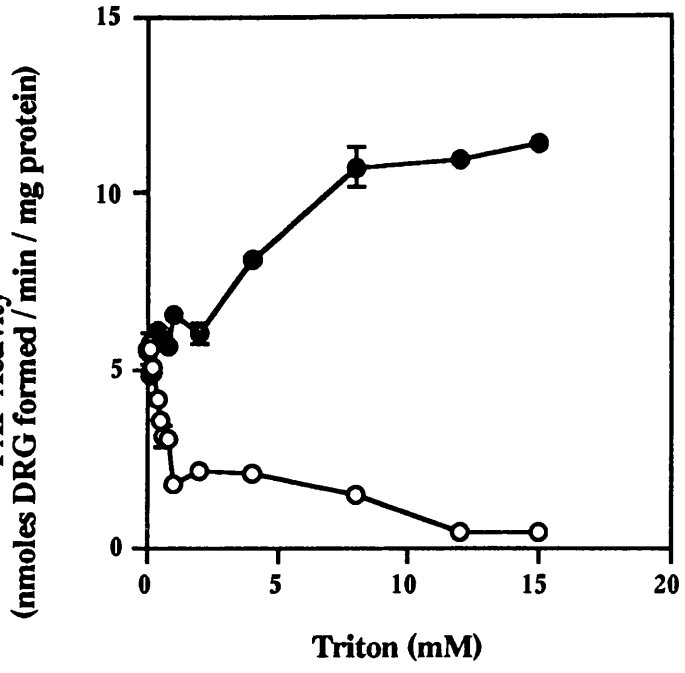


Figure 2.5 Effect of Triton X-100 on PAP1 and PAP2 enzyme activities

PAP activity was measured in the presence of increasing concentrations of Triton X-100 as indicated. Rat liver cytosol (PAP1, ○) and plasma membrane (PAP2, ●) fractions were used as sources of the enzymes using 20μg protein in the assay. Values are means ± SE of triplicate determinations and are representative of two independent observations showing similar results.

degradation of DRG since PAP activity was not affected by addition of tetrahydralipstatin (Table 2.1).

2.3.3 Effect of Substrate Concentration on Enzyme Activity

The rate of DRG production by PAP1 and PAP2 enzymes was measured over a range of concentrations of phosphatidate as shown in Figure 2.6. For any given concentration of substrate, the rate of the reaction for PAP2 was higher than for PAP1. From the straightforward plots of initial velocity against substrate concentration, PAP2 appeared to obey Michaelis-Menton kinetics (Figure 2.6). The hyperbolic graph indicates that the rate of reaction is proportional to lower substrate concentrations, but at higher values a maximum velocity is reached (at 0.6mM phosphatidate). The results for PAP2 were linearised as a double reciprocal plot (1/v against 1/s, where v and s are the rate of reaction and substrate concentration, respectively). This Lineweaver-Burk plot shows that the points are widely scattered about the best fit line (Figure 2.7). The estimated Km and Vmax values for the PAP2 enzyme were 0.09 and 6.4mM, respectively.

For PAP1, the graph of v against s is sigmoidal rather than hyperbolic (Figure 2.6) which is typical of allosteric enzymes. Maximum velocity was reached but substrate concentrations higher than 1.2mM appeared to inhibit the enzyme.

2.3.4 Tissue Distribution of PAP1 and PAP2 Activities

Activities of both PAP1 and PAP2 were measured in a range of normal tissues from nude mice (Figure 2.8). Total PAP activity is highest in brain, lung and kidneys with lowest activity in the muscle (Figure 2.8A) as measured under the conditions of the assay used for the rat liver enzymes. PAP2 activity was generally higher than PAP1 and different patterns of activity of these two enzymes were observed in these tissues. For example, PAP1 activity was high in lung and liver (1.77 and 1.42 nmoles

	PAP Activity (nmoles DRG / min / mg protein)	
	+ THL	-THL
PAP1	4.6 ± 0.3	4.8 ± 0.3
PAP2	4.5 ± 0.4	4.5 ± 0.1

Table 2.1 Effect of tetrahydralipstatin on PAP1 and PAP2 enzyme activities
PAP activity was measured in the presence and absence of 200 μM tetrahydralipstatin (THL) as indicated. Rat liver cytosol (PAP1) and plasma membrane (PAP2) fractions were used as sources of the enzymes using 20μg protein in the assay. Values are means ± SE of triplicate determinations.

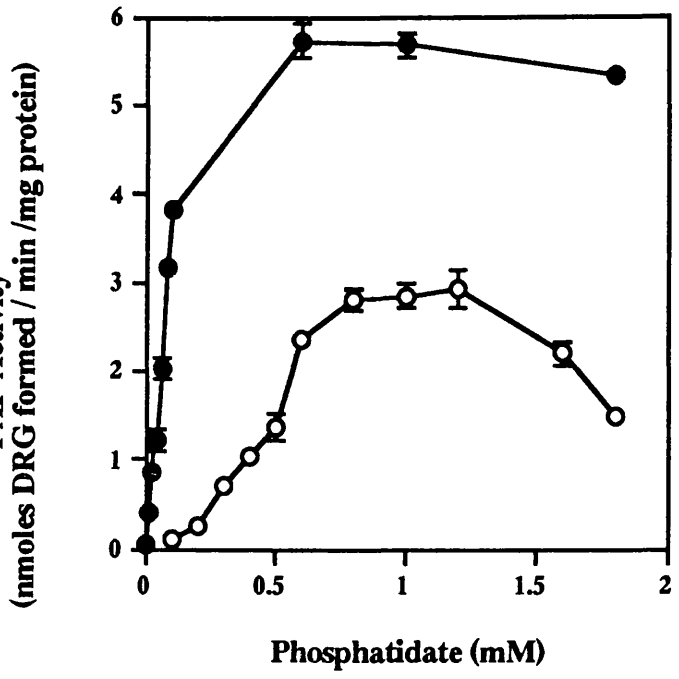


Figure 2.6 Effect of substrate concentration on PAP1 and PAP2 enzyme activities

PAP activity was measured in the presence of increasing concentrations of substrate as indicated. For PAP1, the molar ratio of 3phosphatidate:2PC and 5Mg^{2+}:1phosphatidate was maintained at all levels of substrate used. Rat liver cytosol (PAP1, ○) and plasma membrane (PAP2, ●) fractions were used as a sources of the enzymes using 30μg protein in the assay. Values are means ± SE of triplicate determinations and are representative of three independent observations showing similar results.

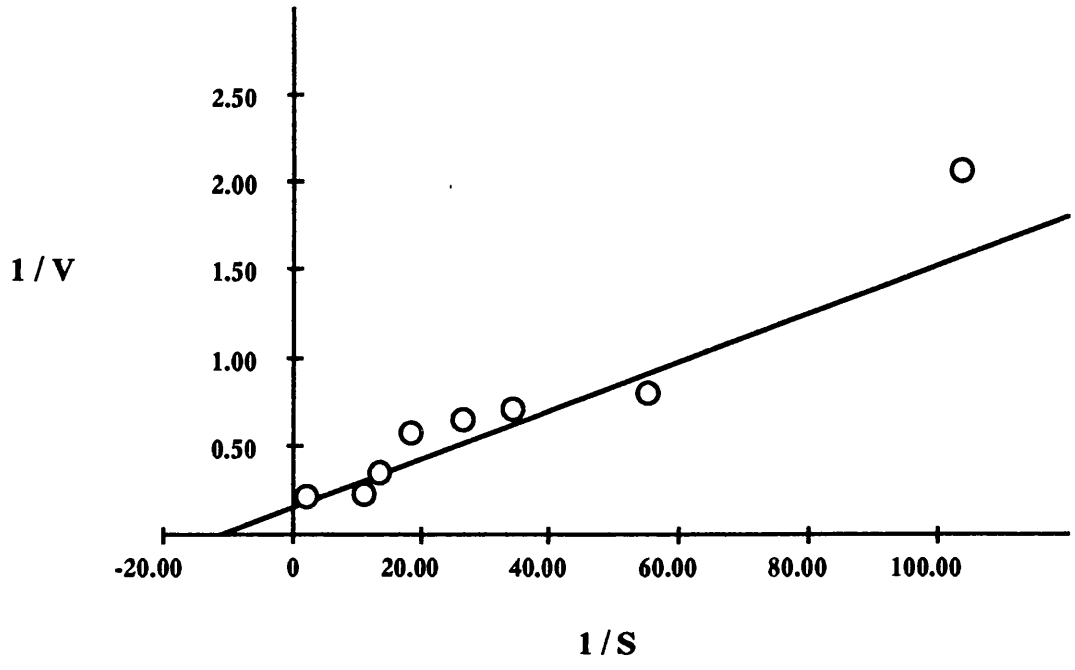


Figure 2.7 The double reciprocal plot of the PAP2 enzyme kinetics

The rate of DRG production of PAP2 enzyme is expressed as a Lineweaver-Burk plot. Rat liver plasma membrane (PAP2) fraction were used as a source of the enzyme using 20μg protein in the assay. Each point is a mean of triplicate determinations and is representative of three observations showing similar results.

DRG formed / min / mg protein respectively) and low in muscle and small intestine (0.33 and 0.29 nmoles DRG formed / min / mg protein respectively). Highest activity for the PAP2 enzyme was in the brain, small intestine and kidney (3.43, 2.89 and 2.76 nmoles DRG formed / min / mg protein respectively) and lowest in muscle (0.34 nmoles DRG formed / min / mg protein) (Figure 2.8B).

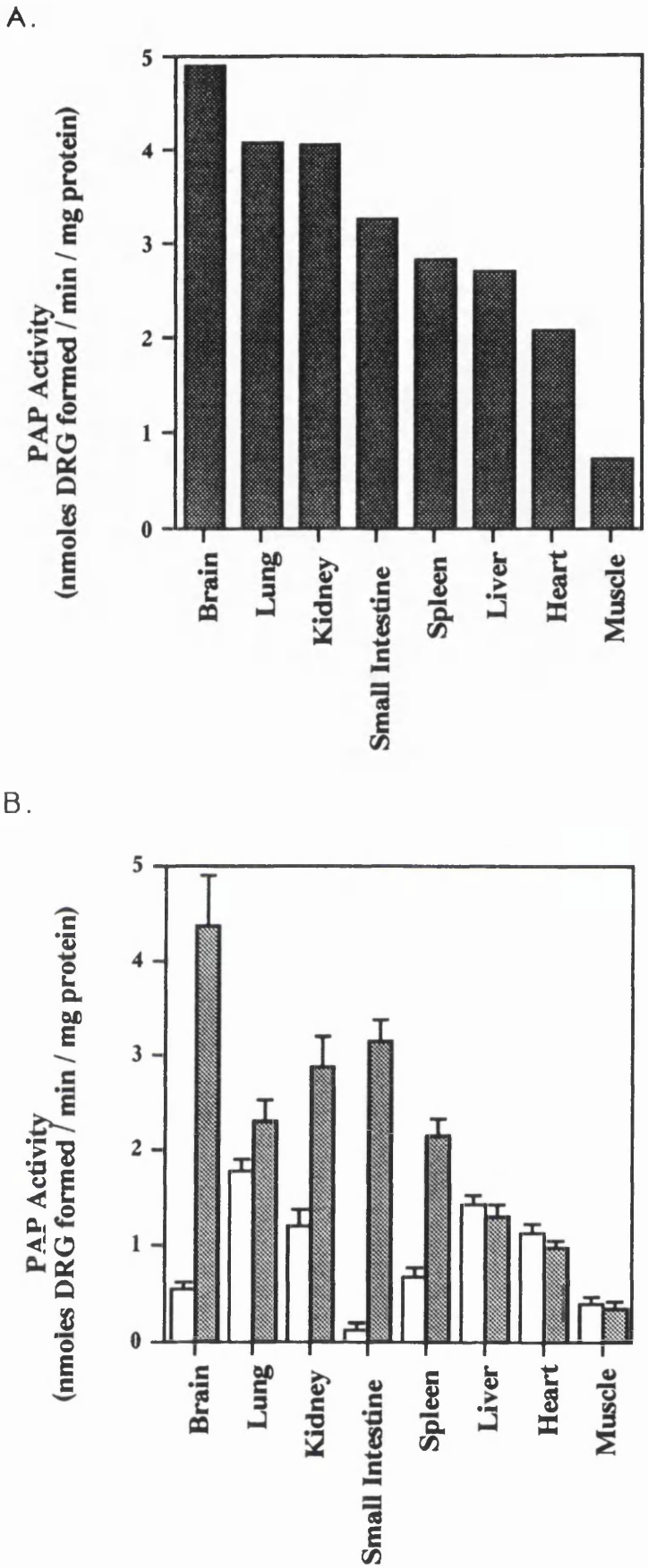


Figure 2.8 PAP1 and PAP2 enzyme activities measured in a variety of tissues from nude mice

PAP activity was measured using 20μg of protein in the indicated tissues. The results are means ± SE of the enzyme activities of three different mice. Total PAP activity is shown in A and B shows PAP1 (□)and PAP2 (▨) activities.

2.4 Discussion

PAP is an enzyme which dephosphorylates phosphatidate to produce DRG and may control the regulation between these two second messengers in signal transduction. An assay to distinguish PAP1 and PAP2 activities has been established and characterised using subcellular fractions from rat liver as crude sources of these enzymes. The activity of PAP was shown to be dependent on both time and concentration of protein. The rate of reaction was linear with protein concentration up to 100μg and the reaction rate for both PAP1 and PAP2 was linear with time up to 60min. These observations were used to define the conditions used for the subsequent assays. Both PAP1 and PAP2 activities were shown to be present in a variety of tissues from mice.

Two activities of PAP were characterised based on their different sensitivity to the thiol-blocking reagent, NEM. PAP1, which is involved in glycerolipid synthesis (Brindley, 1987), was inhibited by this reagent, whilst the activity of PAP2, the signalling form of the enzyme (Jamal *et al.*, 1991), was insensitive to NEM. This work confirms the findings of Professor Brindley's group (Jamal *et al.*, 1991) and forms the basis for the differential assay. In this study an NEM concentration of 4.2mM was chosen as it was previously found that PAP1 was almost completely inhibited at this concentration whilst PAP2 activity was unchanged (Jamal *et al.*, 1991; Martin *et al.*, 1991). Since PAP1 is strongly inhibited by NEM, it would appear that this enzyme is dependent on functional thiol groups. NEM and other such thiol-blocking compounds, for example β-chlormercuribenzoate, have also been shown to inhibit PAP activity in rat liver (Butterwith *et al.*, 1984) and rat lung (Casola & Possamayer, 1981). Inhibition by the latter compound, however, may involve binding of the mercury to a metal-binding site on PAP since mercury chloride has also been shown to inhibit PAP activity from rat lung (Casola & Possmayer, 1981). Although the different sensitivity to NEM would appear to distinguish two separate enzymes, it could be argued that the functional properties of an enzyme may be critically affected

by its environment. This raises the possibility that the activity of PAP in the plasma membrane and cytosol are due to the same enzyme with different properties due to their different locations (Bates & Saggerson, 1979; Ide & Nakazawa, 1985). Physical evidence obtained from gel filtration and anion exchange chromatography on partially purified enzyme preparations, clearly demonstrate that PAP1 and PAP2 have different size and charge characteristics which implies two different forms of PAP (Day & Yeaman, 1992).

Another main difference between the two enzymes is in their dependence on Mg^{2+}. PAP1 was found to have an absolute requirement for Mg^{2+} whereas PAP2 enzyme activity is not dependent on this metal ion for its activity, which confirms previous reports (Jamal *et al.*, 1991). Divalent cations have a profound effect on the packing arrangement and spacing of phosphatidate molecules in membranes (Cullis & DeKruijff, 1979) and this may explain the effects of Mg^{2+} on PAP activity. The optimal concentration of magnesium ions could thus create the appropriate physical conditions in the substrate such that PAP1 is able to interact with and insert itself into the prepared phosphatidate/PC micellar substrate, and then hydrolyse the phosphatidate. The effect of Mg^{2+} is known to be different from other polyvalent cations, such as calcium ions (Ca^{2+}) (Ito & Ohnishi, 1974). Addition of Ca^{2+} to the incubations for the NEM-sensitive PAP resulted in a 5-fold increase in activity, but this increase was less than 10% of that produced by Mg^{2+} (Martin *et al.*, 1987). The PAP1 found in the cytosol has a higher requirement for Mg^{2+} than other cations, however, this dependency is lost when PAP attaches to endoplasmic reticulum (Martin *et al.*, 1987).

PAP2 activity is Mg^{2+} independent and NEM insensitive and has been identified as localised in the plasma membrane (Jamal *et al.*, 1991). However, other studies that have identified a Mg^{2+} independent and/or NEM insensitive PAP activity located in microsomal membranes and cytosol (Martin *et al.*, 1987; Walton & Possmayer, 1985; Walton & Possmayer, 1989). It is possible that there is a Mg^{2+} independent/NEM insensitive PAP activity in the endoplasmic reticulum and/or

cytosolic fractions as well as the plasma membrane. This possibility has been investigated and it was concluded that the NEM insensitive form was predominantly, if not exclusively, in the plasma membrane fraction (Jamal *et al.*, 1991). Alternative locations may be the result of contamination of these fractions with plasma membranes. The NEM-sensitive PAP was mainly found in the cytosolic fraction but can associate with the endoplasmic reticulum (Brindley, 1984) or with mitochondrial membranes (Freeman & Mangiapane, 1989). There is negligible activity found in the plasma membrane (Jamal *et al.*, 1991). The degree of contamination of microsomal membranes with plasma membranes is presumably dependent on the method of homogenising, with different methods fragmenting the plasma membrane to different extents leading to their appearence in different subcellular compartments. With the mild homogenisation procedures used in this study, which have been well characterised in the past (Jamal *et al.*, 1991), it is known that the fractions obtained are largely either plasma membrane or cytosol. Furthermore, PAP activities can be selectively measured using NEM which inhibits PAP1 in the cytosol and endoplasmic reticulum but does not effect PAP2 activity in the plasma membrane. Hence, PAP activities can be measured in a tissue sample without subcellular fractionation (Jamal *et al.*, 1991; Martin *et al.*, 1991).

Membrane-bound enzyme activities are often measured in the presence of detergents as, for example, for DRG kinase (Ohanian & Heagerty, 1994). It was perhaps not surprising, therefore, that at least one of the PAP enzymes, PAP2, had elevated activity when Triton X-100 was used in the assay. This detergent ensures that the protein is present in a detergent micelle, dissociates it from other membrane components and allows it to act on the lipid substrate added to the reaction (Jamal *et al.*, 1991). PAP1 is present in an aqueous environment and the detergent was shown to inhibit this form of the enzyme. PAP1 activity is highest when the substrate is presented as a mixed micelle of the acidic phosphatidate and the zwitterionic lipid, PC. This may provide a substrate resembling the physiological one in the endoplasmic reticulum membrane with which PAP1 can interact.

A potential problem with this assay is the degradation of DRG by lipase action which, if this enzyme is present in large enough amounts, would lead to a decrease in the estimation of PAP activity. However, inclusion of the acylglycerol lipase inhibitor, tetrahydralipstatin, in the assay overcomes this effect (Hadvary *et al.*, 1991; Hermier *et al.* , 1991; Jamal *et al.*, 1991). In the liver fractions, no significant increase in DRG production was observed with tetrahydralipstatin (Table 2.1) suggesting that lipase activity in this tissue is not sufficient to invalidate the determination of the activity of PAP by the rate of DRG formation. However, this assay was required to determine the enzyme activity in other tissues where lipase action may present more of a problem. Thus, the inhibitor was routinely incorporated into the assay so that the enzymes were always measured under similar conditions.

PAP2 enzyme activity displayed Michaelis-Menton kinetics and the results were similar to those achieved for a membrane-associated form found in neutrophils (Taylor *et al.*, 1993). The Km of rat liver and neutrophil membrane forms were 88 and 65μM respectively and the Vmax of the former enzyme was 6.44. nmoles DRG formed / min / mg protein. The Km values of enzymes can range widely, for example, chymotrypsin which acts on acetyl-L-tryptophanomide has a Km of 5000μM whereas lysozyme, acting on Hexa-N-acetylglucosamine has a Km of 6μM (Stryer, 1988). At Km half the active sites of the enzyme are filled and it can be a measure of the strength of the enzyme-substrate complex. A high Km indicates weak binding whereas a low Km indicates strong binding as appeared to be the case with PAP2 enzyme.

Most allosteric or regulatory enzymes have atypical substrate-saturation curves, are very complex in nature and appear to be at variance with classic Michaelis-Menton behaviour. Homotropic regulatory enzymes, that is ones which are believed to contain two or more binding sites for the substrate at least one of which is catalytic, show a sigmoid dependence on substrate concentration. PAP1 showed sigmoidal kinetics and this may reflect a regulatory role for this form of the enzyme as has been suggested (Bell & Coleman, 1980). This sigmoid nature implies that the binding of the first substrate molecule to the enzyme enhances the binding of a

second and thus enhances activity of the enzyme. It is also common for homotropic enzymes to be inhibited by excess substrate such that maximum velocity is obtained at a limited range of substrate concentrations. This was observed with PAP1 enzyme activity which was inhibited at concentrations of substrate above 1.2mM. This has not been shown previously probably due to the limited number of substrate concentrations used (Jamal *et al.*, 1991).

Once the PAP assay had been established it was used to determine enzyme activity in a variety of tissues from mice. PAP activity has already been identified in several tissues including lung (Walton & Possmayer, 1985), adipose tissue (Jamal *et al.*, 1992), heart (Jamal *et al.*, 1992) and brain (McCaman *et al.*, 1965; Vaswanni & Ledeen, 1989). There was a marked variation in the activities of both PAP1 and PAP2 in the various tissues analysed which suggests a difference in importance or function of the enzyme. One striking observation was the high PAP2 activity observed in brain tissue. Interestingly, mammalian brain is also one of the richest sources of PLD (Billah & Anthes, 1990). Signalling enzymes are often found to be highly active in brain, however, the exact functions of these are not fully understood (Farooqui & Hirashima, 1992). It has been postulated that, for example, one of the products of the PLD reaction, choline, is used for the synthesis of the neutotransmitter, acetylcholine (Wurtman *et al.*, 1985). PLD/PAP action may provide a long term increase in DRG, which could modulate the activity of numerous enzymes in the brain (Farooqui & Hirashima, 1992). In contrast, glycerolipid synthesis, which is particularly evident in the liver, does not occur in the brain which supports the lack of PAP1 activity. The range in activities suggests this is not just a constitutive enzyme but has a different role depending on the tissue. This supports the idea that the enzyme may be a suitable target for anticancer agents.

In summary, an assay has now been established and characterised for measuring PAP enzyme activities in rat liver. This can now be used routinely and reliably as a basis for future measurements involving the activity of PAP1 and PAP2.

CHAPTER 3

EFFECT OF Ki-*ras* MUTATION ON PAP ACTIVITY AND LEVELS OF PHOSPHATIDATE AND DIRADYLGLYCEROLS IN NIH 3T3 MOUSE FIBROBLASTS AND HUMAN COLON CANCER EPITHELIAL CELLS

3.1 Introduction

An assay has been established and characterised for measuring PAP1 and PAP2 activities. Both enzymes were found to have a wide range of activities in normal tissue suggesting differential regulation depending on the cellular environment. If they play a role in the control of cell proliferation then it might be expected that activity of these enzymes would be altered as a result of cell transformation. The aim of the studies described in this chapter was to investigate the possible importance of PAP1 and PAP2 activities in proliferation by the use of an experimental model of increased proliferation.

The *ras* oncogene has attracted much attention since *ras* mutations are observed in about 30% of human cancers. Almost all pancreatic tumours contain *ras* mutations and about 50% of colorectal cancers express a mutant *ras* oncogene (Fearon & Jones, 1992; Rodenhuis, 1992). The *ras* gene is highly conserved throughout evolution such that the yeast gene can substitute for the human gene (DeFeo-Jones *et al.*, 1985). Three *ras* genes have been identified in mammals: Ha-*ras*, N-*ras* and Ki-*ras*. These genes encode closely related 21kDa proteins that undergo post-translational modification and localise on the inner surface of the plasma membrane (Willingham *et al.*, 1983). Ras proteins have been implicated in both cellular proliferation and in terminal differentiation (Barbacid, 1987; Hall, 1990). They are thus regarded as a key point in signal transduction and this is supported by the potent transforming ability of mutant *ras* genes, for example, in NIH 3T3 cells (Stacey & Kung, 1984).

Ras is a member of the guanosine triphosphate (GTP) binding protein family. These proteins act as molecular switches in signal transduction pathways and activity is determined by the phosphorylation status of the bound guanine nucleotide (Wittinghofer & Pai, 1991). In the GTP bound state, the protein is active. Ras has intrinsic GTPase activity and hydrolysis of GTP to GDP results in inactivation of the protein (Trahey & McCormick, 1987; Zhang *et al.*, 1992). Activation can be achieved by the activity of a guanine nucleotide exchange factor which dissociates GDP from the ras protein (Mizuno *et al.* , 1991). Since GTP is present at much higher levels than GDP in cells, the nucleotide binding site is rapidly filled by GTP. The majority of transforming *ras* mutations are found in a limited number of positions, codons 12, 13 and 61, and these encode amino acids located in the guanine nucleotide binding site of the ras protein. These mutations result in either increased rates of GDP dissociation or decreased intrinsic GTPase activity, both of which increase the activity of ras (Barbacid, 1987; Vogel *et al.*, 1988). Substitution of Gly with Val at codon 12 is commonly observed in human tumours and this results in a 10-fold decrease in the rate of GTP hydrolysis by the ras protein (Hoshino *et al.*, 1988).

Mouse fibroblasts are readily transformed by transfection with oncogenic *ras* and this property of *ras* is exploited in the NIH 3T3 transfection assay used to detect *ras* mutations in human tumours. Fibroblasts have receptors for epidermal growth factor (EGF), PDGF and insulin and all are mitogenic (Burgering *et al.* , 1991; Gibbs *et al.* , 1990; Satoh *et al.* , 1990). Stimulation of cells with these growth factors leads to activation of the tyrosine kinase activity of the receptors and an increased level of ras-GTP (Kaplan *et al.*, 1990). Transformation of the cells with mutant *ras* results in serum independent proliferation (Hoshino *et al.*, 1988). In addition to increased levels of ras-GTP, these transformed cells have also been shown to have increased levels of DRG, the physiological activator of PKC (Hancock *et al.*, 1988). The source of this DRG is not clear. Activation of the EGF receptor tyrosine kinase activity is known to activate PLCγ which hydrolyses phosphatidylinositol 4,5-bisphosphate (Margolis *et al.*, 1989). Serum-stimulated NIH 3T3 cells have been shown to have increased levels

of DRG and inositol phosphates which is consistent with activation of phosphatidylinositol-specific PLC (Wakelam *et al.*, 1986). However, *ras* transformed NIH 3T3 cells do not have increased levels of inositol phosphates which indicates that, in this case at least, phosphatidylinositol is not the only source of increased DRG (Lacal *et al.*, 1987). Fibroblasts transformed with v-*src* also show increased levels of DRG and this DRG is derived from a pool of cellular lipids that label preferentially with myristate (Song *et al.*, 1991). This suggests that in v-*src* transformed cells, the increased levels of DRG is as a result of an increased breakdown of PC.

PC is the major phospholipid class in mammalian tissues and is known to be the source of DRG produced in response to hormonal stimulation (Exton, 1990; Martinson, 1989; Pelech & Vance, 1989). Furthermore, PC is thought to be the source of the sustained elevation of DRG observed in response to a number of mitogens (Besterman *et al.*, 1986a; Billah *et al.*, 1989; Exton, 1990). If PC is the source of increased levels of DRG in *ras* transformed cells then this would suggest that the activity of an important mitogenic pathway is stimulated in such cell lines. Thus, both *ras* and *src* transformed fibroblasts contain increased levels of DRG and the most likely source is from the breakdown of PC by the action of PLD and PAP. If this is the case, then it would be expected that the activity of PLD and/or PAP might be increased. It has been suggested that PAP is a regulatory point in the pathway for glycerolipid synthesis (Bell & Coleman, 1980; Brindley, 1987). Although it is thought to be PAP1 that is involved in glycerolipid synthesis, a regulatory role for PAP2 in the pathway for DRG production from PC could be postulated. This possibility is supported by the observation that when 1321N1 astrocytoma cells are stimulated via the muscarinic receptor with carbachol, there is a rapid and transient increase in PLD activity but a much slower increase in DRG levels (Martinson *et al.* , 1990). It was suggested that this discrepancy could be the result of a slow conversion to DRG by PAP. However, the kinetics of PLD and PAP derived from bovine endothelial cells suggest that PLD may be the rate-limiting enzyme in the pathway from PC to DRG (Martin, 1988).

Therefore, PAP activity and levels of phosphatidate and DRG, were determined in NIH 3T3 fibroblasts and in fibroblasts transfected with a mutant Ki-*ras* oncogene. The Ki-*ras* oncogene was chosen since this is the most common *ras* mutation observed in colon cancers (Fearon & Vogelstein, 1990). However, one cell line with mutant Ha-*ras* was included for comparison. Fibroblast cell lines are easily transformed by a number of oncogenes and such cell lines are readily available. Furthermore, these cell lines have been widely used in studies of cell signalling pathways (Lowe & Skinner, 1994). However, colon tumours are derived from epithelial cells and the effects of transformation may be cell type specific. In spite of much effort, attempts to establish normal colonic epithelial cells in tissue culture have met with little success to date (Friedman *et al.*, 1981). In contrast, colon tumour-derived cell lines can be established *in vitro* and a number are available which have been characterised for *ras* mutations. The human colonic adenocarcinoma cell line, Caco-2, expresses wild-type Ha-*ras* (Delage *et al.,* 1993). When transfected with Ha-*ras* containing a mutation at codon 12, cells were shown to have increased levels of DRG which is comparable to the effects of mutant *ras* on fibroblasts cells. However, in these cells the DRG appeared to be derived from both phosphatidylinositol and PC since both [^{3}H]arachidonic acid and [^{3}H]myristic acid were able to label the lipids from which the DRG was derived (Delage *et al.*, 1993). Four colon cancer cell lines were also included in this study. Although it is not possible to compare PAP activity in the tumour cell lines with that in normal colon cells, two of the cell lines did not contain a mutant *ras* oncogene. It was, therefore, possible to compare PAP activity in cells with and without a mutant *ras* oncogene. Clearly, the lines probably contain a number of mutations in other oncogenes of relevance to cell signalling.

3.2 Materials and Methods

3.2.1 Chemicals and Reagents

Silica gel 60 thin layer chromatography plates were from Merck (Thornliebank, Glasgow) and the silica gel 150A plates with fluorescent indicator were purchased from Whatman (Maidestone, Kent). DRG kinase from *E.coli* was purchased from Calbiochem-Novabiochem (Nottinghamshire, U.K.); *sn*-1-stearoyl-2-arachidonylglycerol (SAG) and adenosine-5'-triphosphate disodium salt (ATP) were from Sigma (Poole, Dorset); phosphatidylserine was from Lipid Products (Crabhill Lane, Surrey) and [γ-^{32}P]ATP (specific activity 3 Ci/mmol) was from Amersham (Little Chalfont, Buckinghamshire). Tissue culture flasks were obtained from Bibby Sterilin (Stone, Staffordshire) and the 9cm^2 petri dishes were from Falcon (Becton Dickinson Labware, Plymouth). All components of the tissue culture media were purchased from Life Technologies (Paisley, Scotland).

3.2.2 Cell Lines

The source, Ras status and doubling times of the cell lines used in the study are shown in Table 3.1. All epithelial lines were from the American Type Tissue Collection (ATCC). DLD-1 and LoVo contain a mutation in Ki-*ras* whereas HT 29 and Colo 320DM do not (Benhatter*et al.* , 1993; Finkelstein*et al.* , 1993). NIH 3T3 I and NIH 3T3 II are essentially the same cell line but were obtained from the respective laboratories as the parental line for the transfectants. The former was transformed by v-Ha-*ras* (HT3 I) and v-Ki-*ras* (KMSV I) and NIH 3T3 II was transformed by v-Ki-*ras* (Ki 858 II) with a different point mutation to that in KMSVI.

Cell Line	Ras Status	Doubling Time (h)	Source	Reference
NIH 3T3 I	WT for *ras*	20	Dr.A. Balmain, Medical Oncology, Glasgow.	(Copeland & Cooper, 1979; Ellis *et al.*, 1981; Fusco *et al.*, 1987)
HT3 I	v-Ha-*ras* with Gly-Val mutation at codon 12	16	The transformed lines are derived from the NIH 3T3 line.	
KMSV I	v-Ki-*ras* with Gly-Val mutation at codon 12	18		
NIH 3T3 II	WT for *ras*	21	Prof.M. Wakelam, Dept. of Cancer Studies, Univ.of Bir'ham.	
Ki 858 II	v-Ki-*ras* with Gly-Asp mutation at codon 12	17		
HT 29	WT for *ras*	19	adenocarcinoma (ATCC HTB 38)	(Marshall *et al.*, 1977)
Colo320DM	WT for *ras*	21	adenocarcinoma (ATCC CCL 220)	(Quinn *et al.*, 1979)
DLD-1	Ki-*ras* with Gly to Asp mutation at codon 13	21	adenocarcinoma (ATCC CCL 221)	(Cexter *et al.*, 1979)
LoVo	Ki-*ras* with Gly to Asp mutation at codon 13	22	adenocarcinoma (ATCC CCL 229)	(Drewinko *et al.*, 1976; Drewinko *et al.*, 1978)

Table 3.1 Source and Ras status of the murine fibroblast and human colon cancer epithelial cell lines

The *ras* transformed fibroblasts are suffixed with I or II to show which parental NIH 3T3 cell line they are derived from.

3.2.3 Routine Cell Maintenance

All fibroblast cell lines were grown in Dulbecco's modified Eagle's Medium (DMEM) containing sodium bicarbonate (23mM), glutamine (2mM) and donor calf serum (10%, v/v). HT 29 and Colo 320DM were grown in a mixture of Ham's F10 and DMEM (50:50) supplemented with sodium bicarbonate (23mM), glutamine (2mM) and foetal calf serum (10%, v/v). LoVo and DLD-1 were maintained in RPMI 1640 (Northumbria Biologicals, Cranlington, Northumberland) supplemented as for the previous medium. The untransformed fibroblast and epithelial cell lines were passaged at weekly intervals, whereas the transformed lines required passaging twice weekly. They were seeded at an initial density of 10^6 cells per 75 cm^2 flask and incubated at 37°C in an atmosphere of 2% CO_2 in air. Cells were grown for a maximum of 10 passage from frozen stocks which had been stored in liquid nitrogen in growth medium containing DMSO (10%).

Cells were passaged by removal of all medium from the flask then adding 5ml of phosphate buffered saline (PBS) (Dulbeccos A) containing EDTA (1mM) and trypsin (0.25%) to the flask. This solution was removed almost immediately and the cells left for 30s to allow detachment from the surface of the flask. Cells were resuspended into 10ml of medium and counted using a Coulter Counter (Coulter Electronics Ltd., Luton, Bedfordshire) before dilution and plating into fresh tissue culture flasks.

3.2.4 Mycoplasma Testing

All cell lines were free of mycoplasma as confirmed by monthly screening. Cells were fixed with ice-cold glacial acetic acid (25%, v/v) in methanol and then stained with the fluorescent DNA stain Hoescht 3328 (Sigma, Poole, Dorset) at a concentration of 100ng/ml for 15min at room temperature (Chen, 1977). Plates were

then examined under a fluorescent microscope (Polyvar Microscope, Reichert, Leica Ltd., Milton Keynes, England) for visual evidence of infection.

3.2.5 Estimation of Phosphatidate Phosphohydrolase Activity

Cells were plated out at a density of 10^6 cells / dish in $9cm^2$ tissue culture petri dishes and incubated at 37°C in an atmosphere of 2% CO_2 in air for 3-4 days until the cells were confluent. They were washed four times with ice-cold PBS and scraped from the plates in 0.5ml of Tris buffer (20mM, adjusted to pH6.5 with maleate) containing sodium phosphate (5mM), DTT (1mM), NaCl (140mM), leupeptin (1μg/ml) and, for PAP2 measurements, sodium orthovanadate (400μM) and $ZnCl_2$ (1μM) to inhibit activity of protein tyrosine phosphatase. The cells were sonicated four times for 2s then stored frozen at -70°C in aliquots. After thawing, samples were sonicated briefly and protein concentrations determined as described in section 2.2.3. The samples were diluted for use in the same harvesting medium.

Unless stated otherwise, PAP activity was determined at a protein concentration of 20μg/assay. Cells harvested in the absence of vanadate and Zn^{2+} were used for the assay of PAP1 activity. Assays of cells harvested with vanadate and Zn^{2+} all contained a final concentration of 240μM vanadate and 0.6μM Zn^{2+}. The cells were used as sources of the enzymes and PAP activity was measured as described in section 2.2.4.

