Identification of the Membrane Association Domain of the Cyclic AMP-Specific Phosphodiesterase RD1

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Abbreviations:

The abbreviations used in this thesis are in accordance with the recommendations set out in "Instructions to authors", Biochem. J. (1985) **225:**1-26

ADH	Alcohol Dehydrogenase
AKAP	A Kinase Anchoring Protein
ANP	Atrial Natriuretic Peptide
AOX	Alcohol Oxidase
Ba $(OH)_2$	Barium Hydroxide
Ca^{2+}	Calcium ion
CaM	Calmodulin
CAT	Chloramphenicol acetyltransferase
CHO	Chinese Hamster Ovary
C-Terminal	Carboxy Terminal
C-Subunit	Catalytic Subunit
CO_2	Carbon Dioxide
CREB	cAMP Response Element Binding Protein
cAMP	Cyclic AMP
cGMP	Cyclic GMP
cGI-PDE	Cyclic GMP-Inhibited PDE
cGS-PDE	Cyclic GMP-Stimulated PDE
DEAE-Dextran	Diethylaminoethyl-Dextran
DHFR	Dihydrofolate Reductase
d.H_20	Distilled H_20
dNTP	Deoxynucleotide Triphosphate
DMEM	Dulbecco's Modified Eagle's Medium
DPD	Dunce-like Phosphodiesterase
DTT	Dithiothreitol
ECE-1	Endothelin Converting Enzyme 1

EHNA	Erythro-9-(2-Hydroxy-3-Nonyl)-Adenine
ER	Endoplasmic Reticulum
FCS	Foetal Calf Serum
FSH	Follicle Stimulating Hormone
GnT1	Glucoronosyltransferase 1
G-Protein	GTP-Binding Protein
HEK 293	Human Embryonal Kidney 293
HIV	Human Immunodefiency Virus
HL	3-Hydroxy-3-Methylglutaryl-CoA Lyase
IBMX	3-Isobutyl-1-Methylxanthine
IC_{50}	Inhibitory Concentration 50
IDE	Insulin Degrading Enzyme
IGF-1	Insulin-Like Growth Factor-1
IL-6	Interleukin-6
I_{Ca}	Calcium Current
KCl	Potassium Chloride
kDa	Kilodalton
K_m	Michaelis Constant
K_i	Inhibition Constant
LGT	Low Gelling Temperature
LIMP-II	Lysosomal Integral Membrane Protein-II
LMP	Low Melting Point
LP	Liver Phosphorylase
LR1	Linker Region 1
LR2	Linker Region 2
MAP	Mitogen Activated Protein
MAPK	Mitogen Activated Protein Kinase
Mg^{2+}	Magnesium ion
$MgCl_2$	Magnesium Chloride

Mn^{2+}	Manganous ion
MTS	Matrix Targeting Signal
Na^{+}	Sodium ion
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NLS	Nuclear Localization Signal
NO	Nitric Oxide
NMR	Nuclear Magnetic Resonance
N-Terminus	Amino Terminus
ORF	Open Reading Frame
PA	Phosphatidic Acid
PAF	Phosphodiesterase Activating Factor
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDE	Phosphodiesterase
PGE_2	Prostaglandin E_2
PK	Protein Kinase
PKA	Protein Kinase A
PKC	Protein kinase C
PPM.PDE	Peripheral Plasma Membrane PDE
PT	Peroxiaomal 3-Ketoacyl CoA Transferase
PTX	Pertussis Toxin
RD1	Rat Dunce 1
R-Subunit	Regulatory Subunit
ROS-PDE	Rod Outer Segment PDE
ROS-PK	Rod Outer Segment PK
RT-PCR	Reverse Transcriptase-PCR
SCI	Single Colony Isolate

SDS	Sodium Dodecyl Sulphate
Sf9 Cells	*Spodoptera frugiperda* 9 Cells
SH2	Src Homology 2
SH3	Src Homology 3
SIN-1	3-Morpholino-Sydnonimine
SNP	Sodium Nitroprusside
SP	Signal Peptide
STG	Stomatogastric
SV 40	Simian Virus 40
Tα	Transducin α
Tβ	Transducin β
Tγ	Transducin γ
TCR	T-Cell Receptor Complex
TE	Tris EDTA
TEN	Tris, EDTA, NaCl
TFIIIA	Transcription Factor IIIA
TGN	Trans Golgi Network
TM	Transmembrane
Tn9	Transposon 9
TSH	Thyroid Stimulating Hormone
UCR1	Upstream Conserved Region 1
UCR2	Upstream Conserved Region 2
UTR	Untranslated Region
V/GSH	Vanadate/Glutathione
Zn^{2+}	Zinc ion
$ZnSO_4$	Zinc Sulphate

SUMMARY

Many seven transmembrane (7TM) receptors exert their influence upon cellular processes through G-protein stimulated activation of adenylyl cyclase leading to an increase in intracellular concentration of the second messenger cAMP which exists ubiquitously in mammalian cells. The sole role of cAMP is to activate cAMP-dependent protein kinase by binding to its regulatory subunits thus causing their dissociation from and activation of the catalytic subunits. Modulation of cAMP levels has been implicated in a variety of metabolic processes as diverse as muscle contraction, neurotransmission, exocytosis, cell growth and differentiation and platelet aggregation. In addition, reductions in intracellular cAMP concentrations have been observed in a variety of disease states and conditions such as congestive heart failure, diabetes, and cancer as well as a number of inflammatory conditions including asthma, rheumatoid athritis and atopic dermatitis.

It is apparent that distinct intracellular pools of cAMP can exist within a cell due, in part, to the fact that mammalian cells can express at least 9 forms of adenylyl cyclase and several splice variants of the G-protein G_s subunit responsible for the regulation of this enzyme. The sole means of degradation of cAMP is through its hydrolysis to 5'AMP by cAMP phosphodiesterases (PDEs). Of the five families of PDEs known to hydrolyse cAMP the largest and best characterised are the PDE4 cAMP-specific phosphodiesterases. To date, four distinct PDE4 genes have been identified and further diversity is added by differential splicing and post-translational processing of some genes. Moreover, these splice variants display distinct regulatory properties, tissue, cell type and spaciotemporal patterns of expression. Recent studies on the members of the PDE4 gene family has revealed that the various splice variants arise as a result of amino terminal domain swaps on a highly conserved central domain which is thought to contain the catalytic site.

The rat PDE4A gene product RD1 (RNPDE4A1) which was originally isolated from a rat brain cDNA library has been shown to be membrane associated upon expression of the cDNA in transfected COS cells. Sequence analysis has shown that the extreme N-terminal 1-23 amino acids of RD1 are exclusive to this enzyme. Deletion of this unique sequence resulted in the generation of a catalytically active PDE species known as Met26 RD1. In contrast to RD1, met26RD1 is exclusively cytosolic thus leading to the contention that the subcellular localization of RD1 is determined by information contained within its unique amino terminal region.

The principle aim of this study was to determine whether the extreme N-terminal region of RD1 was indeed responsible for membrane association or whether its removal merely disrupted a larger structural conformation of the enzyme responsible for its subcellular localization. This was approached by the generation of a novel eukaryotic expression vector that facilitated construction of gene fusions between various N-terminal regions of RD1 and the soluble bacterial reporter enzyme CAT. By employing this strategy I have demonstrated conclusively that the N-terminal 25 amio acids are sufficient to target the normally soluble CAT to the plasma membrane upon expression in COS cells. Moreover, I have shown by site-specific mutagenesis that membrane association is not due to post-translational modification but is determined by the presence of a unique tryptophan-rich motif within the first 25 amino acids of RD1.

During the course of this study I have also developed a novel assay for the investigation of membrane association based on expression of novel chimeric protein species in a cell-free coupled transcription/translation system.

CHAPTER 1

Introduction

1.1 The Role of cAMP as a Second Messenger

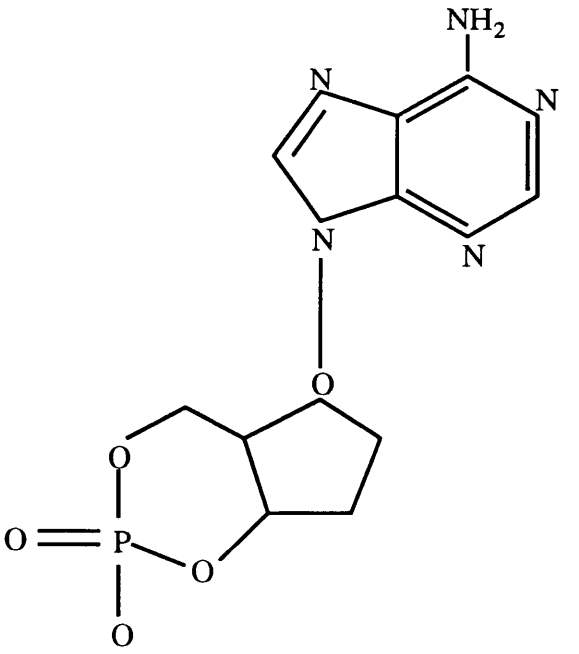
In 1957 Rall et al (Rall et al. 1957), determined that activation of liver phosphorylase (LP) in cell homogenates by epinephrine and glucagon was due to the production of a heat-stable factor. This was associated with the particulate fraction of the homogenate which, in turn, stimulated the activation of LP in the supernatant fractions of homogenates on which the hormones themselves were inactive. Such a factor, which was only produced when the hormones were incubated in the presence of ATP and Mg^{2+}, was subsequently identified as 3',5'-cyclic AMP (Rall and Sutherland 1985) and Fig. 1.1. Cyclic AMP, which was shown to be generated from ATP by the enzyme adenylyl cyclase, was then proposed to serve as a second messenger, able to mediate the effects of a variety of hormones, with the hormones themselves acting as the first messengers (Hall 1963; Sutherland 1972; Sutherland et al. 1968). This was termed the 'Second Messenger Concept' for which cAMP served as the paradigm.

1.2 G-Protein Coupled Signal Transduction

The hormone receptor-producer system for cAMP was originally thought to be carried out by one and the same molecule until it was observed that these two entities could be separated. Later it was shown that these units could be resolved into a receptor and adenylyl cyclase unit following membrane solubilization (Rodbell et al. 1971). In addition, it was shown that there existed a requirement for GTP (Rodbell et al. 1971). The studies of Ross and Gilman (Ross and Gilman 1977) have since shown that the β-adrenergic receptor is coupled to adenylate cyclase through interaction with a third component now known to be a G-protein. It is now clear that a large group of receptors elicit their responses through their interactions with G-proteins, including receptors for amines (catecholamines, acetylcholine and serotonins), proteins

Figure 1.1: Cyclic AMP

This figure represents the structure of the second messenger molecule cAMP.


NH2
N
N
N
N
O
O
O
P
O
O

(glucagon, leutinizing hormone) and light (rhodopsin). Moreover, the application of molecular biology has revealed that these receptors display a high degree of structural similarity and membrane topology. Structural studies of the β_2-adrenergic receptor for example, has revealed the presence of 7 hydrophobic segments of 24-48 amino acids which, it has been suggested, serve as transmembrane spanning α helices that are connected by a series of extracellular and cytoplasmic loops (O'Dowd et al. 1989). The third cytoplasmic loop has been identified as the site of interaction with G-proteins (O'Dowd et al. 1989).

Hetrotrimeric G proteins are a subset of a superfamily of proteins which bind GTP with high affinity (Linder and Gilman 1992). They consist of three distinct subunits α, β and γ in descending order of molecular weight and are broadly defined by the nature of the α subunit (Gilman 1987; Kaziro et al. 1991) which contains the site of guanine nucleotide binding. The α subunit also interacts with the $\beta\gamma$ subunits through its N-terminus (Neer et al. 1988).

Hetrotrimeric G proteins are located at the plasma membrane of cells by virtue of a number of post-translational modifications such as palmitoylation, myristoylation and isoprenylation (Degtyarev et al. 1994; Higgins and Casey 1994; Parenti et al. 1993; Simonds et al. 1991; Spiegel et al. 1991a Wedegaertner et al. 1995). To date twenty subtypes of α subunits, five β subunits and seven γ subunits have been identified and isolated which play a pivotal role in transmembrane signalling as they transduce tramsmembrane signals between a wide variety of extracellular signals and intracellular responses (Milligan et al. 1995).

Upon hormone binding the receptor undergoes a conformational change which promotes the exchange of G protein bound GDP for GTP which, in turn causes dissociation of the α subunit from the hetrodimeric $\beta\gamma$ subunits. The activated α subunit then interacts with adenylyl cyclase whilst $\beta\gamma$ subunits

themselve have been implicated in ras and MAPK signalling pathways (Faure et al. 1994; Inglese et al. 1995; Mattingly and Macara 1996) and modulation of some adenylyl cyclases (Tang and Gilman 1991) and phospholipase C (Camps et al. 1992). The interaction between the α subunit and adenylyl cyclase is terminated by the intrinsic GTPase activity of the α subunit hydrolysing bound GTP to GDP thus promoting re association of now inactive α subunit with the $\beta\gamma$ subunit heterodimer.

1.3 Generation of cAMP by Adenylyl Cyclases

1.3.1 Structural Features of Adenylyl Cyclase

A major site for the control of the level of cAMP within cells is determined by its synthesis and therefore a great deal of attention has been focused on adenylyl cyclase the enzyme responsible for the intracellular conversion of ATP to cAMP. Progress in this field was hindered for many years by the low levels of expression of the protein (0.01-0.001% in mammalian cells) and its considerable lability in detergent-containing solutions (Taussig and Gilman 1995). However, this problem was to some extent circumvented by the observation of Pfeuffer and Metzger (Pfeuffer and Metzger 1982) that mammalian adenylyl cyclases could be activated by the diterpine forskolin, and the subsequent discovery that this compound could be utilised as an affinity medium by covalently linking it to an agarose matrix. This approach was used by Krupinski and co-workers to purify sufficient amounts of a calmodulin sensitive adenylyl cyclase from bovine brain to allow a partial amino acid sequence to be determined (Krupinski et al. 1989). An oligonucleotide probe was synthesised and used to isolate a cDNA clone of ~ 11.5 kb from a bovine brain library.

Hydropathy analysis of this clone suggested a protein of approximately 120-kDa which could be divided into two alternating sets of hydrophobic and hydrophilic domains. Each hydrophobic domain was characterised by six

transmembrane spans (M_1 & M_2) and separated by a large cytoplasmic domain of approximately 40kDa (C_1) with a second cytoplasmic domain (C_2) located after the second transmembrane domain (Krupinski et al. 1989). To date at least eight different adenylyl cyclase clones have been reported in the literature (Bakalyar and Reed 1990; Cali et al. 1994; Feinstein et al. 1991; Gao and Gilman 1991; Krupinski et al. 1992; Premont et al. 1992; Yoshimura and Cooper 1992) and there is some evidence for the existence of splice variants (Wallach et al. 1994). Comparison of the amino acid sequence amongst the different adenylyl cyclase isoforms is approximately 50%, however the cytoplasmic domains C_1 and C_2 appear to be more highly conserved with greater that 90% similarity (Taussig and Gilman 1995).

1.3.2 Regulation of Adenylyl Cyclases

Amino acid sequence comparison has suggested that types II, IV, VII and IX adenylyl cyclases are more closely related to each other than to others (Gao and Gilman 1991; Krupinski et al. 1992) and such criteria suggest that a similar relationship exists between the type V and VI isoforms while types I, III and VIII are equally dissimilar from the others (Cooper et al. 1994). Studies on the regulation of adenylyl cyclases has shown that all isoforms are activated ubiquitiously by both forskolin and the GTP-bound G protein $G_s\alpha$ subunit. However, adenylyl cyclases can also be grouped according to their sensitivity/regulation by Ca^{2+}. For example the type I, III and VIII isoforms are all stimulated by nanomolar concentrations of Ca^{2+} in a calmodulin dependent manner (Cali et al. 1994; Cooper et al. 1994; Tang et al. 1991) whereas types V and VI are inhibited independently of calmodulin (Cooper et al. 1994). The remaining three isoforms, type II, IV, and IX, are Ca^{2+} insensitive (Feinstein et al. 1991; Gao and Gilman 1991; Yoshimura and Cooper 1992). A further level of complexity relates to the observation that G protein $\beta\gamma$ subunits stimulate type II and IV adenylyl cyclases (Cooper et al. 1994; Gao and Gilman 1991; Tang and Gilman 1991) while inhibiting the type I

isoform (Cooper et al. 1994; Tang and Gilman 1991). Furthermore, Jacobowitz (Jacobowitz et al. 1993) and Kawabe (Kawabe et al. 1994) have reported type-specific phosphorylation of adenylyl cyclases upon treatment of cells with phorbol esters.

1.3.3 Tissue Distribution of Adenylyl Cyclase Isoforms

The advent of molecular biology has proved instrumental in determining the tissue distribution of the various adenylyl cyclase isoforms. Krupinski et al (Krupinski et al. 1989) characterised a type I isoform from bovine brain by means of an antisense probe derived from a partial amino acid sequence. This same approach was employed by Bakalyar to demonstrate that type I and II adenylyl cyclases were expressed in high concentrations in brain but absent in the olfactory bulb where the type III adenylyl cyclase was the only isoform expressed in this tissue, implying a role in olfactory signal transduction. That type II adenylyl cyclase is expressed in rat brain was confirmed by Feinstein et al (Feinstein et al. 1991) who were also able to show high levels of expression in rat brain and low levels in olfactory bulb while moderate expression was observed in lung. Type IV adenylyl cyclase has been identified in a wide variety of tissues including brain, heart, kidney, liver and lung but undetected in testis (Gao and Gilman 1991) whereas the type V and VI isoforms have been observed in brain, liver and kidney (Premont et al. 1992). Yoshimura and Cooper have also identified and cloned type VI adenylyl cyclase from the mouse neuroblastoma-embryonic, Chinese hamster brain explant hybrid NCB-20 cell line (Yoshimura and Cooper 1992). A cDNA encoding type VIII adenylyl cyclase was isolated from two rat brain libraries by Cali et al (Cali et al. 1994) who were also able to demonstrate by in situ hybridization that most abundantly expressed in specific cells within the dentate gyrus and hippocampus and less abundantly in the neocortex, thalamus and hypothalamus.

In summary, cAMP is generated from ATP in a reaction catalysed by adenylyl cyclases, a diverse family of enzymes encompassing at least eight

isoforms, some of which demonstrate multiple splice variants and which are expressed in tissue-specific fashion.

1.4 Cyclic AMP-Dependent Protein Kinase

In order to exert its influence within a cell cAMP must first interact with, and secondly activate an effector system, thus converting an extracellular hormonal signal into an intracellular response. The first indication that this response involved phosphorylation was obtained by the the successful purification of a protein kinase from rabbit skeletal muscle which was demonstrated to be completely dependent on cAMP for its activity (Walsh et al. 1968). Cyclic AMP-dependant protein kinase (PK-A) is tightly regulated and maintained in an inactive form in the absence of cAMP (Taylor 1989). However, the enzyme is unique in that the two forms isolated exist as a tetramer comprised of two catalytic (C) subunits and a regulatory (R) dimer (R_2C_2) (Bramson et al. 1984; Taylor 1989). PK-A is activated upon cAMP binding to the R-subunits which initiates the release of the two activated catalytic subunits from the regulatory subunit complex. This allows them to phosphorylate serine residues in a variety of substrate proteins containing the consensus sequence RRXS (Bramson et al. 1984; Hausken et al. 1996).

Genes have been identified in mammalian cells encoding three C-subunits Cα, Cβ and Cγ and four R subunits RIα, RIβ, RIIα and RIIβ (Scott 1991) resulting in two holoenzyme classes of PKA which share a common catalytic subunit but differ in their regulatory subunits. Furthermore, the two holoenzymes can be found in different subcellular locations. This is conferred by the R subunit. Thus PK-AI isoforms are cytosolic whilst PKAII isoforms are predominantly located at a variety of intracellular sites (Cassano et al. 1996; Scott and Carr 1992). Subcellular localization of cAMP-dependent protein kinase will be discussed in more detail in a later section (section 1.6.1).

1.4.1 The Catalytic Subunit

The catalytic subunit displays extensive sequence similarity amongst all eukaryotic protein kinases. In particular, several amino acid residues in the active site have been shown to be highly conserved (Taylor 1989). Affinity labelling studies have revealed the existence of a glycine-rich element with the consensus sequence GXGXXG near the amino terminus of the catalytic subunit which, in conjunction with an invariant lysine (K), is thought to be involved in nucleotide binding, indeed, mutation of this lysine results in the generation of an inactive kinase (Taylor 1989).

Two different isoforms of the catalytic subunit have been isolated, Cα which is present in a wide variety of tissues and Cβ which, although ubiquitously expressed in several tissues, predominates in brain (Showers and Maurer 1986; Uhler et al. 1986a; Uhler et al. 1986b). The two PK-A catalytic subunit isoforms display 91% similarity at the amino acid level (Uhler et al. 1986b) whereas Cβ subunits show greater similarity between Cβ subunits of other species than between Cα and Cβ subunits of the same species (Showers and Maurer 1986).

A conserved amino terminal glycine has been shown to be myristoylated in some species but not in others (Taylor 1989) however this post-translational modification is not thought to be involved in subcellular localization since a murine Cα subunit which has a free amino terminal glycine demonstrates identical properties to the myristoylated forms (Taylor 1989)

1.4.2 The Regulatory Subunit

The function of the regulatory (R) subunit of cAMP-dependant protein kinase is to inhibit the catalytic subunit in the absence of cAMP (Taylor 1989). PK-A is regulated by binding of two molecules of cAMP to two sites, termed A and B, which are found on the R subunit. Binding of one molecule of cAMP to the B site elicits a conformational change and thus exposes the A site to which a

second cAMP molecule binds and causes the dissociation of the R subunit dimer from the two C subunits (Spence et al. 1997). Two distinct classes of R-subunit exist of which α and β isoforms have been identified (Luo et al. 1990), RI can be distinguished from the RII holoenzyme by the presence of a high-affinity binding site for Mg^{2+}ATP, whereas the RII holoenzyme is susceptible to autophosphorylation (Taylor 1989). Both the R1 and RII holoenzymes share a common domain structure in which two subunits are dimerized by means of a disulphide bridge between their amino terminii (Taylor 1989). Despite the observation that the amino terminus is also the site of interaction between the regulatory and catalytic subunits this region demonstrates a degree of variability in amino acid sequence (Taylor 1989). The different regulatory subunit isoforms display differential tissue expression, whilst the RIα subunits can be found in almost all tissues (Bregman et al. 1989; Li et al. 1996; Li and Rubin 1995; Luo et al. 1990). In contrast, RIβ is detected predominantly in brain, testis and placenta (Luo et al. 1990). Similarly, RIIα isoforms are found to be expressed in a wide variety of tissues including heart, skeletal muscle, liver and kidney (Luo et al. 1990) whereas the RIIb isoform demonstrates a more specific pattern of expression, primarily in brain (Li et al. 1996; Li and Rubin 1995; Luo et al. 1990). Within cells, the different R subunit isoforms also display different subcellular localizations, whereas RIα and RIβ isoforms are cytosolic, RIIβ isoforms are bound to specific sites in the cytoskeleton where they are anchored by means of specific interactions with a class of high-affinity binding proteins known as AKAPs (A Kinase Anchoring Proteins) (Bregman et al. 1989; Carr et al. 1993; Coghlan et al. 1994; Glantz et al. 1993; Hirsch et al. 1992; Klauck and Scott 1995; Li et al. 1996; Li and Rubin 1995; Lin et al. 1995a; McCartney et al. 1995; Ndubuka et al. 1993; Scott and Carr 1992). This occurs through an amphipathic α helix binding motif (Carr et al. 1993). Like RIIβ, RIIα isoforms are also able to bind to AKAPS (Li et al. 1996; Li and Rubin 1995).

In summary, cAMP-dependant protein kinases (PK-As) are a family of enzymes which are found in a wide variety of cells and tissue types. They exist under basal condidtions as an inactive tetrameric complex consisting of two binding , or regulatory (R) subunits and two catalytic (C) subunits. Two distinct classes of PK-As have been identified PKA-I and PKA-II each possessing a common C subunit but different R subunits. Binding of cAMP to the R subunits causes dissociation of the R subunit dimer resulting in the release of the now active C-subunit to phosphorylate substrate proteins thus mediating the intracellular effects of cAMP.

1.5 Cyclic Nucleotide Phosphodiesterases

The sole means of degradation of cAMP in cells is by its hydrolysis to 5'AMP by a group of enyzymes known collectively as the cyclic nucleotide phosphodiesterases (PDE). Indeed one of the initial pieces of evidence which pointed to the role of cAMP as a second messenger in cells was the discovery and purification of a PDE which could hydrolyse not only cAMP but also cGMP (Butcher and Sutherland 1962). Subsequent development of assays, using radio-labelled cAMP and cGMP, allowed measurement of PDE activity at near physiological concentrations. These revealed that instead of just one enzyme, there existed a family of PDEs in most tissues (Beavo 1995). Many of these PDEs have since been purified and characterised (Beavo and Houslay 1990). More recently, with the advent and application of molecular biology techniques, it has become evident that PDEs can be divided into at least seven different isoenzyme families based on substrate specificities, kinetic properties and inhibition characteristics (Beavo 1990; Beavo 1995; Beavo et al. 1994; Beavo and Houslay 1990). The different isozyme families are summarised below

PDE1 Ca^{2+}/Cam stimulated,

PDE2 cyclic GMP-stimulated,

PDE3 cyclic GMP-inhibited,

PDE4 cyclic AMP-specific,

PDE5 cylic GMP-specific,

PDE6 photoreceptor specific,

PDE7 cyclic AMP-specific.