3.2.6 Cell Lipid Extraction

Lipid extraction was carried out essentially by the method of Bligh and Dyer (Bligh & Dyer, 1959). Confluent cultures of cells were washed twice with ice-cold PBS and scraped from the petri dish in 0.5ml methanol. This was transferred to a glass test tube and the residue collected with a further 0.5ml methanol. Chloroform (0.5ml) and water (0.4ml) were added and lipids were extracted by addition of a

further 0.5ml chloroform and 0.5ml NaCl (1M) in water. The tubes were vortexed then centrifuged at 250 x g for 10min to separate the phases. The lower chloroform phase from one tube was mixed with that of another to ensure sufficient mass of lipid for accurate determination. This lipid extraction mixture was then split into aliquots of 900µl for phosphatidate mass measurement, 400µl for measurement of DRG mass and 50µl for total phospholipid assay. The lipids were dried under a stream of nitrogen and stored at -70°C until ready for use. The lipids were assayed for DRG and phosphatidate within three days of extraction.

3.2.7 Measurement of Phosphatidate Mass

Phosphatidate was measured using a modification of the method Bocckino *et al* (Bocckino *et al.*, 1987). Lipid extracts were redissolved in 50µl chloroform and applied in 0.5cm bands half way up a plastic thin layer chromatography silica gel 60 plate that had been previously developed in petroleum ether / diethylether (1:1, v/v). Standards of phosphatidate (0.5, 1, 2.5 and 5µg) prepared in chloroform were applied to each plate. They were developed to 95% of their length with chloroform : methanol : ammonium hydroxide (65:35:7.5, v/v/v), air dried and then cut 1.5cm above the origin which removes the neutral lipids and most of the zwitterionic phospholipids. Phosphatidate remained at the origin in this chromatographic system. The plates were then developed in the reverse direction to 95% of their remaining length using chloroform : methanol : acetic acid (9:1:1, v/v/v).

The dried plates were then stained for 1 hour with Coomassie Blue R250 (0.03%) in methanol / water (4:1, v/v) containing NaCl (100mM), destained for 10-15min using methanol / water (4:1, v/v) and dried overnight. The absorbancies of the phosphatidate bands were measured using an LKB 2202 Ultrascan Laser densitometer (Pharmacia LKB Biotechnology, St.Albans, Hertfordshire) in the reflectance mode at 580nm. The output was channelled to an LKB analogue/digital converter and the thin layer chromatographic bands integrated using an LKB CD Plot system. A standard

curve of phosphatidate was constructed from the peak areas for each plate. Triplicate samples from each cell line were analysed on separate plates and the average values calculated.

Figure 3.1 shows a typical standard curve obtained from densitometric analysis (peak areas) of phosphatidate at 0.5, 1.0, 2.5, 5.0 and 10.0 nmoles lipid. This was linear up to 5nmoles of phosphatidate. This represents the mean curve from three t.l.c.plates. Provided staining and destaining of the lipids was carried out for approximately the same time and hence background was a similar intensity, variation between plates for the lipids was within 5%. It is important, however, to compare cell lipid extracts with the standard curve of that particular t.l.c. plate analysis.

3.2.8 Measurement of Diradylglycerol Mass

Solutions:

Incubation Buffer:	Imidazole/HCl	250mM, pH6.6
	$MgCl_2$	62.5mM
	sodium EGTA	5mM
Triton Solution:	Triton X-100	0.06%, w/v
	Imidazole/HCl	25mM, pH6.6
ATP Buffer:	Imidazole/HCl	100mM, pH6.6

It should be noted that many reports often refer to DRG as diacylglycerol (DAG). However, most methods for measuring this lipid cannot distinguish between the various DRGs, that is, *sn*-1,2-diacylglycerol, *sn*-1-alkyl-2-acylglycerol and *sn*-1-alkenyl-2-acylglycerol. Hence, it is more accurate to use the term 'DRG' unless referring to a particular class of the lipid.

Cell lipid extract previously dried under a stream of nitrogen in glass vials was used for measurement of DRG mass. A standard curve of DRG (25 - 500 pmoles), prepared in chloroform, was also set up. The standards were dried under a vacuum in

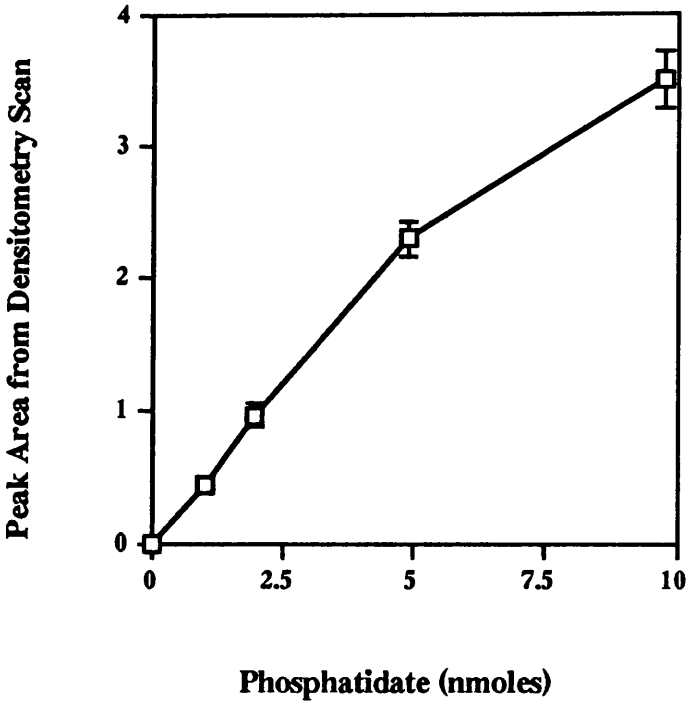


Figure 3.1 Standard curve for the phosphatidate mass assay

The standard curve was generated from three separate mass assays. Phosphatidate was separated from other lipids using thin-layer chromatography and then stained with Coomassie Blue. The area of the peak corresponding to phosphatidate was measured by a densitometric scan. Each point is a mean value ± SE.

a Gyrovap (V.A.Howe, Oxfordshire, England) and both cell extracts and standards were solubilised in 50µl of Triton X-100 (0.6%, w/v) / phosphatidylserine (288µM) by brief vortexing followed by bath sonication for 30min at 4°C (KS100 bath sonicator, Kerry Ultrasonics Ltd., Kent). Following sonication, 10µl DTT solution (100mM) and 20µl incubation buffer were added to each tube. An *E.coli* DRG kinase solution was prepared by mixing 10µl of the purchased enzyme (100m-Units) with 190µl of enzyme mix (885µl water, 17µl Triton solution and 100µl ATP buffer). Ten µl of the final enzyme solution was added to each tube. An ATP stock solution was prepared in ATP buffer ensuring there was sufficient ATP and [γ-^{32}P]ATP (taking into account decay) to obtain a final concentration of ATP of 0.5mM and 1µCi/assay. The final reaction mixture contained Triton X-100 (0.3%, w/v), phosphatidylserine (144µM), imidazole/HCl (50mM, pH6.6), NaCl (50mM), $MgCl_2$ (12.5mM), sodiumEGTA (1mM), DTT (10mM), ATP (0.5mM, 1µCi of [γ-^{32}P]ATP) and *E.coli* DRG kinase (5m-Units) in a volume of 100µl. The reaction was started by the addition of the ATP and samples were incubated at 30°C for 30min.

The reaction was terminated by the addition of 1ml chloroform : methanol : HCl (5N) (150:300:2, v/v/v). The tubes were left to stand for 10min at room temperature and the phases split by the addition of 300µl chloroform and 400µl water followed by centrifugation at 250 x g in a benchtop centrifuge (model CR411, Deva Medical Electronics Ltd., Runcorn, Cheshire) for 10min. The top phase was discarded and the remaining organic phase mixed with 1ml of chloroform : methanol : water (1:1:0.9, v/v/v), centrifuged as before and the top phase discarded. The bottom phase was dried under vacuum. Radiolabelled products were reconstituted in 20µl chloroform and applied to a 5x20cm silica gel 60 t.l.c. plate. The plates were developed in chloroform : methanol : acetic acid (39:9:4.5, v/v/v). The [^{32}P]phosphatidate was identified by autoradiography and the silica corresponding to the phosphatidate scraped, placed in scintillation vials with 3ml scintillation fluid and counted by liquid scintillation counting. DRG in the cell extracts was determined

from a standard curve. Average values were calculated and expressed as pmoles DRG.

Figure 3.2 shows a typical standard curve obtained for conversion of 25-500 pmole *sn*-1-stearoyl-2-arachidonylglycerol to [^{32}P]phosphatidate and indicates that 100% efficiency of conversion of DRG to phosphatidate occured which is essential when mass analysis is used.

3.2.9 Measurement of Inorganic Phosphate

Inorganic phosphate was determined by the method of Van Veldhoven (Van Veldhoven & Mannaerts, 1987). The cell lipid extract for this assay was reconstituted into 50µl chloroform and then split in aliquots of 2x10µl, 2x5µl and 2x2µl into pyrex test tubes. A standard curve of glycerol phosphate (1-100nmole) was prepared in water. All samples and standards were dried in a Techne Dri-block (Fisons Instruments, Loughborough, Leicestershire) at 180°C for 15min or until they were completely dry. Fifty µl of 70% perchlorate was added to each tube and they were then heated to 180°C for 30min, ensuring that the acid did not evaporate by placing a glass marble over each test tube. The tubes were removed and cooled.

To each assay was added 120µl water, 50µl ammonium heptamolybdate.4$H_2$0 (2.5%) and 50µl ascorbic acid (10%). The solution was vortexed then heated to 90°C in a waterbath for 5min. The colour change was from pale yellow to blue in the presence of phosphate. An aliquot of 200µl was removed from each tube and placed in a well of a 96 well plate. Absorbance at 750nm was measured in a plate reader (Emax model, Alpha laboratories, Hampshire, England). Phospholipid content in the cell extract was calculated from a standard curve.

The mass levels of phosphatidate and DRG could then be standardised with reference to nmoles phospholipid in the cell lipid extract.

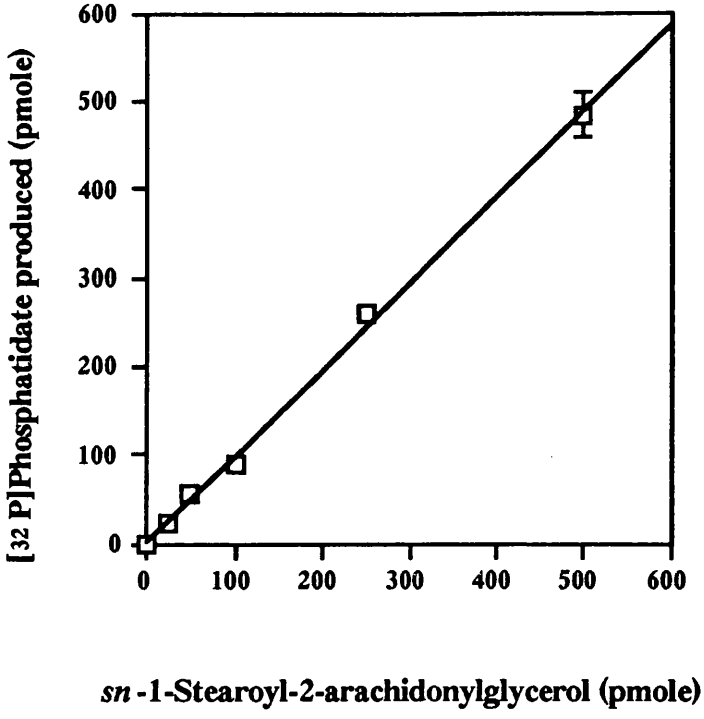


Figure 3.2 Standard curve of conversion of *sn*-1-stearoyl-2-arachidonyl-glycerol to phosphatidate

The result presented is a typical standard curve generated for conversion of 25-500 pmole *sn*-1-stearoyl-2-arachidonylglycerol to phosphatidate. The determination was performed in triplicate and values are expressed as means ± SE. The [^{32}P]phosphatidate was identified by autoradiography and silica corresponding to phosphatidate scraped and counted by liquid scintillation counting.

3.2.10 Statistics

Student's unpaired t-test was used to compare results for the untransformed and transformed fibroblast cells and analysis of variance (ANOVA) was used to compare differences between the colon epithelial cells. "Staview 512+" statistical package for the Apple Macintosh system was used.

3.3 Results

3.3.1 Activity of Phosphatidate Phosphohydrolase in Fibroblast and Epithelial Cells

The activity of PAP1 and PAP2 in control fibroblasts (NIH 3T3 I and II) and the *ras* transformed varients from these lines (KMSVI, HT3I and Ki 858II) as well as the colon cancer epithelial cell lines with (DLD-1 and LoVo) and without (HT 29 and Colo 320DM) a Ki-*ras* mutation, is shown in Table 3.2 and in Figures 3.3 and 3.4. The *ras* transformed cells HT3I and KMSVI have significantly higher PAP1 activity ($p<0.0001$ and $p<0.02$, respectively) than the parental line NIH 3T3 I. Similarly, Ki 858II has significantly higher ($p<0.0018$) activity than its parental line NIH 3T3II (Figure 3.3A). The two parental cell lines had similar PAP1 activities. In contrast, the specific activity of PAP1 was significantly lower ($p<0.0001$) in the colon epithelial cell lines with the Ki-*ras* mutation (LoVo and DLD-1) compared to those without this mutation (Figure 3.4A). Activity of this enzyme was similar in the two cell lines with a Ki-*ras* mutation, but for the two lines with wild type Ki-*ras,* Colo 320DM had significantly higher PAP1 activity than HT 29 ($p<0.0001$).

Overall there was a decrease by approximately 2-fold in the specific activity of PAP2 in the *ras* transformed fibroblast cells. HT3I had a significant 2.1 fold decrease ($P<0.005$) in PAP2 activity, and KMSVI, a 2.4 fold decrease ($p<0.004$) in activity compared to control cells. The Ki 858II cell line had a significant 2.5 fold decrease ($p<0.018$) in PAP2 activity compared to NIH 3T3 II cells (Figure 3.3B). The activity of PAP2 was similar in the two parental cell lines. Specific activity of PAP2 was significantly lower ($p<0.0001$) in the colon epithelial cell lines with a Ki-*ras* mutation compared to those without this mutation (Figure 3.4B).

		nmoles DRG formed / min / mg protein		pmoles lipid / nmole phospholipid	
CELL LINE	***ras* mutation status**	**PAP1**	**PAP2**	**phosphati-date**	**DRG**
NIH 3T3 I	-	1.7 ± 0.1	3.5 ± 0.2	4.7 ± 0.3	1.8 ± 0.2
HT3 I	+	6.4 ± 0.2	1.7 ± 0.0	23.0 ± 2.0	6.3 ± 0.1
KMSV I	+	2.9 ± 0.3	1.4 ± 0.1	23.4 ± 0.9	6.9 ± 0.5
NIH 3T3 II	-	1.4 ± 0.1	3.2 ± 0.1	4.5 ± 0.7	1.7 ± 0.2
Ki 858 II	+	2.5 ± 0.0	1.3 ± 0.1	27.1 ± 0.9	4.6 ± 0.2
HT 29	-	3.4 ± 0.2	3.6 ± 0.2	5.4 ± 0.6	3.4 ± 0.4
Colo 320DM	-	6.7 ± 0.3	3.3 ± 0.2	5.4 ± 0.4	3.7 ± 0.5
DLD-1	+	2.1 ± 0.2	1.9 ± 0.1	33.9 ± 1.1	10.5 ± 0.6
LoVo	+	2.2 ± 0.2	2.0 ± 0.1	75.7 ± 3.5	16.4 ± 0.5

Table 3.2 Specific activities of PAP1 and PAP2 and mass levels of diradylglycerol and phosphatidate in control and *ras* transformed fibroblasts and in human colon epithelial cells

The results for the PAP activities in all cell lines were calculated as specific activities relative to protein for each experiment. The mass levels of lipids were measured relative to total phospholipid. Results are means ± SE for three independent experiments performed in triplicate.

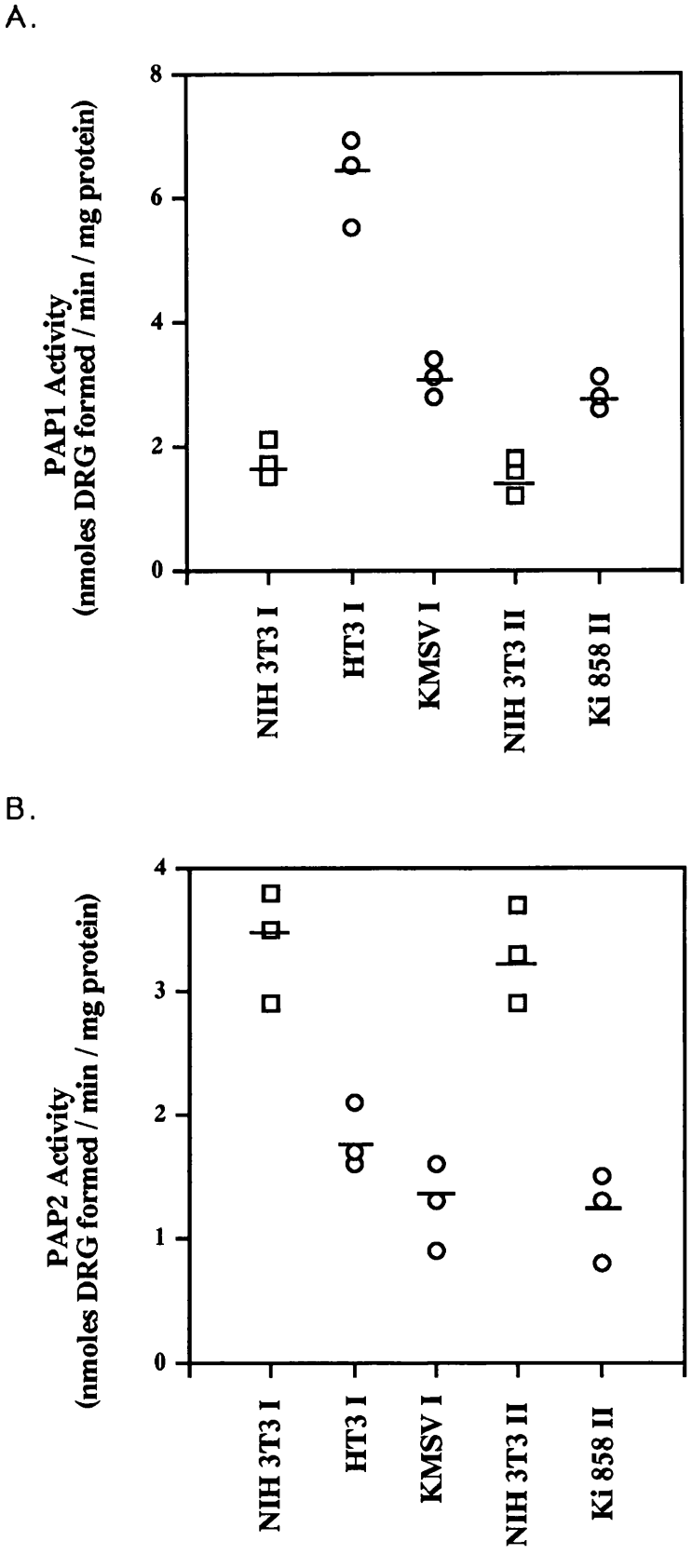
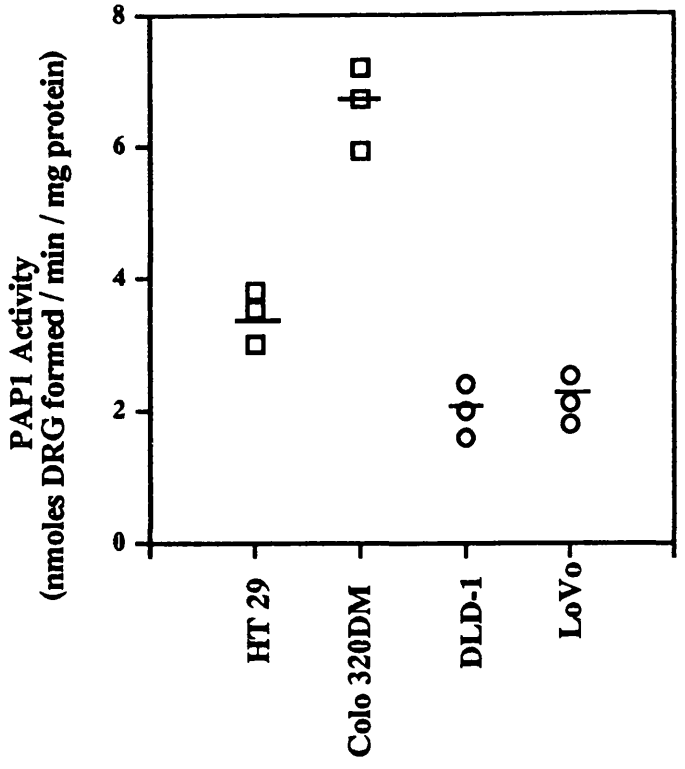


Figure 3.3 Specific activities of PAP1 and PAP2 in control (□) and *ras* transformed (○) fibroblast cells

The results are those from Table 3.2 but the graph shows the mean values from triplicate determinations of each of 3 independent observations. The overall mean is indicated by a bar.

A.



B.

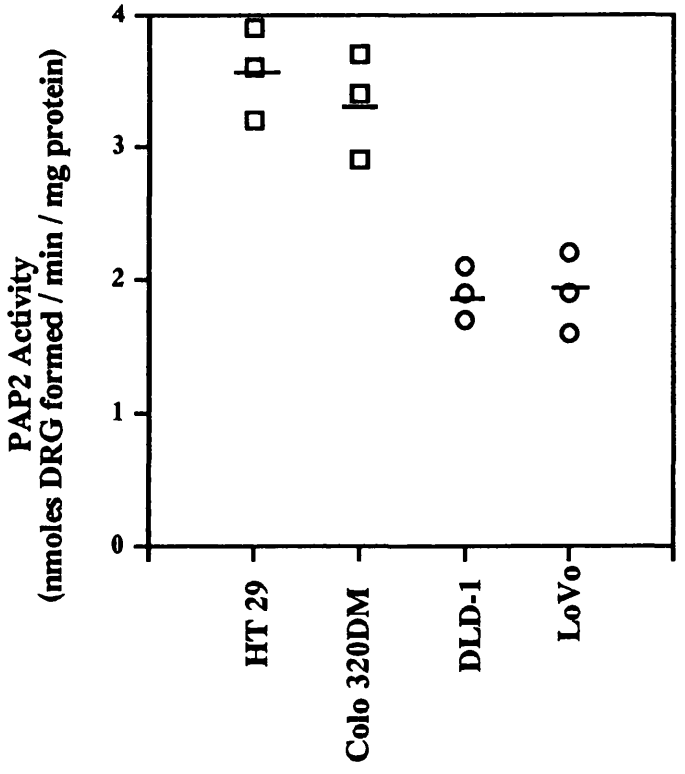


Figure 3.4 Specific activities of PAP1 and PAP2 in colon cancer epithelial cell lines without (□) and with (○) Ki-*ras* mutations

The results are those from Table 3.2 but the graph shows the mean value from triplicate determinations of each of 3 independent observations. The overall mean is indicated by a bar.

3.3.2 Phosphatidate and DRG Mass Levels in Fibroblast and Epithelial Cells

Total phosphatidate and DRG mass levels in the cell lines is shown in Table 3.2 and Figures 3.5 and 3.6. In HT3I and KMSVI *ras* transformed cells, phosphatidate mass was significantly higher by about 4.8-fold compared to the NIH 3T3 I fibroblast line ($P<0.003$ and $p<0.0001$, respectively). Similarly, Ki 858II had a significantly higher phosphatidate mass, by 6-fold ($p<0.001$), compared to NIH 3T3II (Figure 3.5A). DRG mass was also found to be higher in the *ras* transformed cells compared to the parental lines although the increase was not as marked as for phosphatidate (Figure 3.5B). HT3I had a 3.5-fold higher DRG mass than NIH 3T3 I ($P<0.0004$), KMSVI had a 3.9-fold increase ($p<0.0023$) and Ki 858II also had a significantly higher DRG mass by 2.8-fold ($p<0.0023$) than NIH 3T3 II cells.

The colon lines with mutant *ras* also had a much higher phosphatidate mass ($p<0.0001$) than those without the mutation (Figure 3.6A). Whilst phosphatidate mass was similar in the two colon lines with wild type *ras*, LoVo had significantly higher ($p<0.0001$) phosphatidate mass compared to DLD-1. DRG mass was also higher in the two epithelial cell lines with a Ki-*ras* mutation ($p<0.0001$) compared to those without (Figure 3.6B). Again, there was no significant variation in DRG mass between HT 29 and Colo 320DM, however, DRG mass was significantly higher ($p<0.0001$) in LoVo than DLD-1.

3.3.3 Relative Changes in Phosphatidate and DRG Mass

The ratio of total DRG relative to total phosphatidate within the cells is shown in Figures 3.7 and 3.8. This was calculated by dividing the mean mass of DRG by the mean phosphatidate mass from each experiment. The ratio of DRG to phosphatidate was significantly lower in the *ras* transformed fibroblasts compared to their parental lines ($p<0.0001$) (Figure 3.7). Also, in the epithelial cells there was a significant

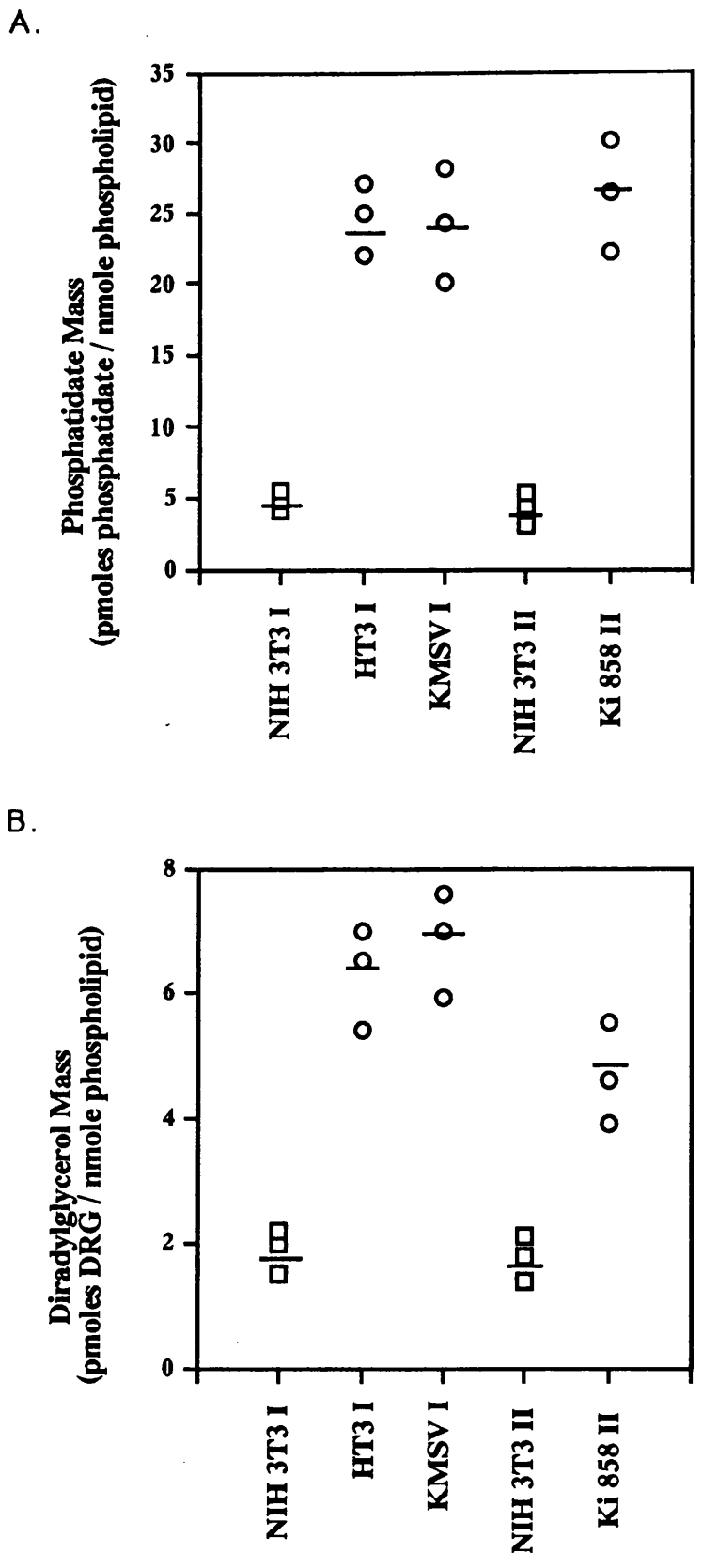


Figure 3.5 **Second messenger mass levels in control (□) and *ras* transformed (○) fibroblast cells**

The results are those from Table 3.2 but the graph shows the mean value from triplicate determinations of three independent observations. The overall mean is indicated by a bar.

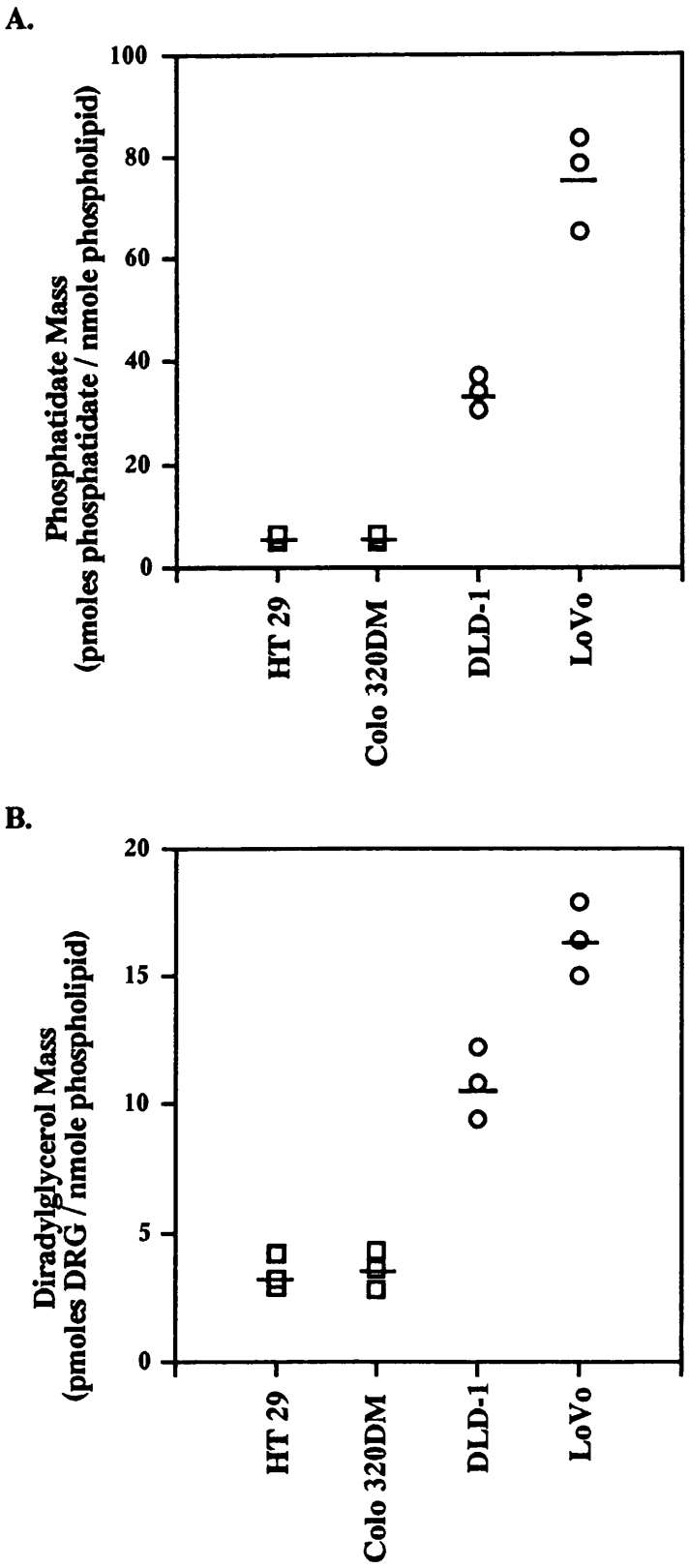


Figure 3.6 Second messenger mass levels in colon cancer epithelial cell lines without (□) and with Ki-*ras* (○) mutations

The results are those from Table 3.2 but the graph shows the mean value from triplicate determinations of the three independent observations. The overall mean is indicated by a bar.

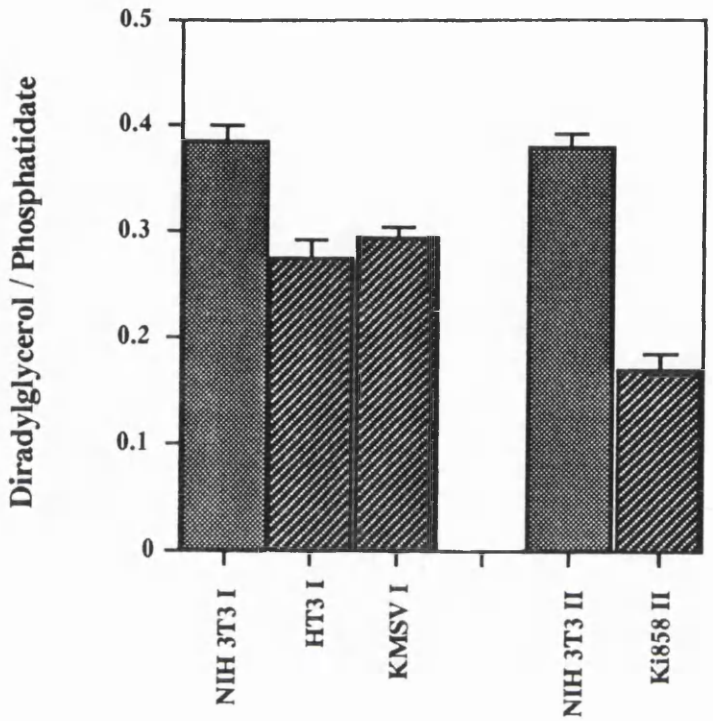


Figure 3.7 Mass of diradylglycerol relative to phosphatidate in control (▩) and *ras* transformed (▨) fibroblast cells

The results are the mean ± SE of three independent experiments. For each experiment the mean diradylglycerol mass was divided by the mean phosphatidate mass.

decreased level of DRG relative to phosphatidate ($p<0.0001$) in the cells with the Ki-*ras* mutation (Figure 3.8).

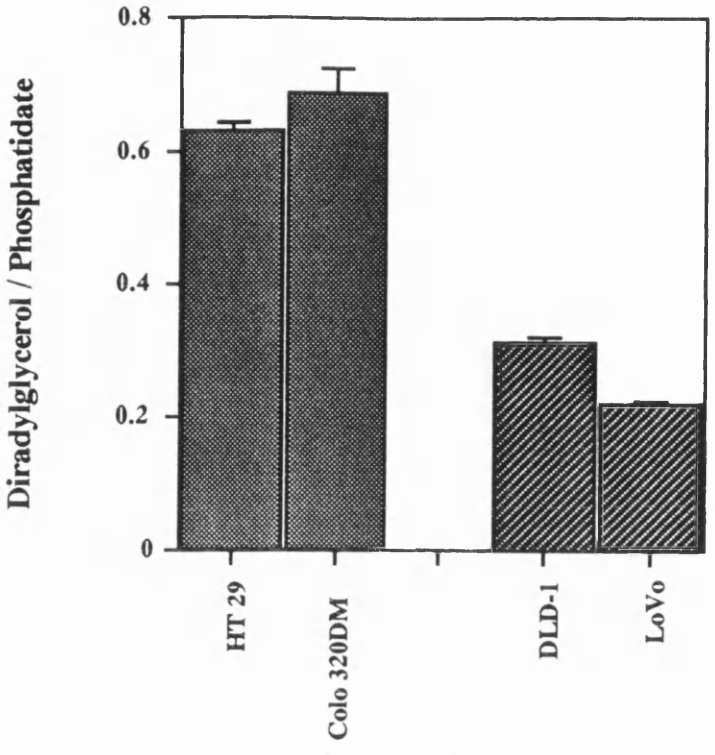


Figure 3.8 Mass of diradylglycerol relative to phosphatidate in colon cancer epithelial cell lines without (▩) and with (▨) Ki-*ras* mutations

The results are the mean ± SE of three independent experiments. For each experiment the mean diradylglycerol mass was divided by the mean phosphatidate mass.