The type 1, 2 and 3 PDE families can hydrolyse both cAMP and cGMP, whereas the type 4 and 7 PDEs are specific for cAMP and the type 5 and 6 PDEs are specific for cGMP. The different isozyme families are described in more detail below.

1.5.1 Nomenclature of cyclic nucleotide Phosphodiesterases

Due to the incredibly complex and diverse nature of the PDE gene family it soon became clear that the original system of naming PDE isoenzymes, based on the observed elution profile from an ion exchange chromatography column, was inadequate to cope with the frequent discovery of new genes as well as novel splice variants and alternative transcripts of existing genes. The current nomenclature (Beavo et al. 1994) is based upon the following criteria. The first two letters indicate the species from which the gene was cloned followed by the designator 'PDE' for cyclic nucleotide phosphodiesterase, next the arabic number defines the gene family followed by a letter to indicate the specific gene, finally the splice variant is identified by an arabic numeral with a single letter representing the original report.

Thus, for example, RNPDE4A1A was isolated from rat (*Rattus norvegicus*), it is a member of the PDE4 gene family, it was the first splice variant identified and is the first of a number of clones of that enzyme.

1.5.2 Structure of cyclic nucleotide Phosphodiesterases

The cyclic nucleotide phosphodiesterases are a large and diverse group of enzymes which have been intensely studied in recent years. At present at least 16 different PDE genes have been identified, many of which demonstrate an additional level of diversity by the presence of multiple splice variants which are expressed in cellular and tissue-specific patterns (Beavo 1995; Beavo et al. 1994; Beavo and Houslay 1990; Rybalkin and Beavo 1996). Amino acid sequence information has now been obtained for many PDEs either from directly sequenced proteins or, through the application of molecular biology, from isolated cDNA clones (Baehr et al. 1991; Bentley et al. 1992; Bolger et al. 1993; Michaeli et al. 1993; Monaco et al. 1994; Novack et al. 1991; Sonnenburg et al. 1991; Swinnen et al. 1991b; Yan et al. 1996). Such analyses have revealed that PDEs contain a modular structure which includes a highly conserved region of 250-270 amino acids within the core of the protein (Fig. 1.2) and which is thought to reflect the catalytic site (Charbonneau 1990; Charbonneau et al. 1986; Charbonneau et al. 1991; Sonnenburg et al. 1995). Such evidence has been obtained from studies employing limited proteolytic digestion, mutagenesis and truncation analysis (Catty and Deterre 1991; Charbonneau et al. 1986; Charbonneau et al. 1991; Kincaid et al. 1985; Sonnenburg et al. 1995; Stroop et al. 1989).

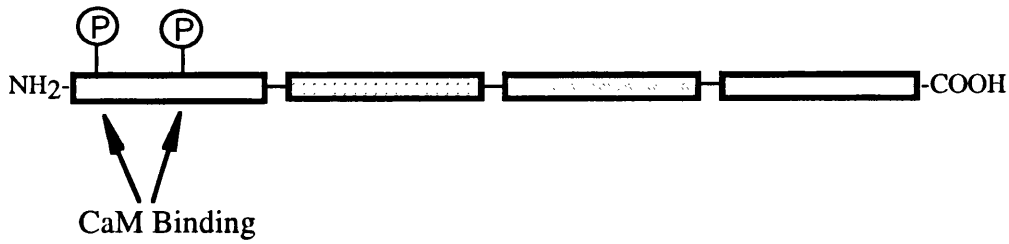
Charbonneau (Charbonneau 1990) has postulated that the relatively large size of PDEs would suggest that they may exist as multidomain proteins. Thus they might comprise a single, homologous catalytic domain linked to a variable non-catalytic N-terminus which may be involved in regulatory functions as well as containing sites for nucleotide binding, membrane interaction and dimerization. Indeed there is a growing body of evidence in the literature to support this hypothesis. For example, studies on the type 2 cGMP-specific PDE and the α and α' subunits of type 6 photoreceptor rod and cone

PDEs have revealed the presence of a second conserved segment of approximately 340 amino acids long which contains two internally homologous repeats and that this region contains a non-catalytic cGMP binding site (Ahn et al. 1991; Charbonneau et al. 1990). In addition, Sonnenburg et al (Sonnenburg et al. 1995) have recently demonstrated that the type 1 PDEs from bovine brain and lung contains a calmodulin binding site and have confirmed an original observation made by Kincaid et al (Kincaid et al.1985) that the N-terminus of the type 1 PDEs contains an inhibitory site. Meacci et al (Meacci et al. 1992) have suggested that, in addition to the catalytic region, the N-terminal region of the type 3 PDE from human sarcoplasmic reticulum may be responsible for conferrment of membrane association. Furthermore, the N-termini of some members of the PDE4 family have been implicated in their membrane and subcellular localization (McPhee et al. 1995; O'Connell et al. 1996; Shakur et al. 1993; Shakur et al. 1995).

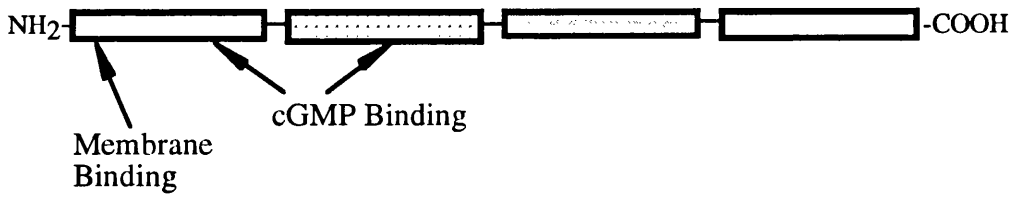
Figure 1.2: Structure of Representative Members of the Cyclic Nucleotide PDE Families

The putative structure of five of the seven PDE families is shown. The different domains are represented by boxes connected by wires corresponding to putative hinge regions. The open boxes represent regulatory domains where several phosphorylation sites and allosteric sites have been mapped. The shaded boxes correspond to the catalytic domains. The letter 'P' in an open circle represents a site of phosphorylation. Putative membrane association regions are marked by arrows.

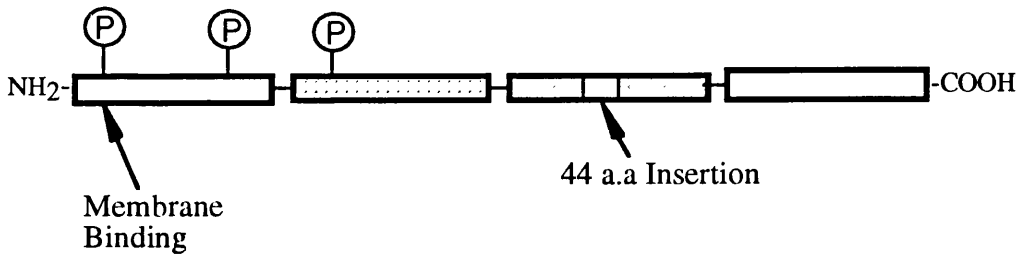
CaM-PDE



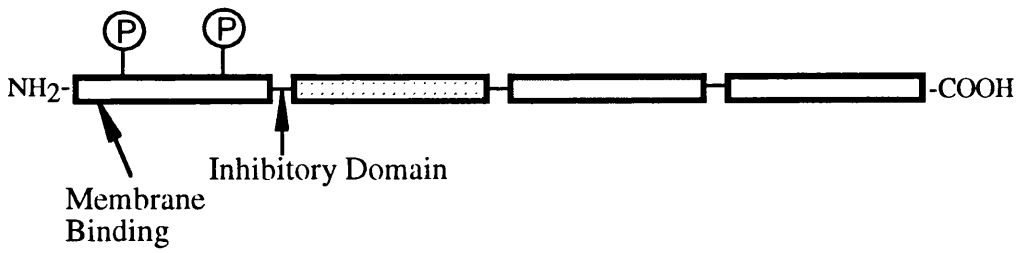
cGS-PDE



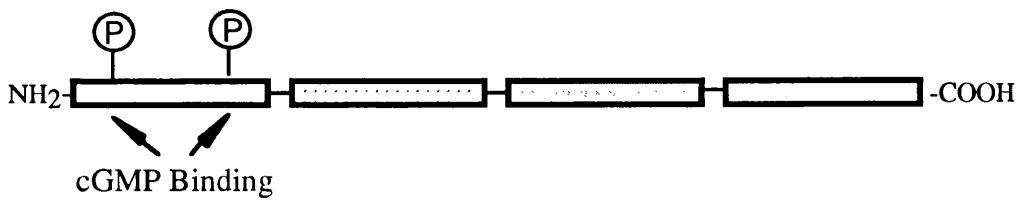
cGI-PDE



cAMP-PDE



cGMP-PDE



In addition to the apparent multi-domain structure observed amongst several PDEs, there is evidence that many of the mammalian PDEs exist as homodimers consisting of two identical catalytic subunits (Beavo 1995; Bloom and Beavo 1996; Kincaid et al. 1985; Shenolikar et al. 1985; Sonnenburg et al. 1995; Stroop et al. 1989). The photoreceptor type 6 PDEs however, are unusual in that they exist as a heterotetrameric protein in an $\alpha\beta\gamma_2$ conformation (Artemyev et al. 1996; Baehr et al. 1979; Beavo 1995; Gillespie 1990; Liu et al. 1996). In addition, Artemyev et al have recently demontrated by means of subunit specific antibodies that the rod cGMP PDE can also exist as distinct $\alpha\alpha\gamma_2$ and $\beta\beta\gamma_2$ isoforms (Artemyev et al. 1996).

1.5.3 PDE1, Calcium/Calmodulin Stimulated PDE

As the name implies, Ca^{2+}/CaM PDEs are activated by Ca^{2+} and calmodulin (Wang et al. 1990). Kakiuchi and Yamakazi in 1970 discovered that in rat brain there existed two cyclic nucleotide PDE activities, the basal activity and the calcium dependent activity (Kakiuchi and Yamakazi 1970). The calcium dependent activity required the presence of a heat-stable non-dialysable factor for which they coined the phrase PAF and was also detected by Cheung (Cheung 1971) . PAF was found in a variety of tissues including human, porcine and rat brain as well as bovine heart, however Cheung demonstrated that PAF purified from one tissue was able to stimulate PDE activity in other tissues but at the same time demonstrating that the effect of the activator was protein specific (Cheung 1971).

The type 1 Ca^{2+}/CaM-stimulated PDEs are a large group of PDEs which originate from three distinct genes (Beavo 1995; Beavo et al. 1994; Rybalkin and Beavo 1996). Moreover, there is evidence for the existence of multiple splice variants for each of the three separate genes (Bentley et al. 1992; Loughney et al. 1996; Polli and Kincaid 1992; Sonnenburg et al. 1993; Sonnenburg et al. 1995; Yan et al. 1996; Yan et al. 1995). The members of this

isozyme family provide the possibility of 'crosstalk' occurring between two different intracellular second messenger systems namely the Ca^{2+} and cAMP-signalling pathways.

1.5.3.1 PDE1A

Sharma and Wang (Sharma and Wang 1986) first isolated a 58kDa enzyme from bovine lung which demonstrated a higher affinity for cAMP than cGMP but a higher V_{max} for cGMP than cAMP. It is now known that this enzyme is a product of the PDE1A gene (Beavo 1995). Similarly the 59kDa bovine heart isozyme islated by Hansen and Beavo can also be classified as derived from a PDE1A gene (Hansen and Beavo 1986; Novack et al. 1991). Sonnenburg et al have isolated a 61kDa isozyme from bovine brain capable of hydrolysing both cAMP and cGMP (Sonnenburg et al. 1993) and has since been shown to be an amino terminal splice variant transcribed from the PDE1A2 gene (Sonnenburg et al. 1995). More recently, Loughney et al (Loughney et al. 1996) have isolated and extensively characterised a CaM-stimulated PDE splice variant PDE1A3 which is expressed in a wide variety of tissue types including brain, heart, skeletal muscle, liver and kidney. It displays, like the 58kDa lung isozyme, a higher affinity for cGMP than cAMP.

1.5.3.2 PDE1B

A second Ca^{2+}/CaM-stimulated PDE gene, PDE1B, has been identified which demonstrates some sequence similarity with the 59/61kDa isozyme but is otherwise entirely distinct, Bentley et al (Bentley et al. 1992) by screening a bovine brain cDNA library with degenerate oligonucleotides based on partial peptide sequences, isolated a cDNA which encoded a 63kDa CaM-stimulated PDE. More recntly Spence et al have described that phorbol ester stimulation of Chinese hamster ovary (CHO) cells leads to the rapid induction of CaM-stimulated PDE activity which was identified as the PDE1B isozyme (Spence et al. 1997).

1.5.3.3 PDE1C

The original evidence for the existence of a third Ca^{2+}/CaM-stimulated gene family was postulated by the experiments of Shenolikar et al (Shenolikar et al. 1985) who reported the purification and characterization of a 74kDa PDE from bovine brain that preferentially hydrolysed cGMP. It has now been shown that this enzyme belongs to the PDE1C isozyme family (Beavo 1995). To date, at least five different splice variants of PDE1C are known to exist and it has been shown that at least one isozyme hydrolyses cAMP as well as cGMP, Yan et al (Yan et al. 1995) have demonstrated the enrichment of this PDE1C2 splice variant in rat olfactory epithelium, thus suggesting a role for this isozyme in odorant desensitization. The same laboratory has also recently isolated a PDE1C1 cDNA from a mouse brain library which, by means of RNAse protection assays and *in situ* hybridization, was shown to have a distinct tissue and cellular expression pattern (Yan et al. 1996). More recently, Loughney et al (Loughney et al. 1996) have isolated cDNAs for human PDE1C1 and PDE1C3 splice variants which are expressed preferentially in brain and demonstrate a higher affinity of cGMP than cAMP. Two further PDE1C splice variants have been identified, PDE1C4 and PDE1C5 (Yan et al. 1996). Unusually though, these two splice variants, whilst having identical coding sequences, differ in their 3'-untranslated regions (3'-UTR). This is the first evidence for 3'- splice variants and it has been postulated that this may be important in post-transcriptional regulation of the isozyme mRNA (Yan et al. 1996)

1.5.3.4 Regulation of Calcium/Calmodulin Stimulated PDE

Kincaid et al (Kincaid et al. 1985) have shown that limited proteolysis of the 59kDa bovine brain PDE increased the 3' ,5'-phosphodiesterase activity of the enzyme in a Ca^{2+}/CaM-independent manner. Similar results have also been obtained by Sonnenburg et al (Sonnenburg et al. 1995) for the PDE1A1 and PDE1A2 splice variants and Loughney et al (Loughney et al. 1996)

observed very low activity when the PDE1A3, PDE1C1 and PDE1C3 splice variants when expressed as full-length proteins in yeast and that activity was not increased upon addition of 10μM calmodulin. However, when amino terminal truncated PDE1A3 and PDE1C1 splice variants were expressed in the yeast system, measurable hydrolysis of cAMP amd cGMP was observed (Loughney et al. 1996). These studies suggest that calmodulim modulates PDE activity by interaction with elements within the amino terminus of the protein, eliciting a conformational change which results in increased phosphodiesterase activiy. That the amino terminus of the PDE1 isozyme family contains calmodulin binding domains has already been discussed above (Section 1.5.1). Indeed Sonnenburg et al (Sonnenburg et al. 1995) have recently confirmed this postulate by identifying two calmodulin binding domains as well as an inhibitory element located within the amino terminus of the PDE1A1 and PDE1A2 isozymes.

Calmodulin activation of PDEs is thought to occur in a two-step process. The calmodulin molecule exists as a dumbell shaped structure comprised of two lobes connected by an α helix (Wang et al. 1990). Each lobe contains two Ca^{2+} binding sites. Upon binding of four molecules of Ca^{2+}, firstly to the high affinity binding sites and finally to the low affinity sites, a conformational change is elicited which allows binding of the Ca^{2+}/CaM complex to the PDE by means of hydrophobic interactions thought to involve specific amino acids within the α helix (Wang et al. 1990).

It has also been reported that several Ca^{2+}/CaM stimulated PDEs can be phosphorylated in a kinase specific manner (Beavo 1995; Florio et al. 1994; Hashimoto et al. 1989; Sharma 1991; Sharma and Wang 1985; Wang et al. 1990). For example, the 59kDa heart PDE1A1 and 61kDA brain PDE1A2 appear to be phosphorylated by cAMP-dependent protein kinase (Florio et al. 1994; Sharma 1991) whereas the 63kDa PDE1B1 isozyme is phosphorylated only by Ca^{2+}/CaM dependent protein kinase II (Florio et al. 1994; Hashimoto et

al. 1989; Wang et al. 1990). The effect of phosphorylation is to decrease the enzymes affinity for calmodulin thereby increasing the amount of Ca^{2+}/CaM required for activation (Wang et al. 1990). Phosphorylation can be inhibited in the case of the PDE1A1 and PDE1B1 isozymes by increasing the concentration of Ca^{2+}/calmodulin (Sharma and Wang 1985), indeed inclusion of Ca^{2+} and calmodulin in the reaction mixture prior to incubation with kinase completely inhibits phosphorylation (Sharma and Wang 1985). This effect for the PDE1A1 and PDE1A2 isozymes is substrate directed. If Ca^{2+} or calmodulin are added to the reaction mixture individually these isozymes are phosphorylated, but the Ca^{2+}/CaM complex phosphorylation is inhibited thus suggesting that the phosphorylation site is in or near the calmodulin binding domain (Sharma 1991; Sharma and Wang 1985). Florio et al (Florio et al. 1994), by a combination of proteolysis and HPLC, have identified serine 120 as the site of phosphorylation of the PDE1A2 isozyme, which would appear to be in excellent agreement with the findings of Sonnenburg et al (Sonnenburg et al. 1995) whose results for this isozyme have identified the region between amino acids 114-137 as a calmodulin binding site. In contrast to this, the binding of calmodulin to the PDE1B1 isozyme did not seem to prevent phosphorylation (Hashimoto et al. 1989). Despite the apparent kinase specificity observed for the PDE1A1, PDE1A2 and PDE1B1 isozyme dephosphorylation, in contrast, is mediated solely by Ca^{2+}/CaM dependent phosphatase (calcineurin) (Beavo 1995; Florio et al. 1994; Hashimoto et al. 1989; Sharma 1991; Sharma and Wang 1985; Wang et al. 1990). Latterly, it has been shown that Ca^{2+}/CaM-stimulated PDE1 activity can be induced in CHO cells due to overexpression of specific PKC isoforms upon incubation in the presence of PMA (Macfarland et al. 1991) and more recently through receptor-mediated stimulation of lipid signalling pathways (Spence et al. 1997).

1.5.4 PDE2, Cyclic GMP-Stimulated Phosphodiesterases

To date only one gene has been identified encoding for the cGMP-stimulated PDE (cGS-PDE) (Beavo 1995; Rybalkin and Beavo 1996). However, several investigators have presented evidence for the existence of at least two amino terminal splice variants (Murashima et al. 1990; Sonnenburg et al. 1991; Yang et al. 1994). Since the original observations of Beavo et al (Beavo 1971; Beavo et al. 1970), cGS-PDEs have been identified in both particulate and soluble fractions in a wide variety of tissues (Macfarland et al. 1991; Manganiello et al. 1990; Martins et al. 1982; Murashima et al. 1990; Whalin et al. 1991).

Martins et al (Martins et al. 1982) have purified a 105-107 kDa protein from bovine aderenal gland which hydrolyses cAMP and cGMP with similar maximal rates. Interestingly, this enzyme characteristically displayed the positive co-operativity also observed by other investigators (Beavo 1971; Rybalkin and Beavo 1996; Sonnenburg et al. 1991; Whalin et al. 1991). Furthermore, in the presence of 1μM cGMP, cAMP hydrolysis was increased some 5 to 6-fold (Martins et al. 1982). The presence of two distinct sites for cGMP binding was suggested by the observation that approximately 10% of the total cGMP bound at a concentration of 7 nM whilst the remaining nucleotide bound at a concentration of around 4 μM. Confirmation of the presence of two cGMP binding sites was obtained in the study by Stroop et al (Stroop et al. 1989) which, by means of photoaffinity labelling and limited proteolytic digestion, identified one binding site in the catalytic region and another allosteric site in the non-catalytic domain.

Murashima et al (Murashima et al. 1990) purified a 100kDa protein from bovine brain cerebral cortex grey matter which showed a 10 to 15-fold increase in cAMP hydrolysis when the enzyme was assayed in the presence of 0.4 μM cGMP. Photoaffinity labelling in the presence of [^{32}P]-cGMP showed that this

increase was inhibited by increasing the cGMP concentration to 100 μM. Comparison of peptide fragments of the particulate bovine brain and soluble liver enzymes demonstrated the tissue-specific distribution of two distinct splice variants (Murashima et al. 1990).

The availability of isozyme-specific antibodies was utilized by Macfarland and co-workers to determine that cGMP-stimulated PDE was preferentially localized within the glomerulosa layer in bovine adrenal gland (Macfarland et al. 1991). This enzyme is responsible for mediating atrial natriuretic peptide (ANP) triggered inductions in aldosterone production through the reduction of cAMP levels (Macfarland et al. 1991).

The PC12 cell line is derived from adrenal medulla and contains adenosine receptors coupled to cAMP synthesis as well as ANP receptors coupled to cGMP synthesis (Whalin et al. 1991). As such this cell line has been extensively used in the study of neurotransmitter release. Whalin et al (Whalin et al. 1991) have identified cGS-PDE in both particulate and soluble fractions of PC12 cells and subsequently purified a 102kDa PDE from the soluble fraction which displayed positive co-operativity. Stimulation of PC12 cells with [^{3}H] adenine resulted in the accumulation of cAMP. In contrast, treatment of PC12 cells with either ANP or sodium nitroprusside (SNP) generated an increase in cellular cGMP levels with a concomitant decrease in cAMP. Whalin et al (Whalin et al. 1991) reported that the decrease in cellular cAMP was due to activation of cGS-PDE and confirmed this by showing that treatment of PC12 cells with HL-725, a potent cGS-PDE inhibitor, reversed the effects of ANP and SNP.

Sonnenburg and co-workers (Sonnenburg et al. 1991) have cloned and expressed a 4.2kb cDNA from a bovine adrenal cortex library which, upon transfection in the cell line S49, resulted in the expression of soluble cAMP hydrolytic activity. This enzyme displayed positive co-operativity with low (1-5 μM) concentrations of cGMP increasing this activity approximately 3-fold.

Sonnenburg et al (Sonnenburg et al. 1991) have also demonstrated the existence of two distinct splice variants in a variety of bovine tissues. RNase protection assays revealed a 452bp band in adrenal cortex and medulla, liver, kidney lung and trachea while the detection of a 268bp band in brain suggests the presence of a specific isozyme in this tissue. Both the 452bp band and the 268bp band were detected in heart . Although unlike brain, heart expressed a substantial amount of the adrenal-like cGS-PDE mRNA as detected by the partial protection of the 268bp antisense riboprobe corresponding to the 5' end of the adrenal cGS-PDE. Furthermore, since the brain isozyme is membrane associated it can be inferred that the N-terminus is likely to be responsible for conferrment of membrane association on this enzyme (Sonnenburg et al. 1991).

Repaske et al (Repaske et al. 1993) have isolated a 350bp fragment of cDNA from rat brain that shows a high degree of homology with the bovine cGS-PDEs. By using this fragment as a probe these investigators have detected a 4.4kb mRNA in rat brain, liver, heart and kidney. Furthermore, regional analysis of brain showed that this isozyme was expressed predominantly in the hippocampus and cortex whilst being virtually absent from cerebellum of hindbrain (Repaske et al. 1993). Interestingly, Repaske et al (Repaske et al. 1993) have pointed out that there is very little expression of cGMP-dependent protein kinase in brain and therefore have postulated that the mode of action for cGMP must be through the hydrolysis of cAMP by cGS-PDE.

Consistent with the observations of Murashima (Murashima et al. 1990) and Sonnenburg (Sonnenburg et al. 1991), which allude to the existence of alternative splice variants amongst the cGMP-stimulated PDEs, Yang et al (Yang et al. 1994) have cloned and characterised a unique isozyme from a rat brain cDNA library. The calculated MW of the rat brain isozyme, at ~ 105kDa, agrees well with that of the bovine adrenal gland enzyme, at ~103kDa. Indeed, the rat brain and bovine adrenal gland cGS-PDEs share 91% amino acid sequence similarity . Hydrophilicity analysis, however , has revealed that the

N-terminal 37 amino acids of the rat brain enzyme, unlike the bovine isozyme, are extremely hydrophobic (Yang et al. 1994). Yang and co-workers (Yang et al. 1994) have also determined that greater than 75% of the rat brain isozyme can be found in the particulate fraction and suggest a role in targeting to membranes for the hydrophobic N-terminal region.

The involvement of a cyclic GMP-stimulated PDE in the regulation of cardiac contractility was first alluded to by the experiments of Fischmeister and Hartzell (Fischmeister and Hartzell 1987) who observed increased Ca^{2+} current (I_{Ca}) in frog ventricular cardiomyocytes perfused with cAMP. This was subsequently shown to be decreased by the intracellular perfusion of the same cells by 20 μM cGMP. More recently, Mery et al (Mery et al. 1993) reported that the increase in I_{Ca} resulted from the phosphorylation of L-type Ca^{2+} channels by cAMP-dependent protein kinase and demonstrated that this effect could be modulated in a biphasic manner by the nitric oxide (NO) donor SIN-1. Whilst SIN-1 made no difference to basal I_{Ca} in frog ventricular cells, an increase of ~40% I_{Ca} was observed when cells stimulated by the cyclase activating compounds forskolin or isoprenaline, were perfused with SIN-1 in the 0.1-10 nM range. Conversely, SIN-1 concentrations in the range 100 nM-1 mM led to a subsequent 85% decrease in I_{Ca} in the same cells. This observed decrease in I_{Ca} is due to activation of the cGMP-stimulated PDE reducing intracellular cAMP (Mery et al. 1993).

Further confirmation that cGMP-stimulated PDE is responsible for decreasing I_{Ca} in frog ventricular cells through cAMP hydrolysis was provide by the observation that EHNA, a potent cGS-PDE inhibitor, could reverse this trend and also block the inhibitory effect of the NO donors SIN-1 and SNP (Mery et al. 1995). Additional confirmation that EHNA acts as a specific cGS-PDE inhibitor has been provided by Haynes et al (Haynes et al. 1996) who showed that EHNA reversed hypoxic pulmonary vasoconstriction in rat lung, a condition originally caused by the cGS-PDE catalysed hydrolysis of cAMP.

Moreover, Michie et al (Michie et al. 1996) have shown that EHNA causes inhibition of a cGS-PDE implicated in the regulation of TCR signal transduction.

In summary, the cyclic GMP-stimulated PDEs are a family of PDEs which can hydrolyse both cAMP and cGMP. Both substrates demonstrate positively co-operative kinetic effects and micromolar concentrations of cGMP stimulate hydrolysis of cAMP. Both particulate and soluble cGS-PDEs have been characterised for a wide variety of tissues and cells. To date only one gene has been identified although there is evidence for the presence of at least two splice variants. The cGS-PDEs have been implicated in catecholamine secretion, calcium channel control, pulmonary vasoconstricion, aldosterone synthesis and T-cell signal transduction while possible involvement in olfaction and central nervous system function has been suggested.

1.5.5 PDE3, Cyclic GMP-inhibited Phosphodiesterases

Cyclic GMP-inhibited phosphodiesterases (cGI-PDEs) have been identified and characterised from a number of different tissues (Beavo 1995; Degerman et al. 1996; Manganiello et al. 1995; Manganiello et al. 1990) including adipocytes, where they have been implicated in modulation of the anti-lipolytic action of insulin (Brechler et al. 1992; Degerman et al. 1990; Gettys et al. 1988; Taira et al. 1993). Cyclic GI-PDEs are also known to play an important role in reversing the aggregatory action of cAMP in platelets (Lopez-Aparicio et al. 1993; Lopez-Aparicio et al. 1992; Macphee et al. 1988).

Glycogenolyis and gluconeogenesis are two processes that occur in hepatocytes due to a glucagon stimulated increase in the level of cAMP. Insulin, by contrast, is known to antagonize the effect of glucagon through the reduction of cAMP levels by activation of cGI-PDEs in these cells (Heyworth et al. 1983; Houslay and Kilgour 1990; Pyne et al. 1987).

Certain agents which exert positive inotropic effects in cardiac tissue are known to potentiate myocardial contractility by the selective inhibition of the

cGI-PDE isozyme family (Brechler et al. 1992; Meacci et al. 1992; Movsesian et al. 1991; Smith et al. 1991).

This isozyme family encodes proteins of 105-135kDa MW which display high affinities for both cAMP amd cGMP with *K*m values in the range of 0.1-0.8μM, however, they show V_{max} values which are approximately 4-10-fold higher for cAMP than cGMP, thereby effectively conferring specificity for cAMP as the preferred substrate (Beavo 1995; Degerman et al. 1996; Manganiello et al. 1990). Unlike the PDE4 isozyme family, hydrolysis of cAMP is inhibited by the presence of cGMP. Pyne et al (Pyne et al. 1987) for example, were able to show that 2μM cGMP caused approximately 50% inhibition of the 'dense-vesicle' PDE isolated from rat hepatocytes, whereas Manganiello et al (Manganiello et al. 1990) have reported that cGMP induced competitive inhibition with an IC50 in the 0.1-0.6μM range.

Initial attempts to purify the cyclic GMP-inhibited PDEs were hindered by the fact PDEs in general are expressed in cells with low abundance, are extremely labile and susceptible to proteolysis and are membrane bound. Thus Heyworth et al (Heyworth et al. 1983) and Pyne et al (Pyne et al. 1987) using a combination of homogenization and subcellular fractionation, demonstrated cGI-PDE activity in both particulate and cytosolic fractions of rat hepatocytes, and further resolved the particulate activity into two distinct enzyme populations, the plasma membrane bound PDE and the 'dense-vesicle' PDE. Subsequent to thse initial studies several groups have managed to purify cGMP-inhibited PDEs (Manganiello et al. 1995; Monaco et al. 1994). The observation that certain cardiotonic agents such as cilostamide and its derivates are specific inhibitors for cGI-PDEs was exploited by Degerman et al (Degerman et al. 1987; Degerman et al. 1990) to purify to homogeneity, the cGI-PDE from rat adipocytes. Lopez-Aparacio and co-workers (Lopez-Aparicio et al. 1992) were also able to purify the human platelet cGI-PDE by immunoprecipitation with specific polyclonal antibodies. Such a strategy has

also been succesfully used by Smith et al (Smith et al. 1993) to prepare cGI-PDEs from myocardium from several mammalian species.

Cyclic GMP-inhibited PDEs are found in both particulate and cytosolic cell fractions (Beavo 1995; Degerman et al. 1996; Manganiello et al. 1995; Meacci et al. 1992; Monaco et al. 1994). Gettys et al (Gettys et al. 1988) in their studies on the activation of the rat adipocyte cGI-PDE by the catalytic subunit of cAMP-dependent protein kinase, demonstrated that limited proteolysis of crude adipocyte microsomes resulted in the transfer of PDE catalytic activity from the particulate to the cytosolic fraction. Moreover, it was observed that this soluble PDE activity was only minimally activated by the addition of the catalytic subunit of PK-A, therefore Gettys and co-workers (Gettys et al. 1988) suggested that proteolysis had resulted in the removal of a regulatory domain from the PDE. Houslay and Kilgour (Houslay and Kilgour 1990) have shown that the cGI-PDE activity in hepatocytes was increased by 2-3-fold by PK-A but only if the cell fraction was initially treated with Mg^{2+} prior to challenge with PK-A. These investigators have shown that the effect of Mg^{2+} was to stimulate endogenous protein phosphatases

The first substantiated evidence regarding the structure of a cGI-PDE was obtained by Meacci and co-workers (Meacci et al. 1992). By using degenerate oligonucleotide probes based on the partial amino acid sequence of the human platelet cGI-PDE, these investigators isolated a cDNA from a human myocardial cDNA library which encoded a 125kDa protein. The deduced amino acid sequence revealed the presence of a highly conserved region which, upon expression as a fusion protein in a bacterial expression system, demonstrated cAMP hydrolytic activity and, furthermore, was immunoreactive to antibodies raised against the platelet enzyme. Interestingly, these investigators also identified the presence of a unique 44 amino acid within the catalytic region which is absent in the other PDE isozyme families (Manganiello et al. 1995; Meacci et al. 1992). Taira et al have subsequently confirmed the

existence of this insert upon isolation of two clones for the rat adipocyte cGI-PDE (Taira et al. 1993).

Hydropathy analysis of the amino acid sequence of the cGI-PDEs has revealed that the N-terminus contains several hydrophobic regions and it has been suggested that these may adopt a transmembrane α helical conformation (Degerman et al. 1996; Leroy et al. 1996; Meacci et al. 1992; Rahn et al. 1996; Smith et al. 1993; Taira et al. 1993). Indeed the experiments of Leroy and co-workers on full-length and truncated forms of the rat adipocyte and human myocardial cGI-PDEs have successfully demonstrated that removal of the hydrophobic N-terminal region results in the transfer of cAMP hydrolytic activity from the particulate to the cytosolic fraction uopn expression of cDNAs for these two isozymes in both NIH 3006 fibroblasts and Sf9 insect cells (Leroy et al. 1996).

The first indication that there may be more than one gene encoding the cGI-PDE isozyme family was provided by the experiments of Taira etal (Taira et al. 1993). The cDNA for RcGIP1 was cloned from a rat adipocyte cDNA library and the putative catalytic domain expressed in *E.coli* where it displayed the inhibition by cilostamide characteristic of the cPI-PDEs. A second distinct, but related, partial cDNA was also isolated which demonstrated a high degree of sequence similarity to the human cardiac cGI-PDE. It has been suggested that this may have beeen derived from RNA from vascular elements within the adipose tissue (Taira et al. 1993). Since these initial experiments, the PDE3A isozyme has been identified as the predominant form in smooth muscle, platelets and cardiac tissue whereas PDE3B, the second gene product, is more abundant in adipose tissue and hepatocytes (Beavo 1995; Degerman et al. 1996).

More recently Reinhardt et al (Reinhardt and Bondy 1996; Reinhardt et al. 1995) have shown that the PDE3A and PDE3B genes display distinct spaciotemporal patterns of expression during embryonic and postnatal

development. Reinhardt and co-workers (Reinhardt et al. 1995) have shown by means of *in situ* hybridization, that PDE3A is the gene expressed in rat adipocytes and hepatocytes during embryogenesis and throughout adult life, whereas PDE3B was highly expressed in rat mycardium throughout myocyte differentiation and into adulthood. This evidence appears to be somewhat confusing as it is in direct contrast to previous reports (Taira et al. 1993). Reinhardt et al (Reinhardt and Bondy 1996) also observed the abundant expression of both PDE3A and PDE3B mRNAs in embryonic rat brain and have subsequently demonstrated that PDE3B is uniformly expressed in rat brain from embryonic day 15 to adulthood, suggesting a fundamentaly metabolic role for this isoform. PDE3A mRNA however, displays three distinct patterns of expression. Firstly, it is highly abundant in primary and secondary neuroepithelial zones suggestive of a role in cell cycle regulation. Secondly, it is transiently expressed during early postnatal development in basal forebrain in concert with IGF-1 expression, suggesting a possible connection between IGF-1 stimulation and neuronal maturation. Finally, the observation that PDE3A mRNA is abundantly expressed in subsets of mature striatal and hippocampal neurons has implicated this isozyme in neuronal signalling (Reinhardt and Bondy 1996).

Evidence has also been presented for the existence of alternative transcripts of the human cardiac cGI-PDE which tends to suggest that these transcripts may be expressed in a tissue-specific manner. Kasuya and co-workers isolated a 4.4kb cDNA from a human placental cDNA library which encodes a protein of approximately 74kDa MW (Kasuya et al. 1995). Comparison of the nucleotide sequence revealed that the 4.4kb transcript is virtually identical to the 3' portion of the 7.6kb transcript corresponding to the 125kDa human cardiac enzyme previously cloned by Meacci etal (Meacci et al. 1992). By means of RNase protection assays and Northern blots and these researchers have identified an alternative promoter present within the cardiac

enzyme mRNA which, when expressed in HeLa cells as an in-frame fusion with the bacterial reporter enzyme CAT demonstrated an approximately 6-fold increase in CAT activity over the native enzyme (Kasuya et al. 1995). Expression of recombinant 80kDa and 125kDa isozymes in Sf9 insect cells also revealed differences in their kinetic properties and subcellular localization. The K_m for cGMP for the 80kDa enzyme was shown to be approximately 7-fold higher than the K_m for the 125kDa isozyme. Furthermore, the 125kDa isozyme was found in the particulate fraction in contrast to the cytosolic location of the 80kDa species (Kasuya et al. 1995). This observation further strengthens the hypothesis that the N-terminus contains elements which confer membrane localization upon members of the cGI-PDE, as previously suggested from several other studies (Degerman et al. 1996; Leroy et al. 1996; Meacci et al. 1992; Rahn et al. 1996; Smith et al. 1993; Taira et al. 1993). It would appear then that alternative transcription within the cGI-PDE isozyme family may provide a mechanism whereby the same gene can by expressed with different kinetic charateristics and subcellular localizations within different tissues.

1.5.5.1 Regulation of PDE3

As early as 1966 Butcher etal (Butcher et al. 1966) suggested that the anti-lipolytic action of insulin in rat adipose tissue may be due, at least in part, to a decrease in cAMP levels. That this process might involve the activation of a PDE was strengthened by the observation that epinephrine-stimulated increases in cAMP in adipose tissue was enhanced by the inclusion of caffeine, a known PDE inhibitor, in the incubation (Butcher et al. 1966). Loten and Sneyd (Loten and Sneyd 1970) demonstrated that the decrease in cAMP in epididymal fat pads was concommitant with an increase in cyclic nucleotide phosphodiesterase activity upon stimulation by insulin (Loten and Sneyd 1970). Some years later Heyworth et al (Heyworth et al. 1983) were able to show that rat hepatocytes contained two distinct membrane associated PDEs which they termed the plasma membrane PDE, presumed to be a PDE4 species, and the

'dense-vesicle' PDE, presumed to be a PDE3 species. These researchers were able to demonstrate that glucagon increased cAMP could be subsequently decreased by the addition of insulin, however, the action of insulin was inhibited by addition of IBMX, a known PDE inhibitor, to the reaction (Heyworth et al. 1983). Pyne et al (Pyne et al. 1987) in 1987 subsequently purified the 'dense-vesicle' PDE and showed that it could be activated by both glucagon and insulin whereas the plasma membrane PDE was activated by insulin in a fashion which could be blocked by glucagon. The 'dense-vesicle' PDE enzyme also observed to be inhibited by cGMP as well as milrinone, a specific inhibitor of cGI-PDEs (Pyne et al. 1987).

The observation that cilostamide acted as a specific inhibitor of the cGI-PDEs was exploited by Degerman and co-workers to purify to homogeneity a cGI-PDE from the particulate fraction of rat adipose tissue. This displayed an 80% increase in cAMP hydrolytic activity upon incubation in the presence of nanomolar concentrations of insulin (Degerman et al. 1987). Degerman et al (Degerman et al. 1990) have since shown that the cGI-PDE from rat adipose tissue can be activated by both insulin and the catecholamine isoprenaline. These processes involve covalent modification, mainly phosphorylation. It has been postulated that thes effects may occur through the action of an insulin-activated serine protein kinase and cAMP-dependent protein kinase at two distinct sites (Degerman et al. 1990).

Insulin-stimulation of the human platelet cGI-PDE has also been reported by Lopez-Aparacio et al (Lopez-Aparicio et al. 1992) who have observed that patients with diabetes mellitus display an increased sensitivity to platelet aggregating agents due to the action of cAMP as an inhibitory second messenger in these cells. Experiments carried out by these investigators demonstrated that incubation of the purified platelet cGI-PDE with insulin was accompanied by a 300-400% increase in phosphorylation of the enzyme. That this covalent modification was responsible for the observed increase in cAMP

hydrolysis was confirmed by a reduction of cGI-PDE activity after inclusion of the serine/threonine protein kinase inhibitor H7 in the reaction (Lopez-Aparicio et al. 1992). Since this study Lopez-Aparacioand colleagues (Lopez-Aparicio et al. 1993) have detected , partially purified and characterised an insulin-stimulated cGI-PDE serine kinase in human platelets. Previous studies have also demonstrated that agents such as prostaglandinds E_1 and I_2 mediate the activity of the human platelet cGI-PDE by a direct cAMP-dependent catalysed phosphorylation (Macphee et al. 1988).

The activation of cGI-PDEs by phosphorylation has been well documented in the literature since the first evidence that the rat adipocyte particulate cGI-PDE was specifically phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (Gettys et al. 1988; Leroy et al. 1996; Lopez-Aparicio et al. 1993; Rahn et al. 1996; Rascon et al. 1994; Taira et al. 1993). Based on the observation that limited proteolysis released an active PDE into the cytosolic fraction which showed no measurable increase in activity upon incubation with the catalytic subunit of cAMP-dependent protein kinase, Gettys and co-workers postulated that the site of phosphorylation was located within the N-terminus of the enzyme (Gettys et al. 1988).

Degerman et al (Degerman et al. 1990) through radiolabelling and phosphopeptide mapping were able to show that insulin and isoprenaline induced serine phosphorylation. However, identification of several cAMP-dependent phosphorylation sites was only achieved upon isolation and characterization of the enzyme, suggesting that serine 427 was the specific residue phosphorylated (Leroy et al. 1996; Lopez-Aparicio et al. 1993; Rahn et al. 1996; Rascon et al. 1994; Taira et al. 1993). Interestingly, Leroy and co-workers (Leroy et al. 1996) have recently demonstrated that although serine 427 was the major site of *in vitro* phosphorylation found using solubilized adipocyte cGI-PDE, the major site of phosphorylation in intact cells treated with insulin, or other agents known to elevate cAMP, was in fact serine 302. This

observation has since been confirmed by the experiments of Rahn etal (Rahn et al. 1996). It has been suggested that the reason for this difference may reflect a difference in structure of the enzyme upon membrane insertion (Leroy et al. 1996).

In addition to the wealth of information regarding hormonal activation of the cGI-PDEs, Brechler and co-workers have presented evidence for the inhibition of cGI-PDE by glucagon (Brechler et al. 1992). Brechler et al (Brechler et al. 1992) observed that, upon addition of glucagon to the particulate fraction of frog ventricle, approximately 40% inhibition of PDE activity was observed in the presence of micromolar concentrations of GTP. No effect was observed for the cytosolic PDE activity. Furthermore, it was noted that treatment of the particulate fraction with pertussis toxin (PTX) resulted in the complete abolition of the inhibitory effect of glucagon. Since no effect was observed upon treatment with cholera toxin, Brechler and colleagues (Brechler et al. 1992) suggested that the inhibition of the cardiac cGI-PDE by glucagon was mediated by membrane receptors via a PTX sensitive G-protein possibly G_i or G_o. Further evidence in support of this theory was provided by the observation that mastoparan, a wasp venom peptide known to activate G_i /G_o directly, mimicked the effect of glucagon.

In summary, cGMP-inhibited phosphodiesterases are 'low K_m' PDEs which specifically hydrolyse cAMP, are inhibited by cGMP and are stimulated by hormones and agents which raise intracellular cAMP and are known to be activated/deactivated by phosphorylation/dephosphorylation. Cyclic GI-PDEs have been identified, and in some cases isolated, from both cytosolic and particulate fractions of adipocytes, hepatocytes, cardiac tissue, smooth vascular muscle and platelets where they have been implicated in lipolysis glycogenolysis and gluconeogenesis, myocardial contractility, blood pressure and clotting respectively. Two distinct genes have been identified for the cGI-PDEs and there is evidence for the existence of alternative transcripts.

Structurally, they show the same domain organisation as other PDE families, with membrane association and regulatory elements contained within the N-terminus and a C-terminal catalytic domain which contains a 44 amino acid insert unique to the cGI-PDE isozyme family.

1.5.6 PDE4, Cyclic AMP-Specific Phosphodiesterases

The PDE4 cyclic AMP-specific PDEs are, to date, the largest and most extensively studied of the seven PDE isozyme families (Beavo 1995; Beavo et al. 1994). At least four separate genes have been identified most of which, to add a further level of complexity, have been shown to possess two or more alternative splice variants (Beavo 1995; Beavo et al. 1994; Bolger et al. 1993; Colicelli et al. 1989; Davis et al. 1989; Engels et al. 1995; Horton et al. 1995; Livi et al. 1990; McLaughlin et al. 1993; Michaeli et al. 1993; Sullivan et al. 1994). This isozyme family specifically hydrolyses cAMP and can be further characterized by the observation that they are uniquely sensitive to inhibition by rolipram (Beavo 1995; Beavo et al. 1994; Beavo and Houslay 1990).

PDE4 was originally classified after the initial observations of Reeves et al (Reeves et al. 1987b) that four distinct PDE activities present in guinea-pig cardiac ventricle could be eluted by increasing salt concentration following separation by DEAE-ion exchange chromatography. Moreover, Reeves and collegues (Reeves et al. 1987a) observed that the compounds rolipram and RO-12-2074 were selective and potent inhibitors of PDE4 activity. However,it should be noted that some years prior to this work, Marchmont and Houslay (Marchmont and Houslay 1981) purified a 52kDa protein which specifically hydrolysed cAMP from rat liver. Subsequent studies by Pyne et al (Pyne et al. 1987) identified this enzyme isolated from rat hepatocytes as the peripheral plasma membrane PDE (PPM.PDE) and showed that, similarily to the guinea-pig PDE4, it could be specifically inhibited by both rolipram and RO-17-2074.

1.5.6.1 Molecular Cloning of PDE4 Isoenzymes

Undoubtedly a major insight in PDE understanding was obtained from early studies done on the fruit fly *Drosophila melanogaster*. Dudai et al (Dudai et al. 1976) had shown as early as 1976 that, unlike normal flies which can learn to avoid an odorant associated with an electric shock, an X-linked mutant failed to display this learning despite being able to sense both the odorant and the electric shock and so named the mutation *dunce* . Subsequent biochemical characterization of flies containing the *dunce* mutation revealed that the defect lay in a gene which encoded a cAMP phosphodiesterase (Byers et al. 1981; Davis and Kiger 1981) and therefore provided the first tangible evidence that memory and learning could have a genetic basis. Chen et al (Chen et al. 1986) have since isolated the gene by means of chromosome walking and identified various associated RNA transcripts. Upon sequencing several cDNA clones originating from the various transcripts, these investigators ascertained that the *dunce* gene product possessed striking amino acid sequence similarity to the amino acid sequence of the Ca^{2+}/CaM PDE enzyme. That researchers have observed such high degrees between primary amino acid seqeuences has led to the postulation that *dunce* is most likely the probable ancestor of the mammalian cAMP PDEs (Chen et al. 1986; Qui et al. 1991). Qui et al (Qui et al. 1991) have provided a further valuable insight into the complex nature of the *dunce* gene by demonstrating that it spans a region of approximately 148 kb of genomic DNA whilst six different mRNA species can be generated by three alternative transcription initiation sites in conjunction with alternative splicing.

The first PDE genes to be cloned from mammalian species were obtained by two different experimental approaches. By exploiting the amino acid similarities observed between the bovine Ca^{2+}/CaM PDE and the *dunce* PDE, Davis et al (Davis 1990; Davis et al. 1989) used a probe derived from the *dunce* gene to screen a rat brain cDNA library. They subsequently cloned a

cDNA, now known as RD1 (RNPDE4A1), which encoded a 610 amino acid open reading frame. This displays significant homology to a conserved domain of approximately 275 amino acids found in most other PDEs . Interestingly, Davis and collegues (Davis 1990; Davis et al. 1989) also isolated a series of other cDNAs with divergent N-terminal regions, RD2 and RD3, (RNPDE4A2 and RNPDE4A3 respectively). Furthermore, RD3 has a 99 bp deletion suggesting the possibility of the existence of alternative transcripts.

The second molecular approach to cloning mammalian cDNA was adopted by Colicelli et al (Colicelli et al. 1989). The yeast *Saccaromyces cerevisiae* contains genes which encode *RAS* 1 and *RAS* 2. When the activated form of *RAS* 2 is present yeast, cells display a loss of growth control and an increased sensitivity to heat shock as a result of the accumulation of cAMP by the uncontrolled activation of adenylyl cyclase (Colicelli et al. 1989). By constructing a rat brain cDNA library in a yeast expression vector, Colicelli et al (Colicelli et al. 1989) were able to isolate and clone and characterize the DPD (Dunce-like Phosphodiesterase) PDE cDNA clone that was able to complement the loss of growth control associated with *RAS* 2 activation in yeast. Biochemical characterization of DPD showed it to be a high-affinity cAMP PDE with approximately 80% amino acid similarity to the dunce gene of *Drosophila melanogaster* (Colicelli et al. 1989). This same approach has also been employed by other researchers to clone cDNAs for PDE4s from humans (Bolger et al. 1993; Michaeli et al. 1993; Sullivan et al. 1994).

The first definitive evidence for the existence of multiple genes encoding PDE4s was presented by Swinnen et al (Swinnen et al. 1989a; Swinnen et al. 1989b). By adopting a similar strategy to that described by Davis et al (Davis et al. 1989) Swinnen and co-workers used a *dunce* derived probe to identify two cDNAs in a rat testis cDNA library which they named ratPDE1 (RNPDE4C) and ratPDE2 (RNPDE4A). By using a probe derived from ratPDE2 a further two clones were isolated from a Sertoli cDNA library, ratPDE3 (RNPDE4D)

and ratPDE4 (RNPDE4B). Sequence analysis of these four clones revealed that although they possessed similar but not identical sequences over a region of approximately 1080 bases they, more importantly, displayed considerable sequence heterogeneity outwith this region. Comparison of the deduced amino acid sequences for each of the four clones demonstrated that each contained the highly conserved region of approximately 270 residues observed in all previously published PDE sequences whilst a great degree of diversity was observed in the sequences corresponding to the amino and carboxy termini (Swinnen et al. 1989a). Bolger et al (Bolger et al. 1993) has independently established the presence of four distinct PDE4 genes homologous to the *dunce* gene product of *Drosophila melanogaster* , in human brain.

Clones corresponding to human PDE4 isoenzymes have also been isolated from human tissues by exploiting the same methodology employed above. Livi et al (Livi et al. 1990) cloned a PDE4A gene (HSPDE4A4) from a human monocyte cDNA library whilst Bolger and colleagues have isolated the cDNA for PDE46 (HSPDE4A5) from a human foetal brain cDNA library.