3.4 Discussion

PAP1 and PAP2 activities and second messenger levels were measured in two cell model systems. The specific activity of PAP1 was shown to be higher in *ras* transformed fibroblast cells whereas PAP2 activity was lower in these cells. Total levels of both the second messengers, phosphatidate and DRG, were higher in the transformed cells. However, the increase in phosphatidate mass was greater than that of DRG such that the ratio of DRG:phosphatidate was lower in the *ras* transformed cell lines. In contrast, both PAP1 and PAP2 activities were lower in the colon epithelial cell lines which contained a Ki-*ras* mutation than in those with wild type Ki-*ras*. Again, both phosphatidate and DRG mass levels were higher. As for the fibroblasts cells, the ratio of DRG:phosphatidate was lower in the lines containing a mutation in the Ki-*ras* gene.

Transformation of fibroblasts by mutant *ras* oncogene results in cells that show increased rates of proliferation, are able to form colonies in agar and produce tumours when injected into mice. Furthermore, the transformed cells do not show increased rates of proliferation in the presence of growth factors (Parries *et al.*, 1987). Since a number of growth factors are known to activate *ras*, substitution of *ras* with a mutant form that remains in the active state is thought to circumvent the requirement for growth factor activation. Consistent with the increased proliferation rates is the observation that the levels of the important second messenger, DRG, is increased in *ras* transformed fibroblasts (Perry *et al.*, 1992; Wolfman & Macara, 1987). All three *ras* transformed fibroblasts used in this study showed increased rates of proliferation (Table 3.1) and increased levels of DRG (Table 3.2). The two major sources of DRG in mitogen stimulated cells are from the breakdown of phosphatidylinositol 4,5-bisphosphate by PLC and from the breakdown of PC either by a PC-specific PLC or by a combined action of PLD and PAP (Cook & Wakelam, 1992). Since there is evidence to suggest that PC is the main source of the sustained levels of DRG observed in cells stimulated with certain mitogens (Cook & Wakelam, 1991a; Cook

& Wakelam, 1991b) as well as the source of DRG in transformed fibroblasts (Lacal *et al.*, 1987), it was proposed that the activity of PLD and/or PAP might be increased.

All three *ras* transformed fibroblast cell lines had increased levels of PAP1 activity (Table 3.2). However, this form of the enzyme is found in the cytosol and is thought to translocate to the endoplasmic reticulum where it becomes activated (Brindley, 1987). The main function of PAP1 is considered to be glycerolipid synthesis rather than as an intermediate in signal transduction. In contrast, activity of PAP2 was significantly decreased ($p<0.004$) in the transformed cell lines (Table 3.2). This form of PAP is thought to be involved in signal transduction and, certainly, its location in the plasma membrane, the source of PC, would support this role (Billah & Anthes, 1990). Although this result was unexpected, the results confirm a recent observation that PAP2 activity is decreased in Rat1 fibroblasts transformed with Ha-*ras* (Martin *et al.*, 1993). They did not determine the steady state levels of DRG but did show an increased rate of formation of both phosphatidate and DRG from cellular lipids labelled with myristate. This would also exclude the possibility that elevated levels of DRG result from an increased breakdown of PC by a PC-specific PLC, although it has been reported that *ras* can activate such an enzyme (Diaz-Laviada *et al.*, 1990). DRG can also be derived from PC by the activity of PLD and PAP. Decreased activity of PAP would suggest that this is not the main pathway for generation of DRG. However, the level of phosphatidate, the intermediate in the PLD/PAP coupled reaction, is increased in the *ras* transformed fibroblasts. Although PLD activity was not measured in this study, previous work has suggested that activity of this enzyme is not increased in *ras* transformed cells (Fu *et al.*, 1992; Huang & Cabot, 1992). In addition, work using an anti-*ras* antibody has shown that *ras* is downstream from the phospholipases in 3T3 cells and it might be expected that an alteration in the *ras* gene would not effect enzymes such as PLD and possibly PAP (Yu *et al.*, 1988). An alternative explanation is that DRG is derived from another pathway. For example, some reports have suggested that *ras* causes an elevation of phosphatidylinositol 4,5-bisphosphate breakdown (Fleischman *et al.*, 1986; Hancock

et al., 1988) leading to increased DRG levels. Phosphatidate may be generated in the transformed cells from an increased activity of DRG kinase which phosphorylates DRG to produce phosphatidate (Kanoh *et al* , 1990).

Both phosphatidate and DRG may be important second messengers and it is unknown which has the predominant effect in transformed cells. It is possible that phosphatidate is the more important messenger and that the decreased PAP2 activity allows only a slow removal of phosphatidate. Furthermore, as the ratio of DRG to phosphatidate was found to be lower in the *ras* transformed cells, this would support the idea that phosphatidate is the more important second messenger. Certainly, in growth factor stimulated cells this would appear to be the case. It has been shown that the mitogenic response of 3T3 cells stimulated by PDGF correlates with the production of phosphatidate rather than DRG (Fukami & Takenawa, 1992). Phosphatidate has been shown to evoke various cellular responses and although the exact cellular functions are unclear, it may be involved in linking growth factor receptor signalling to Ras. Evidence for this comes from the ability of phosphatidate to increase the activity of the *ras* GTPase-inhibiting protein (Tsai *et al.*, 1990) and to inhibit the activity of *ras* GTPase-activating protein (GAP) (Tsai *et al.*, 1989), the effects of which would maintain Ras in its active GTP-bound form. As this lipid can also activate protein kinase C ζ *in vitro* (Berra *et al.*, 1993) and has several mitogenic effects such as increase in arachidonic acid (Murayama & Ui, 1987), phosphatidate may indeed play an important role in cell signalling.

Unlike PAP2, PAP1 activity was increased in the *ras* transformed fibroblasts. Although the activity of this enzyme was increased, it does not appear to be removing phosphatidate. As the *ras* transformed fibroblasts have an increased rate of proliferation compared to the control cells, these have a requirement for a higher rate of synthesis of membrane lipids. The increase in PAP1 activity could account for its involvement in this process. This increase in activity could also account, at least in part, for the observed increase in DRG levels found in the *ras* transformed cells. Interestingly, PAP1 activity was higher in the fibroblasts transformed with Ha-*ras*

compared to Ki-*ras*. As the Ha-*ras* transformed cells also have a higher rate of proliferation, this would seem to corroborate the idea that PAP1 is involved in increased membrane synthesis in these cells.

One of the main differences between the two cell models used in this study was in the activity of PAP1. In the colon epithelial cell lines which contained the *ras* mutation, PAP1 activity was decreased. This suggests that the *ras* mutation itself does not alter PAP1 activity. Unlike the fibroblast cells, there is no difference in doubling time between these four colon lines and other genetic alterations which are probably present in these cells, such as a *src* mutation, may effect cell turnover rate. PAP2 activity was lower in the colon lines with mutant *ras* which was consistent with the findings in the *ras* transformed fibroblasts cells. Likewise, similar results were observed with regards the levels of phosphatidate and DRG, in that presence of oncogenic *ras* was associated with an increased level of both second messengers. Although the results between the two cell models compared well, other mutations may also have been present in the epithelial cells and it cannot definitely be concluded that *ras* was responsible for the observed changes. It could also be argued that as the pairs of colon lines were grown in different media, this may have had an effect on signalling in these cells. However, no evidence has yet been presented that media has an effect on cell signalling. It is probably more likely that the serum, which contains the essential growth factors and fatty acids required to sustain growth of the cells, is the important component of the growth medium. As the same serum was used for all cell lines and the results were similar in the two cell models, then this would suggest that any changes are not due to the fact that different media was used.

The results presented would appear to argue against a role for the PLD/PAP pathway in the formation of increased DRG. It is not certain from this study the exact source of DRG in these cells. Other studies have compared DRG and PKC levels in colon tumours and compared with normal colon tissue. Both were found to be decreased in colon tumour tissue (Guillem *et al.*, 1987b; Phan *et al.*, 1991) which is in contrast to what was observed in the *ras* transformed fibroblast cell model. Although

signalling was also studied in colon tumour derived cell lines, there was not a 'normal' control and, hence, the previously reported colon tumour data cannot be directly compared with these results. However, in the colon tumour cells, mutant *ras* appeared to be having a similar effect on second messengers as in the fibroblast model.

CHAPTER 4

ACTIVITY OF PHOSPHATIDATE PHOSPHOHYDROLASE AND MASS MEASUREMENT OF PHOSPHATIDATE AND DIRADYLGLYCEROL SECOND MESSENGERS IN HUMAN COLORECTAL CANCER

4.1 Introduction

Protein kinase C is thought to play a central role in signal transduction since it is involved in the control of both proliferation and differentiation (Clemens*et al.*, 1992). As a result there has been much interest in the use of PKC as a target for anti-tumour drug development (Gescher & Dale, 1989). However, there are multiple isoforms of PKC which show differential tissue distribution and are involved in diverse signalling pathways (Dekker & Parker, 1994). Furthermore, it has been proposed that certain isoforms may play a role as tumour suppressors. Overexpression of PKCα or PKCβ1 in rodent fibroblasts has been shown to stimulate cell proliferation (Borner *et al.*, 1992; Finkenzeller *et al.*, 1992). In contrast, overexpression of PKCβ1 in the human colon carcinoma cell line HT29 results in decreased rates of proliferation and decreased tumourigenicity in nude mice (Choi *et al.*, 1990).

PKC activity has been shown to be decreased in human colon tumours when compared with the activity present in adjacent normal colon (Guillem *et al.*, 1987a; Kopp *et al.*, 1991). Furthermore, PKC activity was shown to be lower in the normal colon of patients with colon tumours than in the colon of patients without cancer (Sakanoue *et al.*, 1991). Decreased activity of PKC was also detected in benign adenomas (Guillem *et al.*, 1987a). Weinstein and coworkers have suggested that this may indicate a role for PKC in the origin and growth of colon cancer (Weinstein, 1990; Weinstein, 1992). Whereas a mutation in Ki-*ras* is observed in about 50% of colon tumours, nearly all tumours studied had decreased activities of PKC (Guillem *et*

al., 1987a; Guillem *et al.*, 1987b). Clearly, if PKC acts as a tumour suppressor then activators rather than inhibitors of PKC could have a role as chemotherapeutic agents.

Levels of the main physiological activator of PKC, DRG, have also been shown to be decreased in human colon tumours (Phan *et al.*, 1991; Sauter *et al.*, 1990). During growth factor stimulation of cell proliferation, the two main sources of DRG are thought to be from the breakdown of phosphatidylinositol 4,5-bisphosphate by PLC and from the breakdown of PC by the combined activities PLD and PAP (Billah & Anthes, 1990; Wakelam *et al.*, 1991). Reduced activity of the enzymes involved in one or both of these pathways would result in reduced rates of production of DRG. A recent study has reported that expression of PLCγ is increased in human colon tumours (Noh *et al.* , 1994). It is not known if this increased expression is associated with increased activity of PLCγ. Nothing is known about the expression or activity of PLD or PAP in human colon tumours. However, in the previous chapter, PAP activity was shown to be decreased in *ras* transformed fibroblasts and in human colon carcinoma cell lines that express a mutant *ras* oncogene. This suggested that transformation is associated with reduced activity of PAP. Whilst this observation could be used to support the hypothesis that DRG levels are reduced in the human colon tumours due to decreased activity of PAP, it should be noted that the *ras* transformed fibroblasts had increased levels of DRG.

PAP activity was, therefore, determined in human colon cancers and in adjacent pathologically normal colon. There are a number of problems associated with such a study since neither tissue represents a homogenous cell population. The tumour tissue will contain some normal colonic epithelium as well as a proportion of inflammatory cells and connective tissue. The epithelial lining of normal colonic mucosa consists of a sheet of single-cell thickness organised into tubular invaginations or crypts with surface epithelium between the crypt orifices. The proliferative region of the large intestine occupies approximately the basal two-thirds of the crypts (Hamilton *et al.*, 1994). Cells migrate towards the surface of the colonic crypts and are extruded from the mucosal surface. This cell loss is thought to be

caused by induction of apoptosis. Clearly, the process of proliferation and maturation is under tight control and regulated by the expression of specific signal transduction pathways. For instance, in the rat intestine immunofluorescence and western blot analysis has revealed that PKC α, βII, δ, ε and ζ are expressed in all intestinal epithelial cells *in situ* and exhibit distinct subcellular distribution patterns along the crypt-villus unit. Dramatic changes in membrane association and level of expression of these isoforms occur as the cells ceased division in the mid crypt region and begin differentiation (Black & Saxon, 1994). Signalling events such as increased levels of Ca^{2+} and modulation of PKC have been implicated in the apoptotic procedure (Dive *et al.*, 1992; Martin *et al.*, 1994) and these must be regulated in some way, although the biochemical events surrounding this are unknown. Perhaps altered PKC isoform expression in mature cells activate signal transduction pathways leading to transcription of genes which regulate programmed cell death (Martin *et al.*, 1994). Thus, the normal colonic mucosa is composed of cells at different stages of maturation and at each stage it is probable that the activity of various signalling pathways will differ.

Most colorectal cancers develop from adenomas derived from normal colonic epithelium lining the intestine. Adenomas contain a high proportion of replicating cells found all along the crypt column and even at the luminal surface of the gut (Friedman*et al.*, 1981). As a tumour progresses through the adenoma-carcinoma sequence of events (Fearon & Jones, 1992), it invades underlying layers of tissue and can be staged according to how far it has penetrated the bowel wall and nodal status of the tumour. One of most commonly used staging systems is the Astler-Coller modification of the original Duke's system (Astler & Coller, 1954). For example, a Stage A tumour involves the submucosa layer only, it is a small benign tubular type of adenoma or polyp which can increase in size and become more villous. This stage, Stage B, penetrates underlying layers of the bowel, is more aggressive and can contain patches of carcinoma tissue. These patches may grow out to form the third stage, Stage C, which is an invasive carcinoma typically with the development of

pericolic or perirectal nodes. In the case of a Stage D tumour, distant metastases are present. Tumours normally remain undetected until the later stages of development. The relationship between biochemical events in signal transduction and invasivness of a tumour has not been reported.

A total of 35 colon tumours were studied. The tumours were obtained from various sites along the length of the colon and ranged from Stage A to Stage D. Normal colon was defined as macroscopically normal colon as judged by a pathologist. Activities of PAP1 and PAP2 were determined in all samples. Levels of the substrate and product of PAP activity, phosphatidate and DRG respectively, were also determined in 14 of the samples. These 14 were selected on the basis of tissue availability and included tumours with a range of activities of PAP.

4.2 Materials and Methods

4.2.1 Preparation of Human Colon Tissue for Analysis

Surgically resected colorectal tumours were obtained from the Western Infirmary, Glasgow. Immediately following resection, the specimen was opened longitudinally along the antimesenteric border and rinsed thoroughly with saline solution to remove blood clot and debris. Samples of tumour tissue were taken, avoiding obvious areas of necrosis, rinsed again in ice-cold saline solution, placed in plastic sealed tubes and immediately frozen in liquid nitrogen. In addition, samples of macroscopically normal mucosa were taken as close to the resection margin as possible and treated in the same way. All samples were frozen within 10-15min of tumour resection and stored at -70°C until analysis. Detailed information on each patient including tumour site, histology and Duke's staging (Astler-Coller) were obtained.

Samples were prepared for analysis by firstly grinding the frozen tissue into small pieces using a pestle and mortar. The tissue, still frozen, was then ground to a fine powder using a Microdismembrator (Braun Ltd., Buckinghamshire). The powdered tissue was kept at -70°C until use. For measurement of enzyme activity, the tissue was hand homogenised in 3 volumes of ice-cold Tris buffer (20mM, adjusted to pH 6.5 with maleate) containing di-sodium phosphate (5mM), DTT (1mM), NaCl (140mM) and leupeptin (1μg/ml) at 4°C. This was divided into aliquots which could be stored at -70°C. Protein was determined as previously described (Section 2.2.4).

4.2.2 Estimation of PAP Activity in Colon Tissue

PAP1 and PAP2 activity was measured using the method of Jamal and co-workers (Jamal*et al.*, 1991) as described previously (Section 2.2.5). PAP1 and PAP2 activities were determined in triplicate and at three concentrations of protein (10, 20

and 30μg/assay). Values are expressed as nmoles DRG formed / min / mg protein. The standard error of the triplicate samples was always less than 5%. Data was analysed using a Student's paired T-test of the Statview 512+ system for the Apple Macintosh.

4.2.3 Measurement of Phosphatidate and DRG Mass

A lipid extraction was carried out on about 500mg of the powdered tissue essentially using the method of Bligh and Dyer (Bligh & Dyer, 1959). The tissue was hand homogenised in 500μl methanol per 100mg tissue and chloroform, 250μl per 100mg tissue, was added. The tissue was allowed to extract for 15min. A volume of water equal to that of the chloroform was added to the extracted tissue samples and vortexed thoroughly. Samples were then centrifuged at 250 x g in a benchtop centrifuge (Model CR411, Deva Medical Electronics, Runcorn, Cheshire) for 10min to separate the phases. The upper phase was discarded and a further 250μl chloroform and 250μl water were added and the mixture vortexed and centrifuged as before. The lower phase was divided into aliquots of 200μl, 400μl and 50μl for analysis of DRG, phosphatidate and total phospholipid respectively. The aliquots were dried under nitrogen and stored at -70°C.

All samples were analysed for the second messengers within three days of lipid extraction. Total phospholipid was measured as in section 3.2.9 and all results of DRG and phosphatidate mass were expressed per nmole phospholipid. Phosphatidate was measured as before (section 3.2.7) in triplicate measurements where sufficient tissue was available. Each replicate was analysed on a separate t.l.c. plate and the results presented as means ± SE. DRG mass was also measured essentially as described earlier (Section 3.2.8). The lipid extract aliquot for this assay (200μl) was split into 2 x 50μl, 2 x 10μl and 2 x 2μl aliquots and the mass from these was always within the range of the standard curve (25-500 pmoles *sn*-1-stearoyl-2-arachidonyl-glycerol). Phosphatidate and DRG mass measurements were carried out on 14

patients out of the 35 patient samples. Results were analysed as described in section 4.2.2.

4.3 Results

4.3.1 Characterisation of PAP1 and PAP2 in Colon Samples

A total of 35 colorectal carcinomas and paired normal mucosa samples were assayed for PAP1 and PAP2 activities. Clinical pathology reports on the tumour tissue of the 35 patients is given in Table 4.1.

PAP1 and PAP2 activities were characterised in human tissue to determine whether the human enzyme could be measured under similar conditions to those used for the rat liver enzymes. The reaction rates for both forms of the enzyme were proportional to the protein concentration up to 50μg and time up to 60min (Figure 4.1). Hence, the majority of experiments were carried out using concentrations of protein from the linear portion of the curve for a reaction time of 60min.

Figure 4.2 shows that PAP1 activity has an absolute requirement for Mg^{2+} with optimum activity reached at around 3mM Mg^{2+}. Presence of Mg^{2+} made no difference to the activity of PAP2. As with the rat liver enzymes, PAP1 activity was found to be sensitive to NEM (Figure 4.2) whereas the PAP2 signalling enzyme is insensitive to this reagent.

When Triton X-100 was present for the measurement of PAP2, there was a substantial increase in the activity of this enzyme (Figure 4.3) with optimum activity achieved at about 0.5% Triton X-100 in the assay. This is equivalent to 8mM used in the past to measure the rat liver enzyme. The addition of this detergent to the assay for PAP1 inhibited the activity of this enzyme at concentrations where PAP2 activity was stimulated.

4.3.2 PAP Activity in Human Colon Tumour and Adjacent Normal Mucosa

There was a wide range of activities of both PAP1 (0.1 - 2.3) and PAP2 (0.3 - 4.6) in the normal colon samples (Figure 4.4). Similarly, a wide range of both PAP1

Patient	Age (Yr)	Sex (M/F)	Duke's Staging	Location
1	62	F	A	rectal adenocarcinoma
2	85	F	A	rectal adenocarcinoma
3	35	F	A	rectosigmoid carcinoma
4	85	F	A	rectal adenocarcinoma
5	87	M	B_1	sigmoid carcinoma
6	71	M	B_1	rectal adenocarcinoma
7	63	M	B_2	rectal adenocarcinoma
8	75	M	B_2	adenocarcinoma
9	79	M	B_2	adenocarcinoma
10	75	M	B_2	adenocarcinoma
11	77	M	B_2	rectal carcinoma
12	75	M	B_2	adenocarcinoma
13	93	F	C_1	rectosigmoid carcinoma
14	67	F	C_1	sigmoid carcinoma
15	58	M	C_1	adenocarcinoma
16	60	M	C_1	adenocarcinoma
17	52	M	C_1	adenocarcinoma
18	74	M	C_1	caecal carcinoma
19	58	F	C_2	caecal carcinoma
20	49	M	C_2	rectosigmoid carcinoma
21	46	M	C_2	rectal carcinoma
22	75	F	C_2	ascending colon
23	54	M	C_2	caecal carcinoma
24	51	F	C_2	caecal carcinoma
25	83	F	C_2	caecal carcinoma
26	69	M	C_2	rectal carcinoma
27	68	M	C_2	ascending colon
28	70	M	C_2	rectal carcinoma
29	60	M	C_2	rectal carcinoma
30	64	F	C_2	rectal carcinoma
31	65	F	D	ascending colon
32	56	F	D	sigmoid carcinoma
33	83	M	D	caecal carcinoma
34	71	M	D	caecal carcinoma
35	79	M	D	rectosigmoid carcinoma

Table 4.1 Clinical pathology of individual colon samples

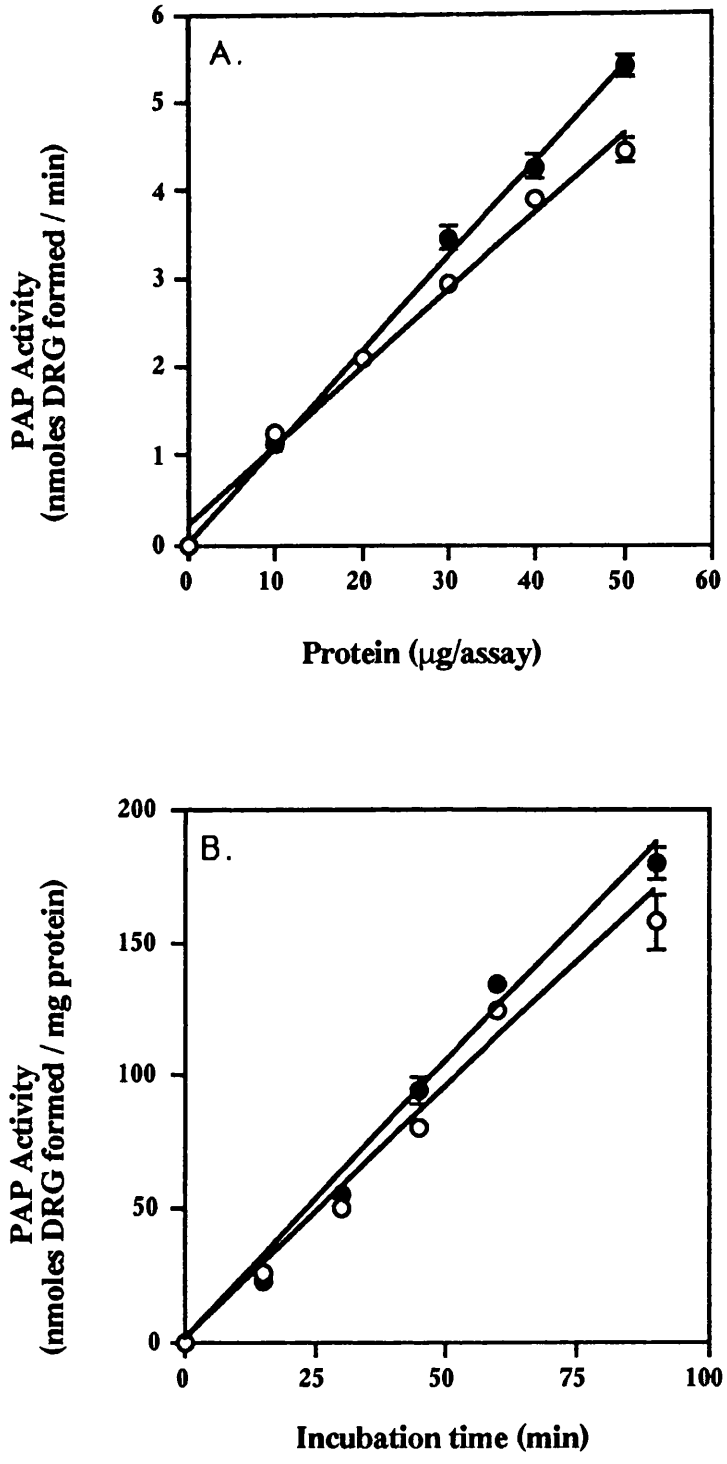


Figure 4.1 Effect of enzyme concentration and incubation time on PAP1 and PAP2 activities in colon tissue

DRG production was measured with various concentrations of normal human colon tissue as a source of PAP1 (O) and PAP2 (●) (A). DRG production was also determined after incubation of samples (20μg) for various times (B). Each point is a mean ± SE of triplicate determinations and is representative of two experiments showing similar results.

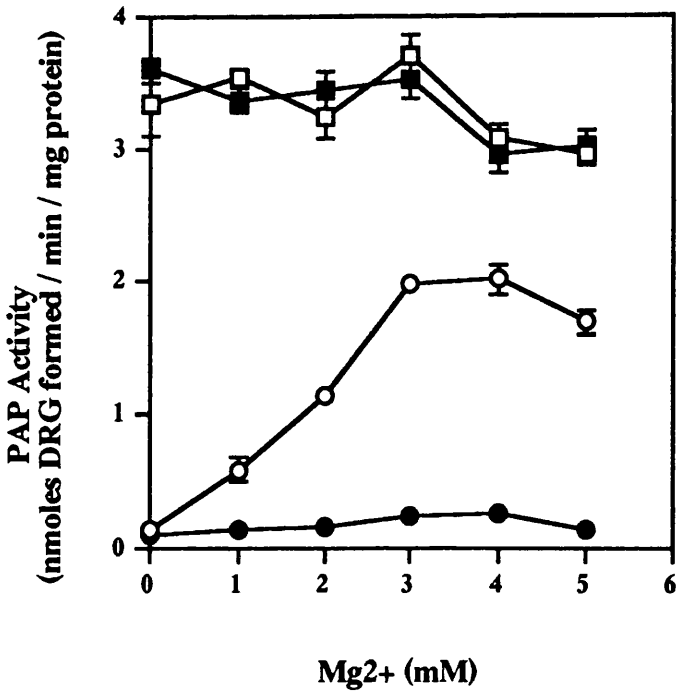


Figure 4.2 Effect of Mg^{2+} and NEM on PAP1 and PAP2 enzyme activities in normal colon tissue

PAP activity was measured in the presence of increasing concentrations of Mg^{2+} as indicated either in the presence (closed symbols) or absence (open symbols) of 4.2mM NEM. Normal human colon tissue was used as a source of the enzyme (20μg) for measuring PAP1 (○, ●) and PAP2 (□, ■) activities. Each point is a mean ± SE of triplicate determinations and is representative of two independent observations showing similar results.

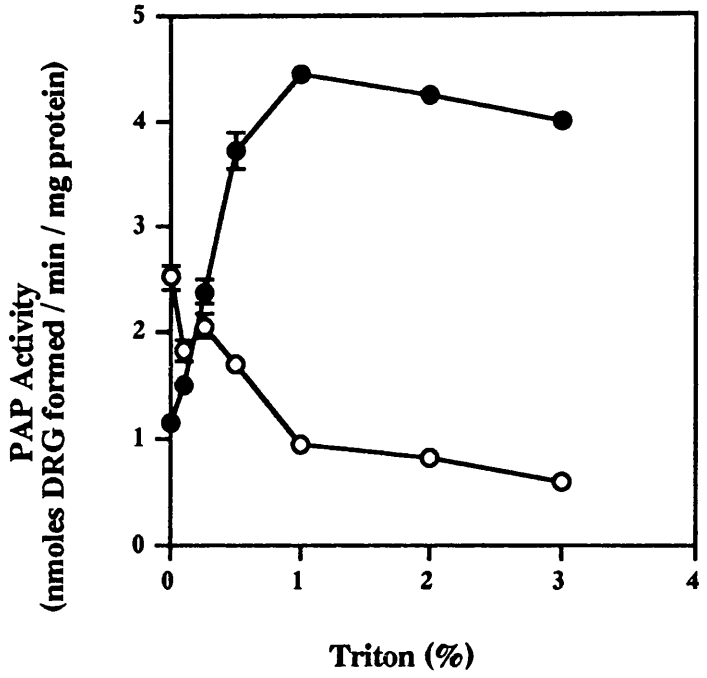
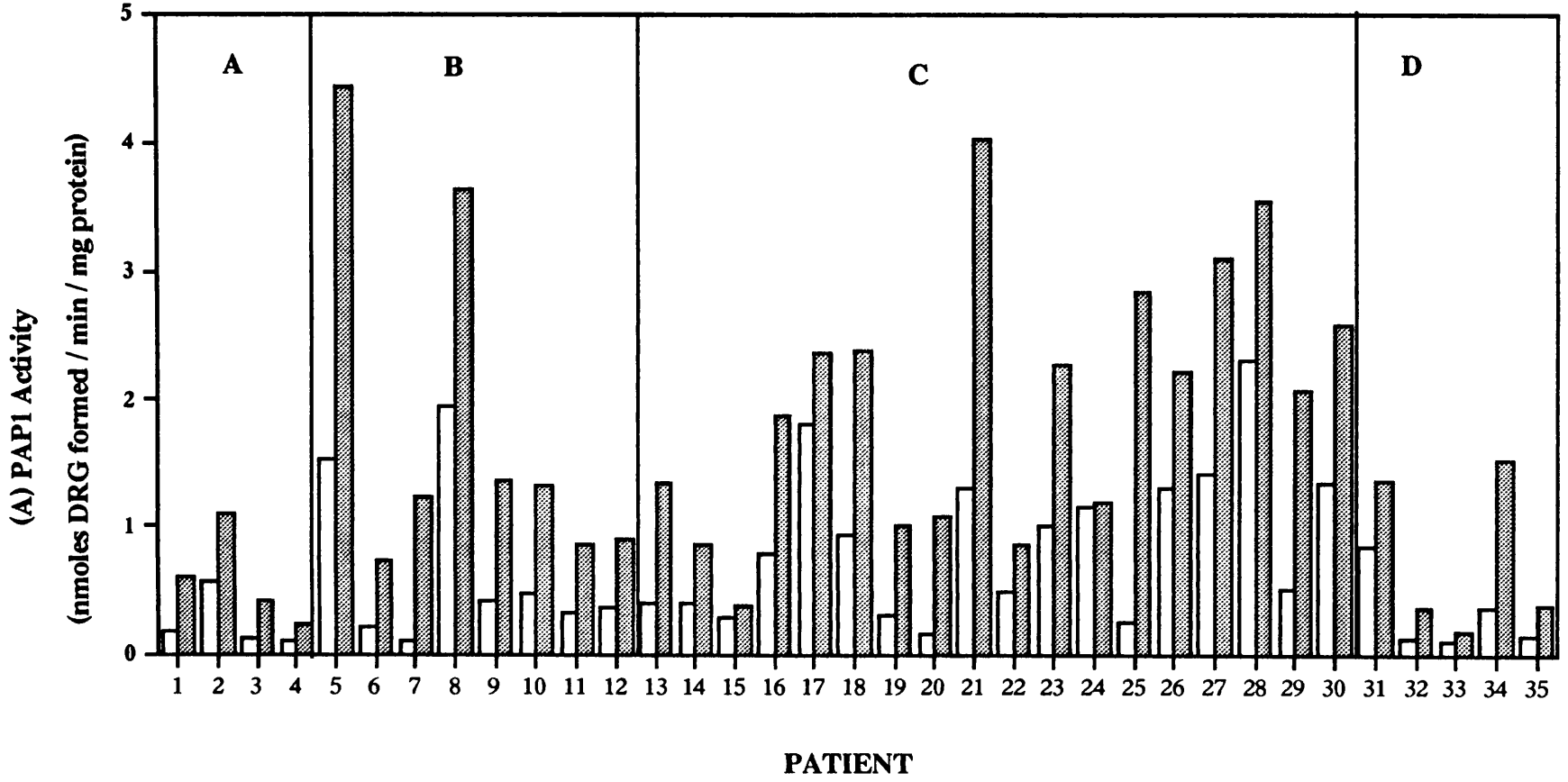


Figure 4.3 Effect of Triton X-100 on PAP1 and PAP2 enzyme activities in normal colon tissue

PAP activity was measured in the presence of increasing concentrations of Triton X-100 as indicated. PAP1 (○) and PAP2 (●) activities were measured in normal human colon tissue using 20μg protein in the assay. Each point is a mean ± SE of triplicate determinations and is representative of two independent observations showing similar results.


(A) PAP1 Activity
(nmoles DRG formed / min / mg protein)
0
1
2
3
4
5
A
B
C
D
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35
PATIENT

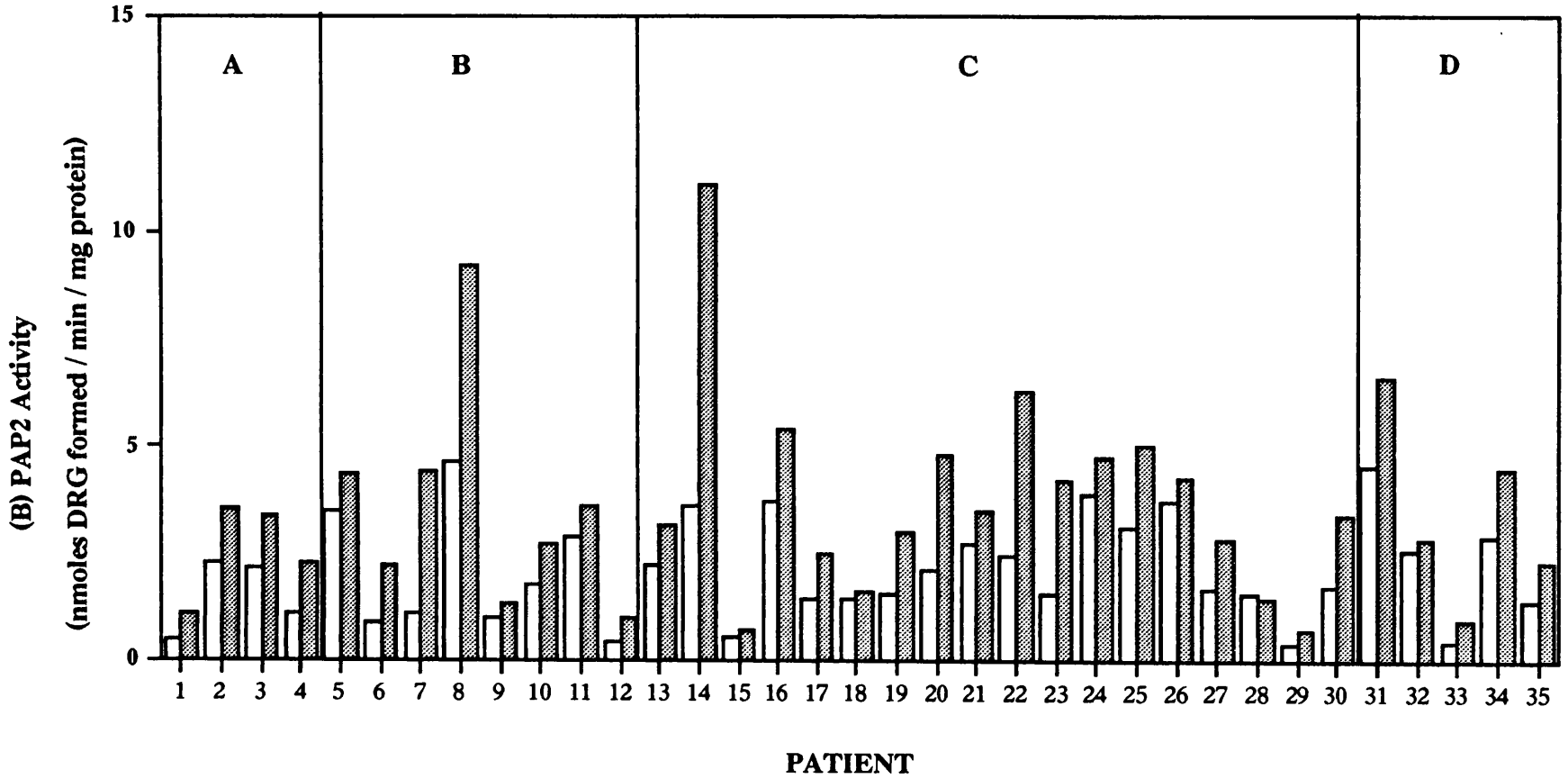


Figure 4.4 Activities of PAP1 and PAP2 in colon tumour tissue and in adjacent normal colon.
PAP1 (A) and PAP2 (B) activities were measured in paired normal (□) and tumour (▨) tissue. Each assay contained 20μg of protein. Duke's staging (A, B, C or D) for the patient samples is indicated in the graphs. Each column represents the mean of triplicate determinations, with SE always less than 5%. There was significant difference ($P<0.02$) between tumour and normal tissue apart from in patient 15 and 24 (PAP1) and in patients 15, 18, 28 and 32 (PAP2).