Since these initial studies several other researchers have cloned and characterized cDNAs representing each of the four PDE4 genes. Sullivan et al (Sullivan et al. 1994) reported the cloning of the PDE4A gene product h6.1 (HSPDE4A7) from a human T-cell Jurkat cDNA library by a combination of PCR screening and reversal of heatshock in yeast and Horton and colleagues (Horton et al. 1995) have isolated the cDNA for 2EL (HSPDE4A8), a novel PDE4A splice variant, from the same Jurkat T-cell library. Interestingly, 2EL whilst possessing sequences in common with h6.1, differs from this gene product by virtue of a distinct 5'-end. It also contains a 34 base insert within the region thought to encode the catalytic domain which effectively renders this PDE inactive presumably by orchestrating a frameshift and thus causing the premature termination of the open reading frame. It has, to date not been possible to prove conclusively that 2EL exists in a physiological context, nor to

assign a role to this splice variant. Curiously, in an earlier study Bolger et al (Bolger et al. 1993) reported a similar observation with the identification of TM3. Horton et al (Horton et al. 1995) have tentatively suggested that such non-functional splice variants may represent an intracellular mechanism for the control of PDE gene expression at the mRNA level.

As mentioned previously, a mutation in the *Drosophila dunce* gene leads to behavioral and learning difficulties (Byers et al. 1981; Davis and Kiger 1981). Interestingly, Horton et al (Horton et al. 1995) by means of *in situ* fluorescence chromosomal hybridization have mapped the human PDE4A gene to the p13.1-q12 region of chromosome 19. Chromosome 19 has also been implicated in susceptibility to late-onset Alzheimers syndrome. It is intriguing, therefore, to consider the possibility that changes in the concentration of intracellular cAMP by this PDE may somehow be implicated in the manifestation of this disease state.

Several studies have established the existence of a second PDE4 gene designated PDE4B (Bolger et al. 1993; Bolger et al. 1994; Colicelli et al. 1989; Monaco et al. 1994; Obernolte et al. 1993; Swinnen et al. 1989a). The first reported cloning of a PDE4B gene was obtained by the studies of Colicelli et al (Colicelli et al. 1989) who isolated and characterized the DPD (RNPDE4B1) gene from rat brain, and Swinnen and colleagues (Swinnen et al. 1989a) who screened a Sertoli cell cDNA library with a probe derived from a rat PDE4A clone and isolated the cDNA for RNPDE4B2A. Comparison of the primary amino acids sequences for these two clones revealed that RNPDE4B2A differed from RNPDE4B1 by the addition of an extra 41 amino acids at the N-terminus thus suggesting that, like the PDE4A isozymes, PDE4B also demonstrated alternative transcripts. Subsequently, several researchers have reported the cloning of cDNA corresponding to PDE4Bs from both rat and humans. Obernolte et al (Obernolte et al. 1993) have cloned a 3.8kb cDNA from a human lymphocyte B-cell line by low stringency screening with a rat PDE4B probe.

This clone exhibited ~86% and ~93% sequence similarity at the DNA and protein levels respectively to the rat PDE4B. Expression of this clone in bacteria revealed a 7.5-13-fold increase of cAMP hydrolysis which was shown to be inhibitable by rolipram, thus confirming its status as a PDE4 isozyme.

Whilst investigating the possibility of developing PDE4 inhibitors as anti-depressant and anti-inflammatory drugs, McLaughlin et al (McLaughlin et al. 1993) have presented evidence for the expression of a second PDE4B gene in human brain. A cDNA for HSPDE4B2A was isolated and characterized from a human frontal cortex cDNA library. Independent confirmation for the existence of this PDE4B gene has also been presented by Bolger and colleagues (Bolger et al. 1993) by utilizing a combination of PCR, hybridization screening and yeast complementation. Moreover, Bolger et al (Bolger et al. 1993) have provided evidence for the existence of a further human PDE4B splice variant TM72 (HSPDE4B1).

Further evidence for the existence of multiple PDE4B splice variants has been presented by Monaco et al (Monaco et al. 1994). Isolation of a genomic rat PDE4B clone from a cosmid library has shown that it is derived from the assembly of 11 exons and 10 introns distributed over ~25 kb. Moreover, the 5'-untranslated region contains the potential to encode multiple short open reading frames which may reduce translation of the full-length PDE4B mRNA in a manner analogous to that suggested previously for the PDE4A splice variant 2EL (Horton et al. 1995) and TM3 (Bolger et al. 1993).

By comparison with the information obtained for PDE4A and PDE4B gene products, relatively little is known about PDE4C. Early reports were successful in identifying and isolating cDNAs for both rat (Swinnen et al. 1989a) and human (Bolger et al. 1993; Bolger et al. 1994). Moreover, since only partial cDNAs which do not encode functional isozymes have been identified, further characterization has not been possible. Engels et al (Engels et al. 1995) have reported the cloning of a putative full-length human PDE4C.

However, it would appear that the approach used to generate this clone resulted in the generation of a composite cDNA from two different mRNA sources, thus any inferences made regarding this clone must necessarily be treated with a certain amount of caution. More recently, Owens and colleagues (Owens et al. 1997) have reported the isolation of a cDNA for a PDE4C gene from the human glioblastoma cell line U87 by means of RT-PCR and 5' RACE. Interestingly, this approach has yielded two alternative 5' end sequences suggesting that PDE4C, like the PDE4A and PDE4B genes, encodes for alternative splice variants. Sequence comparison suggests that, as for the other PDE4s, the human PDE4C is more homologous to the rat PDE4C than it is to other human PDE4s thus implying some conservation of functional significance.

Next to the PDE4A isozyme family the PDE4D gene demonstrates the most heterogeneity with evidence to support the existence of multiple splice variants having been reported in the literature (Baecker et al. 1994; Beavo 1995; Beavo et al. 1994; Bolger et al. 1993; Bolger 1994; Bolger et al. 1994; Jin et al. 1992; Monaco et al. 1994; Nemoz et al. 1996; Sette et al. 1994b; Swinnen et al. 1989a). Swinnen et al (Swinnen et al. 1989b) reported the first cloning of a PDE4D cDNA from a Sertoli cell cDNA library whilst Bolger and co-workers (Bolger et al. 1994) have shown the existence in rat brain of two PDE4D splice variants, RPDE13 and RPDE3 (RNPDE4D1B and RNPDE4D3A respectively). These, while displaying a large degree of sequence similarity in the catalytic region, possess divergent 5'- ends. Evidence has also been obtained for the existence of distinct PDE4D splice variants from a variety of human sources including brain (Bolger et al. 1993), heart (Baecker et al. 1994) and, more recently, peripheral blood mononuclear cells (Nemoz et al. 1996). This suggests the possibility of the involvement of PDE4 isozymes in physiological proccesses as diverse as memory and learning, congestive heart failure and anti-inflammatory conditions.

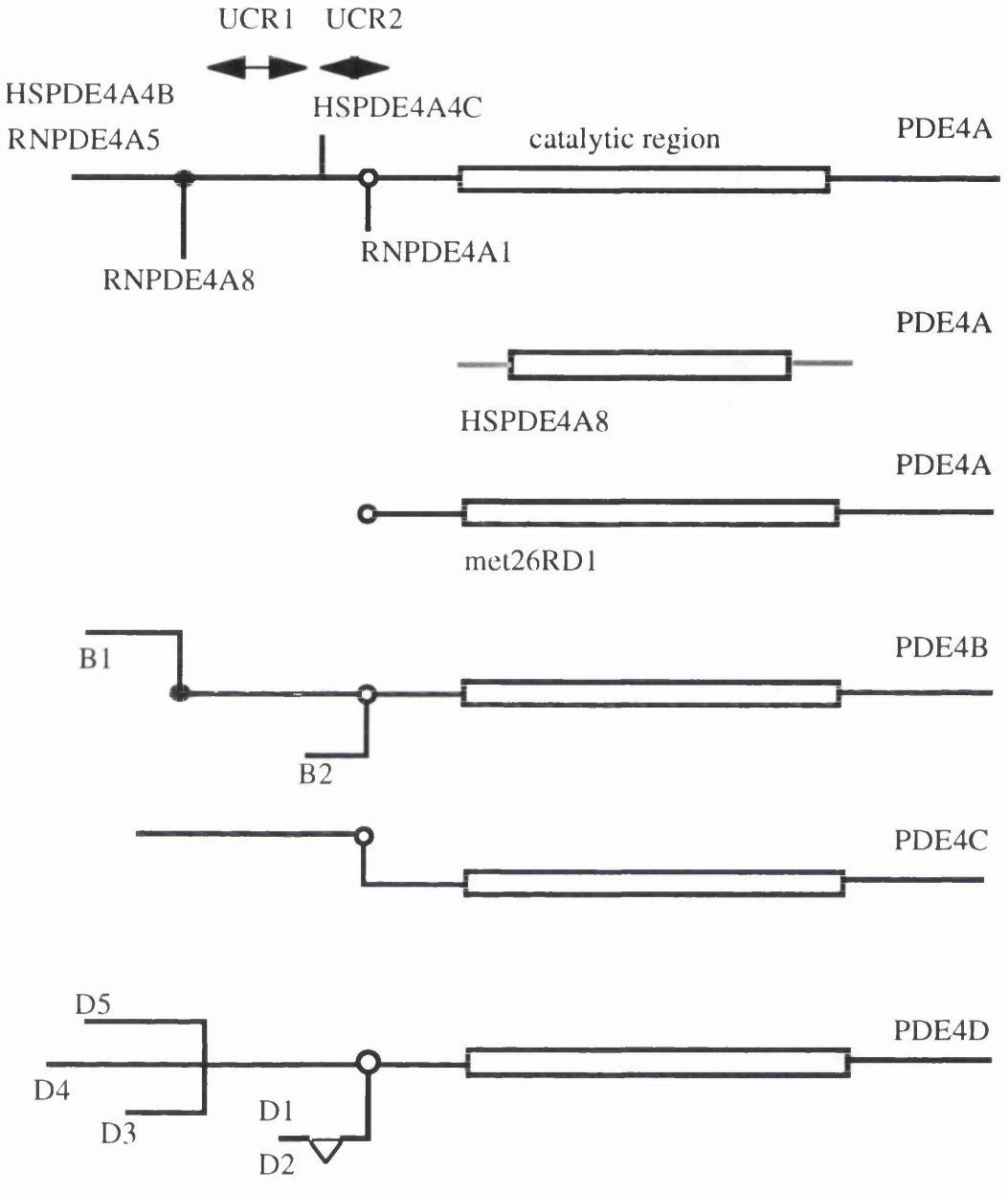
1.5.6.2 Structure of PDE4s

Like most other PDE isozymes studied to date, PDE4s can be regarded as multi-domain proteins composed of a highly conserved core containing the catalytic domain linked to variant N- and C-terminal fragments (Charbonneau 1990). Many groups have subsequently cloned cDNAs for a variety of PDE4 isozymes (Bolger et al. 1996; Bolger et al. 1993; Bolger et al. 1997; Chen et al. 1986; Colicelli et al. 1989; Davis et al. 1989; Engels et al. 1995; Jin et al. 1992; Livi et al. 1990; McLaughlin et al. 1993; Obernolte et al. 1993; Owens et al. 1997; Sullivan et al. 1994) which are consistent with Charbonneau's proposal. Furthermore, such studies have demonstrated that considerable structural similarities exist throughout this region of approximately 270 amino acids.

More interestingly, studies carried out on human homologues of each of the four PDE4 genes by Bolger et al (Bolger et al. 1993) have ascertained the existence of two further regions of amino acid sequence homology. These are known as UCR1 and UCR2 (Upstream Conserved Regions) and have been demonstrated to be unique to the PDE4 gene family (Fig. 1.3). Thus, the PDE enzyme can be classified as either 'long form' PDE4s containing the catalytic domain along with UCR1 and UCR2, or 'short form' PDE4s consisting of the catalytic domain and UCR2 only. Furthermore, it has been observed that all catalytically active PDE4s transcribed from a specific gene have identical C-terminal regions. Each of these are distinct from the C-termini of isozymes transcribed from other PDE4 genes (Erdogan and Houslay 1997; Huston et al. 1996; Lobban et al. 1994; Shakur et al. 1995)

Figure 1.3: Splice Variants Arising from PDE4 Genes

PDE4 splice variants are shown with an open box representing the highly conserved putative catalytic region. Long form splice variants are denoted by closed circles (●) and short form splice variants by open circles (○). Three splice variants of the rat PDE4A gene are shown, the short isoform RNPDE4A1 and the long isoforms RNPDE4A5 and RNPDE4A8. The human long isoform splice variant HSPDE4A5B is also represented. HSPDE4A4C represents the N-terminally truncated clone known as h6.1 (Sullivan et al 1994). In addition an inactive human splice variant HSPDE4A8 also known as 2EL (Sullivan et al 1994) which has short novel sequences (grey lines) spliced onto an N- and C- terminally truncated catalytic region is shown. Met26RD1 denotes the active 'core' PDE4A gene product. Long and short isoform splice variants are also shown for PDE4B and PDE4D genes. PDE4D2 is related to PDE4D1 except for a deletion in the N-terminal splice region.


UCR1
UCR2
HSPDE4A4B
RNPDE4A5
HSPDE4A4C
catalytic region
PDE4A
RNPDE4A8
RNPDE4A1
PDE4A
HSPDE4A8
PDE4A
met26RD1
B1
PDE4B
B2
PDE4C
D5
PDE4D
D4
D3
D1
D2

1.5.6.2.1 The Catalytic Site

Evidence that the 270 amino acid region (Fig. 1.2) found to be highly conserved amongst PDE4 isozymes corresponded to the catalytic domain was provided by the studies of Jin et al (Jin et al. 1992). These researchers demonstrated that it was possible to remove a carboxy terminal 99 amino acid region flanking the conserved domain from RNPDE4D2A and still observe cAMP hydrolytic activity in both bacterial and eukaryotic expression systems. By employing the same strategy to engineer deletion of the N-terminal 97 amino acids of RNPDE4D2A, Jin and co-workers also demonstrated that the N-terminus does not form part of the catalytic domain whereas a larger deletion of 131 amino acids at the N-terminus leads to abolition of cAMP hydrolysis. Interestingly, Swinnen et al (Swinnen et al. 1989a) had previously reported the cloning of RNPDE4D3 which was identical to RNPDE4D2A with the exception that it lacked 78 amino acids at the N-terminus and have shown that this isozyme possesses catalytic activity. More recently, Jacobitz et al (Jacobitz et al. 1996a) provided further confirmation that the highly conserved region common to all PDEs contained the catalytic domain by demonstrating that a truncated PDE4A encompassing only the conserved domain was capable of hydrolysing cAMP and that this activity could be inhibited by the selective PDE4 inhibitor rolipram.

Analysis of the amino acid sequences of all mammalian PDEs revealed the presence of several highly conserved serine, threonine and histidine residues thus implying a possible conservation of function. By means of site specific mutagenesis Jin et al (Jin et al. 1992) have determined that some of these amino acid residues are necessary for cAMP hydrolysis. Substitution of the invariant threonine 349 (numbered for RNPDE4D2A) by either alanine or proline abolished catalytic activity whereas substitution by serine resulted in catalytic activity but with a reduced V_{max}. Conversely, substitutions of serines 305 and 398 had no effect upon cAMP hydrolysis but mutation of histidines 278 and

311, which lie in close proximity to threonine 349, also prevented cAMP hydrolysis. More recently, Jacobitz and co-workers (Jacobitz et al. 1996b) working on a human PDE4A (HSPDE4A4) have shown that not only are histidine residues at positions 433, 437 and 473 necessary for substrate binding, but that, in addition to eliciting a substantial decrease in cAMP hydrolytic activity, mutation of these amino acids resulted in a marked reduction of this enzymes ability to bind the prototypical PDE4 inhibitors [^{3}H](R)-rolipram or [^{3}H]PR 73401.

1.5.6.2.2 UCR1 and UCR2

Cloning of the first human homologues to the *dunce* PDE and alignment of their sequences by Bolger et al (Bolger et al. 1993; Torphy et al. 1992b) revealed the presence of two regions of high homology which were unique to the PDE4 gene family. These were subsquently named UCR1 and UCR2. Analysis of the amino acid sequence of UCR1 revealed a region of approximately 55 amino acid residues divided into a polar N-terminal region and an apolar predominatly hydrophobic C-terminal region which may demonstrate the ability to adopt an amphipathic α helix. Interestingly, the polar region in PDE4D3 contains a PKA consensus phosphorylation site RRES situated within a region composed predominantly of positively charged amino acids. Phosphorylation of this serine may thus elicit a conformational change in the region which may have functional implications. UCR2 consists of approximately 76 amino acids and is, in contrast to UCR1, a very hydrophilic structure. Secondary structural predictions suggest that UCR2 is composed of three α helices the first and last of which display strong negative charges whilst the middle helix is slightly positively charged and thus UCR2 may exist as an amphipatic helix (Torphy et al. 1992b).

UCR1 and UCR2 are separated from each other by the so called Linker Region 1, LR1 (Houslay et al. 1997b), a short region which exhibits very little primary amino acid sequence homology between individual members of the

PDE4 isozyme family but which do appear to display some gene specific similarity. While LR1 of the PDE4A genes products consists of alternating hydrophobic and hydrophilic regions, LR1 of PDE4B is exclusively hydrophilic. The LR1 of PDE4C although predominantly hydrophilic possesses a hydrophobic N-terminal region while the LR1 region in PDE4D is located at its C-terminus. UCR2 is in turn separated from the catalytic domain by the so called Linker Region 2 (LR2) (Houslay et al. 1997b) which varies in size from 10 to 28 amino acids all of which are hydrophobic, although whilst the LR2 of PDE4C contains some negatively charged residues the others consist only of positively charged amino acids. Moreover, it would seem that LR2 may form a predominantly random coil structure.

Whilst specific functional significance has yet to be attributed to the predicted structures within URC1 and UCR2, Bolger (unpublished data) has observed that UCR1 can associate with UCR2 in a yeast two-hybrid assay designed to detect protein-protein interactions. It may be then, that association of these two distinct regions serves in some regulatory manner specific to the PDE4 isozyme family. It should be noted however, that this observation was based upon studies using isolated UCR1 and UCR2 regions and it remains to be seen whether this can be replicated by the full-length PDE4 gene products.

1.5.6.3 Tissue Distribution of PDE4 Isozymes

Members of the PDE4 gene family have been identified in a wide variety of cell and tissue types by a variety of experimental approaches. Marchmont and Houslay (Marchmont and Houslay 1980) have demonstrated the presence of PDE4 activity in rat liver plasma membranes and later in hepatocytes (Houslay et al. 1992). Meanwhile, Swinnen and colleagues (Swinnen et al. 1989a; Swinnen et al. 1989b) isolated cDNA clones for the first mammalian homologues of the *Drosophila dunce* PDE from rat testis. Such data implies that the action of PDE4 species may be initmately involved in the regulation of cAMP levels in cells implicated in cellular development and differentiation. It

has also been shown (Swinnen et al. 1989b) that whilst RNA transcripts for all four PDE4 genes are present in germ cells, Sertoli cells appear to contain only RNA corresponding to PDE4D. which, it was observed, increased by more than 100-fold upon stimulation of the cells by FSH. More recently, Bolger et al (Bolger et al. 1996) demonstrated the specific expression of a PDE4A splice variant RPDE39 (RNPDE4A8) in testis by RNase protection assay and immunoblotting with a specific PDE4A polyclonal antibody. Evidence has also been presented for the existence of PDE4 isozymes in mammalian heart (Baecker et al. 1994; Reeves et al. 1987a). Furthermore, the numerous reports of PDE4 in lymphocytes (Erdogan and Houslay 1997; Obernolte et al. 1993; Sullivan et al. 1994), monocytes (Livi et al. 1990; Nemoz et al. 1996; Verghese et al. 1995) and thymocytes (Michie et al. 1996) suggests that regulation of cAMP levels by PDE4 may be important in a variety of inflammatory diseases and conditions.

Selective inhibition by the anti-depressant drug rolipram has implicated the PDE4 isozyme family in mood regulation, memory and learning. As such, many studies have described the specific expression of PDE4 gene products and splice variants in brain. Following the initial isolation of the cDNA from RD1 (RNPDE4A1A) Shakur and co-workers (Shakur et al. 1995) have demonstrated by immunoblotting with a PDE4A specific antiserum, that RD1 is expressed predominantly in the cerebellum in rat brain. McPhee and co-workers (McPhee et al. 1995) have since shown that RD1 is widely expressed in other regions of rat brain as well as cerebellum, namely cortex, hypothalamus and striatum and to a lesser extent, hippocampus. Furthermore, this study has confirmed that the PDE4A splice variant RPDE6 (RNDPE4A5), which was previously identified by Northern blotting and RNase protection assay (Bolger et al. 1994), is also differentially expresssed in rat brain. However, unlike RD1, it appears to be absent from cerebellum and brain stem.

Several other studies have suggested the expression of other PDE4 genes in brain. Colicelli (Colicelli et al. 1989) described the first cloning of a PDE4B gene from a rat brain cDNA library and Lobban et al (Lobban et al. 1994) have subsequently confirmed the existence of two PDE4B splice variants in rat brain by immunoblotting with specific antisera. Other researchers have also demonstrated the expression of PDE4B genes in human (McLaughlin et al. 1993) and rat (Monaco et al. 1994) brain respectively. More recently, Engels and colleagues (Engels et al. 1995) reported the cloning of a PDE4C cDNA from human substantia nigra, while Owens et al (Owens et al. 1997) have identified the existence of PDE4C in the glioblastoma U87 cell line.

1.5.6.4 Regulation of PDE4

Analysis of the available literature reveals that PDE4 isozymes can be regulated in a number of ways by hormones and growth factor action. These include phosphatidic acid (Disanto et al. 1995; Disanto and Heaslip 1995; Nemoz et al. 1997; Savany et al. 1996) and phosphorylation (Alvarez et al. 1995; Lenhard et al. 1996; Pyne et al. 1989; Sette and Conti 1996; Sette et al. 1994a; Sette et al. 1994b; Swinnen et al. 1991a; Swinnen et al. 1989b) as well as at the level of mRNA transcription (Manning et al. 1996; Sette et al. 1994a; Sette et al. 1994b; Verghese et al. 1995).

1.5.6.4.1 Regulation of PDE4 by Phosphatidic Acid

Several studies have reported that elevated levels of phosphatidic acid (PA) can be observed in a range of cells associated with inflammatory responses (Disanto et al. 1995; Disanto and Heaslip 1995; Nemoz et al. 1997; Savany et al. 1996). As it is well known that cell cycle progression and proliferation of T-lymphocytes can be influenced by changes in cAMP then PA action on PDE4 activity may play a regulatory role (Michie et al. 1996; Savany et al. 1996). Indeed, as lymphocytes lack an adenylyl cyclase coupled inhibitory receptor (Savany et al. 1996) then it would seem reasonable to suggest that control of intracellular cAMP levels and hence lymphocyte function

may be modulated by cAMP phosphodiesterase activity. Savany and co-workers (Savany et al. 1996), by means of anion exchange chromatography, were able to resolve the cyclic nucleotide PDE activity in rat thymocytes into five peaks and demonstrated that 50-60% of the total PDE activity was found in peaks 2 and 3 which were inhibited by rolipram with IC_{50} values of 0.35 μM and 0.60 μm respectively. Upon addition of physiological concentrations of various phospholipids Savany et al observed that peak 3 PDE activity was activated by approximately 50% above basal by anionic phospholipids whilst peak 2 PDE activity was inhibited by phospholipids possessing saturated fatty acid side chains. More recently, Nemoz et al (Nemoz et al. 1997) studied the effect of PA upon various recombinant PDE4 isoform expressed in Sf9 and MA10 cells. These researchers showed that PA could activate PDE4 activity in a splice variant specific fashion where the long form splice variants PDE4A5, PDE4B1 and PDE4D3 were activiated by anionic phospholipids whereas the short forms PDE4A1, PDE4B2 and PDE4D1 and 4D2 were not affected. Moreover, Nemoz et al observed that PA increased the V_{max} of PDE4D3 whilst leaving the K_m of the enzyme for cAMP unchanged. Why various isoforms of PDE4 should be differentially activated by PA remains unclear. However, one possible explanation that is suggested by the above observations refers to the fact that the long form PDE4s are known to contain the highly conserved region known as UCR1 (Bolger et al. 1993) which is absent in the short forms. The splice variant PDE4D3 has been shown to be phosphorylated on serine 54 within UCR1 by cAMP-dependent protein kinase A (Sette and Conti 1996) and Nemoz et al have shown that PDE4D3 can be activated by both cAMP-PKA and PA to the same extent. Moreover, the observation that activation of PDE4D3 by both PA and PKA mediated phosphorylation is not additive (Nemoz et al. 1997) suggests that both may interact at a similar site on the enzyme. This argument is further strengthened by the observation that only anionic phospholipids, which possess a free phosphate group, are capable of activating PDE4 isozymes.

While the exact nature of the mechanism of action of PA in activating PDE4 remains a matter of speculation, experiments carried out by DiSanto et al (Disanto et al. 1995) showed that an antibody raised against a highly conserved peptide sequence near the catalytic region of PDE4 was able to inhibit PA-stimulated PDE4 activity whilst making no difference to basal PDE4 activity expressed in the monocyte cell line U937. Why this should be the case remains to be ascertained.

1.5.6.4.2 Regulation of PDE4 by Phosphorylation

As early as 1989, Pyne et al (Pyne et al. 1989) observed that the hepatocyte peripheral plasma membrane PDE (PPM.PDE) could be phosphorylated as a result of challenge of cells by insulin and that this phosphorylation event could be inhibited by glucagon. Moreover, by a combination of phosphoamino acid analysis and immunoblotting with specific anti-phosphotyrosine antibodies these workers were able to determine that the site of phosphorylation was tyrosine specific.

More recently, Sette et al (Sette et al. 1994a) observed that incubation of the rat thyroid cell line FRTL-5 with thyroid stimulating hormone (TSH) caused a rapid, albeit transient, increase in intracellular cAMP levels. The transient nature of this elevation in cAMP was explained by the concomitant increase in PDE4 activity observed 3 minutes post hormonal stimulation which reached a maximal level after 15 minutes. Immunoblotting studies with specific antisera showed the presence of an immunoreactive protein species of approximately 100kDa in the TSH stimulated cells, in addition to the 93kDa species observed in both hormone stimulated and control cells. Inclusion of cyclohexamide in the reaction, and the lack of increase in mRNA levels suggested that the larger band was not due to ongoing protein synthesis. Moreover, radiolabelling of quiescent FRTL-5 cells showed that ^{32}P was incorporated into a polypeptide of approximately 90-99kDa which co-purified with the cAMP-PDE acitvity upon hormonal stimulation. Sette and colleagues were then able to demonstrate that

the catalytic subunit of cAMP-dependent protein kinase could phosphorylate the purified PDE4 component from FRTL-5 cells in a cell-free system (Sette et al. 1994a; Sette et al. 1994b). This suggested that the larger immunoreactive band previously observed may represent a phosphorylation mediated conformational change of the enzyme thus altering its rate of migration in SDS acrylamide gels. Furthermore, Sette et al (Sette et al. 1994a) observed that okadaic acid, a potent phosphatase inhibitor, was able to potentiate the effect of TSH. It has subsequently been shown that the PDE4 component activated by cAMP-dependent protein kinase mediated phosphorylation in FRTL-5 cells is the long form splice variant PDE4D3 (Sette et al. 1994b). More recently, Alvarez et al (Alvarez et al. 1995) have demonstrated that the human PDE4D3, expressed in the monocyte cell line U937, can be phosphorylated by cAMP-dependent protein kinase. This occurred upon stimulation of the cells with PGE_2, an agent which increases intracellular levels of cAMP. Moreover, these workers have shown that phosphorylation altered the response of human PDE4D3 to the divalent cation Mg^{2+}, where the normally biphasic response became monophasic upon phosphorylation. Cyclic AMP-dependent protein kinase mediated phosphorylation of PDE4 has also been recently described in bovine vascular smooth muscle cells, although the specific isozyme involved remains to be identified (Ekholm et al. 1997).

Cyclic AMP-dependent protein kinase is known to specifically phosphorylate serine residues contained within the consensus sequence RRXS (Bramson et al. 1984; Hausken et al. 1996). Analysis of the amino acid sequence of PDE4D3 shows that it contains two putative PKA consensus phosphorylation sites around serines 13 and 54 which are contained with UCR1, the structural element identified by Bolger and colleagues (Bolger et al. 1993) as being unique to the long-form PDE4 splice variants. By means of site-specific mutagenesis of serines 13 and 54, Sette et al (Sette and Conti 1996) have identified serine 54 as the site of PKA-dependent phosphorylation which

leads to the activation of PDE4D3. This has been demonstrated by the expression of wild type and mutant proteins in both a cell-free system and in MA-10 cells.

In addition to PKA mediated phosphorylation, Lenhard and colleagues (Lenhard et al. 1996) have recently shown by means of mass spectrometry that the human PDE4B2B splice variant is phosphorylated in both Sf9 cells and yeast. Experiments using bacterially expressed peptide fragments of HSPDE4B2B have identified the phosphorylation site as serine 487 which lies within a serine-proline rich sequence SMIPQSPSPP which corresponds to the PXSP consensus sequence recognised by MAP kinase. Moreover, these researchers were able to demonstrate a certain degree of specificity of phosphorylation by MAP kinase compared to various other serine-proline kinases such as casein kinase and cdc2 kinase. Despite demonstrating that MAP kinase could specifically phosphorylate HSPDE4B2B peptide fragments it remains unclear as to the physiological significance of this modification since it was observed that phosphorylation did not alter either the V_{max} or K_m for cAMP. It should be pointed out, however, that this experiment was carried out on a peptide due to technical difficulties in expressing full-length HSPDE4B2B and therefore may not reflect the the true state of affairs *in vivo*.

1.5.6.4.3 Transcriptional Regulation of PDE4

Interestingly PDE4D3 appears to be the only PDE4D seen expressed in quiescent FRTL-5 cells. However, prolonged exposure of these cells to TSH results in the production of mRNAs for the two short form PDE4D1 splice variants in addition to PDE4D2. This is indicative of regulation at the level of transcription. Indeed transcriptional regulation of PDE4s has subsequently been reported in a number of instances Thus Verghese et al (Verghese et al. 1995) have recently shown that treatment of Mono Mac 6 cells with dibutyryl cAMP causes a transient increase in mRNA levels corresponding to PDE4A, 4B and 4D gene products in a concentration-dependent fashion. Furthermore, this

rise in mRNA levels was accompanied by a concommitant rise in protein levels. In addition to this study, Manning et al (Manning et al. 1996) have demonstrated that treatment of isolated peripheral blood monoctyes with a mixture of salbutamol and rolipram to elicit an increase in intracellular cAMP, is followed by a selective increase in mRNA and protein levels corresponding to PDE4A and PDE4B. However, they also noted that the mRNA for PDE4D, which was detected in untreated control cells, appeared to be down regulated in the treated cells. Similarly, Torphy et al (Torphy et al. 1995) demonstrated that steady-state mRNA levels for PDE4A and PDE4B were elevated in the human monocyte U937 cell line upon treatment with the β-adrenoreceptor agonist salbutamol. This has led these investigators to speculate that increasing PDE4 activity by such a method may exacerbate inflammatory processes.

More recently, the studies of Erdogan et al (Erdogan and Houslay 1997) have shown that PDE4 activity in Jurkat T-cells was increased by treatment of the cells with the adenylyl cyclase activator forskolin, in a manner which could be inhibited by the application of actinomycin D thus implying transcriptional regulation. Moreover, by means of RT-PCR these investigators showed that forskolin had the effect of decreasing mRNA levels corresponding to a novel PDE4A species identified as the sole PDE4 responsible for cAMP hydrolysis in untreated cells whilst increasing transcripts corresponding to PDE4D1 and PDE4D2 splice variants. Thus it may be speculated that dual regulation of PDE activity through transcription as described by the observations of Manning (Manning et al. 1996) and Erdogan (Erdogan and Houslay 1997) may be a mechanism whereby cells can fine tune the hydrolysis of cAMP in a time dependent fashion.

1.5.6.4.4 Regulation of PDE4 by Rolipram

In addition to regulation by phosphatidic acid, phosphorylation and mRNA transcription the type 4 PDE enzymes are unique in that they can be specifically inhibited by the anti-depressant rolipram (Baecker et al. 1994;

Disanto and Heaslip 1995; Houslay et al. 1997a; Huston et al. 1996; Jin et al. 1992; Kelly et al. 1996; Livi et al. 1990; McLaughlin et al. 1993; Nemoz et al. 1997; Sette et al. 1994a; Verghese et al. 1995; Wilson et al. 1994). However, it should be mentioned here that the Dunce PDE isolated from *Drosophila* is unusual in that it is the only type 4 PDE enzyme which is not inhibited by rolipram. Whilst the reason for this apparent anomaly remains to clarified it has been suggested that it may be as a result of species-related codon bias in or around the region of the enzyme involved in interacting with rolipram (Heery et al. 1997; Jacobitz et al. 1996b). Moreover, the ability of rolipram to inhibit PDE4 isozymes appears to vary according to the expression system studied, the intracellular location as well as the conditions used to purify the enzyme (Houslay 1996; Houslay et al. 1997a; Muller et al. 1996; Schneider et al. 1986). The studies of Wilson et al (Wilson et al. 1994) have shown that the human PDE4 splice variant h6.1 can be found in both particulate and cytosolic fractions when expressed in yeast and while the particulate fraction displayed a K_i of 0.7 μM, in keeping with the value of 0.6 μM reported for a similar isozyme by Livi et al (Livi et al. 1990) the soluble enzyme had a K_i of 0.4 μM. A similar situation has also been observed in rat where the IC_{50} for rolipram was greater for the membrane bound form of RPDE6 (RNPDE4A5A) than the cytosolic form (McPhee et al. 1995). More recently, Bolger and collegues (Bolger et al. 1996) made a similar observation for another PDE4A splice variant, PRDE39 (RNPDE4A8) demonstrating an IC_{50} value of 0.5 μM for rolipram for the particulate enzyme and 1.0 μM for the soluble fraction. The recent study carried out by Huston and colleagues (Huston et al. 1996) showed that when the human PDE4 splice variant h46 (HSPDE4A4B) was transiently transfected into COS cells the vast majority of the enzyme was cytosolic with an IC_{50} value for rolipram of 1.6 μM but there was also a small but significant amount of PDE activity associated with the particulate fraction which gave an IC_{50} value of 0.195 μM. These results have been explained by suggesting that

the soluble enzyme exhibited simple competitive inhibition kinetics, whilst the particulate enzyme displayed partial competitive inhibition (Huston et al. 1996).

As mentioned above the cell system used to study and characterise PDE enzymes can have marked effects upon the results. For example Owens and collegues (Owens et al. 1997) recently demonstrated that a PDE4C splice variant gave markedly different inhibition characteristics depending on whether the PDE gene was expressed in COS cells or yeast. A K_i of 0.6 μM for rolipram was observed upon expression of the gene in COS cells whereas this was noted to be 2.6 μM if the same gene was expressed in yeast. Earlier results obtained by Torphy et al (Torphy et al. 1992a) have demonstrated that PDE4 expressed in rat brain, heart and human monocytes was inhibited by rolipram with a K_i of 1 μM whereas in yeast this was approximately 0.06 μM. These workers have speculated that this may reflect a conformational change in the protein dependent upon the expression system studied.

In addition to the above mentioned differences in the inhibition of PDE4 mediated cAMP hydrolysis other factors have been observed to affect the efficacy of rolipram. DiSanto and Heaslip (Disanto and Heaslip 1995) have observed that the phosphatidic acid (PA)-stimulated PDE4 activity in U937 cells could be inhibited by nanomolar concentrations of rolipram in a PA dependent fashion. This, however, could be reversed by increasing the PA concentration whereas inhibition by micromolar rolipram concentrations proceeded in a PA independent manner. Moreover, such observation were apparent regardless of whether intact cells, cell homogenates or purified PDE4 was used. More recently, Sette and Conti (Sette and Conti 1996) demonstrated that inhibition of PDE4D3 in FRTL-5 cells by rolipram was altered by phosphorylation. In the absence of hormone stimulation, rolipram exhibited a biphasic inhibition with a low affinity and high affinity component in the micromolar and nanomolar range respectively but TSH-mediated phosphorylation of PDE4D3 demonstrated only the high affinity inhibition. The fact that these findings

could be replicated using purified recombinant PDE4D3 ruled out the possibility that this was caused by the presence of a mixture of different PDE4 isoforms. All these data might then imply that rolipram inhibition is highly sensitive to a conformational state of the PDE4 isoform. Thus changes in phosphorylation or, for example, interaction with intracellular components may be expected to elicit changes in rolipram sensitivity.

The contention that rolipram may bind to more than one site on PDE4 has been suggested by the observations of several investigators (Disanto and Heaslip 1995; Jacobitz et al. 1996a; Jacobitz et al. 1996b; Kelly et al. 1996; Souness et al. 1992; Torphy et al. 1992a). The results of Schneider et al (Schneider et al. 1986) suggested that rolipram bound with high affinity and in a saturable manner to sites in rat brain although the identity of this binding site has not been demonstrated. The studies of Souness et al (Souness et al. 1992), on guinea-pig eosinophil PDE4 activity, showed that incubation with vanadate/glutathione (V/GSH) was accompanied by an increase in cAMP hydrolysis which could be potently inhibited by rolipram. Moreover rolipram was 10-fold more potent on solubilized than on membrane-bound PDE4 activity, which led these workers to postulate that V/GSH caused a conformational change in the enzyme which exposed a high affinity rolipram binding site. Evidence for the existence of such a site has been presented by the work of Torphy and colleagues (Torphy et al. 1992a) who observed that a human monocyte PDE4 bound rolipram in the nanomolar range but that inhibition only occurred when rolipram was present in micromolar concentrations. This apparent lack of correlation between high affinity binding and inhibition of cAMP hydrolysis has since been noted by several investigators (Barnette et al. 1995b; Disanto and Heaslip 1995; Kelly et al. 1996). New evidence for the existence of two rolipram binding sites has been demonstrated by the studies of Jacobitz et al (Jacobitz et al. 1996a) who demonstrated that a recombinant human PDE4A, which had been N- and C-terminal truncated,

could be inhibited by rolipram at micromolar concentrations, but that it could not bind [^{3}H]-rolipram with the same high affinity observed for the non-truncated form. Furthermore, these workers were able to narrow down the location of the high affinity binding site to a region encompassing amino acids 265-332 and demonstrated that binding to this site was not a pre-requisite for inhibition. In addition, there is some evidence to suggest that high affinity rolipram binding may contribute to some of the side effects such as nausea, observed upon administration of rolipram as an anti-depressant. Barnette and co-workers (Barnette et al. 1995a) have observed that in isolated rabbit gastric glands, increased acid secretion was a function of high affinity rolipram binding in the presence of histamine. Although a satisfactory explanation for the phenomenon of high affinity rolipram binding remains elusive, some researchers (Souness et al. 1992; Souness and Scott 1993) have tentatively suggested that high affinity rolipram binding may act as an allosteric regulator in a manner similar to that observed for cGMP on PDE2 isozymes (Beavo 1995; Rybalkin and Beavo 1996). However, other investigators consider this to be unlikely (Kelly et al. 1996).

A further level of confusion surrounding the subject of rolipram binding and inhibition has been provided by the observation that, in some systems at least, there is a marked stereoselectivity for the different enantiomers of rolipram whilst in others no such selectivity has been observed. Since the original observation by Schneider et al (Schneider et al. 1986) that R-rolipram was more potent for binding than S-rolipram in rat brain, Torphy and co-workers (Torphy et al. 1992a) have reported a similar result for the human monocyte PDE4 although this observation could not be repeated with the purified enzyme. When this isozyme was expressed in yeast, R-rolipram inhibited cAMP hydrolysis with an apparent K_i of 61 nM in contrast to 390 nM as noted for the S-enantiomer. In contrast, Owens et al (Owens et al. 1997) have shown that the PDE4C gene product expressed in yeast displayed no

stereoselectivity for either enantiomer but that there was a marked selectivity for R-rolipram when this isozyme was expressed in COS cells.

Clearly, the binding and inhibition of PDE4 by rolipram is a complex subject which is complicated by the diverse results observed by a number of investigators studying a wide range of PDE4 splice variants in a variety of cell systems. Whether some PDE isoforms exist in two conformational states as suggested by some researchers (Huston et al. 1996; Kelly et al. 1996; Owens et al. 1997; Sette and Conti 1996; Souness et al. 1992; Souness and Scott 1993) remains to be further elucidated, as does the contention that only the long-form PDE4 splice variants display high affinity rolipram binding (Disanto and Heaslip 1995; Sette and Conti 1996).

1.5.7 PDE5, Cyclic GMP-Specific Phosphodiesterases

The PDE5 phosphodiesterases are a group of enzyme which are characterised by their ability to bind and hydrolyse cGMP (Beavo 1995; Beltman et al. 1995; Burns and Pyne 1992; Burns et al. 1992; Francis 1990; Francis et al. 1994; Ito et al. 1996; McAllister-Lucas et al. 1993; Miyahara et al. 1995; Saeki et al. 1995; Thomas et al. 1990).

To date only one gene for PDE5 has been identified. McAllister-Lucas et al (McAllister-Lucas et al. 1993) used a PCR generated probe to screen a bovine lung cDNA library and isolated and subsequently cloned a cDNA encoding a 99.5kDa protein. This, upon transfection into COS7 cells, was observed to produce a protein able to bind and hydrolyse cGMP. More recently it has been suggested that at least two alternative splice variants may exist in bovine lung (Beavo 1995). Comparison of the sequence of the cloned gene suggested that, in addition to the highly conserved core of approximately 250 amino acids common to all known mammalian PDEs, PDE5 enzymes also contained a second region. This region showed most similarity to the cGMP-binding region found in the PDE2 and PDE6 PDE gene families. Furthermore, this region was also observed to contain two internal repeats and has been

speculated as representing an allosteric site (McAllister-Lucas et al. 1993). It has also been suggested that in its native conformation, PDE5 is a homodimer (Francis 1990; McAllister-Lucas et al. 1993).

Whilst no detailed studies have been carried out on tissue distribution of PDE5, it has been shown to exist in rat and human platelets, rat spleen, guinea-pig and bovine lung, vascular smooth muscle, sea urchin sperm (Beavo 1995; Ito et al. 1996; McAllister-Lucas et al. 1993) and, more recently, porcine and human aortic smooth muscle (Miyahara et al. 1995; Saeki et al. 1995). Moreover, PDE5 has been implicated in ANP-mediated Na^{2+} loss and diuresis observed in kidney (Beavo 1995).

Kinetic studies have determined that the PDE5 enzyme from bovine lung has a K_m for cGMP of 5 μM while half-maximal binding occurs at 0.2 μM (McAllister-Lucas et al. 1993) and that Mg^{2+} or Mn^{2+} is required for catalysis (Francis 1990). There is also some evidence that the bovine lung PDE5 has a requirement for Zn^{2+}. Francis et al (Francis et al. 1994) have identified two tandem amino acid sequences which contain some similarity to the Zn^{2+} binding motif in the catalytc site of thermolysin, a known Zn^{2+} hydrolase. Moreover, these investigators have determined that Zn^{2+} in the range 0.05-1 μM, supports cGMP catalysis. As mentioned above, the cGMP-specific PDEs contain a high-affinity non-catalytic cGMP binding site (Beavo 1995; Burns and Pyne 1992; Burns et al. 1992; McAllister-Lucas et al. 1993; Thomas et al. 1990). As yet no function has been attributed to this site although a possible function as an allosteric site has been suggested. However, it has been observed that upon binding of cGMP to this site, the bovine lung PDE5 becomes a substrate for phosphorylation by cGMP-dependent protein kinase (Beavo 1995; Thomas et al. 1990). Thomas et al (Thomas et al. 1990) have identified serine at position 92 as the site of phosphorylation, while other investigators have observed that guinea-pig lung PDE5 can be phosphorylated on the same serine by the catalytic subunit of cAMP-dependent protein kinase (Burns and Pyne 1992; Burns et al.

1992). These observations are somewhat surprising since the consensus phosphorylation sites differ for each kinase. One possible explanation may lie in the fact that these studies were carried out on a partially purified enzyme which may have co-purified some factor which allowed activation by PKA whereas this factor would be absent in the pure enzyme preparation (Beavo 1995).

1.5.8 PDE6, Photoreceptor Phosphodiesterases

The photoreceptor PDE6 enzymes are involve in attenuation of light activated visual transduction. Upon excitation of rhodopsin by light, the G-protein transducin undergoes an exchange of GDP for GTP followed by dissociation of the Tα subunit from Tβγ subunits (Beavo 1995; Gillespie 1990). Tα in turn, activates a membrane associated PDE through dissociation of its inhibitory subunits from the cataltyic core causing cGMP hydrolysis which affects a cGMP dependent ion channel thus mediating Na^{2+} influx. Photoreceptor PDEs specifically hydrolyse cGMP and K_m values of 20-150 μM have been reported in the literature (Baehr et al. 1979; Gillespie 1990) although, more recently, a K_m for cGMP in the range of 17-80 μM has been suggested (Granovsky et al. 1997). Multiple genes have been isolated for the PDE found in rod and, in addition, a gene has been identified for a cone PDE which shows that these isozymes are distinct but highly related (Beavo 1995).

Whilst most PDEs are thought to exist *in vivo* as homodimers, the photoreceptor PDEs are unusual in that they are heterotetramers composed of one of each of two catalytic subunits (α and β) of approximately 90kDa and two inhibitory γ subunits of 10kDa (Deterre et al. 1988; Ovchinnikov et al. 1986). Recently it has been discovered that $\alpha\alpha\gamma_2$ and $\beta\beta\gamma_2$ species can exist in addition to the more common $\alpha\beta\gamma_2$ species (Granovsky et al. 1997). Comparison of the primary amino acid sequence for the catalytic α and β subunits has revealed that they are highly related (72%) products of separate genes with the α' subunit sharing >60% similarity with the α and β subunits

(Artemyev et al. 1996). Both rod and cone PDE catalytic subunits contain a highly conserved region of approximately 250 amino acids conserved in all mammalian PDEs. In addition they contain a high affinity non-catalytic cGMP binding site common to the other cGMP hydrolysing PDEs (Artemyev et al. 1996; Beavo 1995; Charbonneau et al. 1990; Cote et al. 1994; Gillespie 1990; Gillespie and Beavo 1988; Lipkin et al. 1988). The α and β subunits contain C-terminal CAAX sequences which are modified by farnesylation and geranyl-geranylation respectively. This may play a role in determining the membrane localization of the photoreceptor PDEs (Artemyev et al. 1996; Catty and Deterre 1991).

Some investigators have reported that the photoreceptor PDEs can be activated by limited proteolytic digestion by trypsin and suggested that this treatment removed the inhibitory γ subunits (Gillespie and Beavo 1988; Gillespie and Beavo 1989). However, it has since been demonstrated that trypsin treatment caused release of the PDE from the membrane by degradation of the β subunit C-terminus while the γ aubunits remained bound to the catalytic core (Catty and Deterre 1991). Since the early work of Ovchinnikov and colleagues (Ovchinnikov et al. 1986) first determined the primary structure of the γ subunit. Lipkin et al (Lipkin et al. 1988) observed that the inhibitory subunit contained a polycationic sequence of amino acids (residues 24-45) which were essential for interaction with transducin and that inhibition of the catalytic core was via the C-terminus. Brown et al (Brown and Stryer 1989) later extended this observation to implicate the C-terminus in the interaction of the γ subunit with the catalytic subunits as well as transducin. More recently, by means of photolabelling, it has been demonstrated that Cys68 is critical in this interaction and, furthermore, that it accelerated the rate of GTP hydrolysis by the Tα subunit (Granovsky et al. 1997; Liu et al. 1996). Moreover, these investigators have shown that inhibition of the α subunit by the γ subunit is by

binding to the catalytic site at a region which contains the NKXD guanine ring binding motif found in G-proteins (Granovsky et al. 1997).

There is some evidence to suggest that the photoreceptor PDEs can be regulated by a negative feedback mechanism involving the noncatalytic cGMP binding site. Cote et al (Cote et al. 1994) have shown that binding of cGMP to this site slows the rate of PDE activation and that activation by transducin decreases cGMP binding to the noncatalytic site in addition to increasing its rate of dissociation by approximately 10-fold. Following removal of the inhibitory γ subunits the rate of disociation was observed to increase >50-fold which has led these investigators to suggest that this may constitute a mechanism for generating faster but smaller photoresponses which are characterisic of light-adapted photoreceptors.

It would appear that the phosphoinositol signal transduction pathway may also be involved in mediating visual transduction. Udovichenko et al (Udovichenko et al. 1994) have reported that protein kinase C purified from bovine rod outer segments (ROS-PKC) can phosphorylate the inhibitory γ subunit on a specific threonine residue (thre35) and thus increase its ability to inhibit the catalytic subunit which may, in turn, play a role in desensitization of the photoreceptor PDEs.

1.5.9 PDE7, Cyclic AMP-Specific Phosphodiesterase

The first evidence for the existence of a PDE7 gene family was inadvertantly obtained by Michaeli et al (Michaeli et al. 1993) who isolated a cDNA, which they referred to as HCP1 (HSPDE7A1). This was derived from a human glioblastoma cDNA library by means of a sensitive yeast complementation assay. Comparison of the amino acid sequence revealed significant homology to the cAMP-specific PDE, with approximately 50% similarity including a core region of ~300 amino acids found to be conserved in all mammalian PDEs. Upon characterisation of the pharmacological properties of HCP1 these investigators observed that while HCP1 displayed a high affinity

for cAMP, with a K_m of 0.2 μM there was no detectable cGMP hydrolytic activity. This was unlike the cAMP-specific PDE4 isozymes. Indeed HCP1 was insensitive to inhibition by the archetypal PDE4 specific inhibitors rolipram and RO20-1724. Independently, Ichimura and Kase (Ichimura and Kase 1993) reported expression of an unknown cAMP-specific PDE which accounted for ~35% of the total activity in certain T-lymphocyte cell lines which shared the same inhibition profile as HCP1, although it should be noted that their findings were based on kinetic studies rather than immunological methods. Interestingly some years earlier, Lavan et al (Lavan et al. 1989) reported that rat liver contained an IBMX-insensitive cAMP hydrolysing PDE component which accounted for approximately 30% of the total cAMP hydrolysing PDE activity. This species may represent a liver PDE7. However, it should be noted that in addition to exhibiting a K_m for cAMP of 25 μM, this PDE also hydrolysed cGMP with a K_m of 237 μM. Based on Northern blot analysis of various human tissues Michaeli et al (Michaeli et al. 1993) demonstrated that HCP1 mRNA was abundant in skeletal muscle and also detectable in heart and kidney.