(0.2 - 4.4) and PAP2 (0.7 - 11.1) activities were observed in the colon tumours. The activities of PAP1 and PAP2 were higher in the colon tumour tissue than in the adjacent normal mucosa in the majority of the 35 paired samples (Figure 4.4). The mean activity of PAP1 in the tumour tissue (1.6± 0.2 nmoles DRG formed /min /mg protein) was significantly higher (2.3 fold, $p<0.0001$) than that in the normal mucosa (0.7±0.1 nmoles DRG formed /min /mg protein). Likewise, the mean activity of PAP2 in the tumour tissue (3.9±0.4 nmoles DRG formed /min /mg protein) was significantly higher (1.8 fold, $p<0.0001$) than that in the normal mucosa (2.2±0.2 nmoles DRG formed /min /mg protein). PAP activity was never lower in the tumour than in the normal colon. However, patients 15, 18, 28, and 32 had similar activities of PAP2 in normal and tumour tissue and patients 15 and 24 had similar activities of PAP1 in normal and tumour tissues. There was no correlation between PAP1 and PAP2 activities and either sex, age, Duke's Staging or location of tumour.

4.3.3 Phosphatidate and DRG Mass in Colon Samples

Mass levels of phosphatidate and DRG were measured in the majority of the patients. However, there was not sufficient tissue to measure phosphatidate mass in patient 12. There was a wide range of DRG mass in both normal colon (1.2 - 10.2) and in colon tumours (0.8 to 7.0) (Figure 4.5). A smaller range of levels of phosphatidate mass was observed in both normal colon (1.2 - 5.5) and in colon tumours (0.8 - 4.0) (Figure 4.6). In general, DRG mass was about twice that of phosphatidate mass.

Both second messengers were lower in the tumour tissue than in adjacent normal (Figures 4.5 and 4.6). The mean DRG mass in the tumour tissue (3.2±0.6 pmoles DRG /nmole phospholipid) was significantly lower (1.6 fold, $p<0.0005$) than that in the normal colon (5.0±0.8 pmoles DRG/nmole phospholipid). Only 1 patient (27) had a higher DRG mass in the tumour than in the normal colon. Phosphatidate mass in colon tumour tissue (2.5±0.3 pmoles phosphatidate/nmole phospholipid) was

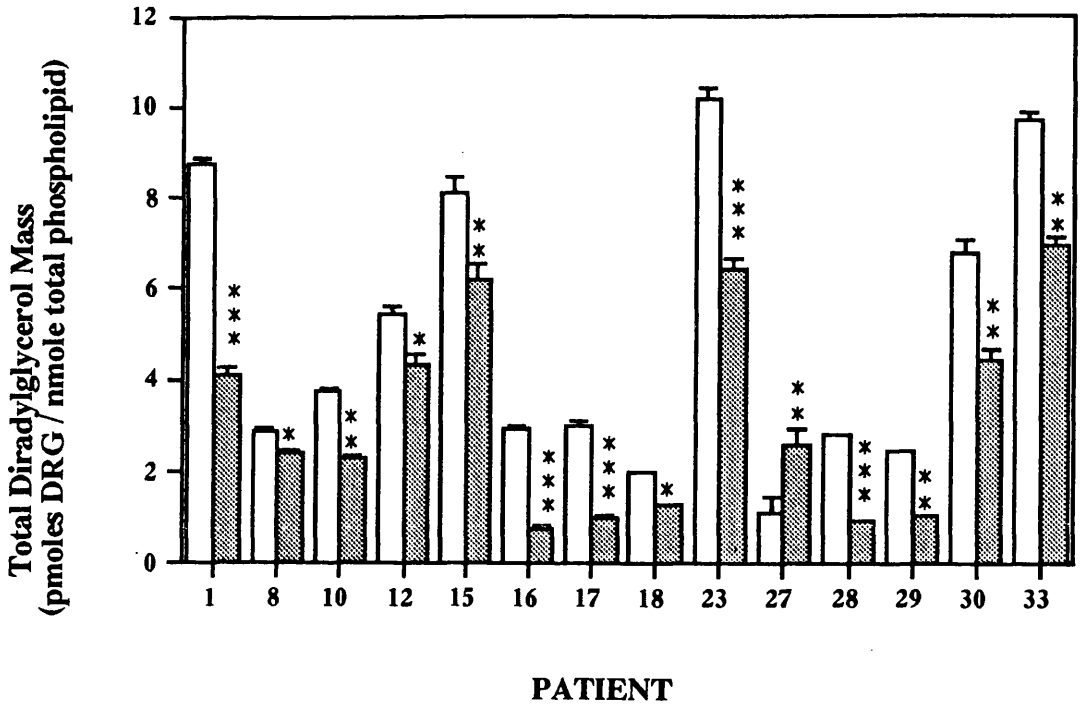


Figure 4.5 Diradylglycerol mass in paired human colon and colon tumour
DRG mass was measured in paired colon tumour (▨) and normal (□) colon from fourteen patients. Conversion of DRG to [^{32}P]phosphatidate was measured in sample lipid extracts and the results compared to a standard curve of *sn*-1-stearoyl-2-arachidonylglycerol. Determinatons were performed in triplicate and the values expressed as a mean ± SE per nmole phospholipid. Asterisks represent statistically significant differences (Student's *t* test; * $p<0.02$, ** $p<0.01$, ***$p<0.001$).

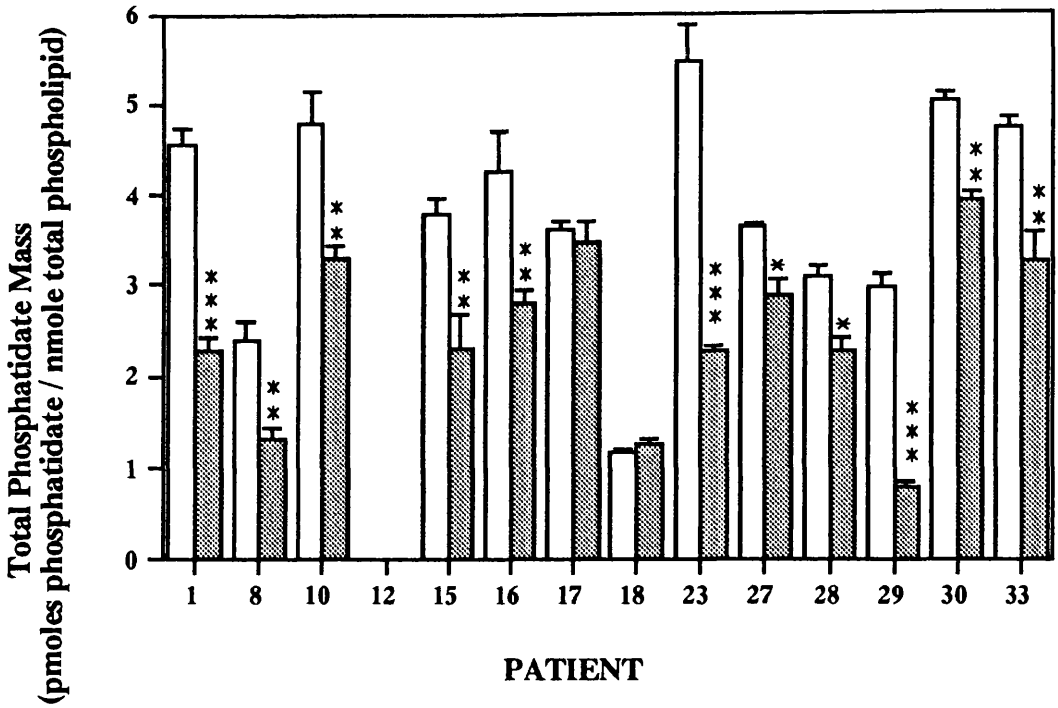


Figure 4.6 Phosphatidate mass in paired human colon and colon tumour Phosphatidate mass was measured using thin-layer chromatography in paired colon tumour (▨) and normal (□) tissue from thirteen patients. Determinatons were performed in triplicate and the values expressed as a mean ± SE per nmole total phospholipid. Asterisks represent statistically significant differences (Student's *t* test; * $p<0.02$, ** $p<0.01$, ***$p<0.001$).

also significantly lower (1.5 fold, $p<0.0001$) than in adjacent normal mucosa (3.8±0.3 pmoles phosphatidate/nmole phospholipid). The ratio of levels of the product of PAP activity (DRG mass) to that of the substrate (phosphatidate mass) was similar in the colon tumours and in the normal colon (Figure 4.7).

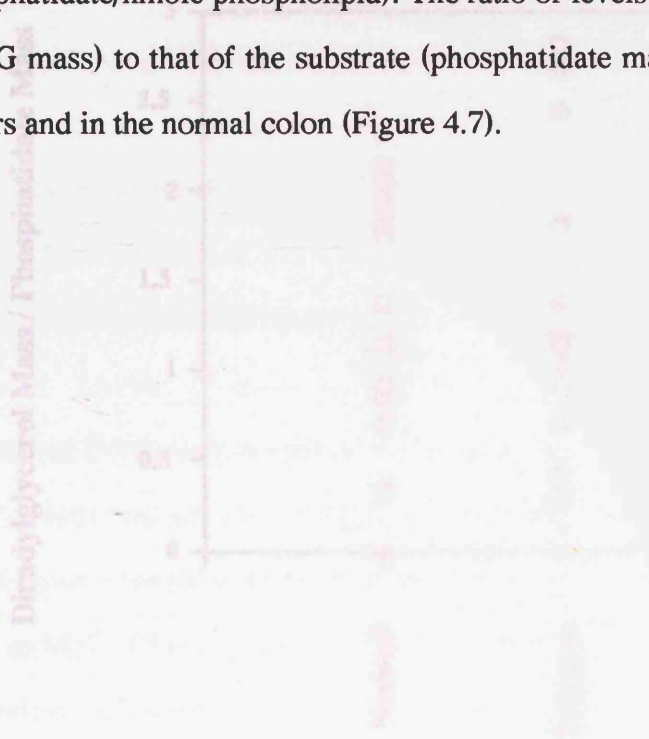


Figure 4.7 Mean of DRG relative to phosphatidate mass in normal colon mucosa and colon tumour

The mean DRG mass for each patient has been divided by the corresponding phosphatidate mass. The overall mean for the normal and tumour tissue is represented by a small bar.

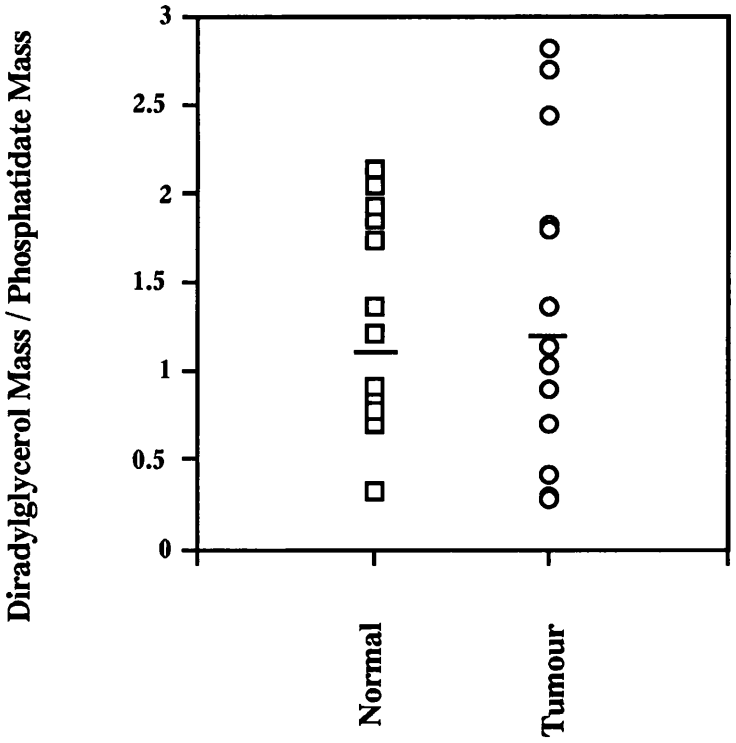


Figure 4.7 Mass of DRG relative to phosphatidate in paired human colon and colon tumour

The mean DRG mass for each patient has been divided by the mean phosphatidate mass. The overall mean for the normal and tumour tissues is represented in each case by a small bar.

4.4 Discussion

PAP activity was determined in 35 human colon tumours and in the adjacent normal colonic mucosa. The activities of human PAP1 and PAP2 were characterised and shown to have similar properties to those of the rat liver enzymes. A wide range of activities of both PAP1 and PAP2 were observed in the normal colonic mucosa. Furthermore, activities of both forms of PAP were increased in the majority of the colon tumours. Levels of both the substrate and product of PAP activity, phosphatidate and DRG, were decreased in the majority of patients.

PAP activity has not previously been analysed in human tissue. Both an NEM sensitive and insensitive form were identified and these two activities showed similar sensitivities to Mg^{2+} (Figure 4.2) and Triton X-100 (Figure 4.3) as observed for the rat liver activities (Chapter 2, Figures 2.3 and 2.5). PAP1 and PAP2 activities have now been measured in human, rat and mouse tissues. These enzymes were active under the same assay conditions and displayed similar properties, such as in their sensitivity to NEM. This suggests that these enzymes are highly conserved in mammals and may play important roles in their tissues. There is limited information available regarding measurement of PAP activity in other species, however, two forms of PAP have been purified from the yeast, *Saccharomyces cerevisiae* (Lin & Carman, 1989). As the mammalian forms have not been purified it is not possible to compare the degree of homology between yeast and animal forms. However, the conditions used to measure PAP activity in animal and yeast are similar (Jamal *et al.*, 1991; Morlock *et al.*, 1991). For example, as with mammalian PAP1 activity, the cytosolic form of PAP from yeast is dependent on Mg^{2+} (Lin & Carman, 1989).

A wide range of activities of both PAP1 and PAP2 were found in the normal colon (Figure 4.4). This was not unexpected for an enzyme that is involved in glycerolipid synthesis such as PAP1, since the fat content of the diet can vary markedly. Evidence from epidemiological studies has, in fact, suggested that high fat diets increase the risk of colon cancer (Reddy *et al.*, 1992). It has been proposed that

specific bacteria in the lumen can metabolise dietary lipid to DRG. This may then enter the intestinal epithelium and activate PKC, thus stimulating proliferation (Morotomi *et al.*, 1990). In fact, Friedman and coworkers have shown that DRG can enhance proliferation of colonic adenoma and carcinoma derived cells in culture (Freidman *et al.*, 1989). It appears that DRG, produced both intracellularly and extracellularly, can act both as a mitogen and second messenger in the colon. If the diet is the cause of increased risk of cancer, for example, by increasing the rate of proliferation of colon cells, then this may also effect the normal tissue of these patients. This may explain why PAP1 activity is so varied amongst different patients in both their normal and tumour tissues. It would be of interest to determine if there is such a variation in PAP activity in the colon of individuals without cancer.

Activity of PAP2 also varied markedly between patients. This form of the enzyme is commonly thought to be involved in signal transduction and the interpatient variability was, therefore, rather surprising. It is not known if such variability in activity would be observed in the normal colon since it is possible that the so-called normal mucosa of the cancer patients is abnormal. It has already been reported that PKC activity is decreased in the histologically normal colon of patients with colon cancer (Guillem *et al.*, 1987b). On the other hand, DRG levels were similar in the colon of patients without cancer and the normal colon of patients with cancer (Kopp *et al.*, 1991).

Both PKC activity and DRG levels have been reported to be decreased in colon tumours (Guillem *et al.*, 1987a; Phan *et al.*, 1991; Sauter *et al.*, 1990). Since PAP is involved in the formation of DRG from PC, it was proposed that the decrease in DRG mass could be due to a decreased activity of PAP. However, both PAP1 and PAP2 activities were significantly increased in colon tumours when compared with activities in adjacent normal colon (Figure 4.4). This was an unexpected finding and difficult to interpret. Not all signalling pathways lead to mitogenesis. It is possible that the PLD/PAP pathway is involved in other cellular functions such as motility or membrane trafficking. The observed increase in PAP activities may be due to an

increase in synthesis of lipid membranes (PAP1) and membrane proteins (PAP2) which are processed through the golgi. For example, the function of the GTP binding protein, ARF, as a regulator of organelle structure and membrane traffick has recently been elucidated, although it is unknown how this protein is regulated (Donaldson & Klausner, 1994). ARF has been shown to activate PLD which suggests that this enzyme may be involved in membrane trafficking (Kahn *et al.,* 1993).

Mutations in Ki-*ras* have been identified in a high proportion of colon tumours and this mutation has been implicated in the progression of the tumour (Fearon & Jones, 1992). However, PAP activity was shown to be decreased in fibroblasts transfected with a mutant Ki-*ras* oncogene (Chapter 3, Figure 3.3). Furthermore, the fibroblasts expressed increased levels of DRG whereas the levels of this lipid were decreased in colon tumours. There is an apparent discrepancy between the cell models of increased proliferation and the tumour tissues. This can be accounted for by one of several reasons. It is possible, for instance, that when cells are removed from their tissue environment and placed in culture conditions, that regulation of enymes and lipids in the cells is altered. This could mean that measurements made in the tissue is more representative of the physiological biochemical events. However, analysis of signalling in cell models represents a homogenous population of cells whereas observed changes in tissue samples is a measurement in a variety of cell types. If there is a decrease in enzyme activity within the epithelial cells of the tissue, this could be masked by the changes present in the tissue as a whole. It could also be argued that any changes in PAP activity or second messengers in tumours are nothing to do with the *ras* oncogene. Other genetic events, such as mutations in *src,* could also effect these.

The increased activity of PAP was very consistent in that only 4 of the 35 patients had no significant increase in PAP2 activity in their tumours and only 2 of the 35 had no significant increase in PAP1 activity. Since PAP has not yet been purified it is not possible to quantify expression of PAP at the level of either transcription or translation. Clearly, increased activity could be the result of increased

levels of the protein but it is also possible that the increase is due to the presence of a mutant form of PAP. PAP catalyses the conversion of phosphatidate to DRG and a change in PAP activity could alter the levels of these two lipids. Both phosphatidate and DRG have been shown to be mitogenic *in vitro* (Freidman *et al.*, 1989; van Corven *et al.*, 1992). Total phosphatidate and DRG mass was estimated in a representative sample of the colon tumours. DRG mass was significantly decreased in 13 of the 14 tumours studied (Figure 4.5) and this confirms previous reports (Phan *et al.*, 1991; Sauter *et al.*, 1990). In addition, phosphatidate mass was also shown to be decreased in 11 of the 13 tumours studied (Figure 4.6). Thus, the levels of these two lipids, which are thought to play a key role in mitogenic signal transduction (Billah & Anthes, 1990; Moolenaar *et al.* , 1986; van Corven *et al.*, 1992), are decreased in this rapidly proliferating tumour.

Measurement of the total mass of phosphatidate and DRG is potentially misleading. Both lipids are important components of cell membranes and are composed of a number of distinct molecular species (Divecha *et al.*, 1991; Pessin *et al.* , 1990; Pettitt & Wakelam, 1993). The specificity of PAP for particular molecular species of phosphatidate is not known. It is possible that this enzyme is specific for a limited number of phosphatidate species and its activation may generate specific forms of DRG. These could be involved in, for example, activation of particular isoforms of PKC. Although phosphatidate could have been converted to another second messenger, lysophosphatidate, by phospholipase A activity (Moolenaar, 1994), increased activities of both PAP1 and PAP2 could explain, at least in part, the decrease in phosphatidate mass. The decrease in DRG mass suggests that there is an increase in the metabolism of this lipid, probably by metabolising enzymes such as DRG lipase and kinase.

The observation of increased activity of PAP in colon tumours is novel but the importance of this change is unclear. Clearly, it does not explain the decreased levels of DRG but does indicates that signalling pathways are disrupted in this tumour type. Results are not consistent with effects of mutant *ras* on lipid signalling in fibroblasts

or with the human colon cancer cell lines. Whilst *ras* mutations are common in colon cancer, clearly this factor alone does not appear to account for the observed changes in cell signalling pathways.

CHAPTER FIVE

COMPARISON OF PHOSPHATIDATE PHOSPHOHYDROLASE ACTIVITY AND Ki-*ras* MUTATIONS IN HUMAN COLORECTAL TUMOURS

5.1 Introduction

In Chapter 3, a clear increase in DRG and phosphatidate mass levels was observed in *ras* transformed cells in culture. There was also a consistent decrease in PAP2 activity which suggests that a mutation in the *ras* oncogene may cause down-regulation of this enzyme. A decrease in PAP2 activity could account for the elevated levels of phosphatidate, a lipid which may have an important role in the regulation of proliferation in cells. Although similar changes were observed in colon tumour cell lines that contain a mutant *ras* oncogene, this was not the case when colon tumour tissue was compared with normal colon. In the tumour samples DRG and phosphatidate were decreased and PAP1 and PAP2 activities were increased. This could be explained by differences in regulation of the lipids and enzymes under cell culture conditions when compared to the more physiological environment of the tumour sample. However, it is also possible that the *ras* oncogene is not the only important oncogene mutation present in colon cancer and that some other oncogene, such as *src*, may be responsible for changes in PAP activities and lipid levels.

A previous study attempted to correlate changes in DRG mass levels in colon tumours with presence of a mutated *ras* oncogene (Phan *et al.*, 1991). They showed that DRG mass was decreased in tumour tissue compared to normal, regardless of the *ras* mutation status. However, these workers limited their study to mutations in codon 12. Four out of 15 tumours contained a mutation which is a lower frequency than had previously been reported for colon tumours (Bos *et al.*, 1987a; Vogelstein *et al.*, 1988). Furthermore, the presence of a mutation was associated with some of the biggest changes in DRG levels (Phan *et al.*, 1991). It is possible, therefore, that if they

had also identified mutations at codon 13, they may have been able to relate changes in DRG to the presence of mutant *ras*. Clearly, this is not the same relationship as that observed in colon cancer cells in culture, but it is nonetheless of interest. Since there was a marked variation in the magnitude of the changes in PAP activity and levels of DRG and phosphatidate, an attempt was made to relate these changes to the presence of a *ras* mutation.

Different methods have been used in the past to detect point mutations in the *ras* genes. Originally, activation of the *ras* oncogene was detected by transfection of genomic DNA into NIH 3T3 mouse fibroblasts. These cells undergo malignant transformation when a mutated *ras* gene is incorporated into their genome. This method is too laborious for large scale screening. Furthermore, false negative results can arise due to the DNA from human tumours being degraded, either because of necrosis or due to degradation which can occur during surgery and subsequent handling of the samples.

Several highly sensitive methods are now available for measuring Ki-*ras* mutations. These include analysis of single stranded conformation polymorphism (Orita *et al.*, 1989) or large deletions/insertions that disrupt or create recognition sequences of restriction enzymes can be detected by Southern blotting following digestion with appropriate enzymes (restriction fragment length polymorphism) (Sugimura *et al.*, 1990). However, these methods cannot identify specific point mutations. An alternative method which was used in this study was developed by Bos and co-workers using direct hybridisation of oligonucleotides to amplified regions of DNA (Bos *et al.*, 1987b). Polymerase chain reaction (PCR) (Mullis & Faloona, 1987) was used for DNA amplification so that enough DNA can be generated to enhance greatly the signals obtained with these oligonucleotide hybridisations (Verlaan de Vries *et al.*, 1986). For this procedure, DNA is isolated from frozen or fresh tissue samples, and *ras*-specific sequences amplified by PCR are dotted onto hybridisation membranes. The hybridisation procedure involved hybridising a collection of oligonucleotides complementary to possible mutations in codons 12, 13 or 61 of the

Ki-*ras* oncogene. Stringent washing along with strict temperature control were used to obtain fully matched hybrids. After stringent washing, only the completely matching oligonucleotides remain hybridized and give a signal on autoradiographs.

The structure of the human Ki-*ras* gene from a lung cell line has been determined (Shimizu *et al.*, 1983) and the regions containing codons 12, 13 and 61 can be amplified (Figure 5.1) by choosing amplimers complementary to sequences at the 5' and (in opposite orientation) 3' ends of the region of interest. Codons 12 and 13 are located on exon 1, whilst codon 61 is found in exon 2 and the 'KA' and 'KB' amplimers flank these regions (Verlaan de Vries *et al.*, 1986). Identification of point mutations by this method is very sensitive since amplification of mutant alleles can be detected from a small number of cells (Ehlen & Dubeau, 1989). Therefore this method is perfectly suited for the analysis of mutational events in solid tumour samples which will contain both neoplastic and normal cells. The knowledge and expertise for use of these techniques are also available in the department.

PCR and hybridisation techniques have been employed in the present study for detection of Ki-*ras* mutations in codons 12, 13 or 61 of colon tumour and normal tissue. Fourteen paired samples were chosen as these had been used in previous studies to measure PAP activity and second messenger levels. In this way, direct comparisons could be made between these and the presence of a mutation in *ras*.

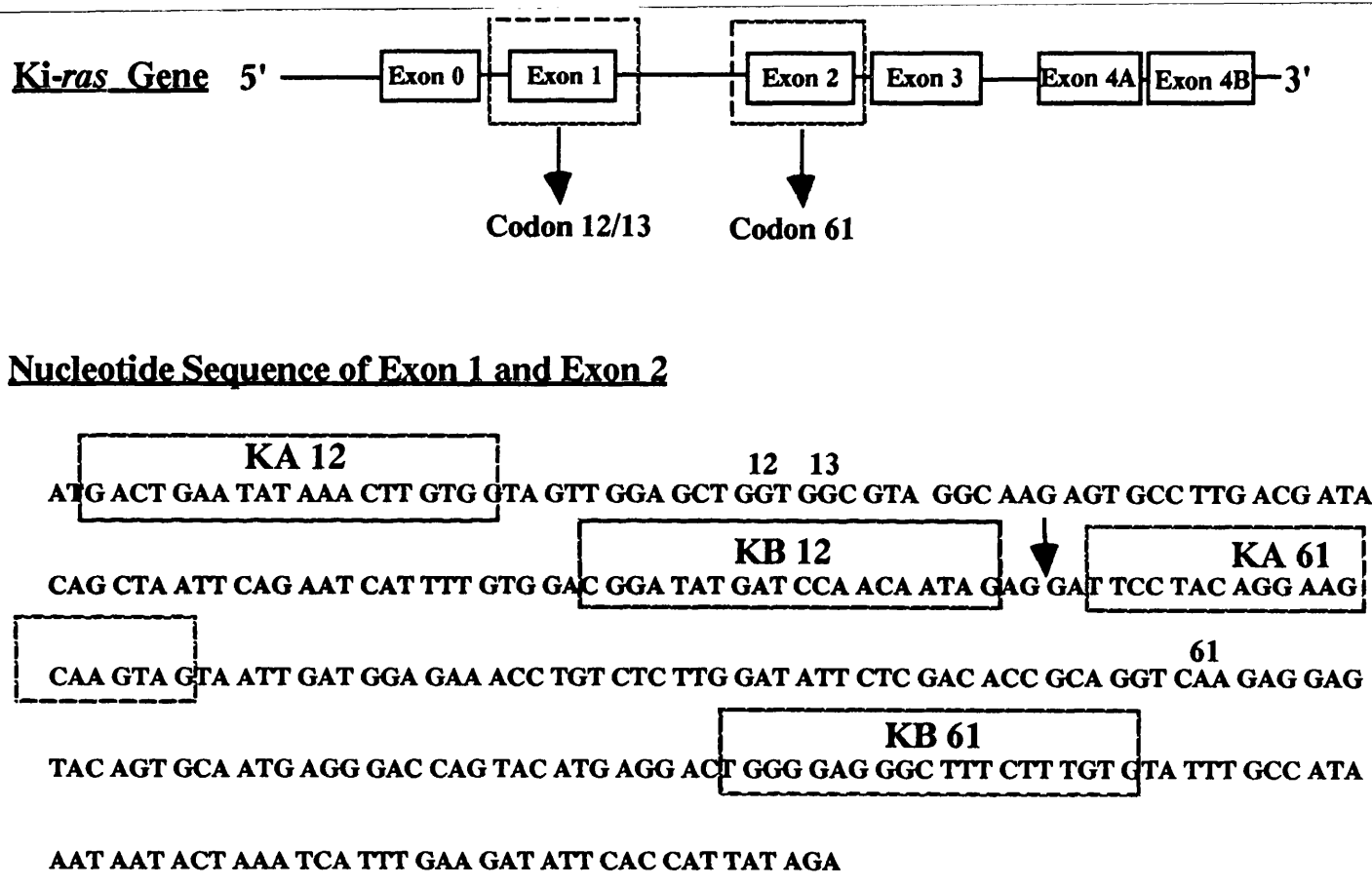


Figure 5.1 Structure and nucleotide sequence of exons 1 and 2 of the human Ki-*ras* gene

The nucleotide sequence includes location of the primers used for detection of codons 12/13 and codon 61 which are found in exons 1 and 2 respectively (first panel). The wild type nucleotide sense sequence is GGT and GGC for codons 12 and 13 respectively. Primers KA 12 and KB12 were designed to amplify this region. The wild type nucleotide sense sequence for codon 61 is CAA with the chosen primers, KA61 and KB61, flanking this region as indicated. Exons 1 and 2 are separated by the arrow in panel two.

5.2 Methods

5.2.1 Chemicals and Reagents

DNA was extracted from tissue samples using the Nucleon I extraction kit from Scotlab (Paisley, Scotland). PCR was carried out using the GeneAmp PCR Reagent kit from Perkin Elmer (Buckinghamshire, England) which contained Ampli*Taq* DNA polymerase. Synthetic amplimers were produced at the Beatson Institute, Glasgow using reagents from Cruachem (West of Scotland Science Park, Glasgow) and then deprotected at 55°C overnight and purified on an OPC cartridge (Cruachem) according to the manufacturers' instructions. Oligonucleotides used for hybridisation were from Clontech Laboratories Inc. (Palo Alto, California). Other purchases included: tetramethylammonium chloride (TMAC) (Sigma), G-50 sephadex Nick columns (Pharmacia LKB, St.Albans, Hertfordshire), T4 polynucleotide kinase and 123bp DNA ladder were from Life Technologies (Paisley, Scotland).

5.2.2 Tissue Preparation and DNA Extraction

Solutions:

Tris/HCl Buffer:	Tris/HCl	400mM (2M, pH 8)
	EDTA	60mM
	NaCl	150mM
	SDS	1%, v/v
TBE Buffer:	Tris/Borate	89mM, pH8.0
	EDTA	2mM

Frozen colon tissue was ground to a fine powder as described in Section 4.2.1 and approximately 100mg of this tissue was used for DNA extraction. Samples of

tissue for analysis were suspended in 340µl sterile Tris/HCl Buffer in 1.5ml microtubes. The suspension was vortexed briefly, 100µl sodium perchlorate (5M) added and the solution was continually agitated using a rotary mixer (Matburn Surgical Equipment, Portsmouth, England) at 37°C for 20min. These tubes were then incubated in a shaking waterbath at 65°C for 20min to deproteinise the tissue. DNA was extracted according to the method described in the DNA extraction kit. Briefly, 580µl chloroform was added which had been stored at -20°C and this was mixed with constant agitation as before at room temperature for 20min. The contents of each tube were transferred to a nucleon eppendorf tube which is included in the kit. This has been specially designed with an insert at the upper end, through which the contents of the tubes flow. These were then centrifuged in a microcentrifuge (Microcentaur model, Scotlab) at 1300 x g for 1min. Nucleon silica suspension (45µl) was added to each tube which goes into the insert and after further centrifugaton for 4min at 1300 x g, the DNA phase becomes separated. Without disturbing the silica, the DNA phase above the insert was poured off into another clean test-tube. DNA was precipitated by addition of 880µl ethanol and samples were left at -20°C for 1-2 hours. DNA was pelleted by centrifugation at 4000 x g for 5min and the supernatent discarded. The pellet was washed using 1ml of 70% ethanol at 4°C. This was removed and the pellet allowed to air dry before addition of between 100 and 200µl sterile water, depending on the size of the pellet. DNA extracted samples were stored at 4°C until used.

The quality of the extracted DNA was analysed by agarose gel electrophoresis using approximately 2µl extract on agarose gel (0.8%) prepared in TBE buffer and ethidium bromide (0.5µg/ml). A Hybaid Electro-4Gel Tank was used to run the gel in TBE buffer at 100v for 2 hours and the ethidium bromide staining visualised by ultraviolet trans-illumination. One µl of the DNA extract was sufficient for amplification by PCR.

5.2.3 Amplification of Ki-*ras* Specific Sequences by PCR

Amplification was carried out in a volume of 100µl PCR reaction mix with the following final concentrations: Tris/HCl (10mM, pH 8.3), KCl (50mM), $MgCl_2$ (1.5mM), gelatin (0.001%, w/v), deoxynucleoside-triphosphates (dNTPs; dATP, dTTP, dCTP, dGTP; 10mM each), amplimers (0.5µg each), *Taq* polymerase (5U/ml) and DNA extract (1µg; 1µl of sample extraction). Two different sets of amplimers were used to amplify the Ki-*ras* oncogene, each set amplifying regions around codons 12 and 13 (KA12 and KB12, Table 5.1) and codon 61 (KA61 and KB61, Table 5.1). Before amplification, paraffin oil (50µl) was overlaid on the amplification mix in each tube to prevent the samples drying. A negative control containing no DNA was included in the reactions to check for contamination.

Amplification was carried out for 35 cycles as previously described (Breivik*et al.* , 1994) at the following temperatures: 94°C for 1min, 58°C for 1min and 72°C for 1min, in a Dri-block device (LEP scientific, Linford Wood, Milton Keynes). A final incubation was carried out following the 35 cycles for 10min at 72°C. Presence of a PCR amplified product was detected as before on a 2% agarose gel using 10µl of amplified sample. One µg of a 123bp DNA ladder was also placed in each gel to check the size of the PCR product. The amplified DNA was stored at -20°C.

5.2.4 Preparation of Oligonucleotide Probes

Radioactive oligonucleotide probes were prepared for each possible point mutation in the Ki-*ras* gene. The exact nucleotide sequences of each probe for point muations in codons 12, 13 and 61 are given in Table 5.2.

A modification of the method described by Berent (Berent*et al.* , 1985) was used to 5' label each probe as follows: in a reaction volume of 30µl was added Tris/HCl (60mM, pH 7.8), 2-mercaptoethanol (15mM), $MgCl_2$ (10mM), ATP (0.35µM), oligonucleotide (50ng), 50µCi [γ-^{32}P]ATP and T4 nucleotide kinase (5U).

Amplimer	Sequence	Target	Fragment Amplified (bp)
KA12	G ACT GAA TAT AAA CTT GTG G	Ki-*ras*	108
KB12	C TAT TGT TGG ATC ATA TCC G	12/13	
KA61	T TCC TAC AGG AAG CAA GTA G		128
KB61	C ACA AAG AAA GCC CTC CCC A	Ki-*ras* 61	

Table 5.1 Amplimers used for Ki-*ras* codons 12/13 and codon 61

The first letter of the target sequence represents the 5' end and the last letter the 3' end. All amplimers are 20 nucleotides long. The fragment length includes the amplimer sequences.

Gene/Codon	Mutation	Amino Acid	Hybridisation Pools
Ki-*ras* 12	GGA GCT GGT GGC GTA GGC AA	gly (w.t)	
	AGT	ser	Pool 12A
	GTT	val	
	GAT	asp	
	TGT	cys	Pool 12B
	GCT	ala	
	CGT	arg	
Ki-*ras* 13	GGA GCT GGT GGC GTA GGC AA	gly (w.t.)	
	AGC	ser	Pool 13A
	GTC	val	
	GAC	asp	
	TGC	cys	Pool 13B
	CGC	arg	
	GCC	ala	
Ki-*ras* 61	ACC GCA GGT CAA GAG GAG TA	gln (w.t.)	
	GAA	glu	Pool 61A
	AAA	lys	
	CAT	his-1	
	CTA	leu	Pool 61B
	CCA	pro	
	CGA	arg	
	CAC	his-2	

Table 5.2 **Primer oligonucleotides used for detection of point mutations in the Ki-*ras* gene**

The incubation was carried out at 37°C for 60min. Labelled oligonucleotide was separated from the unincorporated nucleotides using Nick columns containing G-50 DNA grade sephadex. The top and bottom caps were removed from the columns and the storage buffer allowed to drain off. The column was then washed with 400µl TE buffer (Tris/HCl (10mM, pH 7.8), and EDTA (1mM)). The labelled product from the above reaction (30µl) was placed on the column which was flushed with a further 400µl TE buffer. The column was placed over a microtube and 400µl of the same buffer was drained through the column into the tube, with unincorporated nucleotides remaining in the column. The purified labelled probe was stored at -20°C and used within 48h of preparation.