In a more recent study, Bloom and Beavo (Bloom and Beavo 1996) have reported the isolation of a novel alternative splice variant of PDE7 from a murine skeletal muscle cDNA library (MMPDE7A2). Comparison of this clone with HSPDE7A1 revealed that while the two shared >90% amino acid similarity, the human splice variant contained a longer N-terminus which was considerably more hydrophilic than the mouse PDE. Moreover, RNAse protection showed the mRNA for this clone to be most abundant in skeletal muscle. Interestingly, the sequence analysis of HSPDE7A1 was thought to suggest originally that this clone was truncated at the 5' end. However, Western blot data obtained with an antibody raised against a C-terminal antibody of HSPDE7A1 by these investigators appeared to confirm that the clone is indeed full-length. This antibody was also used to probe the human T-cell line Hut78 which previously was suggested to express PDE7 activity

(Ichimura and Kase 1993). Indeed these investigators identified an immunoreactive protein species of approximately 55kDa in both the soluble and particulate fractions. This antibody could also detecte HSPDE7A1 in transfected Sf9 cells, where it exhibited a K_m for cAMP in the submicromolar range, which was absent in mock transfected cells.

Based on the above studies it is evident that PDE7 splice variants are expressed in a cellular and tissue specific manner. However, there appears to be some difficulty in correlating the presence of mRNA with detectable levels of protein in some cases. What the physiological role of PDE7 is remains, as yet, to be elucidated but it has been suggested that such PDEs may be involved in immune response. This is clearly an area requiring much further study.

1.6 Compartmentalization of Cyclic AMP Response Elements

As a second mesenger, cAMP has been implicated in a diverse range of physiological and metabolic processes including synaptic transmission, ion channel and transporter activities, gluconeogenesis, glycogenolysis, cell growth and differentiation, gene transcription, learning and apoptosis (Beavo 1995; Houslay and Milligan 1997; Rubin 1994). The mechanism by which cAMP is involved in so many intracellular activities has intrigued many investigstors for many years due to the fact that the sole role of this molecule is to cause the activation of cAMP-dependent protein kinase (PKA). This has led to the hypothesis (Houslay and Milligan 1997; Scott 1991) that cAMP signalling is restricted to specific intracellular compartments within a given cell type due to localized expression of adenylyl cyclase, PKA and PDE enzymes. Such an inference has been strengthened by the discovery (Houslay and Milligan 1997) that at least nine isoforms of adenylyl cyclase and over thirty isoforms of PDE can be expressed within a cell due to the existence of multiple genes and alternate splicing of individual genes.

The basal level of cAMP in a cell is ~1 μM but activation of cAMP-dependent protein kinase will not occur until this is increased to approximately 10 μM (Houslay and Milligan 1997). This can be brought about by either increased production of cAMP by adenylyl cyclase, inhibition of phosphodiesterase mediated cAMP degradation or a combination of both. Thus, in a situation where two different receptors can elicit entirely different responses through PKA mediated phosphorylation, it would seem likely that a non-uniform concentration of cAMP would be established within any given cell rather than a uniform increase in the concentration of cAMP which, having occurred, would result in the activation of the entire PKA pool. Indeed, there are a number of pieces of evidence to support such a contention. For example, Hempel and colleagues (Hempel et al. 1996) have analysed the spatio-temporal distribution of cAMP in intact neurons of the lobster stomatogastric network (STG) by means of indicator dyes and confocal microscopy. By doing this these investigators obtained evidence for specific localized increases in cAMP concentration mediated by a variety of neruomodulators. Prior to this study, Barsony and Marx (Barsony and Marx 1990) observed that for epithelial cells and fibroblasts localized fluctuations in cAMP concentrations occurred in specific subcellular compartments which was hormonally mediated. Whereas isoproternol and PGE_2 increased cAMP at the plasma membrane, calcitonin resulted in a perinuclear localization.

In order that a non uniform gradient of cAMP be generated and maintained in a compartmentlised manner within a given cell, the various components of the cAMP signalling pathway, namely adenylyl cyclase, cAMP-dependent protein kinase and cAMP-specific PDE4, would have to be targeted to specific intracellular locations. It has been well established that adenylyl cyclase is located in the plasma membrane by virtue of its hydrophobic transmembrane spanning M_1 and M_2 domains and this has been discussed at length in a previous section (Section 1.3.1). The remainder of this chapter will

be devoted to a review of the mechanism of membrane localization of cAMP-dependent protein kinase and PDE4 isoforms as well as attempting to provide an overview of the subject of subcellular localization/targeting.

1.6.1 Intracellular Targeting of cAMP-Dependent Protein Kinase

Protein kinases represent a large and diverse group of enzymes of which to date approximately 300 members have been identified (Faux and Scott 1996). The activity of these enzymes is tightly regulated and demonstrates a restricted substrate availability. Clearly conformational constraints provide the major means of achieving substrate selectivity. However, it has been postulated that one additional mechanism for selective activation of kinases may involve their compartmentalization to specific subcellular localizations (Faux and Scott 1996). As mentioned previously (Section 1.4), PKA is a hetrotetrameric molecule composed of two catalytic (C) and two regulatory subunits (R) (Bramson et al. 1984; Taylor 1989). Moreover, the R subunits can be further characterised into RI and RII and it is these which confer selective tissue distribution upon the different PKA isoforms (Rubin 1994; Scott and Carr 1992). In mammalian brain at least 70% of PKA catalytic subunits are found associated with the particulate fraction via interaction with the RIIβ subunit. However, analysis of their amino acid sequence initially revealed the absence of any obvious classical targeting signal. Subseqeuently, Sarkar et al (Sarkar et al. 1984), whilst purifying RIIβ co-purified a contaminating 75kDa protein from bovine brain which displayed a high affinity for RIIβ (P75). The subsequent isolation of a cDNA corresponding to P75 by Hirsch et al (Hirsch et al. 1992) resulted in this protein being renamed AKAP75 (A Kinase Anchoring Protein 75). Since these initial studies several investigators have reported the isolation of a number of AKAPs from a variety of tissues and cell types, thus suggesting the existence of a large and diverse family of proteins which bind not only RIIb subunits but also PKAII holoenzymes (Bregman et al. 1989; Coghlan et al. 1994; Lester et al. 1996; Lin et al. 1995a; McCartney et al. 1995).

There now appears to be a wealth of evidence implicating AKAPs in compartmentalization of PKAII to specific subcellular localizations (Faux and Scott 1996; Hausken and Scott 1996; Rubin 1994; Scott and Carr 1992). For example, Coghan et al (Coghlan et al. 1994) have isolated and characterised AKAP95 from rat pituitary gland and observed its localization to the nuclear matrix, McCartney and colleagues (McCartney et al. 1995) have identified AKAP100 in the sacroplasmic reticulum of human cardiac and sketetal muscle and, more recently, Lester et al (Lester et al. 1996) have isolated AKAP220 from rat pituitary gland. They have observed the association of AKAP220 with the peroxisomal fraction where it may be involved in controlling cAMP-mediated androgen biosynthesis. Lin and co-workers (Lin et al. 1995a) have characterized S-AKAP84 , a novel AKAP from mammalian sperm which appears to be developmentally regulated. Moreover, immunofluorescence analysis demonstrated that S-AKAP84 is co-localized with mitochondria in the flagellum possibly by means of a putative mitochondrial targeting signal located within its N-terminal 30 amino acids.

As the same function is shared by AKAPs, namely the ability to bind RII, it is reasonable to expect that they may share other features in common. However, analysis of several AKAPs cloned in recent years has revealed a distinct lack of homology at the primary amino acid level (Hausken and Scott 1996). There is, however, a certain degree of conservation at the secondary structural level. All AKAPs studied to date appear to contain a C-terminal RII binding site which some investigators believe can adopt an amphipatic α helical conformation (Carr et al. 1993; Coghlan et al. 1994; Faux and Scott 1996; Hausken et al. 1996; Hausken and Scott 1996; McCartney et al. 1995; Scott and Carr 1992). However, this view is not shared by all investigators. Rubin (Rubin 1994) has suggested that such studies based solely upon computer predictions require more detailed experimental confirmation.

In addition to binding RII subunits or PKAII holoenzymes, AKAPs must also contain information which dictates targeting to specific intracellular locations (Hausken and Scott 1996). Glantz et al (Glantz et al. 1993) have provided evidence that AKAP75 contains two non-contiguous domains, T_1 and T_2, located at its N-terminus and which are responsible for conferrment of cytoskeletal asociation in HEK293 cells. Subsequent research has shown that deletion or replacement of key amino acids within T_1 or T_2 caused a translocation of AKAP75 to the cytoplasm (Li et al. 1996). It has also been reported that AKAP95 contains two zinc finger motifs similar to the zinc binding regions of the transcription factor TFIIIA (Coghlan et al. 1994; Rubin 1994). When considered in conjunction with the fact that AKAP95 is localized to the nuclear matrix it is reasonable to speculate that PKAII mediated phosphorylation may be involved in activation of cAMP-mediated transcription through elements such as CREB (Rubin 1994).

Until recently it has been thought that AKAPs were responsible for targeting of individual kinases to specific subcellular locations however, there is evidence to suggest that AKAP79 binds Ca^{2+}/CaM-dependent phosphatase 2B, calceneurin and the α and β isoforms of PKC and targets these enzymes to the postsynaptic densities in neurons (Hausken and Scott 1996). This has led to the suggestion that reversible phosphorylation may be involved in cAMP-mediated synaptic receptor/channel transmission (Hausken and Scott 1996).

In addition to studying the RII/PKAII binding site of various AKAPs, some researchers have investigated the self-interaction of the RII subunit. It has thus been shown that dimerization of the RII subunit is a prerequisite for anchoring (Hausken and Scott 1996; Scott and Carr 1992) and studies using truncation mutations of RIIβ have narrowed down the site of interaction to a region within the N-terminal 1-50 amino acids (Luo et al. 1990). More recently, Li et al (Li and Rubin 1995) ascertained that large bulky side chains which are a feature of amino acid residues Leu^{13} and Phe^{36} play pivotal roles in

both dimerization and tethering of RIIβ dimers to AKAP75. Hausken et al (Hausken et al. 1996) have recently extended this observation to include the RIIα subunit of PKAII. In this instance substitution of isoleucines at positions 3 and 5 for either leucine of phenylalanine caused a 24-fold decrease in AKAP binding whilst replacement with serine of asparginine resulted in a complete abolition of AKAP binding.

In summary, cAMP-dependent protein kinases are a group of enzymes characterised by their widespread distribution and substrate specificity. Intracellular expression of PKAs is restricted by means of protein-protein interactions with members of a family of anchor proteins known as AKAPs by virtue of intracellular localization to various subcellular compartments.

1.6.2 Compartmentaliztion of cAMP-hydrolysing phosphodiesterases

In addition to targeting of PKA, maintenance of specific intracellular pools of cAMP may be influenced by the intracellular localization of cAMP hydrolysing PDEs. In total, five of the seven PDE isoenzyme families are responsible for cAMP degradation. Thus members of the PDE1,2 and 3 families can hydrolyse both cAMP and cGMP whereas PDE4 and 7 enzymes are specific for cAMP (Beavo 1995). There is evidence to suggest that at least some of the cAMP PDEs expressed in a variety of cells and tissues are localized in specific subcellular compartments (Pyne et al. 1987; Pyne et al. 1986; Pyne et al. 1989). Examples of this will be described in more detail below.

Yang et al (Yang et al. 1994) have shown that cGS-PDE clones from bovine adrenal gland and rat brain are splice variants of the same gene which display divergent 5' ends with the N-terminal 37 amino acids of the rat enzyme demonstrating no homology to the bovine isozyme. Moreover, analysis has shown that this region is extremely hydrophobic. Since more than 75% of the cGS-PDE activity in both rabbit and bovine brain is associated with the particulate fraction it has been postulated that the hydrophobic amino terminal

may confer membrane targeting upon cGS-PDEs in a tissue specific manner (Yang et al. 1994).

Members of the cGMP-inhibited PDE3 family have been isolated from both cytosolic and particulate fractions from several tissues (Degerman et al. 1996; Manganiello et al. 1995). Early studies by Gettys et al (Gettys et al. 1988) on rat adipocytes demonstrated that cGI-PDE activity was associated with the particulate fraction of microsomes, moreover, incubation with trypsin resulted in a transfer of cAMP hydrolytic activity from the particulate to the soluble fraction. Interestingly, these investigators also observed that the soluble PDE3 activity exhibited minimal activation by the catalytic subunit of PKA suggesting the possibility that membrane association was required for regulation of cGI-PDE. Since this initial reseach, cGI-PDE activity has been isolated from mammalian myocardial sarcoplasmic reticulum (Meacci et al. 1992; Movsesian et al. 1991; Smith et al. 1993). Although the elements responsible for conferment of membrane association of the cGI-PDEs have not been conclusively identified, analysis of the primary amino acid sequences has revealed that the N-termini of these enzymes contain several stretches of hydrophobic residues (Degerman et al. 1996; Leroy et al. 1996; Manganiello et al. 1995; Meacci et al. 1992; Smith et al. 1993; Taira et al. 1993). Meacci et al have suggested that these hydrophobic regions may constitute a series of transmembrane α helices. Interestingly, recent studies have shown soluble cGI-PDEs to be N-terminally truncated thus implying that membrane association is determined by information contained within this region. (Leroy et al. 1996; Manganiello et al. 1995).

Early indications that cAMP-specific PDE4 activity localizes to distinct subcellular membrane fractions was obtained from studies on rat liver. Marchmont and Houslay (Marchmont and Houslay 1980) first demonstrated that insulin could stimulate cAMP hydrolytic activity associated with the plasma membrane fraction in a broken cell system. Later, Heyworth et al (Heyworth et

al. 1983) extended this observation to intact hepatocytes where cAMP PDE activity was identified in two separate fractions, referred to as the peripheral plasma membrane PDE (PPM.PDE), a PDE4 isoform, and the 'dense-vesicle' PDE, a PDE3 species, associated with microsomes. In recent years a great deal of interest has been shown in compartmentalization of PDE4 isoforms (Bolger et al. 1996; Houslay 1995; Houslay 1996; Houslay et al. 1992; Houslay et al. 1997a; Houslay et al. 1995; Houslay and Tipton 1973; Huston et al. 1996; Lobban et al. 1994; McPhee et al. 1995; Nemoz et al. 1996; O'Connell et al. 1996; Pooley et al. 1997; Shakur et al. 1993; Shakur et al. 1995; Souness et al. 1992; Wilson et al. 1994). What has become apparent is that this area has been made more complicated by the existence of multiple genes and alternative splice variants for PDE4 (Bolger et al. 1993; Bushnik and Conti 1996).

Of all the PDE4 isozymes studied to date, the most significant advances in understanding of subcellular compartmentalization relate to members of the PDE4A gene family. As mentioned earlier cAMP-specific PDEs are transcribed from four separate genes (Section 1.5.6.1) all of which share a conserved catalytic core and C-terminal region but which possess distinct N-terminal domains (Beavo 1995; Bolger 1994; Houslay 1995). Moreover, individual splice variants can be further characterised as either 'long-form', containing UCR1 and UCR2 in addition to the catalytic core, or 'short-form' containing UCR2 only (Beavo 1995). Initial studies carried out by Shakur et al (Shakur et al. 1993) focussed on the rat brain PDE4 isoform called RD1 (RNPDE4A1) (Davis et al. 1989). When a cDNA clone for RD1 was transiently transfected into COS cells approximately 85% of the PDE4 activity was found in the high-speed particulate fraction. In contrast to this and N-terminally truncated species called Met26RD1 was exclusively cytosolic. As Met26RD1 was missing the first 25 amino acids it was suggested that this region may contain the information required for compartmentalization of RD1. Subsequent studies confirmed the existence of RD1 as being solely in the membrane fraction of rat

brain cerebellum (McPhee et al. 1995; Shakur et al. 1995). In addition these studies also showed that in COS cells transfected with a plasmid encoding RD1 all of the enzyme was in fact membrane associated and that the small cytosolic activity detected was due to co-expression of Met26RD1 (McPhee et al. 1995; Shakur et al. 1995). More recently, Pooley et al (Pooley et al. 1997) demonstrated that human thyroid carcinoma FTC cells stably transfected with cDNA for RD1 showed the entire pool of RD1 to be not only membrane associated but sequestered specifically to the Golgi apparatus.

Specific intracellular targeting of PDE4A isozymes is not restricted solely to RD1. The long-form splice varant RPDE6 (RNPDE4A5) has been identified as partitioning between the particulate and soluble fractions both in transfected COS cells and also expressed natively in brain (McPhee et al. 1995). Bolger et al (Bolger et al. 1996) have also reported recently the identification of a testis specific PDE4A splice variant pRPDE39 (RNPDE4A8) which, upon expression in COS cells, is distributed between the high speed P2 pellet fraction and the supernatant. Huston and colleagues (Huston et al. 1996) have also reported that the human PDE4A splice variant PDE46 (HSPDE4A4B), when expressed in COS cells, was associated with both the soluble and particulate fractions. Moreover, they observed that the particulate enzyme could not be solubilized by either the non-ionic detergent Triton X-100 nor high salt concentrations. This might indicate cytoskeletal association. That particulate association of PDE46 was a function of its N-terminus was confirmed by the observation that h6.1 (Sullivan et al. 1994), which lacks the N-terminal extension of PDE46, was exclusively soluble when expressed in COS cells (Huston et al. 1996).

One valuable insight into targeting of PDE4A has been gained from the work of O'Connell et al (O'Connell et al. 1996). These investigators observed that RPDE6 (RNPDE4A5) was able to interact with the Src homology (SH3) domain of the tyrosyl kinase Src. In addition it also displayed varying affinities

for the SH3 domains of other tyrosyl kinases, namely c-Abl, Crk, Csk, Lck, Lyn and Fyn. This interaction is thought to have been mediated through a proline-rich motif contained within the N-terminus of RPDE6. It has been postulated that such an interaction between Src and a PDE may serve to prevent inhibition of the kinase by cAMP-mediated phosphorylation (Houslay and Milligan 1997; Houslay et al. 1997a). The PDE4B gene product PDE4 (RNPDE4B2A) has also been suggested to occur exclusively associated with selective membrane compartments in brain (Lobban et al. 1994). This is thought to occur by virtue of its alternatively spliced N-terminal region. In contrast, the splice variant DPD, which lacks this region was completely cytosolic.

While there is a growing body of evidence that PDE4 isozymes are located within specific subcellular compartments the mechanism involved in each case remains to be elucidated as does the biolological function. It does appear, however, that such interactions are mediated through information contained within the splice variant specific N-terminal regions of the individual PDE isozymes (O'Connell et al. 1996; Shakur et al. 1993).

It is known that proteins can be targeted to various intracellular locations by a number of different mechanisms. Outlined below are some of the methods involved in targeting of proteins to such subcellular compartments as the plasma membrane, endoplasmic reticulum, Golgi apparatus, peroxisomes and lysosomes.

1.7 Protein Targeting Signals

Signals required for the intracellular targeting of cytoplasmic proteins are often found at their amino or carboxy termini. Moreover, these signals may or may not be removed following the initial targeting of the protein (Boyd and Beckwith 1990; Szczesna-Skorupa et al. 1988). In contrast, nuclear targeting signals may be internally located and not removed upon achieving correct

targeting. Targeting signals involved in directing proteins to the peroxisomes tend to be either internal or carboxy terminally located and remain uncleaved. An additional feature of protein targeting signals is based upon the observation that they can, in some instances, act in an independent context to their protein of origin by targeting otherwise normally soluble foreign proteins to specific intracellular locations (von Heijne 1996).

1.7.1 The Signal Peptide

The signal peptide (SP) mediates the entry of proteins into the secretory pathway (Andrews et al. 1992; Green et al. 1989; Kaiser and Botstein 1990; Lyko et al. 1995; Milstein et al. 1972; Szczesna-Skorupa et al. 1988; von Heijne 1990; von Heijne 1996). Analysis of the amino acid sequence of various signal peptides has failed to reveal any conservation of sequence. However, such sequences usually possess a characteristic tripartite structure consisting of a basic amino terminus, known as the n-region, and invariantly contains lysine and arginine residues, followed by a central hydrophobic core and, finally, a C-terminal polar region consisting of small uncharged amino acids which constitute the site of cleavage by signal peptidase (Lyko et al. 1995; von Heijne 1990; von Heijne 1996). The n- and h-regions are responsible for membrane targeting, however, the n-region is also thought to mediate surface binding through interactions between the basic amino acids and anionic phospholipids in the lipid bilayer. The h-region would appear to adopt an α helical conformation which may or may nor span the lipid bilayer (von Heijne 1996). Following translocation and cleavage of the signal peptide by signal peptidase, both the signal peptide and the protein are released into the lumen of the endoplasmic reticulum (ER) (Andrews et al. 1992).

In addition to the cleaved signal peptide, a second class of translocation signal has been identified. Known as a signal anchor, this sequence shares some of the features of the signal peptide in that they initiate translocation but unlike signal peptides, they are not cleaved from the protein (Andrews et al.

1992; Sakaguchi et al. 1987; Szczesna-Skorupa et al. 1988; von Heijne 1990). The signal anchor can also be found in tandem with a third class of topogenic elements known as a stop transfer signal. This functions to halt translocation of the polypeptide into the ER lumen and thus establishes it as an integral ER membrane protein (Andrews et al. 1992; Davis and Model 1985; Sakaguchi et al. 1987; Szczesna-Skorupa et al. 1988).

1.7.2 Endoplasmic Reticulum Retention/Retrieval Signals

Many of the proteins found in the ER lumen are in transit to various regions within the cell. However, some proteins are permanent residents in the ER itself and as such, must somehow be distinguished from proteins which will be secreted. This may happen by one of two mechanisms either by direct retention or by retrieval of the protein from distal compartments of the secretory pathway, these proteins must possess an endoplasmic reticulum retention signal (Pfeffer and Rothman 1987). In the first instance this function has been identified for many proteins as the carboxy terminally located tetrapeptide sequence KDEL, originally identified by Munro and Pelham (Munro and Pelham 1987) and Warren (Warren 1987). The KDEL sequence has now been shown to occur in several mammalian proteins (Andres et al. 1991; Hsu et al. 1992; Lewis and Pelham 1992b; Mazzarella et al. 1990; Tang et al. 1993; Yun and Eipper 1995). Furthermore, a variant, HDEL, is known to determine ER retention in yeast (Hsu et al. 1992; Lewis and Pelham 1990). Moreover it has been suggested that proteins with a KDEL sequence are retrieved from the Golgi complex to the ER by a mechanism involving a KDEL receptor which cycles between the Golgi complex and the ER (Lewis and Pelham 1992b; Pelham 1988; Warren 1987). To this effect, several researchers have isolated and characterised cDNAs for these receptors in both humans and yeast (Lewis and Pelham 1990; Lewis and Pelham 1992a; Lewis and Pelham 1992b; Semenza et al. 1990). Furthermore, it has been shown in at least one instance that human KDEL receptor resides in the Golgi complex until challenged by

high concentrations of KDEL-bearing ligand whereupon it redistributes to the ER (Lewis and Pelham 1992a). While the sequence KDEL is highly conserved amongst mammalian proteins, there is some degeneracy allowed. For example, it has been shown (Andres et al. 1991) that mutagenesis of KDEL to QDEL, KEDL and KDEI prevented secretion of human prepro-neuropeptide Y whereas substitution of the leucine for valine, to generate KDEV, prevented ER retention of this protein.

The second mechanism whereby proteins are localized to the ER is less well understood. While soluble proteins are retrieved from the Golgi complex and retained in the ER by means of the KDEL tetrapeptide, integral membrane proteins have been observed to employ a dibasic motif (Calakos et al. 1994; Cosson and Letourneur 1994; Jackson et al. 1990; Szczesna-Skorupa et al. 1995; Vincent et al. 1998). Nilsson and colleagues (Nilsson et al. 1989) have demonstrated that the adenoviral E3/19K gene product, a type 1 transmembrane protein, is retained in the ER by virtue of information contained within its C-terminal 15 amino acid residues. Deletion of the extreme C-terminal 6 amino acids revealed that the ER localization signal was contained within the sequence DEKKMP (Nilsson et al. 1989). Moreover, addition of this sequence to the C-terminii of CD4 and CD8, two T lymphocyte expressed type 1 cell surface localized proteins, resulted in their retention in the ER (Nilsson et al. 1989). Subsequent to these initial observations Jackson et al (Jackson et al. 1990) have identified by site-specific mutagenesis two consensus motifs KKXX and KXKXX in which an invariant lysine at position -3 from the C-terminus are sufficient for ER localization. Furthermore, these researchers were able to demonstrate that the di-lysine motif functioned independently of the surrounding amino acid composition. Moreover, the ability of this motif to direct ER retrieval is not simply a feature of charge as substitution of either lysine residue by arginine or histidine causes translocation to the cell surface (Jackson et al. 1990). While the mechanism by which di-lysine motifs direct

retrograde transport to the ER remains to be elucidated, there has been a suggestion that coatomer interaction may be involved (Cosson and Letourneur 1994; Vincent et al. 1998).

A similar strategy to that outlined above was employed by Schutze et al (Schutze et al. 1994) in demonstrating that a double-arginine motif located at the N-terminus of the human invariant chain lip33, a type II transmembrane protein, was involved in its retrieval to the ER. Moreover, the position of the double-arginine motif within the N-terminus was shown to be essential for its function with positions 2 and 3, 4 and 5, 2 and 4 or 3 and 5 the only combinations being tolerated.

While the examples outlined above represent some of the ways in which proteins can be retrieved by the ER these are by no means the only mechanisms which are exploited for this function. For example, influenza virus haemagglutinin, which is normally excluded from coated pits at the cell surface, was internalized to the perinuclear region upon mutation of a specific cysteine located within its cytoplasmic domain to tyrosine in a manner which did not involve phosphorylation of the tyrosine (Lazarovits and Roth 1988).

Interestingly, it would appear that in addition to its involvement with the secretory pathway the ER also functions as site for the retention and subsquent degradation of misfolded and mutated proteins (Klausner and Sitia 1990). The plasma membrane Ca^{2+} ATPase has an acidic region in its cytoplasmically exposed C-terminus (Zvaritch et al. 1995). A truncated protein terminating with this sequence is retained in the ER, moreover, mutation of the acidic amino acids in this region to basic reidues resulted in its expression at the cell surface thus suggesting that the Ca^{2+} ATPase has a masked ER retention signal in its C-terminus (Zvaritch et al. 1995)

Moreover, it has also been shown that proteins which do not themselves contain ER retention signals can be localised within the lumen of the endoplasmic reticulum by associating with a protein which does possess such a

signal. An example of this (Zhen et al. 1995; Zhen et al. 1993) is given by β-glucuronidase where a significant fraction can be sequestered to the ER lumen by virtue of the interaction of this protein with egasyn.

1.7.3 Golgi Retention Signals

Upon leaving the endoplasmic reticulum, proteins are transported to the Golgi complex where they undergo a variety of modifications such as addition of carbohydrate structures, tyrosine sulphation and O-linked sugars (Munro 1991; Pfeffer and Rothman 1987). Such modified proteins then progress through the trans-golgi network (TGN) prior to export to various intracellular locations (Munro 1991; Pfeffer and Rothman 1987). Several modifying enzymes such as asialoglycoprotein receptor subunit H1 (Wahlberg et al. 1995), GnT1 (Burke et al. 1994; Burke et al. 1992), α-2,6-sialyltransfrerase (Munro 1991) and β-1,4-galactosyltransferase (Yamaguchi and Fukuda 1995) are retained by the Golgi complex in addition to the viral glycoproteins E2 and G1 of Rubella virus and Punta Tora virus respectively (Hobman et al. 1995; Matsuoka et al. 1994; Matsuoka et al. 1996).

The mechanism of Golgi retention remains poorly understood in comparison to other retention signals such as the carboxy terminal KDEL motif of the endoplasmic reticulum. However, there is a growing body of evidence which suggests the involvement of a single amino terminal transmembrane (TM) domain (Burke et al. 1994; Burke et al. 1992; Matsuoka et al. 1996; Munro 1991; Nilsson et al. 1991; Pearce et al. 1991; Pelham and Munro 1993; Youakim et al. 1994). Analysis of the primary amino acid sequences of various transmembrane domains reveals a distinct lack of homology. However, they all appear to be approximately 17 residues long and composed predominantly of hydrophobic amino acids (Hobman et al. 1995; Pelham and Munro 1993; Wahlberg et al. 1995; Yamaguchi and Fukuda 1995; Youakim et al. 1994). Munro (Munro 1991) has demonstrated that increasing the length of the transmembrane domain to 23 amino acid residues results in export of the

peptide to the cell surface. It has been suggested that 17 amino acids would be more suited to traversing the thinner Golgi membrane than longer sequences (Pelham and Munro 1993). More recently, some investigators have reported results which implicate the cytoplasmic domain of Golgi retained proteins as being important for retention. Matsuoka et al (Matsuoka et al. 1996) have shown that mutation of a proline within the first 10 amino acids of the cytoplasmic domain of the Punta Tora virus G1 glycoprotein causes its translocation to the cell surface. Prior to this Munro (Munro 1991) suggested that three lysine residues immediately downstream of α-2,6-sialyltransferase may function as a stop-transfer signal in a fashion similar to that observed in the endoplasmic reticulum (See section 1.7.2).

It has previously been suggested that oligomerization may also be an important factor in Golgi retention by generating large protein complexes which may be difficult to export (Nilsson et al. 1994; Pelham and Munro 1993). Whether this proves to be the case remains to be seen. However, some recent research has identified that at least some Golgi proteins form dimers *in vivo*. Yamaguchi and colleagues (Yamaguchi and Fukuda 1995) have demonstrated that β-1,4-galactosyltransferase forms a homodimer mediated by the interaction of specific cysteine and histidine residues located in its transmembrane domain, while Hobman et al (Hobman et al. 1995) have shown that Rubella virus glycoproteins E1 and E2 form a heterodimer. Interestingly, these investigators have also shown that the transmembrane and cytoplasmic domains of E1 are not required for Golgi retention. Such studies demonstrate that two integral membrane proteins can be retained by a single signal (Hobman et al. 1995).

1.7.4 Endosomal/Lysosomal Targeting Signals

It has been shown that the major histocompatability class II molecules associate with Iip31, a type II transmembrane protein found in the endoplasmic reticulum, prior to translocation to a specialized endosomal compartment. When the complex reaches this compartment, Iip31 is released prior to MHC II

appearing at the cell surface (Pond et al. 1995). Endosomal targeting of Iip31 is mediated by virtue of information contained within the first 16 amino acids of its cytoplasmic tail (Lotteau et al. 1990). Site specific mutagenesis has identifed this signal as a di-leucine motif which also requires the presence of an acidic amino acid approximately 4-5 residues N-terminal to the di-leucine motif (Pond et al. 1995). Di-leucine related motifs have also been identified in a variety of receptors and other proteins which are targeted to lysosomes including T-cell antigen receptor CD3 chains (Leteourneur and Klausner 1992), lysosomal integral membrane protein (LIMP) II (Ogata and Fukuda 1994), interleukin-6 (IL-6) signal transducer gp130 (Dittrich et al. 1996), macrophage IgG Fc receptors (Hunziker and Fumey 1994) and the insulin receptor (Haft et al. 1994). In addition, a di-leucine motif has been implicated in internalization and lysosomal targeting of the T lymphocyte integral cell surface glycoprotein CD4 mediated by the human immunodeficiency virus (HIV) Nef gene product (Aiken et al. 1994).

1.7.5 Peroxisomal Targeting Signals

Peroxisomes are small organelles comprised of a single membrane that are found in most eukaryotic cells and which are involved in a variety of processes (Glover et al. 1994). Peroxisomal proteins are synthesised in the cytosol on membrane-free polysome prior to translocation to pre-existing peroxisomes (Fransen et al. 1995; Miyazawa et al. 1989; Tsukamoto et al. 1994). Early studies by Gould et al (Gould et al. 1989) revealed that firefly luciferase was targeted to peroxisomes by information contained within its carboxy terminus. This was identified as the tripeptide sequence serine-lysine-leucine (S-K-L). In addition, these investigators observed that fusion of the SKL tripeptide to the carboxy terminus of chloramphenicol acetyl transferase (CAT) directed this normally cytosolic protein to the peroxisomal fraction of transfected mammalian cells (Gould et al. 1989; Gould et al. 1990). Moreover, Gould and colleagues (Gould et al. 1989) have shown that serine (S) can be

replaced by alanine (A) or cysteine (C) and lysine may be substituted for by histidine (H) or arginine (R) without loss of targeting. Since these intial studies several peroxisomally targeted proteins have been identified that contain carboxy terminal tripeptide targeting signals, such as rat liver alcohol oxidase (AOX) (Miyazawa et al. 1989), insulin degrading enzyme (IDE) (Eitzen et al. 1995) and 3-hydroxy-3-methylglutaryl-CoA lyase (HL) (Authier et al. 1994). While the mechanism for the peroxisomal retention of these proteins has not been elucidated, there is evidence to suggest that it may involve a protein-protein interaction. Fransen et al (Glover et al. 1994) have recently isolated a cDNA clone from a rat liver cDNA library which binds to the SKL peroxisomal targeting signal by using this peptide as bait in a yeast two hybrid assay.

Peroxisomal targeting and retention has also been reported for proteins that lack the C-terminal tripeptide SKL signal. For example, peroxisomal 3-ketoacyl-CoA thiolase (PT) has been shown to possess an amino terminal peroxisomal targeting signal peptide which is cleaved upon successful targeting (Swinkels et al. 1991; Tsukamoto et al. 1994). Moreover, the analogous enzyme in yeast is targeted by information within its amino terminal 16 amino acids. This is in contrast, to the rat enzyme, where such a region is not cleaved following peroxisomal translocation (Glover et al. 1994). More recently, Eitzen et al (Fransen et al. 1995) have isolated the cDNA for the yeast gene Pay2p which encodes a peroxisomal integral membrane protein that may be targeted by virtue of a hydrophobic N-terminal transmembrane domain.

1.7.6 Mitochondrial Targeting Signals

Greater than 90 % of the proteins found in the mitochondria are encoded by nuclear DNA and synthesised in the cytoplasm whereupon, following import, they are sorted to one of four distinct compartments; the outer or inner membranes, the intermembrane space or the matrix (Glaser et al. 1990; Hahne et al. 1994; Kaput et al. 1982; Shore et al. 1995; Steenaart and Shore 1997; van Loon et al. 1986). As import of these proteins is mediated in most instances by

the same mechanism, then specificity of sorting must be encoded by different topogenic domains contained within the precursor protein (Steenaart and Shore 1997). Indeed, it would appear that many mitochondrial proteins are synthesised with a presequence which directs the peptide into the mitochondrion whereupon the presequence is cleaved, prior to translocation to a specific site within the mitochondrion (Glaser et al. 1990; Kaput et al. 1982; van Loon et al. 1986).

The matrix targeting signal (MTS) is the most well studied of the mitochondrial targeting signals having been characterised for a variety of proteins including yeast alcohol dehydrogenase (ADH) III and cytochrome c_1 (van Loon et al. 1986), NADH-cytochrome b5 reductase (Hahne et al. 1994) and pre-ornithine carbamoyl transferase (Steenaart and Shore 1997). Matrix targeting signals are typically rich in basic and hydroxylated amino acid residues and have been postulated to form amphiphilic helices (Hahne et al. 1994; Steenaart and Shore 1997). In contrast, targeting of proteins to the mitochondrial outer, inner and intramembrane compartments is less well understood, although it is thought to involve stretches of hydrophobic amino acids which may act as transmembrane domains (Shore et al. 1995). Steenaart et al (Steenaart and Shore 1997) have shown that the yeast protein Tom70p is targeted and inserted into the mitochondrial outer membrane with its N-terminal located in the intermembrane space while its C-terminus is exposed to the cytoplasm. This orientation is achieved by means of an N-terminal anchoring sequence comprised of two domains, an N-terminal hydrophilic domain and a transmembrane domain. Moreover, it has been shown that increasing the hydrophobicity of the transmembrane caused the Tom70p localization signal to target dihydofolate reductase (DHFR) to the mitochondrial inner membrane (McBride et al. 1992; Steenaart and Shore 1997). Further confirmation of the importance of hydrophobic domains in mitochondrial inner membrane targeting has been presented by Glaser et al (Glaser et al. 1990) who found that deletion

of a C-terminal hydrophobic transmembrane domain of yeast cytochrome *c* oxidase subunit Va resulted in its translocation to the matrix. It has also been demonstrated (van Loon et al. 1986) that the presequence of yeast cytochrome *c* can target DHFR to the inner membrane, where it is anchored by a 19 amino acid hydrophobic transmembrane domain. The presence of two basic amino acid residues following the hydrophobic domain has been suggested by these investigators as fulfilling the function of a stop-transfer sequence.

More recently, Lin et al (Lin et al. 1995a) have identified a putative transmembrane domain in the protein kinase A anchoring protein S-AKAP84 which may serve as a mitochondrial targeting signal. In addition, immunofluorescence studies have shown (Lin et al. 1995a) that S-AKAP84 co-localizes with flagellar mitochondria where it may be involved in PKA mediated activation of flagellar movement in mature spermatozoa.

Targeting of proteins to the intermembrane space is thought to involve a two-step process whereby proteins are first targeted to the mitochondrial inner membrane and subsequently released into the intermembrane space (Gasser et al. 1982; Kaput et al. 1982). Kaput and colleagues (Kaput et al. 1982) have shown that yeast cytochrome c oxidase (CCP) is synthesised with an amino terminal 68 amino acid presequence (preCCP) which would appear to anchor the peptide in the inner membrane, exposing only the extreme N-terminus to the matrix. Mature, soluble CCP is then generated from this transmembrane intermediate upon cleavage of the presequence thus releasing the protein into the intermembrane space.

Thus is would seem that targeting of proteins to specific mitochondrial compartments, while not as clearly defined as targeting to other intracellular locations, is mediated by a variety of factors including hydrophobicity and the presence of charged amino acid residues. Moreover, it has become apparent that several proteins contain targeting signals for more than one mitochondrial

location (Steenaart and Shore 1997) and that their position within the peptide relative to each other can determine the final submitochondrial localization.

1.7.7 Nuclear Localization Signals

Signals responsible for targeting of proteins to intracellular locations such as peroxisomes, the Golgi apparatus and the endoplasmic reticulum are characteristically located at either the amino or carboxy terminus of a protein. In marked contrast, nuclear localization signals (NLS) may be present at any point along the polypeptide chain and, unlike for example signal peptides, are not removed by cleavage following targeting (von Heijne 1996).

While no consensus sequence exists for nuclear localization signals, it has been observed that they are short (4-21 residues) amino acid sequences dominated by the presence of the basic amino acids lysine and arginine (Kalderon et al. 1984; Smith et al. 1985; von Heijne 1996). One such example is characterized by the sequence PKKKRKV found in the nuclear localization signal of SV 40 large T antigen (Smith et al. 1985). Variations upon such signals have been identified in a variety of proteins including nucleoplasmin (Dingwall et al. 1987), the transcription factors NGF1-A (Matheny et al. 1994), NF-κB (Lin et al. 1995b), SW15 (Jans et al. 1995), the DNA binding protein human lymphoid enhancer factor-1 (hLEF-1) (Prieve et al. 1996), C-193, a novel cytokine induced nuclear protein (Chu et al. 1995) and ubiquitin-activating enzyme of yeast (Dohmen et al. 1995). Interestingly, Ono et al (Ono et al. 1994) have recently a identified a nuclear localization signal, KKRKK, close to the amino terminus of the actin- modulating protein cofilin. This is responsible for causing the translocation of this protein to the nucleus upon exposure to metabolic insults such as heat shock or dimethyl sulphoxide. It has been suggested (Ono et al. 1994) that such treatments may alter the structural conformation of the protein thus exposing the nuclear localization signal which would normally remain buried.

A second type of nuclear localization signal demonstrating a bipartite motif composed, usually of two regions of basic amino acids separated by a short spacer region, has also been identified (Matheny et al. 1994). This type of nuclear localization signal has been identified in proteins as diverse as the plant transcription factor Opaque2 (Hicks and Raikhel 1995) and the mammalian zinc-binding protein parathymosin-α which contains the sequences RKR and PKRQKT separated by a 9 amino acid spacer (Trompeter et al. 1996). Zhu et al (Zhu et al. 1995) have shown that the nuclear phosphoprotein mitosin contains a rather unusually spaced bipartite nuclear localization signal consisting of the sequences KRQKX(20)KKSKK. Interestingly, it should be noted that bipartite nuclear localization signals show a greater degree of variation in their amino acid sequence than the more familiar linear nuclear localization signals. It may be in these instances that a bipartite signal is required bring together sufficient positively charged amino acids to allow nuclear import, although it is emphasised that this is pure speculation and would require experimental investigation.

1.7.8 Membrane Association of Proteins by Post-translational Modification

One method of targeting of proteins to the plasma membrane involves acylation which involves the covalent attachment of one or more fatty acid molecules at sites located within either the N- or C- terminus of the protein. The addition of lipids to proteins has been well documented (Chapman et al. 1992; Gaudin et al. 1991; Grand 1989; O'Brien and Zatz 1984; Okubo et al. 1991; Perez et al. 1991; Schlesinger et al. 1980; Soly and Meighen 1991; Spiegel et al. 1991b) and normally occurs through one of two mutually exclusive pathways involving palmitic acid or myristic acid. Myristoylation has been observed for numerous proteins including G protein α subunits (Linder et al. 1991; Spiegel et al. 1991a; Wildman et al. 1993) and ADP-ribosylation factor (Haun et al. 1993; Kahn et al. 1992) where it is linked

through an amide bond to a highly conserved amino terminal glycine residue (Han and Martinage 1992; Linder et al. 1991; Spiegel et al. 1991a). Analysis of the amino acid sequence of RD1 revealed the presence of a proline at position 2 rather than the required glycine so it was therefore deemed unlikely that myristoylation was the mechanism by which the subcellular localization of RD1 was determined. Palmitoylation is a modification which has also been observed for many proteins (Beranger et al. 1991; Glomset et al. 1990; Hancock et al. 1989; Kloc et al. 1991; Leevers et al. 1994; O'Dowd et al. 1989; Okubo et al. 1991). Such modified proteins containing covalently bound palmitate are strongly anchored in the plasma membrane (Grand 1989). In most examples the palmitate moiety is attached via a thioester bond to a cysteine which is located within the consensus sequence CAAX found only at the carboxy terminus (Beranger et al. 1991; Cox and Der 1992; Glomset et al. 1990; Grand 1989; Hancock et al. 1989; Kloc et al. 1991; Leevers et al. 1994). However, in recent years proteins have been identified which are palmitoylated on cysteine located close to their amino terminus (Parenti et al. 1993; Sudo et al. 1992; Widmer and Caroni 1993). Indeed it has been reported that the neuronal protein GAP-43, which is palmitoylated on two N-terminal cysteines, becomes only partially membrane-associated upon prevention of acylation of either cysteine (Zuber et al. 1989).

1.8 Intracellular Localization of the cAMP-Specific Phosphodiesterase RD1 (RNPDE4A1)

Nuclear encoded proteins synthesised in the cytoplasm are transported to various intracellular locations through the secretory pathway via the endoplasmic reticulum (ER) and Golgi apparatus/trans Golgi network (TGN) (Pfeffer and Rothman 1987). In the absence of specific retention signals for

these organelles, proteins are transported by default to the cell surface (Pfeffer and Rothman 1987).

Previous studies have shown that when the cDNA for the cAMP-specific phosphodiesterase splice variant RD1 (RNPDE4A1) is transiently expressed in COS cells, the resultant gene product is predominantly associated with the plasma membrane (McPhee et al. 1995; Shakur et al. 1993). Plasma membrane association of RD1 has also been demonstrated in the synaptosomes of rat brain cerebellum (Shakur et al. 1995). Studies carried out by Shakur et al (Shakur et al. 1993) have shown that deletion of the extreme amino terminal 25 amino acids of RD1 results in translocation of the enzyme from the plasma membrane to the cytosol thus suggesting plasma membrane association of RD1 is mediated by information contained within this region. Extensive database searches have failed to reveal any significant homology between the N-terminus of RD1 and the targeting signals of various plasma membrane associated proteins thus suggesting that this region may contain a novel, previously unidentified, membrane targeting signal. The aim of this thesis was to determine whether the extreme amino terminus of RD1 did indeed, contain a targeting signal or whether this region formed part of a larger structural element required for plasma membrane localization.

CHAPTER 2

Methods and Materials

2.1 CHEMICALS AND SUPPLIERS

Supplier	Reagent
Promega U.K.,	dNTP mix
Southampton, U.K.	Wizard Miniprep Kits
	Wizard PCR Prep Kits
	Wizard Maxiprep Kits
	Taq DNA Polymerase
	Xba1 (10 units/µl)
	Xho1 (10 units/µl)
	Nar1 (10 units/µl)
	Cla1 (10 units/µl)
	BamH1 (10units/µl)
	T4 DNA Ligase
	SP6 TnT ™ System
Sigma Chemical Co.,	Ampicillin
Pool, Dorset, U.K.	Ethidium Bromide
	Chloramphenicol
	TMPD
	AMP
	BSA
Life Technologies Ltd.,	DMEM
Renfrew, U.K.	Pen/Strep soln.
	L-Glutamine
	F.C.S.
	pSV.SPORT1

Bio-Rad Laboratories Ltd Herts, U.K.	Bradford Protein Assay Reagent
Pharmacia Biotech Milton Keynes, U.K.	pSVL
Perkin-Elmer U.K Warrington, U.K.	Taq Dye Deoxy™ Terminator Kits Phenol/H_2O/$CHCL_3$
Cambridge Bioscience Cambridge, U.K.	Anti-CAT Antibody
Amersham International Amersham, U.K.	ECL Detection Kits Hyperpaper-ECL [2-^{3}H] AMP (21Ci/mmol) [^{14}C] Chloramphenicol (54Ci/mmol) L-[^{35}S] Methionine (>1000Ci/mmol)

All other reagents were purchased from B.D.H. Ltd., Poole Dorset, U.K.

2.2 CONSTRUCTION OF RECOMBINANT PLASMIDS

2.2.1 Generation of PCR Fragments

The following reagents were assembled in a sterile 0.5 ml Eppendorf tube, 1µg template DNA, 25pmol each primer, 0.2mM each dNTP, 50mM KCl, 10mM Tris-HCl (pH9.0), 0.1% Triton X-100 (v/v), 1.5mM $MgCl_2$ and 2.5units of Taq polymerase 1 in a final volume of 50µl. reactions were overlayed with 40 µl mineral oil and subjected to the following conditions in a Techne PHC-3 thermocycler; denaturation for 1' at 94°C, annealing for 2' at 37°C, extension for 3' at 72°C for one cycle followed by denaturation for 1' at 94°C, annealing for 2' at 60°C, extension for 3' at 72°C for 30 cycles. Upon completion, the reaction was extracted with an equal volume of chloroform to remove residual mineral oil and the reaction product precipitated by incubation at -70°C for 15 minutes upon addition of 1/10th volume (v/v) of 3M sodium acetate (pH 5.2) and 3 volumes (v/v) absolute ethanol. Finally the PCR fragment was centrifuged at 12,000 g for 15 minutes at 4°C in a Jouan MR18.12 bench-top microcentrifuge, the pellet washed with 1 ml 70% ethanol and centrifuged as above before air drying at room temperature for 15 minutes.

2.2.2 Restriction Digestion of DNA and PCR Fragments

Fragments were digested by the restriction enzymes either for 2 hours or overnight at 37°C according to the manufacturers instructions for the specific enzyme; Xba1 and Xho1 in 6mM Tris-HCl (final pH7.9), 150mM NaCl, 6mM $MgCl_2$ and 1mM DTT , Cla1 in 10mM Tris-HCl (final pH7.9), 50mM NaCl, 10mM $MgCl_2$ and 1mM DTT and Nar1 in 50mM Tris-HCl (final pH8.2), 5mM $MgCl_2$, BamH1 in 6mM Tris-HCl (final pH7.5), 100mM NaCl, 6mM $MgCl_2$ and 1mM DTT. Final volumes were either 50 µl or 100 µl.

2.2.3 Ligation of PCR Products and DNA Fragments

All ligations were carried out according to Sambrook *et al* (Sambrook et al. 1989). Essentially, insert and vector DNA at a molar ratio of 1:1 was incubated at 14°C for 16 hours in a final volume of 10 µl containing 3 Wiess units T4 DNA ligase 30mM Tris-HCl (final pH7.8), 10mM $MgCl_2$, 10mM DTT and 5mM ATP. Ligation reactions were then directly transformed into competent bacteria for propagation and selection.

2.3 BACTERIAL EXPRESSION OF PLASMIDS

2.3.1 Preparation of Competent Bacteria

The *Escherichia coli* strains JM109 (*recA* 1 *supE* 44 *endA* 1 *hsd R* 17 *gyr A* 96 *rel A* 1 *thi* Δ (*lac-pro*Ab)) and HB101 (*sup E* 44 *hsd S* 20 (r_B m_B) *rec A* 13 *ara* -14 *pro A* 2 *lac Y* 1 *gal K* 2 *rps L* 20 *xyl* -5 *mtl* -1) used for propagation of recombinant and stock plasmids, were made competent by a modification of the calcium chloride method (Sambrook et al. 1989). Essentially, 5ml of 2TY broth was inoculated with 10 µl of a glycerol stock solution and incubated at 37°C while shaking. After 18 hours, 1 ml of this seed culture was used to inoculate 100 ml 2TY broth in a 500 ml conical flask which was incubated at 37°C as before. Bacterial growth was assayed by removal of 1 ml of culture at 30 minute intervals and measuring absorbance at 600nM in a visible wavelength spectrophotometer. The bacteria were grown until the cells were in mid log phase ($^{A}600$ of 0.3-0.4) then placed immediately at 0°C for 30 minutes to arrest metabolic activity. Cells were harvested by spinning in a bench top centrifuge (Jouan Instruments C3.12) at 3,500 g for 5 minutes at ambient temperature. The cells were washed to remove any residual growth medium by re suspending the pellets in 15 ml ice cold 0.1M $CaCl_2$ and centrifugation as above. The washed cell pellets were then re suspended in 50 ml 0.1M $CaCl_2$ and incubated at 0°C for 30 minutes followed by centrifugation as before and finally re suspended in 4 ml 0.1M $CaCl_2$.