5.2.5 Selective Hybridisation of Mutation-Specific Oligonucleotides

Solutions:

1x SSC Buffer:	NaCl	150mM
	Sodium citrate	15mM, pH7.6
1x SSPE Buffer:	NaCl	180mM
	NaH_2PO_4	8mM
	EDTA	1mM
5x Denhardt's Solution:	Ficoll	0.1%
	BSA	0.1%
	polyvinylpyrrolidine	0.1%
TMAC Wash Solution:	TMAC	3M
	Tris/HCl	50mM, pH8.8
	EDTA	2mM
	SDS	0.1%

Amplified DNA samples were spotted onto Hybond N transfer membranes (Amersham Int.) using a Dot blot manifold (BRL Hybridot, BRL Life Technologies,

Paisley, Scotland). To do this the membrane was first washed with 2 x SSC solution before being placed into the manifold apparatus. To prepare the amplified DNA for application to the membrane, 5μl of amplified DNA per replicate (2 replicates per membrane) was diluted with one-fifth volume of NaOH and left at room temperature for 30min. Each replicate was diluted with 100μl 10 x SSC and 100μl was added to the manifold which was attached to a vacuum unit. Following application of the DNA, the membrane was washed twice with 20 x SSC and the membrane then air-dried, wrapped in cling-film and, with DNA side down, placed on a pre-callibrated uv trans-illuminator for 5min to cross-link the DNA to the membrane.

For this study it was not necessary to study specific point mutations, only presence or absence of Ki-*ras* was of interest and, hence, up to four of the radioactive probes were pooled for the hybridisation procedure. The membranes were prehybridised for 2h at 37°C on a shaking waterbath in 25ml hybridisation mix per membrane (5 x SSPE, 5 x Denhardt's solution, sodium dodecyl sulphate (0.5%) and sodium pyrophosphate (0.1M)). Pools of the labelled probes as indicated in Table 5.2 (the wild type probe was used on its own) were added to each membrane at a final concentration of 5 x 10^6 CPM/25ml hybridisation mix and this was hybridised for 2h at 37°C in a shaking waterbath. The hybridisation mix was removed and the filter washed with 100ml 6 x SSC for 15min at room temperature followed by 100ml of TMAC (3M) wash solution at 61°C for 30min with constant shaking. Finally, each filter was washed in 6 x SSC at room temperature. Autoradiography was carried out on the filters at -70°C for 1-4 hours. Sometimes longer exposure times were required, depending on the level of radioactivity retained on the membranes. The dot blot results are presented in a table indicating the wild type result and that after hybridising with pools of the labelled oligonucleotides.

The hybridisation procedures described above were those recommended by the manufacturer of the oligonucleotide probes (Clontech). The dissociation temperature (T_m) is used to determine the hybridisation temperature. The T_m depends

on the base composition of the oligonucleotides and increasing the G-C content stabilises the duplex formed. T_m can be estimated by:

T_m(°C) = 2°C per A-T + 4°C per G-C (Suggs *et al.*, 1981).

The T_m of the oligonucleotides was approximately 63°C. Hybridisation to amplified regions of genomic DNA was carried out at 37°C which was about 25°C below the T_m of a fully matched hybrid. This results in hybridisation of oligonucleotides to both wild type and mutant sequences and, hence, mismatch pairing of oligonucleotides and target DNA. Washing of membranes was carried out in the presence of 3M TMAC (Wood *et al.* , 1985) so that different oligonucleotides can be used at the same time. At this concentration the G-C and A-T base pairs have similar stabilities, so that oligonucleotides of the same length have the same T_m, regardless of the base composition. Washing in TMAC was carried out at a temperature (61°C) which allows only fully matched hybrids to remain.

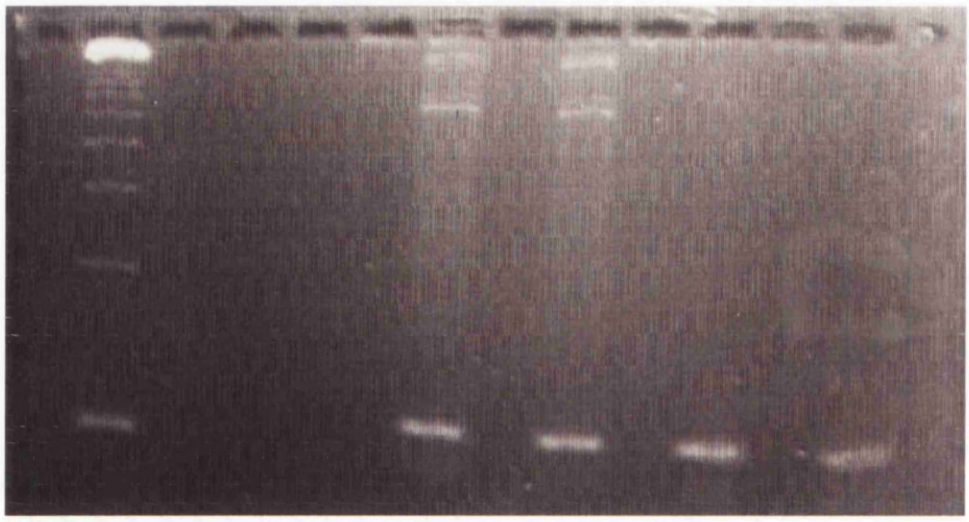
5.3 Results

5.3.1 DNA Amplification

Detection of *ras* involved amplification of regions of DNA containing codons 12/13 and 61. Figure 5.2 shows the PCR product obtained following amplification of codon 61 in tumour and normal tissue from two patients. The size of the amplified region for codon 61 was 128bp as determined by comparison with a 123bp ladder. The control lane indicates no amplified DNA as expected.

5.3.2 Ki-*ras* Mutations

In order to minimise the number of hybridisation reactions necessary for the detection of each possible mutation, multiple pools of probes were used for detection of mutant Ki-*ras* and the pools used are indicated in Table 5.2. Mix 12A, 12B, 13A and 13B each contained three possible point mutations in the first or second base of each codon. Mix 61A and 61B contained 3 or 4 probes for detection of point mutations in either of the three bases. Figure 5.3 displays the results for Ki-*ras* mutation detection. None of the patients had a *ras* mutation within the normal tissue. However, 7 of the 14 tumours tested had a Ki-*ras* mutation. Mutant *ras* was detected in codon 12 of 4/7 patients and the rest were found in codon 13, with no mutations in codon 61. In 6 tumours, the point mutations were found in the pool of oligonucleotide probes which would detect a glycine to valine, serine or aspartate mutation. One patient had a point mutation in codon 12 which would result in a glycine to cystine, alanine or arginine alteration.

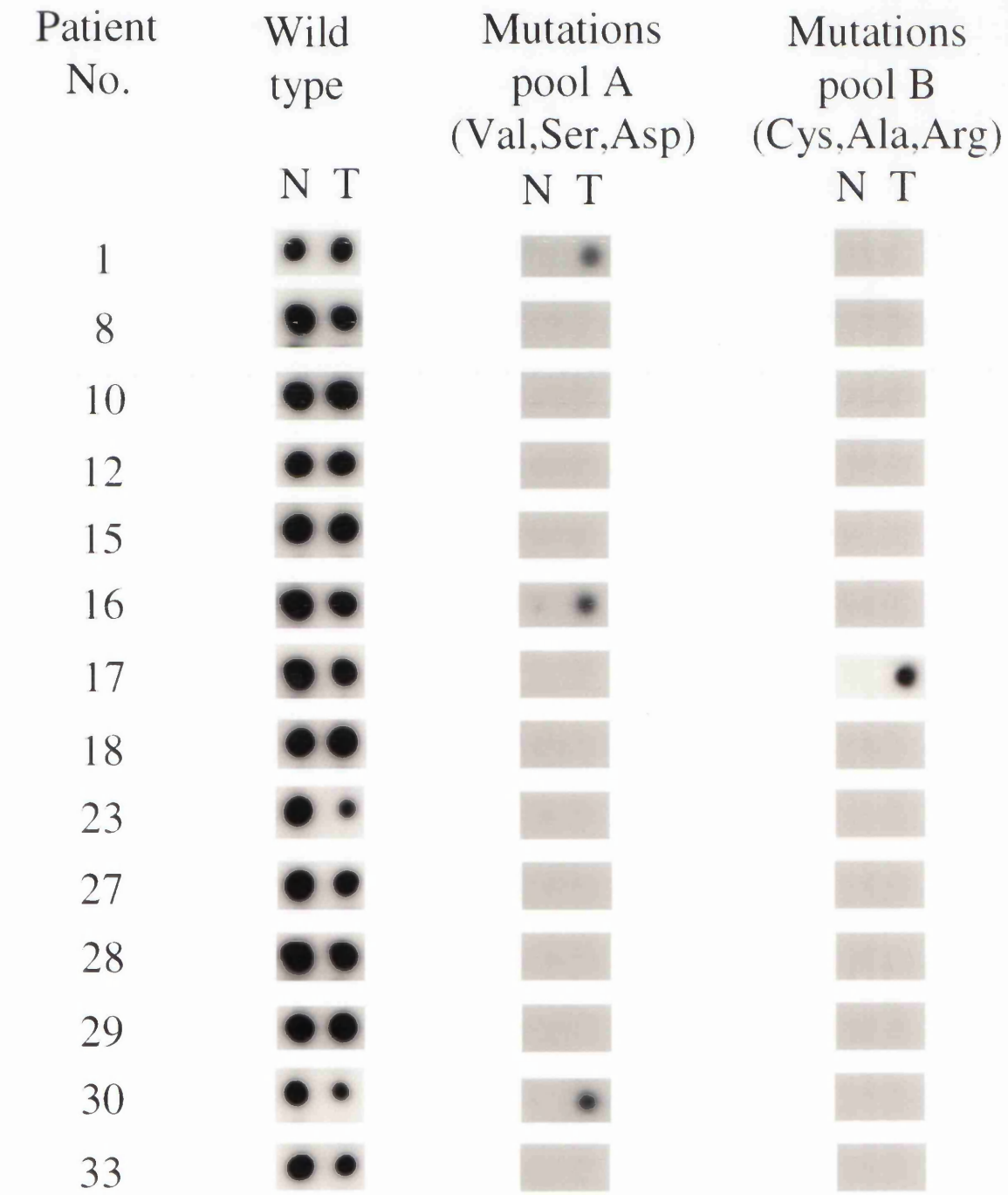


1 2 3 4 5 6

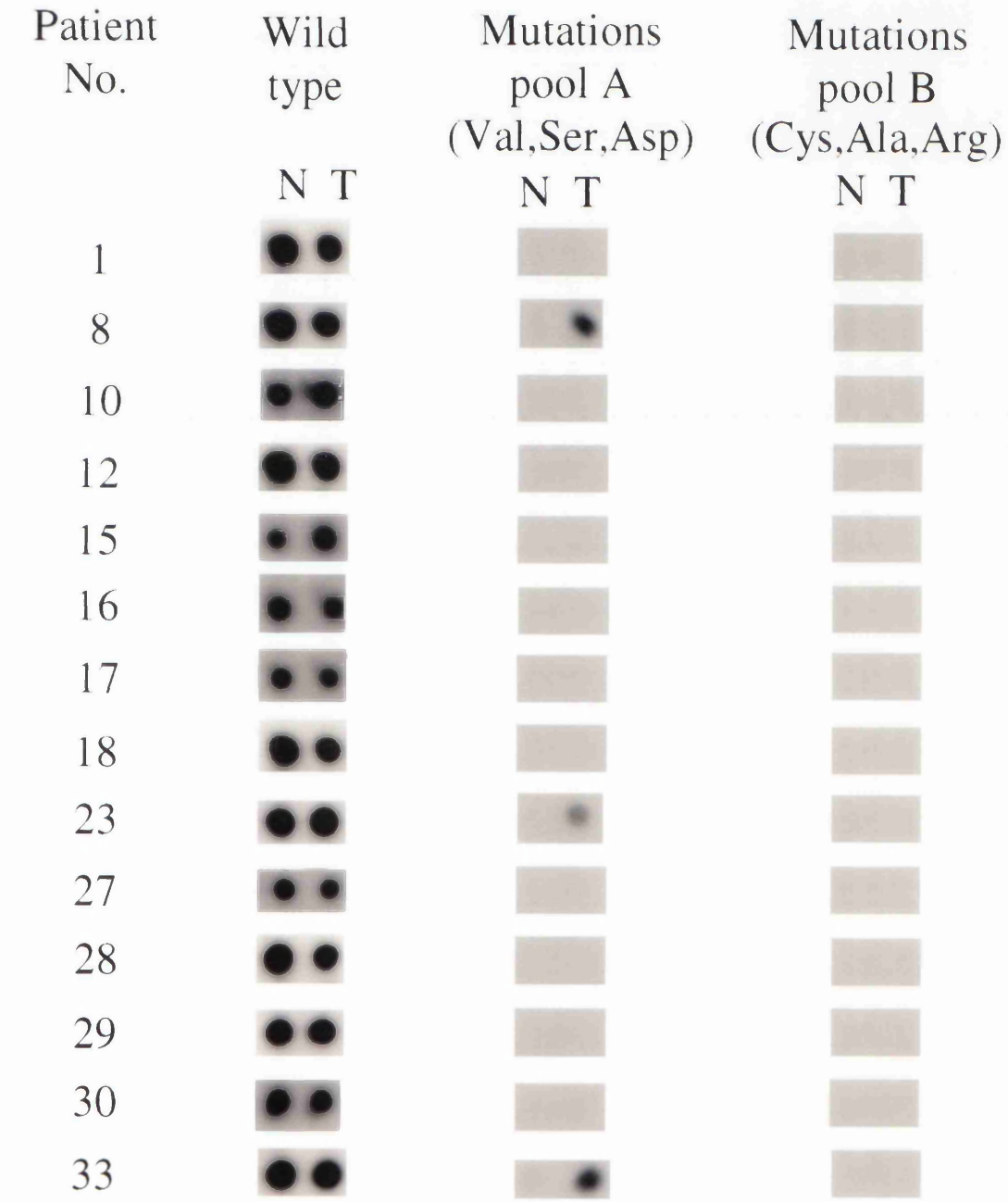
Figure 5.2 Ki-*ras* gene-specific amplification of genomic DNA from tumour and normal tissue

Lane1:123bp DNA ladder,Lane 2:Control lane with no DNA, Lane3, 4, 5 & 6:PCR products from extracted DNA following amplification using KA61 and KB61 primers in normal (3,5) and tumour (4,6) colon tissue.

(A) Codon 12


Patient No.
Wild type
N T
Mutations pool A (Val,Ser,Asp)
N T
Mutations pool B (Cys,Ala,Arg)
N T
1
8
10
12
15
16
17
18
23
27
28
29
30
33

(B) Codon 13


Patient No.
Wild type
N T
Mutations pool A (Val,Ser,Asp)
N T
Mutations pool B (Cys,Ala,Arg)
N T
1
8
10
12
15
16
17
18
23
27
28
29
30
33

(C) Codon 61

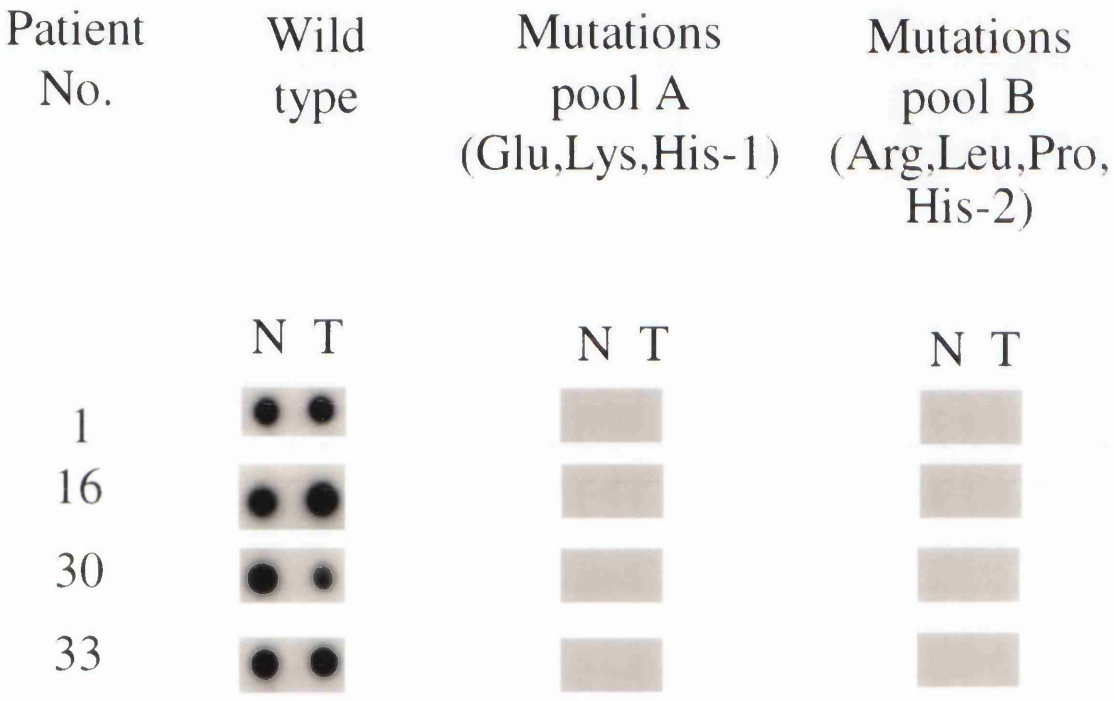


Figure 5.3 Dot blot hybridisation of oligimer probes to amplified DNA in tumour and normal colon tissue

DNA from the indicated patients was amplified using the Ki-*ras* amplimers in Table 5.1 for each of codons 12, 13 & 61. The DNA was spotted onto Hybond N transfer membrane and hybridised to pools of oligimers as shown in the table. Each tissue sample was spotted in duplicate and one of the replicates is displayed. The hybridisation results for all patients are shown for codons 12 & 13, however, as all patients were negative for codon 61 only a representative sample is included.

5.3.3 Relationship Between Presence of *ras* Mutation and PAP Activity

The mutations found in the 7 tumours were distributed according to Duke's Staging as follows: 1 Duke's A, 1 Duke's B, 3 Duke's C and 2 Duke's D. There was no correlation between age, sex or location of the tumour and the presence of a Ki-*ras* mutation. Table 5.3 summarises the changes in PAP1 and PAP2 activities and DRG and phosphatidate levels in the 14 tumours. There was no clear relationship between any of these changes and the presence or absence of a mutation in the Ki-*ras* gene. However, when activated Ki-*ras* was present in the tumours, there was a tendancy towards a higher PAP2 activity but this did not reach statistical significance ($p=0.06$) (Table 5.4).

Patient No.	PAP1	PAP2	Phosphatidate	DRG	Ki-*ras* Status
1	+3.5	+2.3	-2.0	-2.1	13
8	+1.9	+2.0	-1.8	-1.2	12
10	+2.8	+1.5	-1.5	-1.6	wt
12	+2.5	+2.4	ND	-1.3	wt
15	+1.4	+1.3	-1.6	-1.3	wt
16	+2.4	+1.5	-1.5	-3.8	12
17	+1.3	+1.8	1.0	-3.0	12
18	+2.6	+1.1	+1.1	-1.5	wt
23	+2.3	+2.8	-2.4	-1.6	13
27	+2.2	+1.7	-1.3	+2.2	wt
28	+1.5	-1.1	-1.4	-3.0	wt
29	+4.0	+2.1	-3.8	-2.3	wt
30	+1.9	+2.0	-1.3	-1.5	12
33	+1.8	+2.3	-1.4	-1.4	13

Table 5.3 Summary of results for changes in PAP activity, second messenger levels and Ki-*ras* status in colon samples

The results have been summarised as the fold change in enzyme activity or second messenger levels calculated as that in the normal tissue divided by that in the tumour tissue. The presence of a *ras* mutation is indicated by identification of the codon containing the mutation.

Ki-*ras* mutation	PAP1	PAP2	Phosphatidate	DRG
+ n = 7	2.0 ± 0.5	3.8 ± 1.1	2.8 ± 0.3	3.7 ± 0.9
- n = 7	1.9 ± 0.5	1.6 ± 0.3	2.2 ± 0.3	2.7 ± 0.6

Table 5.4 Summary of results for PAP activity, second messenger levels and presence or absence of Ki-*ras* mutation in colon tumours

Results of enzyme activity and mass levels of phosphatidate and DRG are presented as means ± SE from the tumours found to contain the Ki-*ras* oncogene and those without this mutation.

5.4 Discussion

Of the fourteen tumours analysed, half were found to contain a mutation in Ki-*ras*. In the majority of these a glycine at codon 12 or 13 was replaced by one of three amino acids; valine, serine or aspartate (Figure 5.3). The presence of activated *ras* did not correlate with the sex or age of the patient or with the location of the tumour. Changes in PAP activities and second messenger levels were independent of the presence of a Ki-*ras* mutation (Table 5.3). However, whilst PAP2 activity was increased in most tumours, the increase tended to be greater in those with a Ki-*ras* mutation (Table 5.4).

PCR and hybridisation techniques were utilised to make a qualitative analysis of 14 patient tumours for the presence of mutations in the Ki-*ras* gene. Mutations in Ha-*ras* have not been detected in colon tumours (Forrester *et al.*, 1987) and, therefore, this was not analysed in the present study. However, N-*ras* mutations could account for about 14% of all the *ras* mutations in colorectal cancer (Vogelstein *et al.*, 1988) and thus the present study could be a slight underestimate of the number of *ras* mutations in the 14 patient tumours. Mutations in Ki-*ras* have been reported to occur in about 40-60% of colon carcinomas (Capella *et al.*, 1991) which reflects the frequency of 50% found in this study. Of the mutations occuring at codons 12 and13, the G to A transitions resulting in the replacement of glycine by aspartate are the most common (Breivik *et al.*, 1994; Capella *et al.*, 1991; Farr *et al.*, 1988). Although the exact mutation was not identified in this study, the majority of mutations were detected by the oligonucleotide pool which included a change to an aspartate (Figure 5.3). Mutations in the *ras* oncogene family are known to be early events in the tumour progression in animal models of chemical carcinogenesis (Balmain & Brown, 1988). Whilst individual carcinogens have been shown to induce specific mutations, the G to A transition at the second position of codon 12 of each of the three *ras* genes is the most common (Balmain & Brown, 1988; Zarbl *et al.*, 1985). This supports the suggestion that the mutations found, at least in some cancers, are caused by

carcinogens. No mutations were found in the adjacent normal tissue from any of the patients (Figure 5.3). Since the method used was highly sensitive this suggests that the tissues did not contain any tumour cells.

In the preceeding chapters, decreased activity of PAP2 was shown to be associated with *ras* transformation of fibroblast cells and with presence of the Ki-*ras* mutations in colon cancer epithelial cell lines (Chapter 3, Tables 3.2). Furthermore, this decreased PAP2 activity accompanied a decrease in the ratio of DRG relative to phosphatidate (Chapter 3, Figures 3.7 and 3.8). The colon cell lines had a Ki-*ras* mutation of Gly to Asp in codons 12 and 13 and it has now been shown that this is likely to be the mutation present in these tumours. However, the majority of these tumours showed increased levels of PAP and decreased levels of DRG and phosphatidate when compared to adjacent normal tissue. Furthermore, it is now clear that these changes occured regardless of whether a Ki-*ras* gene mutation was present. This confirms the conclusion that the observed changes in enzyme activity and lipid second messengers in the colon were not a direct result of a mutation in the *ras* oncogene. This was not surprising since *ras* mutations are only one of many genetic changes that occur during tumour progression (Vogelstein *et al.*, 1988).

A previous report also found no correlation between the presence of Ki-*ras* mutations and DRG levels in colon tumours (Phan *et al.*, 1991). Since these authors limited their study to mutations at codon 12, it was possible that they might have missed a relationship. In this study, mutations at codons 12, 13 and 61 were investigated. Although 50% of the tumours had a mutation at codon 12 or 13, all but one of the tumours had decreased levels of DRG compared to the normal colon and there was no relationship between the presence of a mutation and the level of DRG or the size of the decrease in DRG levels (Tables 5.3 and 5.4). Whilst PAP2 activity was increased in the majority of tumours, there was a tendancy for the activity to be highest in those tumours with a mutant *ras* oncogene (Table 5.4), although this did not reach statistical significance. Furthermore, it is not clear how to interpret this observation.

Several reports have suggested that biochemical and molecular changes in colorectal tumours could be used as markers of tumour stage. For instance, PKC activity has been suggested as a possible marker for risk of developing colorectal cancer. A consistent lower PKC activity has been observed in cytosolic and particulate fractions in normal-appearing colon of tissue in patients with colon tumours compared to the normal colon of patients without cancer (Sakanoue *et al.*, 1991). This suggests alterations in PKC activity may play a role in the early stages of malignant transformation (Guillem *et al.*, 1987a). It is not possible to say whether PAP activity could be a marker of increased risk. Whilst this activity was increased in tumour compared to normal colon, it is not known if the activity in the normal colon is higher than patients without cancer. Determination of the Ki-*ras* point mutation has been suggested as a prediction for tumour aggression, with more aggressive tumours being classed as highly invasive and potentially metastatic (Finkelstein *et al.*, 1993). This report showed that tumours containing a gly to val mutation in codon 12 or a gly to asp mutation in codon 13 were associated with early stage tumours. However, other mutations such as a mutation in gly to arg in codon 12, accounted for the majority of more aggressive and invasive tumours. This group suggested that an understanding of the role played by specific point mutations may lead to prediction of the degree of tumour aggressiveness and, in addition, individualisation of treatment. Unfortunately, as the specific point mutations were not determined in the tumours in the present study, these could not be related to the tumour stage to corroborate the previous report.

In summary, Ki-*ras* mutations have now been measured in 50% of patients in which PAP activity and second messenger levels have already been measured. The observed changes in signalling were independent of the *ras* mutation status of the tumour. Although this study was more complete than previously reported correlations between signalling and *ras* mutations, nonetheless it confirms earlier conclusions.

CHAPTER SIX

METABOLISM OF DIRADYLGLYCEROL IN HUMAN COLON TISSUE

6.1 Introduction

In Chapter 4, PAP1 and PAP2 enzyme activities were found to be increased and phosphatidate and DRG levels decreased in human colon tumour tissue compared to adjacent normal mucosa. Although the increased enzyme activity could account for the decreased level of phosphatidate, it does not explain the observed decrease in DRG mass.

DRG is an intermediate in the biosynthesis and degradation of glycerolipids in eukaryotic cells as well as functioning as an intracellular second messenger (Bishop & Bell, 1988). There may be discrete pools of this lipid in the various cell compartments which are highly regulated for these functions. It is not known if there is exchange of DRG between cell compartments or if the different enzyme isoforms, identified in various compartments, allow discrete pools of lipid to be maintained. During *de novo* glycerolipid synthesis DRG is generated by dephosphorylation of phosphatidate by PAP activity, probably the PAP1 form of the enzyme. DRG is known to be formed in the endoplasmic reticulum since this is where the enzymes involved in glycerolipid synthesis are located (Brindley, 1987). DRG is also formed in the plasma membrane where it is involved in activaton of PKC (Hannun *et al.*, 1985). Evidence for this comes from agonist stimulation of cells. For example, in PDGF-stimulated Swiss 3T3 cells, DRG is produced within seconds of cell stimulation (Plevin *et al.*, 1991). The DRG is derived from both phosphatidylinositol and PC through receptor-linked responses in the plasma membrane, where the enzymes involved are found (Majerus *et al.*, 1990; Wakelam *et al.*, 1991).

DRG may play different roles depending on cell type. For example, activity of PAP1 is relatively high in the liver where it is controlled by hormonal and nutritional

factors (Brindley, 1985). Here it is involved in the generation of DRG as an immediate precursor of PC and triacylglycerols which are then utilised for the assembly of complex structures such as membrane bilayers, serum lipoproteins and for energy storage (Bishop & Bell, 1988; Brindley, 1987). DRG is also present in high levels in the brain (Farooqui & Hirashima, 1992) which may provide long-term activation of PKC (Farooqui *et al.*, 1988). In particular, the PKCγ isoform is found exclusively in the brain and spinal cord (Nishizuka, 1989). Although the precise function of this isoform is unknown, it may be involved in the control of neurone-specific physiological processes such as release of certain neurotransmitters. One source of this DRG may be from the PLD/PAP2 pathway, the latter enzyme of which was found to have high activity in this study in murine brain (Chapter 2, Figure 2.8). However, the phosphatidylinositol cycle is also active in the brain which involves recycling of both inositol trisphosphate and DRG, both of which can be generated from hydrolysis of phosphatidylinositol 4,5-trisphosphate (Berridge, 1987; Rana & Hokin, 1990). It is of interest to note that inositol trisphosphate recycling is inhibited by low levels of lithium ions which are widely used in the treatment of manic depressives (Rana & Hokin, 1990).

DRG is comprised of molecular species which are probably formed and catabolised at different rates due to the existence of complex enzyme systems which govern the fatty acid composition of the acylglycerols (Holub & Kuksis, 1978). There are three major classes of DRG, that is, *sn* 1,2-diacylglycerol (DAG), *sn*-1-alkyl-2-acylglycerol (AAG) and *sn*-1-alkenyl-2-acylglycerol (ALG). These classes also have their component molecular species which can be analysed by reverse phase high performance liquid chromatography (HPLC) of the dinitrobenzoyl derivatives. In response to bombesin-stimulation of Swiss 3T3 cells, only certain species of the DAG class in the plasma membrane are altered (Pettitt & Wakelam, 1993). Of these, 1-stearoyl-2-arachidonyl-*sn*-glycerol (SAG), generated from phosphatidylinositol 4,5-bisphosphate hydrolysis, was produced within the first few seconds following stimulation. Other changes in DAG species occured at later time points and these

species appeared to be derived from PC (Pettitt & Wakelam, 1993). The exact function of the specific DAG species is not known but it is possible that they may activate specific PKC isoforms. Other DRG classes such as AAG are poor activators of PKC (Cabot & Jaken, 1984; Heymans *et al.*, 1987) and may perform other functions such as generation of triacylglycerols in the endoplasmic reticulum for energy storage.

DRG, generated in the cell from various pathways, is subsequently metabolised by a number of enzymes. There are two major pathways for the metabolism of DRG (Figure 6.1). It can be phosphorylated by DRG kinase leading to production of phosphatidate, the reverse reaction to that catalysed by PAP (Kanoh *et al.*, 1990). Alternatively, DRG may be metabolised to glycerol and free fatty acids by the sequential action of DRG and monoacylglycerol (MG) lipases (Bishop & Bell, 1988). Free fatty acids can be used for membrane formation or can activate PKC (McPhail *et al.*, 1984). It should be noted that previous reports have referred to these enzyme activities as 'diacylglycerol' kinase and lipase. However, as they are not specific for this particular class of DRG, they have been referred to as DRG kinase and lipase in this thesis. It is not known whether the DRG kinase or lipase pathway is the predominant route of metabolism in cells and it may be cell type dependent. For example, the metabolism of [^{3}H]DRGs by kinase and lipase enzymes was investigated in human platelets. Although these cells could attenuate the DRG signal by the action of both enzymes, the primary metabolic fate of DRG was by conversion to phosphatidate by kinase activity (Bishop & Bell, 1986). In other cell types it appears that lipase action predominates over DRG kinase. For example, DRG metabolism was measured in vascular smooth muscle cells in which lipase activity was found to be 30-34 fold greater than that of DRG kinase (Severson & Hee-Cheong, 1989).

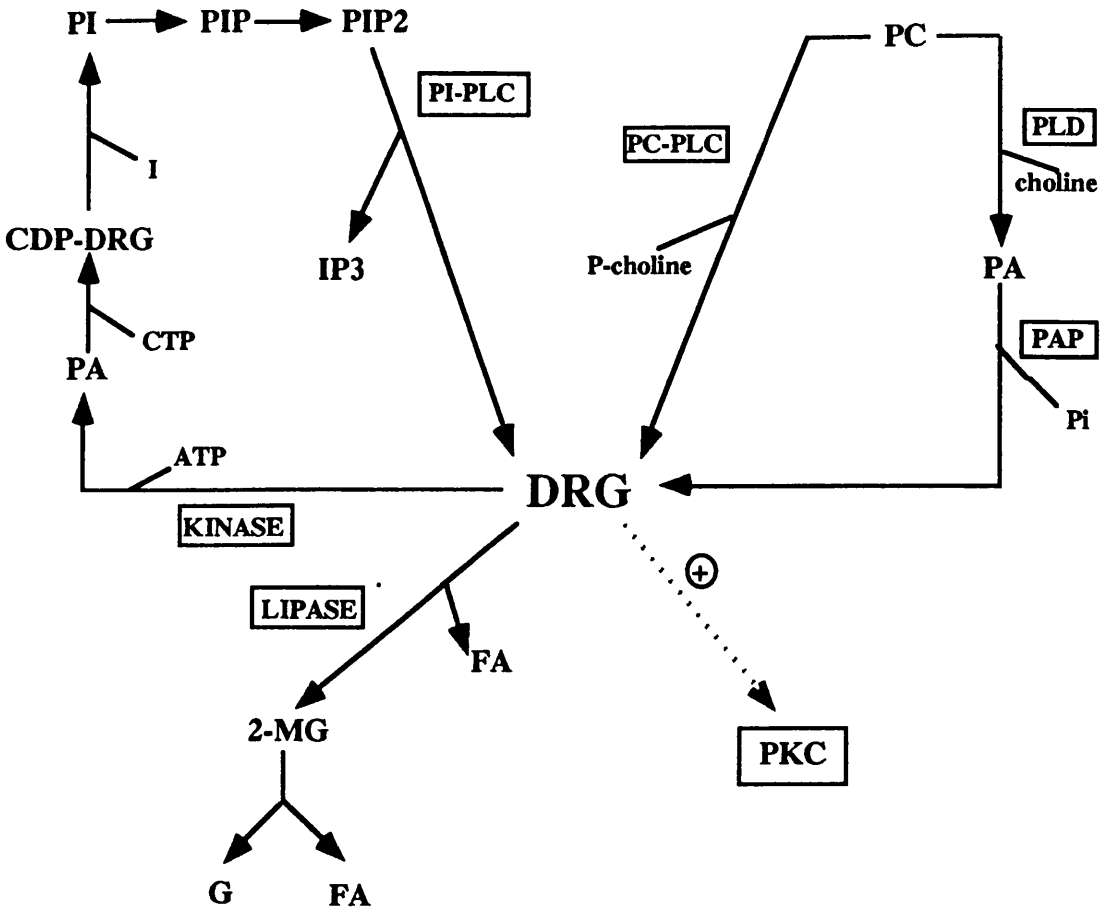


Figure 6.1 Scheme for formation and metabolism of DRG second messenger

DRG is produced by hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phosphoinositide (PI)-specific PLC and by degradation of phosphatidylcholine either by a PC-specific PLC or by the concerted action of PLD and PAP. DRG can activate PKC. It is metabolised by DRG kinase to form phosphatidate. Metabolism by DRG lipase results in the release of fatty acid (FA) from *sn*-1 position followed by hydrolysis of 2-monoglyceride (MG) intermediate by monoacylglycerol lipase to produce glycerol (G) and another FA.

6.1.1 DRG Kinase

DRG kinase converts DRG to phosphatidate. It was first purified from porcine brain cytosol (Kanoh *et al.*, 1983) but the amino acid sequences have also been described for porcine thymus (Sakane *et al.*, 1990), human (Schaap *et al.*, 1990) and rat (Goto & Kondo, 1993) forms. These have molecular weights around 80kDa and are more than 80% identical. There may be a family of DRG kinase isoenzymes since a variety of activities have been described in animal tissues. For example, purification of DRG kinase from rat brain has identified two isoforms of size 80 and 110kDa in a soluble fraction (Kahn & Besterman, 1991; Kato & Takenawa, 1990) and a 150kDa form which is membrane-bound (Kato & Takenawa, 1990). More recently, a 58kDa membrane-bound DRG kinase has been purified from bovine testes (Walsh *et al.* , 1994). It has been suggested that these DRG kinase isoforms have distinct enzymological properties. Evidence for this comes from the work of Lemaitre and coworkers who have recently identified a membrane-bound DRG kinase activity in baboon tissues that has a specificity for arachidonyl-containing DRGs as opposed to a soluble DRG kinase activity which has no apparent substrate specificity (Lemaitre *et al.*, 1990). The 58kDa bovine DRG kinase also has specificity for arachidonyl-containing DRG species (Walsh *et al.*, 1994). A similar activity has also been detected in fibroblasts where an arachidonyl-specific DRG kinase was shown to phosphorylate DRG formed in the plasma membrane from phosphatidylinositols (MacDonald *et al.*, 1988a; MacDonald *et al.*, 1988b). Receptor-stimulated phosphorylation of DRG derived from phosphatidylinositol-specific PLC activity is thought to be important in the resynthesis of phosphoinositides (Berridge & Irvine, 1989). This involves generation of arachidonyl-rich DRG species such as SAG, which is phosphorylated by the specific DRG kinase enzyme to arachidonyl-phosphatidate and this is then converted back into phosphatidylinositol. This has been termed the phosphatidylinositol-phosphatidate cycle (MacDonald *et al.*, 1988b). The function of this cycle is not known but it may help to maintain the arachidonyl-rich lipid species

which are found in the plasma membrane. The soluble DRG kinase has no substrate specificity and will probably metabolise DRG derived from any source. This form of the enzyme will metabolise DRG generated from PC hydrolysis (Kanoh *et al.*, 1990; Sakane *et al.*, 1990). This could theoretically occur in the plasma membrane upon translocation of the cytosolic form. It has already been shown that DRG and phorbol ester can induce translocation of DRG kinase activity from the cytosol to particulate fractions in rat brain and liver (Besterman *et al.*, 1986b) as well as in fibroblasts cells (Maroney & Macara, 1989). Huang and coworkers showed an accumulation of DRG in *ras* transformed rat fibroblasts without any significant change in PLC activity (Huang *et al.*, 1988). Although they found that total DRG kinase activity was not altered in these cells, membrane-bound DRG kinase was actually decreased. They suggest that this change in activity could have led to the observed increase in DRG (Huang *et al.*, 1988). This report showed the value of measuring the activity of DRG kinase in individual cellular compartments. However, the membrane-bound kinase activity measured was probably not the SAG-specific form as *sn*-1,2-dioleoylglycerol was used as the substrate (Huang *et al.*, 1988). As well as compartmentalisation of the activities, it would also have been of interest to study their substrate specificities.

It is not known how DRG kinase and PAP are regulated in the cell. Since they catalyse opposite reactions they may work together to control levels of phosphatidate and DRG. It is possible that different isoforms of each enzyme are specific for different phosphatidate and DRG species, such as was shown for arachidonyl-specific DRG kinase (MacDonald *et al.*, 1988a). In this way, different pools of each lipid could be maintained in each cell compartment. There have been several reports analysing the molecular species of phosphatidate and DRG (Divecha *et al.*, 1991; Pessin *et al.*, 1990; Pettitt & Wakelam, 1993). Additional characterisation of the molecular mechanisms of enzyme interaction with lipid substrates would help to elucidate the significance of different enzyme isoforms and lipid species.

6.1.2 DRG Lipase

An alternative route for DRG metabolism is by breakdown to glycerol and free fatty acids as a result of the sequential action of DRG and monoacylglycerol (MG) lipases. DRG lipase catalyses the removal of fatty acids from the *sn*-1 position of the glycerol and the subsequent hydrolysis of the *sn*-2 acyl chain from the intermediate lipid. Studies with partially pure DRG lipase from a soluble subcellular fraction from bovine aorta have established that DRG is hydrolysed by a single lipase (Lee & Severson, 1994). Further purification, however, is required to determine unequivocally if DRG and MG lipase activities result from a single or separate enzymes. The exact subcellular localisation of DRG lipase activity is not known but there is evidence to suggest that it may be associated with the plasma membrane. Stimulation of human platelets by thrombin results in the rapid, within seconds, production of arachidonate from SAG by a DRG lipase activity located in particulate fractions of the cells (Bell *et al.*, 1979). This fatty acid has several functions within the cell including activation of protein kinase C (Price *et al.*, 1989) or regulation of *ras* through an inhibition of GAP (Yu *et al.*, 1990). The isolation of DRG lipase and/or development of specific inhibitors will be necessary to clarify the role of this enzyme. As there is limited information available regarding different lipase isoforms, an alternative approach is to consider using different forms of DRG as substrate. The use of such species as SAG or DOG, which are derived from phosphatidylinositol and PC respectively, would indicate if the enzyme is specific for DRG derived from a particular source.

6.1.3 Measurement of Kinase and Lipase Activities

DRG kinase activity can be estimated as the amount of [^{32}P]phosphatidate formed from DRG and [^{32}P]ATP (MacDonald *et al.*, 1988a). Subcellular fractionation of the colon tissue into soluble and membrane components would allow

detection of DRG kinases within the different compartments of the cell (Lemaitre *et al.*, 1990; MacDonald *et al.*, 1988a). This would indicate whether DRG phosphorylation occurs in the plasma membrane or endoplasmic reticulum. A limited amount of colon tissue was available at this stage of the study and, hence, subcellular fractionation could not be carried out. However, important information could still be gained by the use of different substrates to measure DRG kinase activity. Two activities of DRG kinase can be identified based on substrate specificity. The cytoplasmic form metabolises all species of DRG (Lin *et al.*, 1986), whereas the membrane-bound form shows specificity for arachidonyl-containing substrates, such as SAG (Lemaitre *et al.*, 1990; MacDonald *et al.*, 1988b). Different substrates have been used in the past to study the specificity of different isoforms for DRG species (Florin-Christensen *et al.* , 1992; MacDonald *et al.*, 1988a). A mixed micellar assay system based on that of MacDonald (MacDonald *et al.*, 1988b) was employed to study metabolism of different DRG species by DRG kinase activity present in homogenates from colon tissue. Molecular species analysis has shown that 1-palmitoyl-2-oleoyl-*sn*-glycerol (POG) and *sn*-1,2-di-oleoylglycerol (DOG) are the predominant DRG species generated as part of a sustained increase in DRG mass in fibroblast cells stimulated with α-thrombin, EGF and PDGF (Pessin *et al.*, 1990). One of these, DOG, was chosen as a substrate in this study to represent PC as a source of this lipid. SAG was also used as a substrate since it is derived from phosphatidylinositols which predominantly consist of *sn*-1-stearoyl-2-arachidonyl species. Therefore, DRG kinase activities measured in this study cannot be attributed to different isoforms, however, the source of DRG, from which metabolism occurs, should be indicated.

Activity of DRG lipase can be estimated by determining the release of radiolabelled fatty acid from DRG labelled in either the *sn*-1 or *sn*-2 position (Severson & Hee-Cheong, 1989). Again, as representatives of lipids derived from PC or phosphoinositides, POG and SAG were used. These have been used in the past as substrates for measuring DRG lipase activity (Chuang *et al.*, 1994; Lee & Severson,

1994). Colon tissue itself will contain various DRG species but this contributes less than 2% of the total DRG in the assays.

Thus, substrate specific DRG kinase and lipase activities were determined in colon tumour and adjacent normal colon in 14 of the 35 samples described in Chapter 4. These 14 were chosen as representatives of the changes observed in PAP activity and levels of phosphatidate and DRG.

6.2 Methods

6.2.1 Chemicals and Reagents

1-[^{14}C]palmitoyl-2-oleoyl-*sn*-glycerol (1-[^{14}C]POG) was a kind gift fron Dr.D.L.Severson (University of Calgary, Alberta, Canada) and had been synthesised by them as previously reported (Hee-Cheong *et al.*, 1985). n-Octyl-β,D-glucopyronaside (OBG) was purchased from Boehringer Mannheim (Lewes, East Sussex), 1-stearoyl-2-[^{14}C]-arachidonyl-*sn*-glycerol (2-[^{14}C]SAG) from Amersham (Little Chalfont, Buckinghamshire) and all unlabelled lipids from Sigma (Poole, Dorset).

6.2.2 Preparation of Human Colon Tissue for Analysis

Tumour and adjacent normal colon tissue from the 14 patients previously analysed for PAP activity and second messenger levels, were used to measure DRG kinase and lipase activities, although in the latter case there was only enough tissue from 12 of the paired samples.

Tissue samples were prepared for analysis as in section 4.2.1 by homogenising the powdered colon tissue in a Tris buffer. Aliquots were stored at -70°C and protein determined as in section 2.2.3.

6.2.3 Measurement of DRG Kinase Activity

The detergent, OBG and phosphatidylserine were used to form a mixed micelle with the DRG substrate. Phosphorylation of this substrate was then used as a measure of DRG kinase activity (MacDonald *et al.*, 1988b). Either DOG or SAG were used as substrates. A stock solution of OBG/phosphatidylserine was prepared by drying down 16.05mg of the lipid (purchased stock = 18mg/ml chloroform) under a

stream of nitrogen in a 2ml glass vial. This was then solubilised in 2ml of a 4-morpholinepropanesulfonic acid (MOPS) buffer (112.5mM, adjusted to pH7.2 with NaOH) containing OBG (182.5mM), NaF (50mM) and DTT (2.5mM). This was vortexed and then sonicated (KS100 bath sonicator, Kerry Ultrasonics Ltd., Kent) for 10min at 4°C. SAG or DOG (300µl), prepared as a 10mM stock in 1ml chloroform, was aliquoted into glass test tubes and dried down under a stream of nitrogen. OBG/phosphatidylserine solution (40µl), was added to each tube to solubilise the lipid followed by sonication for 30min at 4°C in a bath sonicator. Colon tissue homogenate (50µg, 50µl) was added and the reaction started by the addition of 10µl [γ-^{32}P]ATP (30mM; 10µCi/µmol) prepared in a MOPS/NaOH buffer (50mM, pH7.2) containing $MgCl_2$ (180mM). Control reactions contained no enzyme preparation. The final reaction mixture contained OBG (73mM), phosphatidylserine (4.48mM), lipid substrate (3mM), MOPS/NaOH (50mM, pH 7.2), $MgCl_2$ (18mM), NaF (20mM), DTT (1mM) and ATP (3mM; 10µCi/µmol). The samples were incubated at 30°C for 30min and the reaction terminated by addition of 1ml chloroform / methanol / conc.HCl (150:300:2, v/v/v). The test tubes were vortexed and lipids left to extract for 15min at room temperature. Following addition of 300µl chloroform and 400µl water, the tubes were vortexed and centrifuged for 10min at 250 x g in a benchtop centrifuge (model CR411, Deva Medical Electronics Ltd., Runcorn, Cheshire). The upper aqueous phase was discarded and the organic phase was washed in 1ml chloroform / methanol / water (1:1:0.9, v/v/v) and then vortexed and centrifuged as before. The upper phase was again discarded and the final lower organic phase was dried under vacuum. Dried lipid was reconstituted in 50µl chloroform and applied to glass silica gel t.l.c. plates. Radiolabelled products were separated by developing in chloroform / methanol / acetic acid (39:9:4.5, v/v/v) and the [^{32}P]phosphatidate identified by autoradiography and quantified by scintillation counting of scraped bands. All samples were assayed in triplicate and the results expressed as a specific activity (pmoles phosphatidate formed / min / mg protein). Statistically significant differences were determined by Student's paired t-test.

DRG kinase activity was characterised in colon tissue by varying both time and protein concentration. Also, optimum substrate concentration was determined with a range of lipid concentrations from 0.2 to 10mM SAG or DOG. This was carried out by drying down different volumes of a stock of lipid (10mM/ml chloroform) and the reaction carried out as before. Optimum ATP was determined with a range of final ATP concentrations from 0.1 to 10mM (10μCi/μmol).

6.2.4 Measurement of DRG Lipase Activity

DRG lipase activity was determined by measuring the release of radiolabelled fatty acid from labelled DRG substrates as described by Lee and coworkers (Lee & Severson, 1994). Co-sonicates of radiolabelled DRG and phosphatidylserine were used as the substrate with the radiolabel as 1-[^{14}C]POG or 2-[^{14}C]SAG.

A stock solution was prepared consisting of 4 parts 1-[^{14}C]POG (4mM; 2500 dpm/nmole in hexane) and 5 parts phosphatidylserine (4mM in chloroform), allowing 18μl per assay. This was dried down in a glass vial and then reconstituted in the ratio of 80μl potassium phosphate buffer (0.25M, pH 7), 20μl water and 1μl Triton X-100 (10%, v/v) per 18μl original solution. This substrate solution was sonicated in a bath sonicator for 10min at 4°C until optically clear. Colon tissue homogenate (50μg; 50μl) was added to each glass test tube of an experiment along with 250μl potassium phosphate buffer (0.25M, pH 7). The reaction was started by addition of 100μl substrate solution. Final assay concentrations were: 1-[^{14}C]POG (80μM; specific activity 2500 dpm/nmole), phosphatidylserine (100μM), Triton X-100 (0.025%) and potassium phosphate buffer (50mM, pH 7). Samples were incubated for 30min at 37°C and the reaction stopped by addition of 3ml of a fatty acid extraction solution consisting of methanol / chloroform / heptane (1.41:1.21:1, v/v/v) and 0.1mM palmitate as carrier. NaOH (1M; 100μl) was added to each tube and these were then vortexed vigourously for 30s and centrifuged at 250 x g for 10min. A sample (0.5ml) was removed from the aqueous upper phase and added to scintillation vials. Samples

were neutralised by addition of HCl to avoid chemiluminesence. Scintillation fluid (80 volumes) was premixed with HCl (2M; 1volume) and 3ml of this was added to each vial. Radioactivity was measured using a 1600TR scintillation counter (Canberra Packard, Berkshire, UK). Release of palmitate was used as a measure of lipase activity.

To measure DRG lipase activity with 2-[^{14}C]SAG as substrate, a stock of radiolabelled lipid (4mM; 2500dpm/nmole) was prepared by mixing SAG (5.54mg), 400µl of 2-[^{14}C]SAG (53µCi/µmol) and 1800µl hexane. The was stored at -20°C. Substrate preparation and incubations were carried out as described above for POG. The reaction was terminated by addition of chloroform / methanol (2:1, v/v; 2ml) and HCl (5M; 0.1ml) with arachidonate (10µg) as carrier. After addition of NaCl (1M; 0.3ml), the samples were centrifuged at 250 x g and the upper phase removed. The lower phase was dried under N_2, resuspended in chloroform / methanol (2:1, v/v; 50µl) and applied to glass t.l.c. plates. After development to 95% of the length of the t.l.c. plate in hexane / diethylether / acetic acid (70:30:1, v/v/v), the position of MG and [^{14}C]arachidonate was determined by iodine staining. The radioactive band was scraped and ^{14}C counted in scintillation fluid. Release of arachidonate was used as a measure of lipase activity.

Specific activities of all enzymes were routinely expressed as nmoles fatty acid (palmitate or arachidonate) formed / min / mg protein. Statistically significant differences were determined using Student's paired t-test.

6.3 Results

6.3.1 Characterisation of the DRG Kinase Assay

Various experiments were carried out to characterise DRG kinase activity in human colon tissue using DOG or SAG as substrate for the enzyme. The reaction rate of the DRG kinase enzymes was proportional to protein concentration up to 50μg (Figure 6.2A) and time up to 30min (Figure 6.2B). For this reason, the majority of experiments were carried out using 50μg protein for a reaction time of 30min.

The effect of increasing substrate concentration on DRG kinase activity from colon tissue was measured at various SAG and DOG concentrations. Optimum activity for both lipids was achieved at a lipid concentration of about 3mM (Figure 6.3). At concentrations above this, there was a slight decrease in enzyme activity. Hence, 3mM SAG and DOG was used in subsequent assays.

The effect of increasing ATP concentration on DRG kinase activity was also determined. Optimum activity was achieved at 3mM ATP (Figure 6.4). At higher concentrations of ATP, there was a slight decrease in enzyme activity and, hence, in subsequent assays, 3mM ATP was used.

6.3.2 DRG Kinase Activity in Human Colon Tumour and Adjacent Normal Mucosa

The activity of DRG kinase determined with DOG as the substrate in 14 paired tumour and normal colon tissues is shown in Figure 6.5A. There was a wide range of activities of DRG kinase in the normal colon (1.1 - 19.2) and in the tumour tissue (2.7 - 22.5). Activity was significantly different in tumour and adjacent normal tissue in 10 patients. Of these, activity was increased in the tumour in 6 patients and decreased in 4 patients. Overall, the mean activity of this non-specific form of the enzyme was not significantly different between normal mucosa (8.0 ± 1.5 pmoles

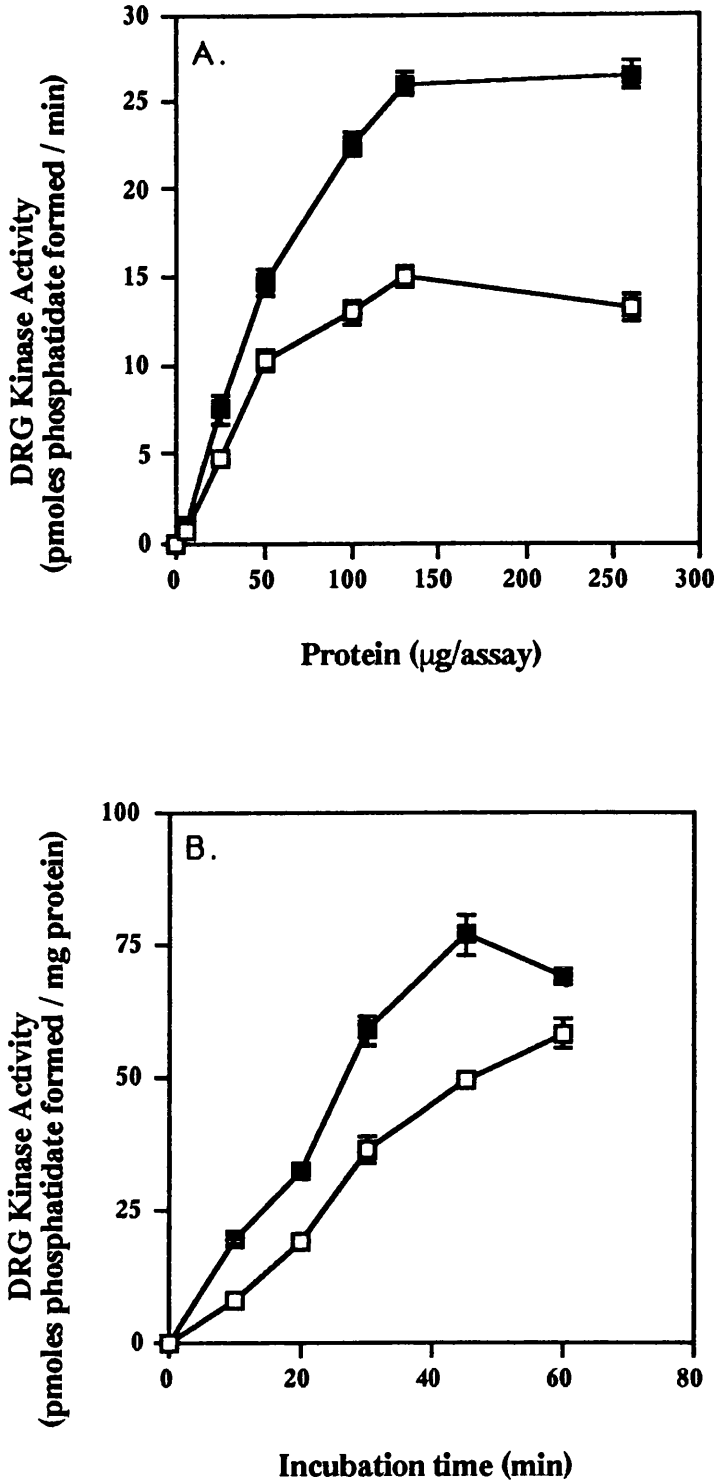


Figure 6.2 Effect of enzyme concentration and incubation time on DRG kinase activity in normal colon tissue

Phosphatidate production was measured with various concentrations of normal human colon tissue with either di-oleoyl-*sn*-glycerol (□) or *sn*-1-stearoyl-2-arachidonyl-glycerol (■) as the substrate (A). Phosphatidate production was also determined after incubation of samples (50μg) for various times (B). ATP and substrate concentration were 3mM. Each point is a mean ± SE from triplicate determinations and is representative of two observations showing similar results.

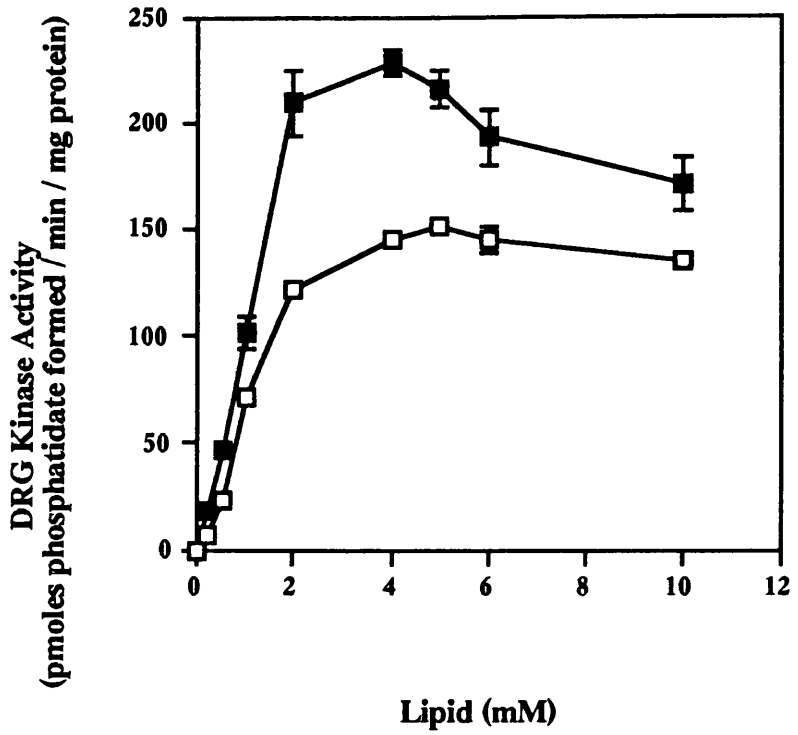


Figure 6.3 Effect of increasing concentrations of lipid substrates on DRG kinase activity from human colon tissue

Phosphatidate production was measured with various concentrations of di-oleoyl-*sn*-glycerol (□) or *sn*-1-stearoyl-2-arachidonylglycerol (■) with human colon tissue (50μg) as a source of the enzyme. ATP concentration was 3mM. Each point is a mean ± SE from triplicate determinations and is representative of two observations showing similar results.

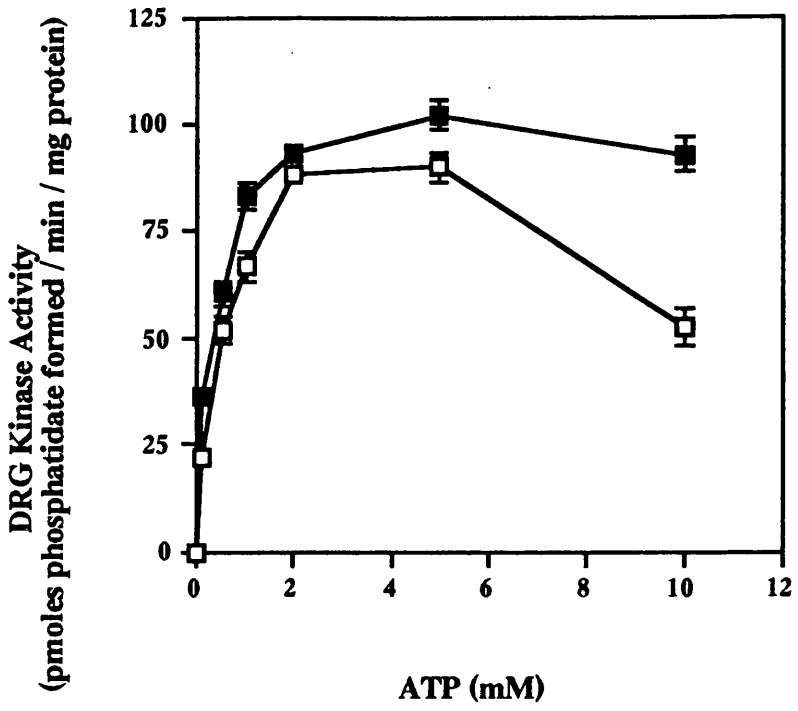


Figure 6.4 Effect of increasing concentrations of ATP on DRG kinase activity from human colon tissue

Phosphatidate production was measured with various concentrations of ATP with human colon tissue (50μg) as a source of the enzyme. Di-oleoyl-*sn*-glycerol (□) or *sn*-1-stearoyl-2-arachidonylglycerol (■) concentrations were 3mM. Each point is a mean ± SE from triplicate determinations and is representative of two independent observations showing similar results.

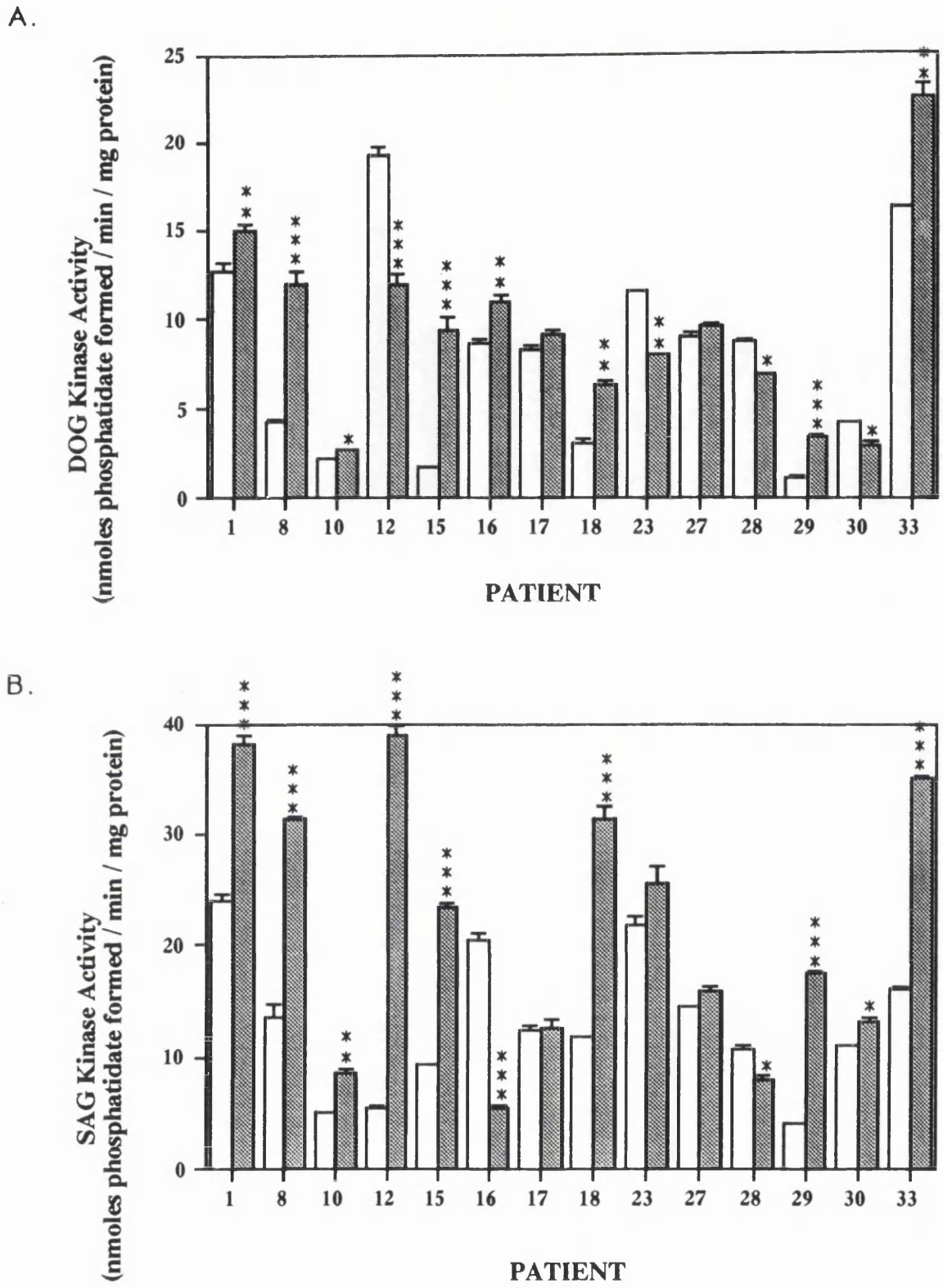


Figure 6.5 Specific activity of DRG kinase in paired colon tumour and adjacent normal tissue

DRG kinase activity in paired normal (□) and tumour tissue (▩) was determined with either (A) di-oleoyl-*sn*-glycerol or (B) *sn*-1-stearoyl-2-arachidonylglycerol as substrate. Each assay contained 50μg protein. Results are the mean ± SE of triplicate determinations. Asterisks represent statistically significant differences (Student's *t* test; * $p<0.02$, ** $p<0.01$, ***$p<0.001$).

phosphatidate formed / min/ mg protein) compared to tumour tissue (9.3 ± 1.4 pmoles phosphatidate formed / min/ mg protein).

DRG kinase activity also showed a marked variation between patients in both normal (4.4 - 24.0) and tumour tissue (5.5 - 39.0) when measured with SAG as the substrate (Figure 6.5B). Activity was significantly higher in the tumour when compared with the normal colon in 8 patients and significantly lower in the tumour in 2 patients (Figure 6.5B). Overall, the SAG-specific enzyme had a significantly higher activity (1.7 fold; $p<0.017$) in colon tumours (21.8 ± 3.1 pmoles phosphatidate formed / min/ mg protein) compared to normal colon tissue (13.0 ± 1.6 pmoles phosphatidate formed / min/ mg protein).

6.3.3 DRG Lipase Activity in Human Colon Tumour and Adjacent Normal Mucosa

Due to the small amount of the radiolabelled lipids and tissue samples available for this part of the study, enzyme activity for both substrates used was not as well characterised in colon tissue as would ideally have been carried out. However, the incubation conditions previously well-characterised for other cells and tissues (Lee & Severson, 1994; Severson & Hee-Cheong, 1989) were used and a limited characterisation of the enzyme was carried out for the colon tissue samples.

The reaction rate for DRG lipase utilising 2-[^{14}C]SAG as substrate was proportional to protein concentration up to 80μg and time up to 30min (Figure 6.6). Measurement of DRG lipase activity was, therefore, carried out using protein concentration from the linear portion of the curve for an incubation time of 30min.

In contrast to DRG kinase, both lipase activities showed only a small variation between patients. DRG lipase activity measured with 1-[^{14}C]POG as the substrate was significantly higher (2 fold; $p<0.0018$) in tumour tissue (12.2 ± 0.7 nmoles palmitate formed / min / mg protein; range 4.9 - 24.7) compared to normal mucosa (6.1 ± 0.4 nmoles palmitate formed / min/ mg protein; range 2.8 - 10.6). Overall,

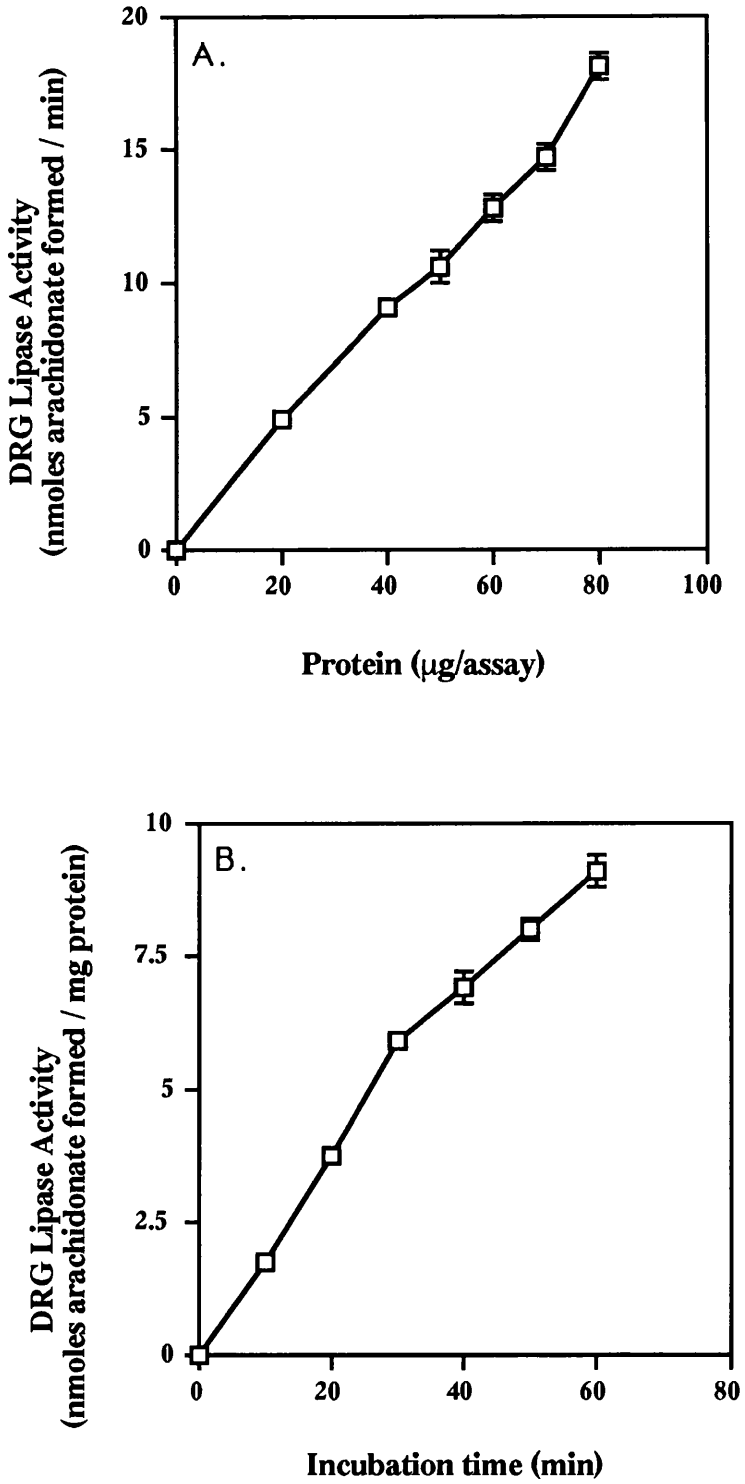


Figure 6.6 Effect of enzyme concentration and incubation time on DRG lipase activity in normal colon tissue

Arachidonate production was measured with various concentrations of normal human colon tissue with *sn*-1-stearoyl-2-arachidonylglycerol as substrate(A). Arachidonate production was also determined after incubation of samples (50μg) for various times (B). Each point is a mean ± SE of triplicate determinations and is representative of two independent observations showing similar results.

DRG lipase activity measured with 2-[^{14}C]SAG as substrate was not significantly different in normal mucosa (2.1 ± 0.2 nmoles arachidonate formed / min/ mg protein; range 1.5 - 3.3) and tumour tissue (1.8 ± 0.2 nmoles arachidonate formed / min/ mg protein; range 0.8 - 3.5) (Figure 6.7B). However, activity of this enzyme was significantly decreased in the tumour tissue in 8 patients and significantly increased in 3 patients.

6.3.4 Relative Changes in PAP, DRG Kinase, DRG Lipase and DRG Mass

The fold change in DRG mass, PAP, kinase and lipase activities in tumour compared to normal mucosa are summarised in Table 6.1. There was no clear correlation between the magnitude of the decrease in total cellular DRG and the changes in activity of the synthesising and metabolising enzymes.

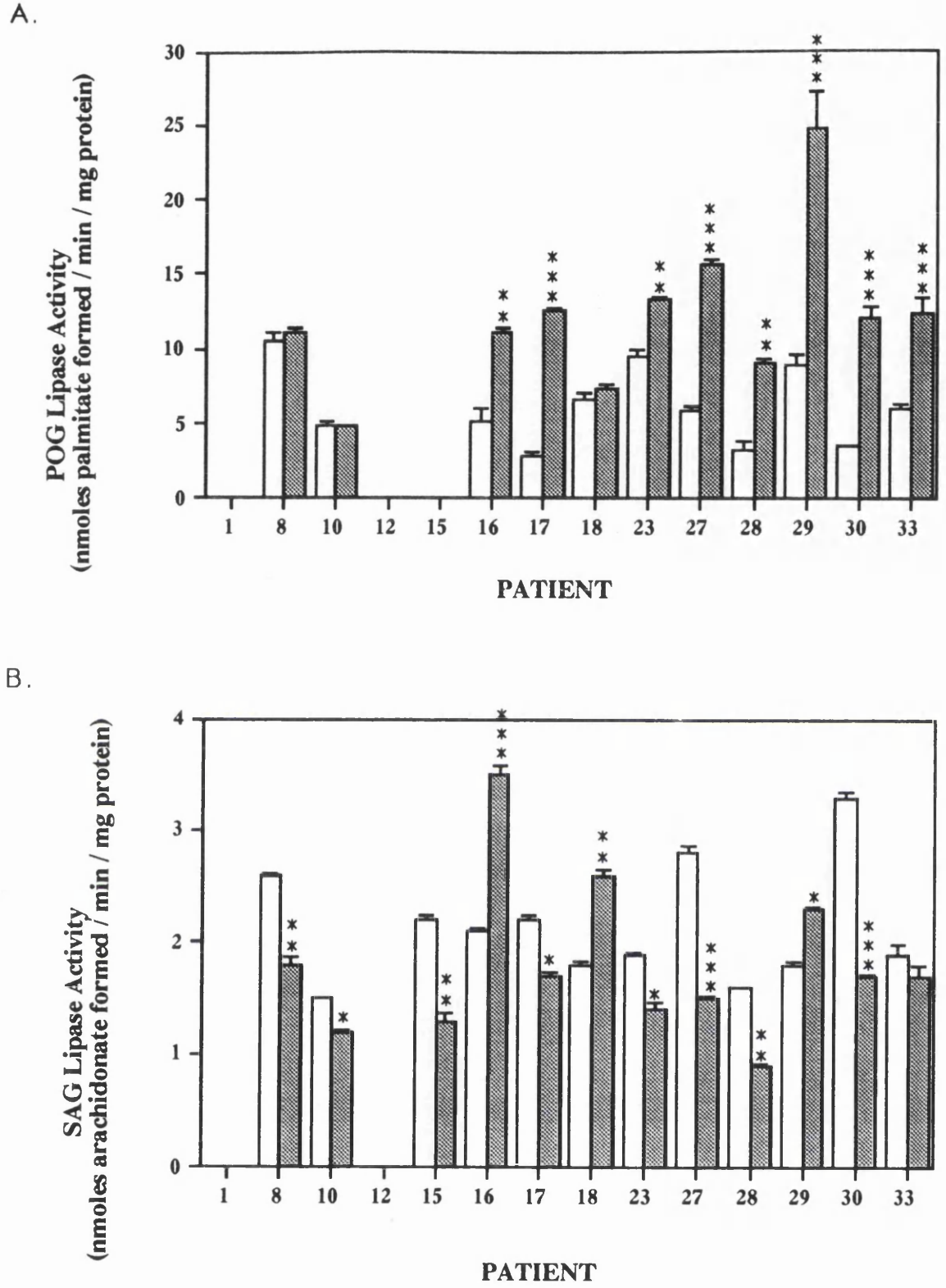


Figure 6.7 Specific activity of DRG lipase in paired colon tumour and adjacent normal tissue

DRG lipase activity in paired normal (□) and tumour tissue (▩) was determined with either (A) palmitoyl-2-oleoyl-*sn*-glycerol or (B) *sn*-1-stearoyl-2-arachidonylglycerol as substrate. Each assay contained 50μg protein. Results are the mean ± SE of triplicate determinations. There was inadequate tissue sample to measure activity in all patients. Asterisks represent statistically significant differences (Student's *t* test; * $p<0.02$, ** $p<0.01$, *** $p<0.001$).

Patient No.	DRG	PAP1	PAP2	SAG kinase	DOG kinase	SAG lipase	POG lipase
1	-2.1	+3.5	+2.3	+1.6	+1.2	ND	ND
8	-1.2	+1.9	+2.0	+2.3	+2.8	-1.4	+1.1
10	-1.6	+2.8	+1.5	+1.7	+1.2	-1.3	1.0
12	-1.3	+2.5	+2.4	+7.1	-1.6	ND	ND
15	-1.3	+1.4	+1.3	+2.5	+5.2	-1.7	ND
16	-3.8	+2.4	+1.5	-3.7	+1.3	+1.7	+2.1
17	-3.0	+1.3	+1.8	1.0	+1.1	-1.3	+4.5
18	-1.5	+2.6	+1.1	+2.6	+2.1	+1.4	+1.1
23	-1.6	+2.3	+2.8	+1.2	-1.4	-1.4	+1.4
27	+2.2	+2.2	+1.7	+1.1	+1.1	-1.9	+2.6
28	-3.0	+1.5	-1.1	-1.4	-1.3	-1.8	+2.8
29	-2.3	+4.0	+2.1	+4.1	+3.1	+1.3	+2.8
30	-1.5	+1.9	+2.0	+1.2	-1.4	-1.9	+3.5
33	-1.4	+1.8	+2.3	+2.1	+1.4	-1.1	+2.0

Table 6.1 Summary of results for changes in DRG mass, PAP, DRG lipase and DRG kinase activities in colon tumour

The results have been summarised as the fold change in enzyme activity or second messenger levels calculated as that in the normal tissue divided by that in the tumour tissue. SAG kinase represents the membrane bound form of the enzyme whereas DOG kinase is the non-specific enzyme which will phosphorylate all forms of DRG. The two lipase activities were measured using SAG and POG which represent substrates derived from phosphoinositides and PC respectively.

6.4 Discussion

DRG kinase and DRG lipase activities in the normal colon varied markedly between patients. In general, DRG kinase activity was increased in the tumour tissue and this increase was most consistent when SAG was used as the substrate. In contrast, DRG lipase activity was increased in the tumour when POG was used as the substrate but no consistent change in SAG-specific activity was observed.

A clear decrease in DRG mass in human colon tumour tissue has been shown in this and other work (Phan *et al.*, 1991). Previously, this was proposed to be the result of a decrease in the generaton of DRG from phosphoinositides or PC. However, the results presented in Chapter 4 indicated a significant increase in PAP2 activity (Figure 4.4) which could have accounted for the observed decrease in phosphatidate mass (Figure 4.6), but not the decrease in DRG. The lower DRG mass in the colon tumour tissue was, therefore, not indicative of decreased production but more a rapid clearance of this second messenger. DRG kinase and lipase activities each play predominant metabolising roles in different cell systems (Florin-Christensen *et al.*, 1993; Florin-Christensen *et al.*, 1992; Hee-Cheong & Severson, 1989), and it was these enzymes that were considered in the present study. It is difficult to make direct comparisons between the kinase and lipase activities measured in this study and those in previous reports. Other workers have utilised subcellular fractions as a source of enzyme activity such that membrane-associated and cytosolic activities could be distinguished. In this study, a colon tissue homogenate was used as a source of kinase and lipase activity and different DRG substrates were used as an indication of the specificity of these activities. Observed differences in enzyme activities based on those substrate specificities may not necessarily indicate different enzyme isoforms and the metabolism cannot be assigned to particular subcellular compartments.

In signalling, DRG is derived from phosphoinositides or PC (Wakelam *et al.*, 1991). If PC is a major source, as proposed (Cook & Wakelam, 1991a; Lassegue *et al.*, 1993), then the observed increase in PAP2 activity in colon tumour suggests an

increase in PC derived DRG metabolism is occurring. In this study, DOG was used as a substrate for kinase activity to represent DRG derived from PC. No overall significant increase or decrease in the activity of DRG kinase was observed using this substrate (Figure 6.5). This suggests that this enzyme is not a major route of metabolism of PC derived DRG. A similar result was found in another study using NIH 3T3 cells to examine the metabolic fate of [^{14}C]DOG. This was found to be preferentially converted, not to phosphatidate indicative of kinase activity, but to other lipids such as monoacylglycerol and fatty acid (Florin-Christensen *et al.*, 1992). This may be the case within colon tumour tissue, where DOG could be preferentially metabolised by other enzymes.

One of the predominant DRG species, POG, is probably generated from PC (Pessin *et al.*, 1990) as an intermediate within either signal transduction pathways or glycerolipid synthesis. Metabolism by lipase appeared to be a major route for removal of this DRG species in nearly all patient tumour samples as indicated by the increased activity in lipase using POG as the substrate (Figure 6.7). It is unclear from this study whether this occurs in the plasma membrane or endoplasmic reticulum. DRG metabolism by lipase is unlikely to occur in the plasma membrane, however, as the products from this route are not known to recycle and the membrane would eventually run out of PC. It probably occurs in the endoplasmic reticulum where PC can be produced by the action of choline phosphotransferase (Brindley, 1987). Complete metabolism of POG results in the release of oleate. This fatty acid has been shown to have several effects in the cell, for example, in isolated hepatocytes and perfused rat liver, translocation of PKC from cytosol to membranes was observed at physiological oleate concentrations, in particular, the PKCβ isoform. Oleate and related unsaturated fatty acids can also stimulate PKC activity in colonic epithelial cells (Craven & DeRubertis, 1988).

If phosphatidylinositol is the major source of DRG in signalling, an increase in phosphatidylinositol 4,5-bisphosphate derived DRG would be expected. This may be the case as a recent report showed an increased expression of PLCγ in human

colon tumours (Noh *et al.*, 1994; Park *et al.*, 1994a). It is not known if this increased expression is associated with increased activity of the enzyme, however, there is a possibility that there is an increase in DRG derived from this source. In addition, other work has shown that a decrease in membrane-associated DRG kinase activity in *ras* transformed fibroblast cells may have resulted in an observed increase in DRG mass (Huang *et al.*, 1988). This suggests a change in the activity of this enzyme can effect DRG levels. SAG was used as a substrate to measure both kinase and lipase activities and represents a phosphatidylinositol derived lipid (MacDonald *et al.*, 1988b). The activity of DRG lipase was not uniformly increased in colon tumour and, therefore, could not account for the overall decrease in DRG mass in this tissue (Figure 6.7). However, when DRG kinase activity was measured using SAG as substrate, this enzyme was found to have significantly higher activity in the tumour (Figure 6.5). Hence, a decrease in phosphatidylinositol derived DRG may be explained by the increased DRG kinase activity. This is a similar finding to NIH 3T3 fibroblasts in which [^{14}C]SAG was mainly converted to phosphatidate, indicating kinase activity (Florin-Christensen *et al.*, 1992). This activity in colon tissue may represent the membrane-bound form of DRG kinase which has been found in other tissues (Lemaitre *et al.*, 1990). The membrane-bound enzyme is specific for arachidonyl-containing DRG species (MacDonald *et al.*, 1988b) which may be active in a cycle within the plasma membrane. The phosphatidate formed by this enzyme can be subsequently converted back to phosphatidylinositol (MacDonald *et al.*, 1988a; MacDonald *et al.*, 1988b), and this raises the possibility that membrane-bound DRG kinase contributes to the unique fatty acid composition of phosphatidylinositols observed in animal cells (Berridge & Irvine, 1989). Not only would this maintain levels of arachidonyl-phosphatidylinositols for use in signalling (Rana & Hokin, 1990) but also other products are generated in the cycle. For example, arachidonyl-phosphatidate produced by the action of DRG kinase which may itself be metabolised by phospholipase A_2 activity to arachidonate. This can have several functions within the cell including activation of specific PKC isoforms (Asaoka *et al.*, 1992).

There are other routes for conversion of DRG which have not been considered in this study. For example, DRG can be converted to triacylglycerols by the action of DRG acyl transferase. This could occur in both the plasma membrane or endoplasmic reticulum. For instance, when [^{14}C]DOG was incorporated into fibroblast plasma membranes by a liposomal fusion technique, 23% of total cellular radioactivity was recovered in triacylglycerols (Florin-Christensen *et al.*, 1992) and use of kinase and lipase inhibitors did not influence this conversion. The increase in activities, therefore, of kinase and lipase enzymes in colon tumour tissue may not solely account for the observed decrease in total DRG mass.

There was a large interpatient variation in the activity of both the kinase and lipase enzymes. This does not reflect variation between assays since one patient sample was included in every experiment as a quality control. Even within the normal tissue there was a wide range in activity. For example, when DRG kinase activity was measured with DOG as substrate there was a 6-fold range in activities observed in the normal colon tissue. It is not clear why there is such a range in activity but it could be related to the diet of each patient. For instance, a high fat diet in some patients could be correlated to requirement for higher levels and activity of metabolising enzymes. It is not known if kinase and lipase activities are controlled by nutritional factors as has been shown for PAP1 activity (Brindley, 1985).

Using SAG as a substrate should represent a total kinase activity since both the SAG-specific and non-specific forms will utilise this lipid. Subsequently, kinase measurements with DOG as substrate represent only the non-specific form of the enzyme and activity in this case is expected to be lower than using SAG. This was not always the case, for example, in the normal colon tissue of patient 12 there was a much higher activity with DOG as substrate. It is possible, however, that the non-specific form in colon cannot utilise SAG to any great extent and that the two specific and non-specific activities should be regarded as separate entities. Overall, kinase activity was found to be higher than lipase in the colon tissues. This has also been observed in human platelets (Bishop & Bell, 1986). However, as these enzymes were

measured under optimal conditions in the test tube, it is difficult to be certain that the difference in activity between kinase and lipase enzymes would be physiological. An alternative method to measure these would have been to assay for both enzymes at the same time. This could have been carried out by determining release of [^{14}C]palmitate and formation of [^{14}C]phosphatidate in the presence of [^{14}C]POG and ATP/Mg^{2+}. In this way, the two enzymes would have to compete for substrate, which may have been a more physiological reflection of their activity. Using a similar technique, lipase activity had much higher activity in rabbit aortic smooth muscle cells (Severson & Hee-Cheong, 1989). This method may not be valid, however, as each enzyme cannot be measured under optimal conditions in the one assay.

It is not possible to correlate DRG mass results directly to enzyme activity. For example, a 2 fold increase in enzyme activity will not necessarily equate with a 2 fold increase in DRG mass. It is possible, however, to relate a change in enzyme activity to alterations in DRG. For example, the significant increase in some of the metabolising enzymes such as lipase, which was found to hydrolyse a predominant DRG species (POG), may account for at least some of the observed decrease in DRG mass. In contrast, as SAG is a fairly minor component of total DRG species (Pessin *et al.*, 1990), then increased metabolism by DRG kinase specific for SAG would probably account for only a minor part of the total DRG decrease.

In summary, the present data suggest that a DRG kinase activity may be eliminating DRG derived from phosphatidylinositols in the membrane and that lipase activity might be an important method for metabolising DRG from PC, probably in the endoplasmic reticulum. This appears to reflect changes in both signalling and glycerolipid synthesis and can, at least in part, explain the decrease in DRG mass in colon tumour tissue. The role of PAP in DRG production is far from clear at the moment because of the difficulty in understanding the relative contributions of several other pathways that generate or utilise DRG. When a clearer, unambiguous understanding of the sources and functions of DRG species is available, the role played by both PAP1 and PAP2 will hopefully be a lot easier to define. In addition,

colon tumours will have multiple changes as a result of several genetic alterations which occur during the progression of the colon tumour (Fearon & Vogelstein, 1990). It is unlikely that only one of these changes, such as a mutation in *ras*, is driving signalling abnormalities. This study has provided an insight into signalling in colon tumours, however, it is still unclear how important the observed changes are to the progression of the tumour. Other second messengers, such as cAMP, and signalling enzymes, such as the MAP kinase cascade, have not been considered here but these may well play important roles in colon tumour development.

CHAPTER SEVEN

MODULATION OF PAP ACTIVITY BY CATIONIC AMPHIPHILIC AGENTS

7.1 Introduction

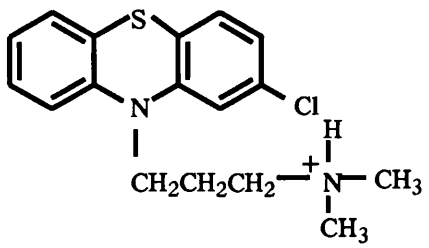
One of the underlying aims of this thesis is to determine whether PAP is a suitable target for anticancer drug development. The studies described in the previous chapters attempted to elucidate the role, if any, of PAP in the control of signal transduction in transformed cells. In both *ras* transformed fibroblasts and in human colon cancer epithelial cells which have a *ras* mutation, a decrease in PAP2 activity was observed. In contrast, when compared with the normal colon, PAP2 activity was increased in colon tumours. Neither system is ideal for the study of PAP activity but both show that PAP activity can be perturbed. A much cleaner approach would be to alter, directly, the activity of PAP in the cell. Since PAP has not yet been purified, it is not possible to overexpress the enzyme nor is it possible to reduce expression either by antisense oligonucleotides (Calabretta *et al.* , 1992) or by gene knockout (Capecchi, 1994). These approaches are all currently in use to study the role of various PKC isoforms (Dekker & Parker, 1994). However, cellular enzyme activities can be modulated by the use of specific activators or inhibitors. For example, the fungal metabolite, Wortmannin, has been used in cells to irreversibly inhibit phosphatidylinositol 3-kinase activity in mammalian cells. When added at nanomolar concentrations, this compound does not effect other kinases (Okada *et al.*, 1994). Wortmannin has been used in several studies such as inhibition of neutrophil activation by chemotactic-peptide (Okada *et al.*, 1994) and for both G protein and tyrosine kinase regulated phosphatidylinositol 3-kinase activities (Stephens *et al.*, 1994). The PKC inhibitor, Ro 31-8220, has been used by several workers to study the dependency of signalling pathways on PKC. For example, the activation of PLD

catalysed PC breakdown in EGF stimulated Swiss 3T3 cells was found to be independent of PKC activition. However, EGF induced PLD induction was shown to be coupled to tyrosine phosphorylation since the tyrosine kinase inhibitor, AG18, selectively inhibited the reaction (Cook & Wakelam, 1992).

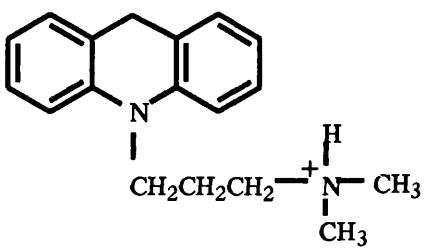
In the past, a number of cationic amphiphilic agents (CAAs) have been used to inhibit PAP. These compounds are characterised by having a hydrophobic domain and an ionizable nitrogen atom which is positively charged (Figure 7.1). One of these, propranolol, is used clinically as a β-adrenergic receptor anatgonist (Stiles *et al.*, 1984) but has also been shown to inhibit PAP activity (Jamal *et al.*, 1991). Efforts to study the role of this enzyme in biochemical pathways have often used synthetic CAAs such as propranolol to interfere with the PLD pathway at the level of PAP. For example, Perry and coworkers studied second messengers involved in activation of the respiratory burst in formyl methionyl leucyl phenylalanine (FMLP)-stimulated human neutrophils (Perry *et al.*, 1992). They used the CAAs propranolol and chlorpromazine to inhibit PAP in order to study the importance of DRG generation from the PAP/PLD pathway. Use of these agents resulted in an increase in phosphatidate and a decrease in DRG levels and a reduction in oxygen consumption. They suggested that DRG, derived from the PAP/PLD pathway, was essential for the respiratory burst in neutrophils. However, propranolol, and probably other CAAs, also effect other enzymes such as PKC (Sozzani *et al.*, 1992) and phosphatidylinositol-specific PLC activity (Das, 1988). It is not clear how the effects of the compounds on these enzymes would influence DRG levels. Furthermore, propranolol is not specific for PAP1 or PAP2, and hence *de novo* synthesis of DRG could also be effected in these cells. Although Perry and co-workers were aware of these problems, no attempt was made to asses if the CAAs effected any of these enzymes *in vitro*. Using a similar approach, comparable conclusions were made in a study of chemotactic-peptide stimulation of the same cells (Billah *et al.*, 1989).

There is a group of natural CAAs known as the sphingoid bases (Lavie *et al.* , 1990). These form the backbone of sphingolipids, such as sphingomyelin, which are

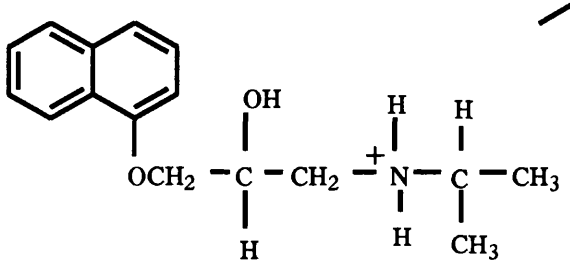
SYNTHETIC CAAs



Chlorpromazine

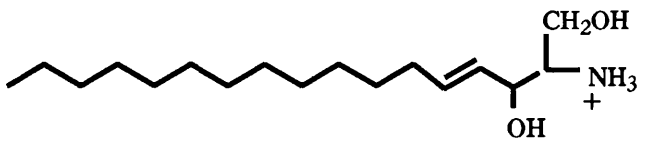


Imipramine

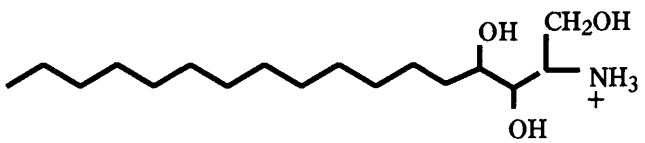


Propranolol

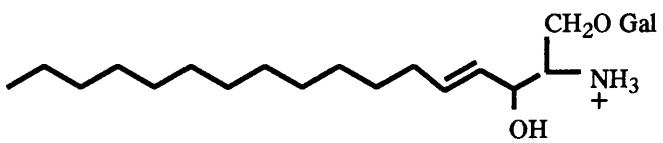
SPHINGOID BASES



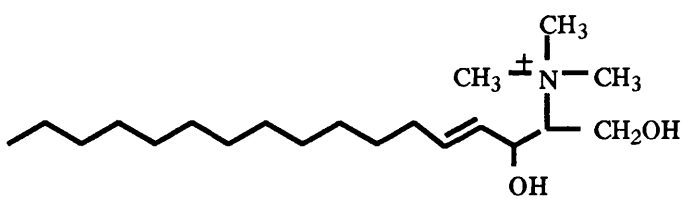
Sphingosine



Dihydro-sphingosine



Psychosine



N,N,N-trimethyl-sphingosine

Figure 7.1 **Structures of the synthetic and natural cationic amphiphilic agents (CAAs)**

important membrane constituents and are particularly concentrated in brain and nerve tissue. The sphingoid bases have a typical 2-amino 1,3-diol hydrophilic head structure and a long hydrophobic alkyl chain (Figure 7.1). They are regarded as a new class of bioregulatory molecules (Hannun & Bell, 1989; Merrill *et al.*, 1989) and endogenous sphingolipids are known to play a role in the regulation of cell growth, differentiation and neoplastic transformation (Liscovitch & Lavie, 1990; Merrill & Stevens, 1989; Michell & Wakelam, 1994). Sphingosine has been shown to inhibit both PAP1 and PAP2 activity when included in the same cell free assay system described in Chapter 2 (Gomez-Munoz *et al.*, 1992; Jamal *et al.*, 1991) and has been used to inhibit PAP in whole cells (Lavie *et al.*, 1990; Mullmann *et al.*, 1991). However, these compounds, like the other CAAs, are not specific for PAP. DRG was shown to be decreased following treatment of NG108-15 neural-derived cells with sphingosine (Lavie & Liscovitch, 1990). Since PKC activity is also decreased in these cells it was proposed that inhibition of PAP reduced the level of DRG and this in turn resulted in down-regulation of PKC activity. However, as this compound can also activate an 80kDa DRG kinase enzyme in the same cells (Sakane *et al.*, 1989), sphingosine may also decrease DRG levels by a concomitant stimulation of DRG metabolism. In addition, sphingosine can inhibit PKC activity directly through interference with the binding of PKC to the ternary complex formed at the plasma membrane between calcium, phosphatidylserine and DRG (Hannun & Bell, 1987). Thus, sphingosine could reduce DRG levels both by inhibition of PAP activity and by stimulated DRG kinase and inhibit PKC activity by both direct and indirect mechanisms.

Work on the CAAs carried out some time ago showed that the mechanism of PAP inhibition is related to the interaction of the amine with the anionic substrate, rather than a direct interaction with the enzyme (Bowley *et al.*, 1977). This was concluded from enzyme kinetic studies that used chlorpromazine to inhibit PAP activity. The inhibition was found to be of a competitive type in which chlorpromazine interacted with phosphatidate, such that it was a poor substrate for PAP. If this is the mechanism of action, then it would explain why these compounds

effect the metabolism of a wide range of acidic lipids and modify the activity of enzymes that are regulated by acidic lipids. For example, propranolol effects PKC activity by modifying the ability of phosphatidylserine to activate the kinase (Sozzani *et al.*, 1992).

It may be possible to produce analogues of lead compounds which have increased selectivity for the target enzyme. This can be through the simple use of a single isomer of a particular compound. For example, the L-isomer of propranolol is much more active as a β-blocker than the D-isomer (McEvoy *et al.*, 1990). A more complicated approach has been to alter the chemical structure of the parent compound. Modification of the PKC inhibitor, staurosporine, which itself is not selective for PKC, has resulted in the production of agents which are more specific for this enzyme. One derivative of staurosporine produced by Ciba Ceigy, CGP 41 251, shows a high degree of selectivity towards PKC and, in addition, antitumour activity both *in vitro* and *in vivo* (Meyer *et al.* , 1989). In fact, this compound is about to enter clinical trial in Glasgow as an anticancer agent in 1995. A number of staurosporine analogues have been developed by Roche and these are widely used as tools to study the involvement of PKC in various signalling pathways (Uings *et al.*, 1992). A similar approach can be envisaged for PAP inhibitors. Compounds such as propranolol or sphingosine, which are already used as PAP inhibitors, might be potential lead compounds for the development of more specific analogues. These compounds would not only provide useful laboratory tools for studying the function and importance of PAP in cells, but might also be potential anticancer agents.

The CAAs were therefore investigated as potential inhibitors of PAP. A range of synthetic and naturally occuring CAAs (Merrill & Stevens, 1989) were examined for their ability to inhibit PAP activity *in vitro*. Whilst this approach should identify compounds that are potent inhibitors of PAP, it takes no account of the potency of the compound in the whole cell. Clearly, this will also be determined by the stability of the agent and its ability to enter the cell and to gain access to the enzyme within the cell. The CAAs were, therefore, tested for their ability to inhibit cell proliferation *in*

vitro. Two assays in routine use in the Department of Medical Oncology were chosen. A tetrazolium-dye based microtitration assay, modified from that used by the USA National Cancer Institute to screen for potential antitumour agents, was used (Plumb *et al.*, 1989). This assay includes a fixed drug exposure time and a regrowth period in drug free medium. The regrowth period allows cells to recover from any short-term non-lethal damage. However, since inhibitors of signal transduction pathways may cause cytostasis rather than cell death, the assay may not detect their activity. Thus a short term assay, [^{3}H]thymidine incorporation, was also used. The cell lines used were the NIH 3T3 fibroblasts and one of the *ras* transformed variants shown to express increased activity of PAP1 but decreased activity of PAP2 (Chapter 3, Table 3.2).

7.2 Materials and Methods

7.2.1 Chemicals and Reagents

Cis-diammine-dichloroplatinum II (cisplatin), tamoxifen, bis-chloroethylnitrosourea (BCNU), etoposide (VP-16), psychosine, sphingosine, dihydro-sphingosine, propranolol, chlorpromazine, imipramine and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (Poole, Dorset). N,N,N-trimethyl-sphingosine was a gift from Professor Igarashi (University of Washington, Washington D.C., U.S.A). [^{3}H]Thymidine was from Amersham (Little Chalfont, Buckinghamshire).

7.2.2 Cell Lines

The cell lines used were the mouse fibroblast cell line, NIH 3T3 I, and the sub-line transformed with v-Ha-*ras* (HT3 I). Details of these cell lines and their routine maintenance is described in section 3.2.3.

7.2.3 Drugs

Chlorpromazine, propranolol, imipramine, sphingosine and the anticancer agents were all prepared as 30mM stock solutions in dimethylsulphoxide (DMSO). The other sphingoid bases were also prepared in DMSO at a stock concentration of 10mM. In the viability assays, propranolol, imipramine, psychosine and BCNU were used at a range of concentrations between 0 and 200μM by diluting the stock solution in culture medium. The other compounds were diluted in the same way and used at a concentration range between 0.1 and 100μM. A solvent control was used in the assays.

7.2.4 Cytotoxicity Assay

Cytotoxicity was determined using a tetrazolium-based microtitration assay (Plumb*et al.*, 1989). Cells were seeded (10^3 cells/well) into 96 well microtitration plates (Linbro, Flow Laboratories, Irvine, Scotland) in 200µl of medium (DMEM + DCS, 10%). The first and last row of 8 wells contained medium only. Plates were incubated for 48h at 37°C in an atmosphere of 2% CO_2 in air to allow the cells to attach and grow. Medium from the wells was then replaced with medium containing a range of drug concentrations. Eight concentrations were used for each drug and four wells per concentration. After a 24h incubation period, drugs were removed and cells fed daily for a further three days. At the end of the growth period the cells were fed with fresh medium and MTT (50µl; 5mg/ml PBS) was added to each well. Plates were wrapped in aluminium foil and incubated for a further 4h. Medium and MTT were removed from the wells and the formazan crystals dissolved in 200µl DMSO added from a dispenser (Welltech Wellfill 3: Denley, Sussex, England). Sorenson's glycine buffer (25µl; glycine (0.1M) plus NaCl (0.1M), adjusted to pH 10.5 with NaOH (1M)) was added to adjust the final pH and the absorbance recorded at a wavelength of 570nm in a plate reader (Model 3550 ELISA plate reader, Bio-Rad Laboratories Ltd., Watford, England). The first and last rows of 8 wells which contained medium and MTT only were used to blank the plate reader. Results were expressed as the IC_{50} concentration which was determined as the drug concentration required to reduce the absorbance to half that of control untreated cells.

7.2.5 [^{3}H]Thymidine Incorporation

Cells were seeded at a density of 10^3 cells/well into 96 well plates in 200µl medium. The cells were allowed to attach and grow for three days. Drugs were added to triplicate wells at a range of concentrations in fresh medium. Control wells contained medium only. [^{3}H]Thymidine (0.1µCi/well) was added immediately after

drug addition. After a 24h incubation period, drug and radiolabel were removed, the cells washed twice in ice-cold PBS and 50µl of trypsin solution (0.2% in PBS) was added to each well. The cells were incubated for 30min at 37°C and an LKB cell harvester (1295-001 cell harvester, Pharmacia LKB, St.Albans, Hertfordshire) was used to transfer the contents of each well to filter paper. Each filter contained the contents from one 96 well plate and was mixed with 10ml scintillation fluid in a sealed plastic bag. Radioactivity was determined with a Beckman 1205 Betaplate scintillation counter. Results are expressed as the IC_{50} concentration which was calculated as the amount of drug required to reduce the counts per minute (CPM) to half that of the control untreated cells.

7.2.6 Effect of Modulators on PAP Activity

Cytosolic and plasma membrane fractions from rat liver were used as a source of PAP1 and PAP2 enzymes. Preparation of the fractions has already been described in Section 2.2.3. The drugs were used at a range of concentrations between 0 and 1.5mM. The DMSO concentration was always less than 5% in the assay and a solvent control was used. PAP1 and PAP2 enzyme activities were measured as described in section 2.2.4. The drug to be tested was included in the reaction mixture and following addition of the enzyme preparation, samples were preincubated as described for 10min at 37°C. The reaction was then started by addition of the substrate. Enzyme kinetic analysis was carried out on PAP2 using four concentrations of substrate (0.02, 0.06, 0.2, 0.6 mM) and various concentrations of sphingosine (0, 0.1, 0.3, 0.8, 1.2 and 1.5 mM).

Samples were assayed in triplicate and the inhibition values expressed as a % of the rate measured in the absence of drug. Enzyme kinetic data was analysed by the "Enzyme Kinetics" package as described in Chapter 2.

7.3 Results

7.3.1 Effect of the CAAs on Cell Proliferation

The cytotoxicity of various CAAs towards NIH 3T3 I and *ras* transformed fibroblast cells (HT3I) is shown in Table 7.1. Toxicity of a range of established anticancer agents was included for comparison. The CAAs were all toxic to cells with IC_{50} values in the micromolar range which were comparable to those of the anticancer agents. Sphingosine and dihydro-sphingosine were the most active of the CAAs in both types of assay whilst psychosine showed least activity. The two cell lines were equally sensitive to the majority of CAAs when sensitivity was determined by MTT reduction. However, differences were apparent when sensitivity was determined by [^{3}H]thymidine incorporation. For example, HT3I was less sensitive to sphingosine and dihydro-sphingosine ($p<0.0026$ and $p<0.0054$, respectively) than NIH 3T3I. The opposite was true of N,N,N-trimethyl-sphingosine where the IC_{50} was lower in the transformed cells ($p<0.021$). Otherwise, the MTT assay and [^{3}H]thymidine incorporation assay gave similar results except for N,N,N-trimethyl-sphingosine where both lines appeared more sensitive when assessed by [^{3}H]thymidine incorporation ($p<0.0032$).

7.3.2 Effect of CAAs and Anticancer Drugs on PAP Activity

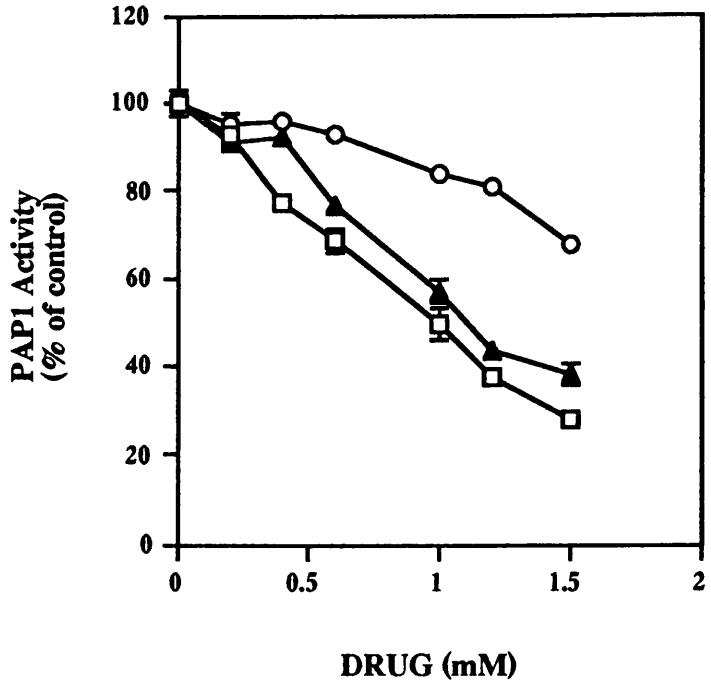
The effects of propranolol, imipramine and chlorpromazine on PAP1 and PAP2 activities are shown in Figure 7.2. Partial inhibition of these enzymes was observed with the synthetic CAAs but at the concentrations used in this study, complete inhibition was not achieved. The sphingoid bases were all strong inhibitors of the PAP1 enzyme activity (Figure 7.3A). Psychosine and N,N,N-trimethyl-sphingosine were the most potent with IC_{50}s of 75μM and 80μM respectively. At similar concentrations, these drugs had no affect on PAP2 activity (Figure 7.3B).

	ID_{50} (μM)			
	MTT		[^{3}H]Thymidine	
DRUG	**NIH 3T3 I**	**HT3 I**	**NIH 3T3 I**	**HT3 I**
Cisplatin	8.6 ± 0.7	10.1 ± 0.7	7.2 ± 1.3	3.0 ± 0.7
Tamoxifen	23.1 ± 0.5	29.8 ± 0.9	10.5 ± 1.5	25.5 ± 1.6
BCNU	19.8 ± 1.4	41.5 ± 1.4	87.3 ± 4.3	31.2 ± 2.1
VP-16	8.5 ± 0.7	10.7 ± 0.9	6.0 ± 1.3	3.1 ± 0.5
Psychosine	96.0 ± 2.5	102.4 ± 2.8	79.2 ± 8.7	70.4 ± 5.8
Sphingosine	3.3 ± 1.0	4.7 ± 0.4	9.1 ± 0.9	27.6 ± 2.2
Dihydro-sphingosine	7.1 ± 0.7	5.3 ± 0.5	4.1 ± 0.5	15.8 ± 1.5
Trimethyl-sphingosine	56.5 ± 4.5	45.3 ± 2.7	27.6 ± 2.5	11.5 ± 1.8
Propranolol	45.6 ± 2.8	63.3 ± 2.3	40.5 ± 2.3	120.2 ± 3.1
Chlorpromazine	/	/	17.5 ± 2.1	24.1 ± 2.1
Imipramine	34.1 ± 2.4	49.3 ± 2.9	21.2 ± 1.5	60.8± 6.6

Table 7.1 **Sensitivity of untransformed (NIH 3T3 I) and *ras* transformed (HT3 I) fibroblast cells to various cationic amphiphilic agents and anticancer drugs**

Cells were exposed to the indicated agents for 24h and cytotoxicity was determined either by MTT dye reduction or by [^{3}H]thymidine incorporation. Results are the mean ID_{50} ± SE of triplicate experiments.

A.



B.

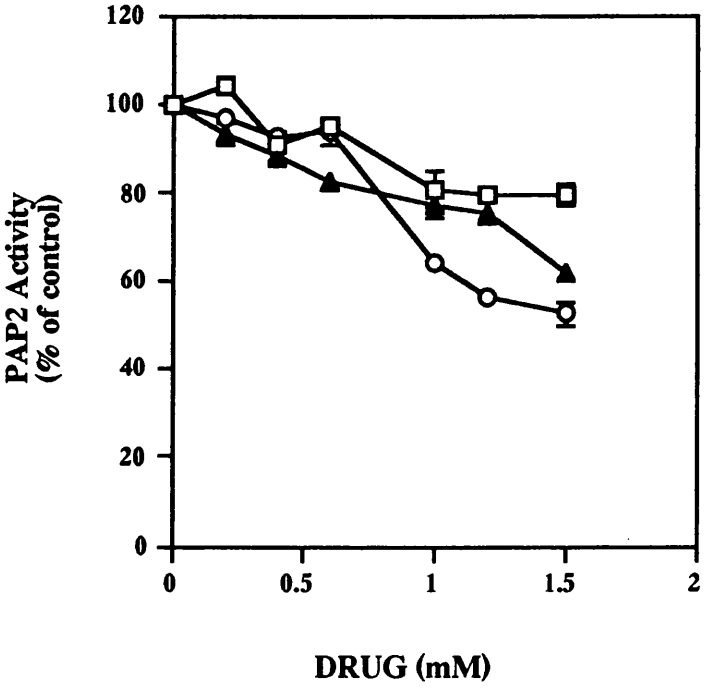
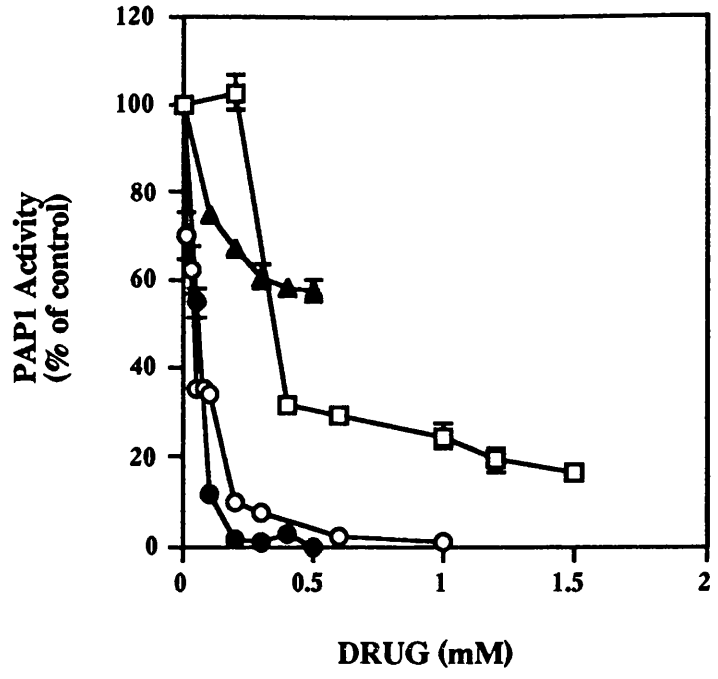


Figure 7.2 Effect of synthetic cationic amphiphilic agents on PAP1 and PAP2 enzyme activities

PAP activity was measured in the presence of increasing concentrations of propranolol (□), chlorpromazine (▲) and imipramine (○) as indicated. Rat liver cytosol (PAP1) (A) and plasma membrane (PAP2) (B) fractions were used as sources of the enzymes using 20μg protein in the assay. Each experimental determination is the mean ± SE of triplicate measurements and is representative of two independent observations showing similar results. Results are expressed as % of control.

A.



B.

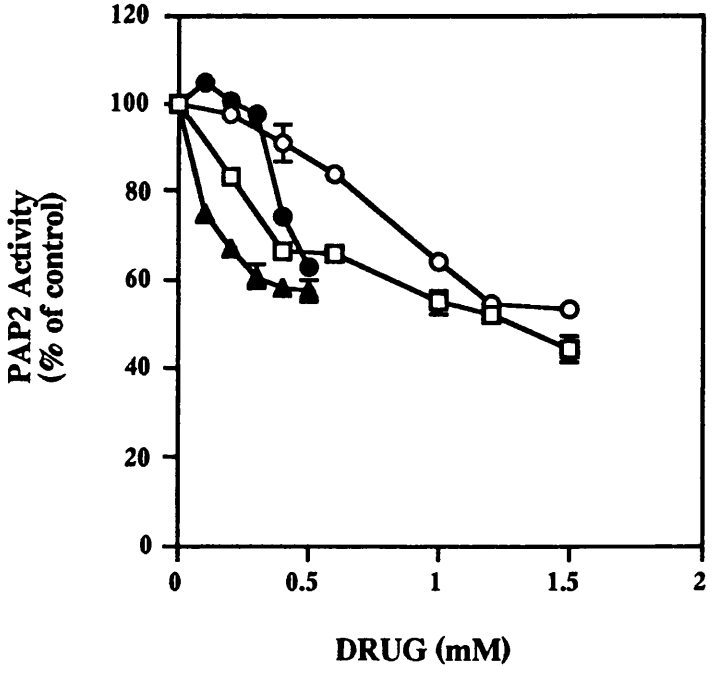
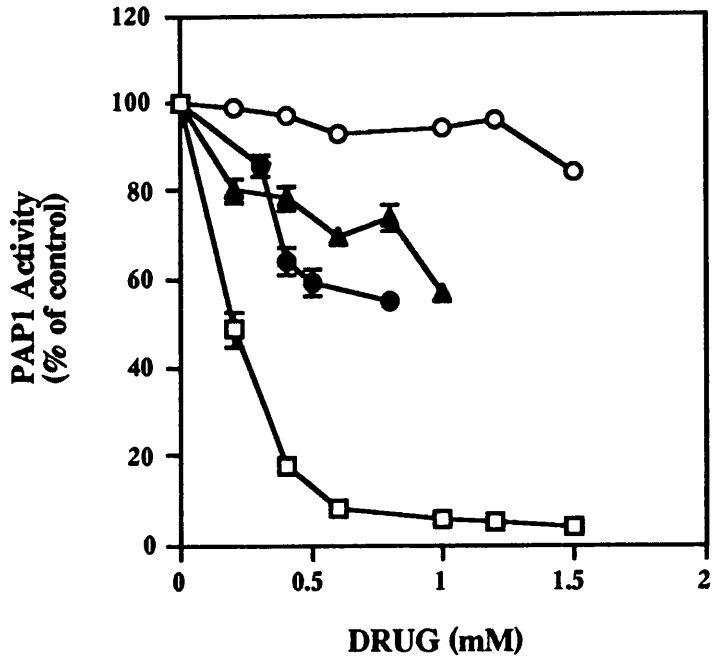


Figure 7.3 Effect of sphingoid bases on PAP1 and PAP2 enzyme activities
PAP activity was measured in the presence of increasing concentrations of sphingosine (□), dihydro-sphingosine (▲), psychosine (○) and trimethyl-sphingosine (●) as indicated. Rat liver cytosol (PAP1) (A) and plasma membrane (PAP2) (B) fractions were used as sources of the enzymes using 20μg protein in the assay. Each experimental determination is the mean ± SE of triplicate measurements and is representative of two independent observations showing similar results. Results are expressed as % of control.

Sphingosine and analogues of sphingosine only partially inhibited PAP2 activity, by about 40%, at concentrations which inhibited almost totally PAP1 activity. Overall, this group of compounds were stronger inhibitors of both PAP1 and PAP2 than the synthetic CAAs. None of the established anticancer agents significantly inhibited PAP2 activity, however, tamoxifen and VP-16 partially inhibited PAP1 enzyme activity and cisplatin completely inhibited this enzyme at a concentration of 1mM (Figure 7.4).

Addition of sphingosine reduced the rate of the reaction of PAP2 at all substrate concentrations used (Figures 7.5A). Furthermore, the magnitude of the inhibition increases with increasing concentrations of sphingosine. From this limited study, it appears that sphingosine does not reduce the Vmax of the reaction. This observation is supported by the Lineweaver-Burk plot (Figure 7.5B), although it should be noted that at high concentrations of sphingosine the data does not transform to a linear plot. This is further demonstrated in the Eadie-Hofstee plot (Figure 7.5C). At the lowest concentrations of the inhibitor used (0.1 and 0.3mM) the slope (Km) is unchanged but the Vmax (y intercept) is decreased. This contrasts with the interpretation of the double reciprocal plot. However, at higher concentrations of sphingosine, the Eadie-Hofstee plot is non-linear and cannot be interpreted.

A.



B.

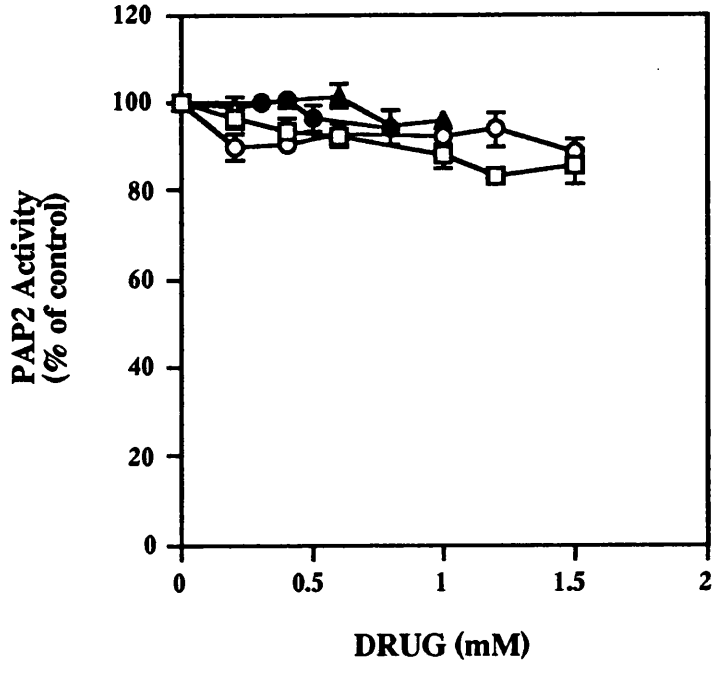
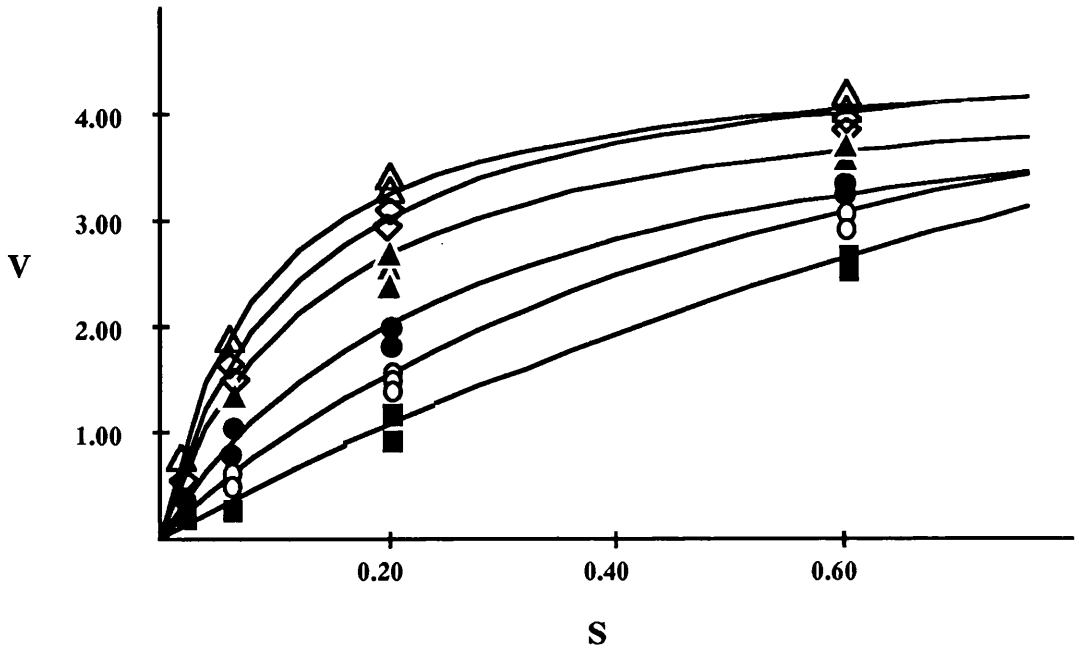


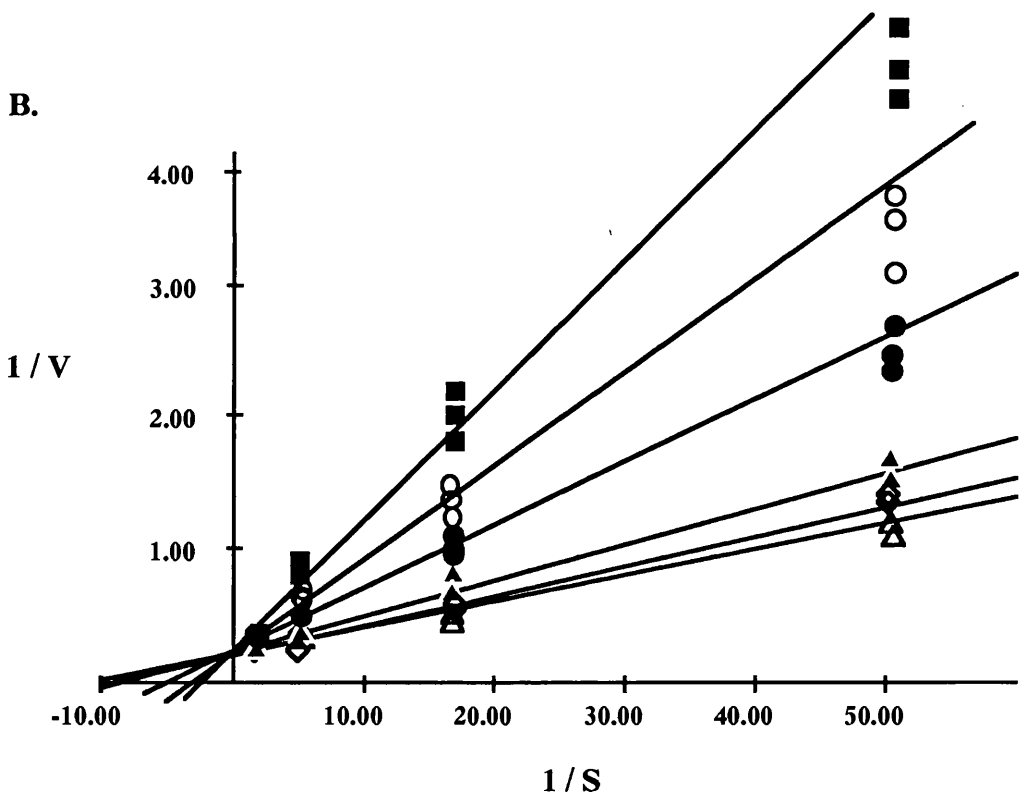
Figure 7.4 Effect of various anticancer agents on PAP1 and PAP2 enzyme activities

PAP activity was measured in the presence of increasing concentrations of cisplatinum (□), VP-16 (▲), BCNU (○) and tamoxifen (●) as indicated. Rat liver cytosol (PAP1) (A) and plasma membrane (PAP2) (B) fractions were used as sources of the enzymes using 20μg protein in the assay. Each experimental determination is the mean ± SE of triplicate measurements and is representative of two independent observations showing similar results. Results are expressed as % of control.

A.


4.00
3.00
V
2.00
1.00
0.20
0.40
0.60
S

B.


4.00
3.00
1 / V
2.00
1.00
-10.00
10.00
20.00
30.00
40.00
50.00
1 / S

C.

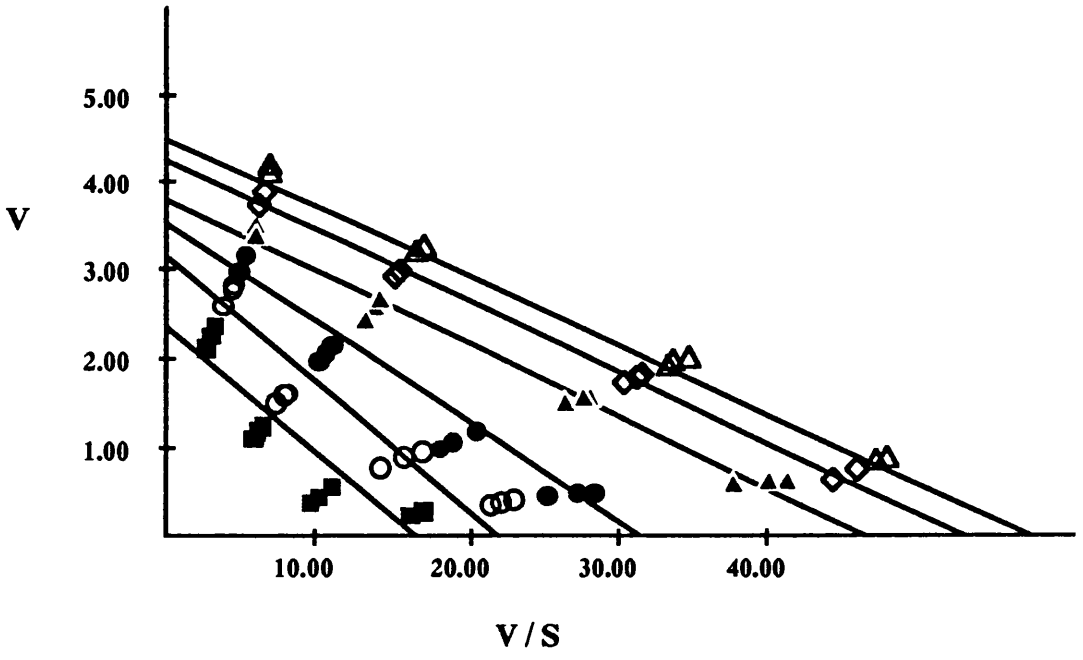


Figure 7.5 Effect of increasing concentrations of sphingosine on the rate of reaction of PAP2

PAP2 activity was measured at a range of substrate concentrations in the presence of increasing concentrations of sphingosine (mM): 0 (Δ), 0.1 (◇), 0.3 (▲), 0.8 (●), 1.2 (○) and 1.5 (■). In A, rate of reaction, v (nmoles DRG formed / min / mg protein), is plotted against substrate, s (mM), whereas B represents a Lineweaver-Burk plot (1/v against 1/s) and C is an Eadie-Hoftsee plot (v against v/s). This graph shows triplicate determinations from one of two independent observations showing similar results.

7.4 Discussion

The CAAs have been used in the past to inhibit PAP activity in whole cells. All the CAAs were shown to be cytotoxic at concentrations comparable to standard anticancer agents. The sphingoid bases were the most potent inhibitors of cell growth and were also shown to be potent inhibitors of both PAP1 and PAP2 enzyme activities.

The cytotoxic properties of the CAAs were determined in an attempt to establish whether inhibition of PAP effects cell growth. MTT and [^{3}H]thymidine incorporation assays were used to screen the compounds and the CAAs showed activity in the two assays at concentrations of between 3 and 100μM (Table 7.1). Although neither assay indicates whether the compounds were cytostatic or cytotoxic, it does show that they are somehow affecting fibroblast cell growth.

On the whole, the two assays gave similar results but there were some inconsistencies. N,N,N-trimethyl-sphingosine was apparently more active when cytotoxicity was determined by the [^{3}H]thymidine incorporation assay (Table 7.1). This could be explained if the drug was causing a cell cycle block at G0/G1 thus preventing cells from entering S phase and incorporating [^{3}H]thymidine into their DNA. In the MTT assay there is a three day regrowth period during which the cells can recover from the initial effect of the drug. This observation suggests that N,N,N-trimethyl-sphingosine is perhaps antiproliferative rather than cytotoxic. It might be expected that drugs which act by inhibiting signal transduction pathways would have a cytostatic rather than a cytotoxic effect. That is, they block proliferation but do not necessarily kill tumour cells in the same way that is associated with classic anticancer drugs. If the drug had been cytotoxic this would result in a terminal reaction and no recovery would occur in the MTT assay. Compounds such as cisplatin or dihydrosphingosine were probably cytotoxic and there was no significant difference between short term and long term assays.

It has been suggested that CAA compounds act by competing with the enzyme for a binding site on the substrate (Bowley *et al.*, 1977). If the reaction involves either a direct or indirect interaction with PAP in whole cells, the observed difference between enzyme activity and phosphatidate levels in the NIH 3T3I and HT3I lines might be reflected by differences in the growth inhibitory effects of the compounds. In this case, the CAAs would be expected to have more effect in the cell line with the highest PAP activity and phosphatidate levels (HT3I). The majority of the CAAs were actually less active in the transformed cells (Table 7.1). This could be explained if the inhibition of cell growth by these compounds is through mechanisms other than PAP. Alternatively, the main inhibitory effect may be through PAP2 activity which is lower in HT3I cells than in the NIH 3T3I cells (Chapter 3, Table 3.2). One of the CAAs, N,N,N-trimethyl-sphingosine, was more active in the *ras* transformed cell lines in both assays. This indicated that at least one of the mechanisms of action of this compound could be through PAP1.

Sphingosine and psychosine are structurally very similar compounds differing only in that psychosine has a galactose group on the positively charged amphiphilic head group. This made a significant difference in activities since psychosine was much less effective than sphingosine in terms of growth inhibition (Table 7.1). Psychosine was, however, the more potent in terms of PAP1 inhibition. This activity may not have been observed in the whole cells due to poor uptake of the drug by the fibroblasts. Endo and co-workers showed a stronger inhibitory effect of N,N,N-trimethyl-sphingosine compared to sphingosine in the *in vitro* [^{3}H]thymidine incorporation assay (Endo *et al.*, 1991). This was seen for HT3 I cells in this study but not for the NIH 3T3 I cells. These investigators used different cell lines in their study but they also presented the drugs to the cells in a different manner. In this study, the drug was dissolved in DMSO and subsequently diluted in medium for application to cells and this is a common practice (Franson *et al.* , 1992; Hannun *et al.* , 1986; Jamal *et al.*, 1991; Mullmann *et al.*, 1991). However, other groups have prepared the sphingoid bases in a complex with BSA (Aridor-Piterman *et al.*, 1992; Endo *et al.*,

1991; Merrill & Stevens, 1989). This keeps the drug in suspension and is thought to aid in uptake into cells. Hence, a difference in drug presentation or cell lines may account for the observed differences between this study in which N,N,N,-trimethyl-sphingosine was found to be less potent than sphingosine and another study in which the opposite was observed (Endo *et al.*, 1991).

In addition to screening the activity of these compounds in whole cells, the specificity of the drugs for PAP1 and PAP2 was also investigated by incorporating them into the enzyme activity assays. In the cell free PAP assay, there are no problems of access in which a drug has to penetrate the plasma membrane to reach the enzyme. In addition there would not be the same problems with competition for other binding sites as in whole cells. For example, in whole cells the CAAs may also inhibit PKC by a direct interaction with this enzyme (Sozzani *et al.*, 1992). One might expect, therefore, that an enzyme measured in isolation of cells would be inhibited at even lower concentrations than in whole cells if that compound was acting through PAP. However, milli molar concentrations of the majority of the compounds were required to inhibit the enzymes in the PAP assay (Figures 7.2 and 7.3). This is probably due to the fact that concomitantly high concentrations of drug, substrate and enzyme are required to optimise conditions for measuring PAP activity in the cell free assay. This makes relating the results to whole cells difficult. However, the enzyme assay does indicate whether the compounds directly effect PAP, and if so, how specific they are for PAP1 or PAP2. For instance, N,N,N-trimethylsphingosine and sphingosine inhibit both forms of the enzymes in the PAP assay with IC_{50}s of 80 and 200μM for PAP1 respectively (Figure 7.3), which is a similar result to those previously reported for sphingosine (Jamal *et al.*, 1991; Lavie *et al.*, 1990). The concentrations required for inhibition of cell growth *in vitro* are much lower as shown in this and other work (Endo *et al.*, 1991; Park *et al.* , 1994b). It is not known whether the observed inhibitory affect in the PAP assay would occur in whole cells. These compounds probably produce their effects through a variety of antisignalling events. One of the mechanisms of action of these drugs was proposed to be through

modulation of PKC activity (Igarashi *et al.* , 1989). Thus, the effects of the compounds on cell proliferation support the proposed lack of specificity of these lead compounds.

Anticancer agents with known mechanisms of action were incorporated into the assay for PAP activity as controls. For example, cisplatin causes cell damage through production of inter-strand and intra-strand DNA adducts at micro molar concentrations. Surprisingly, this drug was also found to inhibit PAP1 activity with an even lower IC_{50} value than propranolol (Figure 7.4). This mechanism of interaction with PAP or its substrate is unknown, although it may involve the platinum integrating into the substrate, perhaps displacing Mg^{2+}. This inhibition is unlikely to occur in cells. HT3I has a much higher PAP1 activity compared to NIH 3T3I but is not any more sensitive to cisplatin which indicates that this drug does not inhibit PAP1 in intact cells (Table 7.1).

Enzyme kinetic studies have shown that chlorpromazine is competitive with PAP for the binding site on the substrate and it was suggested that other synthetic CAAs may act in the same way. The reaction is thought to involve a direct interaction between the positively charged head group and the negatively charged phosphatidate substrate (Koul & Hauser, 1987; Pappu & Hauser, 1983) thus preventing the enzyme-substrate complex forming. Since sphingosine was one of the few compounds that inhibited PAP2 activity, this sphingoid base was used in enzyme kinetic studies in an attempt to determine if the mechanism of inhibition of this enzyme is the same as the other CAAs. Sphingosine shares important structural features with propranolol, such as a positively charged amine group and a hydrophobic domain and thus its mechanism of PAP inhibition could be similar. It has already been shown that the PAP1 enzyme does not follow Michaelis-Menton kinetics (Chapter 2, Figure 2.6) and further inhibition studies were not carried out on this enzyme. One of the most commonly reported methods for estimating Km and Vmax is the double-reciprocal Lineweaver-Burk plot (1/v against 1/s) as described previouly (Chapter 2), however, this is not the only graphical method. The Eadie-Hofstee (Eadie, 1942; Hofstee, 1952)

(v against v/s) plot is another common method for linearising the data. Inhibition of PAP2 activity by sphingosine resulted in kinetic profiles which were difficult to interpret. It appeared that at high drug concentrations the best fit line became non-linear (Figure 7.5). Like PAP1, there may be factors in the assay which interfere with the kinetics of PAP2. It is unknown, for instance, how Triton X-100 effects the kinetics of PAP2 and it should be noted that the method of substrate presentation to the enzyme is far from ideal when compared with the close association of enzyme and substrate in the whole cells. The observed conflicting competitive and noncompetitive interpretation of the interaction may be accounted for by different forms of interaction between sphingosine and phosphatidate. A detailed kinetic analysis of the PAP enzymes will clearly require purification of these enzymes.

Sphingosine has been shown to inhibit both PAP1 and PAP2 *in vitro* and to inhibit cell proliferation. This compound was therefore chosen as the lead compound in a synthesis programme to develop more specific inhibitors of PAP activity. This is a joint project between the Departments of Medical Oncology and Chemistry at Glasgow University. Sphingosine is recognised as a potent inhibitor of PKC (Aridor-Piterman *et al.*, 1992; Endo *et al.*, 1991; Merrill & Stevens, 1989) and the initial screen of the compounds will therefore concentrate on selection between PKC and PAP inhibition. However, a number of other related enzymes will also be included. Development of a specific inhibitor of PAP will clearly aid in the study of the role of PAP, if any, in the control of cell proliferation. There is also the possibility that such a compound may possess antitumour activity.

CHAPTER EIGHT

GENERAL DISCUSSION

The aims of this thesis have essentially been achieved. The aims were:

(i) to establish an assay for measuring PAP1 and PAP2 enzyme activities and determine their tissue distribution.

(ii) to determine whether PAP activity is altered in *ras* transformed fibroblasts.

(iii) to measure PAP activity in colorectal cancer and in adjacent normal tissue and to relate changes in PAP to levels of the second messengers phosphatidate and DRG.

(iv) to determine the effects of known PAP inhibitors on enzyme activity and cell proliferation.

(v) to establish whether PAP is a potential target for anticancer drug development.

An assay was established for measurement of PAP activity. Two activities can be distinguished mainly based on their sensitivity to NEM. PAP1, thought to be involved in glycerolipid synthesis in the endoplasmic reticulum, is sensitive to NEM whereas PAP2, the signalling form located in the plasma membrane, is insensitive to this compound. It is unclear whether PAP is one enzyme with different properties in the different subcellular compartments, or if PAP1 and PAP2 represent two separate enzymes. Purification and cloning of these enzymes from both the plasma membrane and endoplasmic reticulum will determine this. Partially pure preparations obtained by fractionation of rat liver were used as a source of the enzymes to characterise the different activities. However, the differential sensitivity to NEM allows measurement of the two activities in a cell or tissue homogenate without the need for fractionation.

PAP2 displayed Michaelis-Menton kinetics where activity increased with substrate concentration until a maximum velocity was obtained. However, the kinetics were not so clear for the PAP1 form. This enzyme did not display typical Michaelis-Menton kinetics but showed a sigmoid dependence on substrate. This can

be indicative of an allosteric enzyme and is often associated with the activity of a regulatory enzyme. It has been suggested that PAP is a regulatory enzyme in the pathway for synthesis of glycerolipids (Bell & Coleman, 1980). Although it is not known how this enzyme is regulated, it could involve feedback inhibition by the product, DRG.

The activities of PAP1 and PAP2 were measured in a variety of murine tissues. The wide range in activities observed in the tissues suggests a difference in the relative importance in the function of the enzymes. The highest activity was found in the brain which is also known to show high rates of inositol phospholipid turnover (Farooqui & Hirashima, 1992). Although it was not shown which particular cell type contained this high activity, it would be interesting to study these enzymes in diseases associated with the brain, such as glioma. Other pathological conditions such as spinal cord injury and Alzheimer's disease are associated with massive increases in DRG levels which may in turn cause neuronal cell damage (Farooqui & Horrocks, 1991). The exact source of this DRG has not been determined but may involve changes in some of the enzymes involved in generating this second messenger, including PAP activity.

The role of PAP activity in proliferation was studied in *ras* transformed fibroblasts. This was chosen as a well established model of disrupted cell signalling. These transformed cells have already been shown to express increased DRG levels (Lacal *et al.*, 1987; Price *et al.*, 1989; Wakelam *et al.*, 1986) and PKC activity (Morris *et al.*, 1989). Hence, it was disappointing to find that PAP2 activity was decreased in the *ras* transformed cells. This would suggest that it is not involved in the effects of *ras* on cell growth and that other signalling pathways may be associated with activated *ras*. Recent evidence suggests that *ras* is involved in the activation of phosphatidylinositol 3-kinase (PI 3-kinase) (Kaplan *et al.*, 1990; Kodaki *et al.*, 1994). One of the effects of this is to substantially increase the levels of the phosphatidylinositols. Hence, DRG production in *ras* transformed cells may be derived, not from the PLD/PAP pathway but from other sources such as increased

hydrolysis of phosphatidylinositol 4,5-bisphosphate. This does not rule out PC hydrolysis as an important pathway in signalling. It is possible that the PLD/PAP pathway is generating increased levels of phosphatidate, another important second messenger which was also increased in the transformed fibroblasts. The *ras* oncogene could be involved in down-regulation of PAP2 which would disrupt this pathway, allowing phosphatidate to accumulate. It is unclear whether DRG or phosphatidate is the most important second messenger in cell proliferation and until this has been established the function and importance of PAP2 will remain unclear.

Since signalling in fibroblasts may not be representative of all cell types, these enzyme activities were also measured in epithelial cells. A study of this cell type was particularly useful to this study as the majority of solid tumours are derived from epithelial cells. Normal epithelial cells, however, are difficult to grow in culture and, therefore, a cell model was chosen which allowed a comparison of the effects of mutant *ras* in colon cancer epithelial cells. Although the choice of colon tumour cell lines was based on the presence or absence of a *ras* mutation, there was probably a number of other oncogenic differences between the lines. It was therefore somewhat surprising to obtain results that closely reflected those observed in the transfected cell lines. The main difference between the two cell models was in the activity of PAP1 which was increased in the *ras* transformed fibroblasts but was decreased in the colon cancer cells with the *ras* mutation. The role of PAP1 in these is unclear but differential regulation of this enzyme may allow production of different lipids in the endoplasmic reticulum which may be involved in different processes in the two cell types. The effects of *ras* on PAP2 and second messengers were fairly consistent between the fibroblasts and epithelial models, in that there was a decrease in the activity of this enzyme accompanying increased levels of DRG and phosphatidate. As for the fibroblasts, *ras* may have a similar role in these cancer lines in the constitutive activation of other enzymes such as PI 3-kinase.

One of the most exciting results came from the study of PAP2 activity in human colon tumours. Both PAP1 and PAP2 activities were increased in the majority

of tumours when compared with activities in adjacent normal colon. Since the underlying aim of this thesis was to determine whether PAP activity is a suitable target for anticancer drug development, this was a promising result. In addition to altered PAP activity, levels of two second messengers, phosphatidate and DRG, were decreased in the tumour tissue. These changes are clearly very different from those induced by *ras* transformations in the fibroblasts cell lines. Although mutations in the *ras* gene are common in colon tumours (Rodenhuis, 1992), only 50% of the tumours used in this study had this mutation. A number of genetic changes have been identified in colon tumours and the relative importance of these changes in the development of the tumour is not known (Fearon & Vogelstein, 1990). Clearly, it would be important to know whether the changes in PAP activity are an early event that can be detected at the adenoma stage.

PAP catalyses the conversion of phosphatidate to DRG. Both phosphatidate and DRG are important second messengers and a change in PAP activity might be expected to alter the balance between these two lipids. The observed decrease in phosphatidate levels is consistent with the increased activities of PAP. However, levels of DRG were also decreased in the tumours but this could be explained in part by an increase in activity of specific isoforms of the metabolising enzymes, DRG lipase and DRG kinase. Clearly, mass measurements of lipids could be misleading since phosphatidate and DRG both consist of a family of molecular species, not all of which are involved in signalling pathways (Pessin *et al.,* 1990; Pettitt & Wakelam, 1993).

There was a surprising variability between patients in both enzyme activities and lipid levels in the normal colon. The significance of this observation is not known. There is a strong association between a high fat diet and the incidence of colon cancer (Reddy *et al.*, 1992). It may be that dietary fibre influences lipid metabolism in the colon. Whether this variability is a true reflection of a range of activities present in normal colon or whether it is indicative of pre-malignant changes is an interesting area for further study.

In view of the increased activity of PAP in colon tumours, an attempt was made to identify a lead compound for the development of a specific enzyme inhibitor. The sphingoid bases were the most potent inhibitors of both PAP1 and PAP2 enzyme activities and in particular, sphingosine, was also shown to be a potent inhibitor of cell growth. However, the inhibitory effect of this compound on cell growth was probably not just through PAP, for example, sphingosine has been shown to inhibit PKC activity and stimulate PLD (Franson *et al.*, 1992; Merrill & Stevens, 1989). It may be possible to produce analogues of potential lead compounds which have increased selectivity for the target enzyme. Indeed, this project has led to the development of a synthesis programme in which analogues of sphingosine are being tested for their specificity for PAP. A specific PAP2 inhibitor would be provide a useful tool with which to study the importance of this enzyme and the second messengers, phosphatidate and DRG, in signalling in whole cells. For example, inhibition of this enzyme in growth factor-stimulated cells would identify its importance in mitogenesis in the PLD/PAP pathway and if phosphatidate, which would be expected to accumulate, is the important second messenger. Analogues of sphingosine might also be potential antitumour agents. It is possible that these lipid compounds may be poorly absorbed or could pass through the blood-brain barrier to cause neuronal disorders, in which case long-term treatment could not be envisaged. To overcome this, antibody-drug conjugates may be used in which the drug is attached to an antibody directed against a tumour-specific antigen which will bind selectively to those tumour cells. In addition, it should be possible to package the compounds into liposomes which may aid in drug uptake. It has already been shown that liposomal trimethyl-sphingosine reduces toxicity and enhances drug efficacy when compared to free trimethyl-sphingosine *in vivo* (Park *et al.*, 1994). Having obtained evidence of specific drug-target interaction in both *in vitro* and *in vivo* preclinical models, it is also important to screen the drugs in patients for their ability to inhibit PAP activity. Obviously, this is a more difficult task but it may be possible,

for instance, to measure PAP activity in white blood cells before and after treatment. This may determine the specificity of the drug for PAP in patients.

PAP has been an interesting enzyme to study due to its altered activity in both cell and tissue models used in the study. Clearly, purification of both PAP1 and PAP2 are required to study these enzymes in more detail. Pure enzymes would lead to the development of specific inhibitors and a more detailed kinetic analysis could be carried out. In addition, the exact function of this enzyme could be established by cloning a cDNA encoding the PAP enzymes. This could then be transfected into cells to produce cell lines which stably overexpress the enzymes. In this way the role of PAP1 and PAP2 in modulating the expression of genes related to cellular growth could be investigated and the importance of the enzymes in cell proliferation established. The function of PAP2 in cell signalling is not clear at the moment but further work is well worth attempting to establish its importance in the PLD/PAP pathway in generating two second messengers. Although a pure enzyme is required for further studies of PAP, this may be available in the near future since David Brindley's group in Canada is very close to producing a homogenous enzyme (personal communication).

PAP has proved to be a potential target for anticancer agents. One of the main reasons for the development of tyrosine kinase inhibitors is based on the evidence of increased expression of the EGF receptor in various cancers (Arteaga *et al.*, 1991), yet there is no evidence to suggest that growth of the tumours is dependent on this pathway. The fact that the increase in PAP activity occured in almost every patient tumour sample is certainly a more consistent change than has been observed for many other novel targets.

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