2.3.2 Transformation of Competent Bacteria

Plasmid DNA was introduced into competent E.coli by a modification of the method of Sambrook et al (Sambrook et al. 1989). Essentially, 10 µl of TE (10mM Tris-HCL, 1mM EDTA pH8.0) containing 500 ng of plasmid DNA or 10µl ligation reactions were added to 0.2 ml aliquots of competent cells in a pre-chilled 5ml tube (Falcon no.2059) and incubated at 0°C for 30 minutes. Following a heat shock at 37°C for 2 minutes, 2 ml 2TY broth pre-warmed to 37°C was added each tube which was then incubated at 37°C for 1 hour whilst shaking to allow for expression of antibiotic resistance. 0.1 ml aliquots of cells were then spread onto 2TY agar plates (Appendix) containing 50µg/ml ampicillin (final concentration) and incubated at 37°C for 16 hours to facilitate selection of antibiotic resistant plasmid containing colonies. Samples of competent cells incubated in the absence and presence of the plasmid pSVL were plated out as negative and positive transformation controls respectively. All procedures were carried out under asceptic conditions.

2.3.3 Screening of Bacterial colonies for Recombinant Plasmids

Antibiotic resistant colonies were picked from 2TY agar plates by sterile toothpicks and inoculated asceptically into 5 ml cultures of 2TY broth containing 50 µg/ml (final concentration) ampicillin (2TYamp) and the cultures were shaken at 37°C. After an overnight incubation plasmid DNA was isolated from each culture by a modification of the alkaline lysis procedure of Birnboim and Doly (Birnboim and Doly 1979). Essentially, bacterial cells were pelleted from 3 ml of overnight culture by centrifugation at 2500 g in a bench-top microcentrifuge for 5 minutes at room temperature. The supernatant was removed by aspiration and the pellets were re suspended in 0.2 ml 50mM Tris-HCl (pH7.5), 10mM EDTA, 100 µg/ml RNase A. The cells were lysed by the addition of 0.2 ml 0.2M NaOH, 1% SDS with gentle agitation. The lysate was neutralised upon the addition of 0.2 ml 1.32M potassium acetate (pH4.8). Cleared cell lysates were obtained by centrifugation of the samples at 16,000 g for 5 minutes at room temperature. Plasmid DNA was

isolated from the cleared cell lysate by the Wizard Miniprep system (Promega U.K.) which routinely yielded 3-10 μg of supercoiled plasmid DNA. Duplicate samples of putative recombinant plasmid DNA were incubated with and without the restriction enzymes Xba1 and Xho1 as mentioned above. Digested and non digested samples were analysed by electrophorisis through a 1% agarose gel in 89mM Tris-Borate, 89mM boric acid, 2mM EDTA (pH8.0), 0.2 μg/ml ethidium bromide. (Brondyk et al. 1993)

2.4 LARGE SCALE PURIFICATION OF PLASMID DNA

2.4.1 Preparation of a Cleared cell Lysate

For large scale plasmid isolation bacterial glycerol stocks containing the plasmid of interest were streaked onto 2TY agar plates containing 50 μg/ml (final concentration) and grown for 16-18 hours at 37°C. The following day 100 ml 2TYamp was inoculated with a single colony isolate (SCI) from the overnight plate and grown to saturation by incubation at 37°C for 16 hours whilst shaking. Post incubation, cells were pelleted from the overnight culture by centrifugation at 8,000g for 10 minutes at room temperature in Beckman JA2-21 high-speed centrifuge equipped with a JA-14 rotor . After discarding the supernatant the cell pellet was re suspended in 15 ml 50mM Tris-HCl (pH7.5), 10mM EDTA, 100 μg/ml RNase A. Cell lysis was achieved by the addition of 15 ml 0.2M NaOH, 1% SDS followed by inversion of the container until the suspension cleared whereupon neutralisation was effected by the addition of 15 ml 1.32M potassium acetate (pH4.8). The cell lysate was centrifuged at 14,000 g for 15 minutes at room temperature and the supernatant filtered through four layers of muslin to remove denatured protein, chromosomal DNA and other cellular debris.

2.4.2 Purification of Plasmid DNA

Plasmid DNA was precipitated from the cleared cell lysate by the addition of 0.5 volumes of iso-propyl alcohol followed by centrifugation as above. The supernatant was decanted and the pellet re-suspended in 2ml 10mM Tris-HCl

(pH8.0), 1mM EDTA (pH8.0) and purified by Wizard Maxiprep system (Promega U.K.) according to the manufacturers instructions.

2.4.3 Quantification of Plasmid DNA

Plasmid DNA concentrations were determined in each instance from the absorbance reading of a 1/100th dilution made in d.H_2O at 260 nm in a Shimadzu UV-1201 spectrophotometer zeroed against a d.H_2O blank and calculated according to the following formula where an absorbance of 1 OD unit at $^{A}260$ is equivalent to 50.0 µg/ml of double stranded DNA Sambrook et al (Sambrook et al. 1989).

2.5 PREPARATION OF OLIGONUCLEOTIDES

2.5.1 Purification of Oligonucleotides

Synthetic oligonucleotide primers were synthesised on an Applied Biosystems model 381A synthesiser using standard phosphoramidite chemistry. Following synthesis and deprotection, the primers were precipitated from solution by the addition of 1/10th volume (v/v) of 3M sodium acetate (pH 5.2), 3 volumes (v/v) absolute ethanol, incubation at -70°C in a dry ice/methanol bath and centrifugation in a Jouan MR18.12 bench-top microcentrifuge at 12,000 g for 15 minutes at 4°C. The pellet was washed by the addition of 1 ml 70% ethanol, centrifuged as above and air dried for 15 minutes at room temperature before re suspension in 30 µl d.H_2O

2.5.2 Quantification of Oligonucleotides

Oligonucleotide concentrations were determined from the absorbance reading of a 1/100th dilution at 260 nm in a Shimadzu UV-1201 spectrophotometer and calculated according to the following formula where at an absorbance of $^{A}260$, 1 OD unit of single stranded oligonucleotide = 33.0 µg/ml Sambrook et al (Sambrook et al. 1989).

2.6 SITE -SPECIFIC MUTAGENESIS

Mutagenesis was carried out by a modification of the method described by Ho *et al* (Ho et al. 1989) which is outlined in Figure 3 and Chapter 4. PCR conditions were as follows; in a sterile 0.5 ml Eppendorf tube was added 1µg template DNA, 25pmol each primer (A+C or B+D), 0.2mM each dNTP, 50mM KCl, 10mM Tris-HCl (pH9.0), 0.1% Triton X-100 (v/v), 1.5mM $MgCl_2$ and 2.5units of Taq polymerase 1 in a final volume of 50µl with a mineral oil overlay and subjected to the following PCR conditions; denaturation for 1' at 94°C, annealing for 2' at 37°C, extension for 3' at 72°C for one cycle followed by denaturation for 1' at 94°C, annealing for 2' at 60°C, extension for 3' at 72°C for 30 cycles in a Techne PHC-3 thermal cycler. Following completion, reactions were extracted once with an equal volume chloroform to remove residual mineral oil before loading onto a 1.75% low melting point agarose gel and electrophoresis at 100V constant voltage for 75 minutes in 89mM Tris-Borate, 89mM boric acid, 2mM EDTA (pH8.0), 0.2 µg/ml ethidium bromide at 4°C. PCR products were visualised by ultra-violet transilluminator at 312 nm and excised with a sharp scapel. Small fragments (approximately 2-3 mm^3) of each gel slice were combined in a fresh sterile 0.5 ml Eppendorf tube but in this instance the complementary mutagenic primers Band C were omitted in favour of the sense and anti-sense primers A and D only, in a final reaction volume of 100 µl before being subjected to the same thermal cycling conditions as described above. Upon completion of thermal cycling, the mutagenic PCR product was purified directly from the reaction by Wizard PCR Prep system (Promega U.K.).

2.7 SEQUENCING OF PCR PRODUCTS AND MUTATIONS

2.7.1 Taq Cycle Sequencing Reactions

Sequencing reactions were carried out essentially by di-deoxy chain termination according to the method of Sanger *et al* (Sanger et al. 1977) using a Taq DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems) according to the manufacturers instructions. Reaction conditions were as follows; to a sterile 0.5 ml Eppendorf tube was added 1 μg template DNA, 3.2 pmol sequencing primer (SP6), 8.5 ml reaction mixture in a final volume of 20 μl. The samples were subjected to the following conditions in a Techne PHC-3 thermal cycler; denaturation for 30 seconds at 96°C, annealing for 50 seconds at 45°C, extension for 4' at 60°C for 30 cycles. Upon completion of thermal cycling, reaction volumes were increased to 100 μl by the addition of 80 μl d.H_2O and extracted twice with an equal volume of phenol/H_2O/chloroform (68:18:14) before precipitation by the addition of 1/10th volume (v/v) of 3M sodium acetate (pH 5.2), 3 volumes (v/v) absolute ethanol and incubation at 0°C for 15 minutes, centrifugation at 12,000 g for 15 minutes at 4°C in a Jouan MR18.12 bench-top microcentrifuge. The pellets were washed by the addition of 1 ml 70% ethanol and centrfuged as above before drying by incubation at 85°C for 2 minutes.

2.7.2 Gel Electrophoresis of Sequencing Reactions

Pellets were re suspended in 4 μl de-ionised formamide and heated at 90°C for 2 minutes followd by incubation at 0°C. Samples were resolved by electrophoresis on a 6% acrylamide gel containing 8.3M urea run for 14 hours on an Applied Biosystems model 373A automated sequencer. Sequences obtained were analysed directly by Gene Jockey II software (Biosoft).

2.8 COS CELL EXPRESSION OF RECOMBINANT PLASMIDS

2.8.1 Propagation and Transfection of COS cells

The SV40 transformed monkey kidney cell line COS-7 (Gluzman 1981) was propagated in an atmosphere of 5% CO_2 at 37°C in DMEM containing 10% v/v penicillin/streptomycin (100units/ml), 20mM L-glutamine and 10% v/v FCS. Cells were plated on 10cm plates and grown to 75% confluency before transfection. DNA (7µg) was added to the cells in serum- free DMEM containing 50mM Tris/HCl (pH 7.3), 20mM HEPES (pH7.2) and 750µg/ml DEAE-dextran. The plates were incubated at 37°C for 4h. After aspiration of the transfection medium the cells were incubated in 25% glycerol/serum free DMEM for 1' min at 25°C. After rinsing the plates with serum-free DMEM, the cells were then cultured at 37°C for 48h. Cells were also subjected to the same transfection protocol but in the absence of DNA in order to provide a negative control.

2.8.2 Preparation of COS Cell Membrane Fractions

Transfected COS cells were grown as above. After aspiration of the growth medium, the plates were washed with 5 ml sterile PBS before incubation in 1.5ml 40mM Tris/HCl (pH7.8), 10mM EDTA (pH8.0), 120mM NaCl at 25°C for 5min. The cells were scraped from the plate and harvested in an Eppendorf tube by centrifugation at 6000g for 5min at 4°C. The pellets were resuspended in 100µl 10mM Tris/HCl (pH7.8), 0.2mM $MgCl_2$ (TEN) and lysed by 3 cycles of liquid nitrogen, 2min/37°C, 2min. The lysate was centrifuged at 350g for 5min at 4°C to give a P1 pellet fraction and the supernatants aspirated to fresh tubes. The P1 pellets were washed by resuspending in 100µl 40mM Tris/HCl (pH7.8), 10mM EDTA (pH8.0), 120mM NaCl and centrifuging as above. Supernatants were pooled and re-centrifuged at 150,000g in a Beckman Airfuge for 20min to give a P2 pellet fraction with the supernatants designated as S2. Both the low speed (P1) and

the high speed (P2) pellets were resuspended in 25mM Tris/HCl (pH7.8), 5mM EDTA (pH8.0), 0.1mM $MgCl_2$, 60mM NaCl.

2.8.3 Triton X-100 Solubilization

A post nuclear supernatant of transfected COS cells was incubated at 0°C for 30 minutes in the presence of various concentrations of the non-ionic detergent Triton™ X-100. Post incubation, P2 pellet and S2 supernatant fractions were prepared by centrifugation at 150,000g in a Beckman Airfuge for 20 minutes and assayed for CAT activity. The results were plotted as percentage solubilized activity against total activity.

2.9 CHLORAMPHENICOL ACETYLTRANSFERASE (CAT) ASSAY

Chloramphenicol acetyl transferase (CAT) activity was monitored essentially by the method of Seed and Sheen (Seed and Sheen 1988). Duplicate samples of each fraction were assayed in 0.1mM Tris/HCl (pH7.8), 0.25mM butyryl CoA and 0.2µCi [^{14}C] chloramphenicol in a final volume of 100µl by incubating at 37°C for 1hr. Reactions were stopped by addition of 200µl tetramethylpentadecane:xylene (TMPD) (2:1, v/v) and vortexing for 15secs. The organic and aqueous phases were separated by centrifugation at 13,000g in a microfuge before the upper organic phase was transferred to scintillation vials. After addition of scintillation fluid, the samples were then counted for 60secs in a LKB Wallac model 1409 liquid scintillation counter.

2.10 SUBCELLULAR FRACTIONATION

2.10.1 Sucrose Gradients

This was done by a modification of the procedure described previously (Shakur et al. 1993). Forty eight hours post transfection the COS-7 cells were harvested, resuspended in a final volume of TEN buffer and disrupted by forty

strokes of the pestle in a Dounce homogeniser. A post nuclear supernatant was prepared by harvesting transfected COS cells by centrifugation at 6000g for 5 min at 4°C. The pellets were resuspended in 100µl 10mM Tris/HCl (pH7.8), 0.2mM $MgCl_2$ and lysed by 3 cycles of liquid nitrogen, 2min/37°C, 2min. The lysate was centrifuged at 350g for 5min at 4°C to remove cell debris and give a post nuclear supernatant. This was layered on top of a non-linear 0.5 M/1.6 M sucrose step gradient and centrifuged at 50,000 g for 1 hr in a Beckman L8-70M ultracentrifuge equipped with a SW50.1 swing-out rotor. Membranes were harvested from the interface and solid sucrose added to yield a concentration of 1.6M. These were layered under a 0.6-1.6 M continuous sucrose gradient prior to centrifugation at 50,000 g for 18 hr in a Beckman L8-70M ultracentrifuge equipped with a SW50.1 swing-out rotor. After this the gradient was separated into fractions of 180µl which were taken for immediate analysis of CAT and 5'- nucleotidase activity.

2.10.2 Assay for 5'-Nucleotidase Activity

This was carried out essentially according to the method of Newby et al (Newby et al. 1975). 5'-nucleotidase activity was determined by measuring the release of [^{3}H] adenosine from [^{3}H]-AMP. Essentially, 10 µl of particulate fraction were added to a 1.5 ml Eppendorf tube containing 200 µM AMP previously spiked with 3 µCi tritiated AMP in a final reaction volume of 1.0 ml. Samples were incubated at 37°C for 15 minutes before termination by the addition of 100 µl of 0.15M $ZnSO_4$ and 100 µl of 0.15M Ba $(OH)_2$. Following centrifugation at 12,000g for 5 minutes in a Jouan MR18.12 bench-top microcentrifuge at room temperature, 500 µl of supernatant was transferred to a scintillation vial containing 5 ml scintillation fluid. Samples were then counted on an LKB Wallac 1409 scintillation counter.

2.11 WESTERN-BLOT ANALYSIS OF TRANSFECTED COS CELLS

2.11.3 Assay for Protein Concentration

Protein concentration of the various cell fractions was assayed essentially by the method of Bradford (Bradford 1976). Briefly, protein samples were added to 800 µl of d.H_2O in a 1 ml semi-micro cuvette. 200 µl of Protein Assay Reagent (Bio-Rad) was added to the cuvette which was mixed by inversion. Samples were read at 595 nm in a Shimadzu UV1201 UV/visible spectrophotometer. Protein concentrations were determined by reading absorbance values against a standard curve of known concentrations of BSA

2.11.2 SDS-Polyacrylamide Gel Electrophoresis

Separation of proteins was carried out essentially by the method of Laemmli (Laemmli 1970). Briefly, 50-100 µl of sample containing 100 µg of protein was dissolved in an equal volume of 5M urea, 0.18M SDS, 0.38M DTT, 0.05M Tris-HCl (pH8.0) containing bromophenol blue as a tracking dye. Samples were incubated at 100°C for 3 minutes before separation of proteins by electrophoresis through a 10% polyacrylamide slab gel run in 0.025M Tris-HCl, 0.19M glycine and 0.0035M SDS at a constant current of 45mA until the dye front reached the bottom of the gel.

2.11.3 Immunoblotting Analysis

Proteins were transferred to nitrocellulose membrane by incubation in 25mM tris, 192mM glycine, 0.05% SDS, 20% methanol at 1A for 2h in a Hoeffer Transphor blotting system. After blocking the membrane by incubation for 2 hours at room temperature for 45 minutes with 5% dried skimmed milk reconstituted in PBS, the membrane was incubated overnight with a 1:500 dilution of a commercially available anti-CAT polyclonal antibody (5 Prime-3 Prime inc.) in PBS containing 1% dried skimmed milk, 0.2% Nonidet P40, 0.05% thimerosal.

Proteins were detected by subsequent incubation of the blot with a 1: 200 dilution of a horseradish peroxidase conjugated secondary IgG antibody (SAPU) and visualisation by incubation in ECL reagents (Amersham) for 1min. at room temperature followed by exposure to either or both Hyperpaper-ECL (Amersham) and X-ray film.

2.12 MEMBRANE ASSOCIATION OF RD1-CAT CHIMERAS IN A CELL-FREE EXPRESSION SYSTEM

2.12.1 Cell-Free Expression of Recombinant plasmids in a Coupled Transcription/Translation System

Mature native and chimeric proteins were synthesised in an SP6 TnT ™ coupled transcription/translation system (Promega U.K.) according to the manufacturers instructions. Briefly,1 µg of plasmid DNA was added to a sterile 0.5 ml Eppendorf tube containing 25 µl lysate, 2 µl TnT ™ buffer, 1 µl SP6 polymerase, 1 µl amino acid mixture minus methionine and 4 µl [^{35}S] methionine (>1000Ci/mmoll) in a final volume of 50 µl. Protein synthesis was initiated by incubation at 30°C for 90 minutes.

2.12.2 Membrane Association of Proteins Generated in a Cell-Free System

This was carried out essentially according to the method of Scotland and Houslay (Scotland and Houslay 1997). Post translation, duplicate 10µl aliquots were treated by the addition of either 10µl of TEN buffer (no added membranes) or 10µl of TEN containing 0.27mg/ml COS cell membranes (added membranes). Membranes were prepared as described above for transfected COS cells. Samples were incubated at 37°C for 30 minutes and then centrifuged at 150,000g in a Beckman Airfuge for 20 minutes. Supernatants were removed to fresh tubes and pellets were washed by resuspension in 20µl TEN and re-centrifuged as above. The supernatants were removed and pooled with those from the first centrifugation whereupon the pellets were finally resuspended in TEN containing 0.5% Triton X-

100. All samples were subsequently adjusted to give equal volumes, salt and detergent concentrations before running on a 10% SDS-polyacrylamide gel against [^{14}C] labelled molecular weight markers. Gels were visualised on a Fuji Bas 1000 phosphorimager with MacBas software.

CHAPTER 3

Construction and Expression of a Novel Eukaryotic Expression Vector Used in the Study of Membrane Association of the PDE4 RD1

3.1 Introduction

Transfection of COS cells with the eukaryotic expression vector pSVL containing full-length cDNA encoding the PDE4A species RD1 (rPDE-IV$_{A1}$), led to around 85% of the novel PDE activity found to be associated with the plasma membrane fraction and required low concentrations of the non-ionic detergent Triton X-100 to effect its release (Shakur et al. 1993). In contrast, transfection with a plasmid encoding a truncated PDE4A species which lacked the N-terminal first 25 amino acids led to the expression of novel PDE activity which was exclusively cytosolic. It was therefore, suggested that membrane association of RD1 was determined by this unique 25 amino acid N-terminal domain (Shakur et al. 1993). Subsequent cloning of various splice variants of the PDE4A gene has demonstrated that the first 23 residues of RD1 is the alternatively spliced region. Thus it has been suggested that the PDE4A genes encodes a 'core' soluble PDE to which membrane targeting may be conferred by virtue of the N-terminal splice region.

In order to assess whether this N-terminal splice region was indeed responsible for the conferment of membrane association upon a core soluble PDE enzyme I set out to generate chimeric species formed between the N-terminal region of RD1 and the soluble bacterial enzyme chloramphenicol acetyltransferase (CAT) in order to determine if this species would be similarly targeted to the plasma membrane. A eukaryotic expression vector was thus engineered and used as the basis for the generation of a family of plasmids which contained in-frame fusions between various regions of the N-terminus of RD1 and the gene encoding chloramphenicol acetyltransferase (CAT) derived from Transposon 9 (Alton and Vapnek 1979; Gorman et al. 1982; Murry et al. 1988; Shaw et al. 1979). CAT, which was used to successfully determine the mechanism of membrane association of the neuronal protein GAP-43 (Zuber et al. 1989) was chosen as an ideal candidate for a reporter gene because this

bacterial gene in its native form is exclusively soluble and very stable, furthermore, under normal circumstances, CAT is not expressed in mammalian cells. Also, it has been reported that some reporter genes, for example dihydrofolate reductase (DHFR) can encode passenger proteins which contain cryptic subcellular localisation signals (Hurt and Schatz 1987; Janiak et al. 1994).

3.2 Generation of Recombinant Plasmids Containing In-Frame Fusions Between Various Amino Terminal Fragments of RD1 and CAT

3.2.1 Construction of pGS4

Generation of the plasmid pGS4 encoding an in-frame fusion between the CAT gene and the first 100 amino acids of RD1 is outlined in Figure 3.1. The plasmid pBLCAT2 (Luckow and Schutz 1987) is a pUC18 derived eukaryotic expression vector which contains the CAT gene from the bacterial transposon Tn9, as well as the SV40 polyadenylation site. Transient expression of this vector in Chinese Hamster Ovary (CHO) cells is facilitated by the presence of the tk promoter from *Herpes simplex* virus (Luckow and Schutz 1987). This vector was used as the precursor for the generation of an in-frame fusion between the CAT gene and a DNA fragment encoding the first 100 amino acids of RD1. The first 100 amino acids were chosen for this preliminary construct on the grounds that the first 25 amino acids of RD1 may, in fact, constitute part of a larger structural element responsible for membrane targeting whose function was disrupted by the deletion of the extreme N-terminal 25 amino acids.

In order to create an in-frame fusion between the N-terminal 100 amino acid RD1 fragment and CAT, the synthetic oligonucleotide primers

GSOL12, 5'-GCGAGGGAATTCTAGAATGCCTCTGGTT-3'and

GSOL15, 5'-GGCTCCTCGAGCTTCCAGTGTGT-3'

were used in a polymerase chain reaction (PCR) synthesis using full-length RD1 cDNA as the template. A DNA fragment with a predicted length of 321 bp was observed when, post synthesis, a fraction of the reaction mix was analysed by electrophoresis through a 1.75% agarose gel (Figure 3.2). The primers GSOL12 and GSOL15 were designed to include recognition sequences (underlined above) for the restriction enzymes *Xba1* and *Xho1* (underlined) respectively and following an overnight digestion in the presence of the two enzymes a 307 bp DNA fragment was electrophorised through a 1.75% low melting point (LMP) agarose gel and purified as detailed in Materials and Methods (Ch. 2, section 2.6).

The parent plasmid pBLCAT2 (Luckow and Schutz 1987) was digested with *Xba1* and *Xho1* to release the tk promoter region. The digestion products were separated by electrophoresis through a 1.75% LMP agarose gel and the large digestion product purified as described in Materials and Methods (Ch. 2, section 2.6) and subsequently incubated in a ligation reaction with the previously digested PCR product. It was predicted from analysis of the DNA sequence for pBLCAT2 that successful ligation of the RD1 specific fragment into the plasmid would result in the loss of the unique *BamH1* restriction site at position 429 (bp) and would thus prevent linearization upon digestion with *BamH1*. That this was indeed the case is shown in figure 3.3. Plasmid DNA was isolated from two ampicillin resistant colonies picked at random following transformation of ligations into *E.coli* . Duplicate samples of DNA were incubated in the absence (Figure 3.3, lanes 1 and 3) and presence (lanes 2 and 4) of *BamH1* at 37°C for 3 hours followed by electrophoresis on a 1.0% agarose gel. Lane 1 (Fig. 3.3 shows the presence of two bands which correspond to supercoiled and open-circular forms of the plasmid (lower and upper bands respectively) commonly purified from bacteria after several rounds of replication. Incubation in the presence of the restriction enzyme *BamH1* of a duplicate sample of plasmid DNA isolated from the same colony revealed the

presence of a single band which corresponds to linearized plasmid DNA (fig. 3.3, lane 2). This shift from one molecular species to another is dependent on the presence of a *BamH1* site found only in the wild-type plasmid therefore indicating that this was pBLCAT2 and as such contained no insert DNA. Incubation of plasmid DNA isolated from the second colony revealed the presence of supercoiled and open-circular forms of the plasmid when incubation was carried out either in the absence (Fig. 3.3, lane 3) or presence of *BamH1* (Fig. 3.3, lane 4) which could only occur upon successful ligation of an insert between the *Xba1* and *Xho1* sites of the parent plasmid. Further confirmation of the presence of an insert and thus successful generation of an in-frame fusion between the first 100 amino acids of RD1 and CAT was obtained by Taq cycle sequencing. The construct was named pGS4.

3.2.2 Construction of the Eukaryotic Expression Vector pGS7

The plasmid vector pGS4 was generated to create an in-frame fusion between the first 100 amino acids of RD1 linked to the N-terminus of the bacterial enzyme CAT, in order to investigate the role of the N-terminal region of RD1 in determining membrane targeting. Previous work (Shakur et al. 1993) analysing the expression of full-length RD in COS cells was done by transient transfection in a pSVL plasmid (Pharmacia Biotech) with expression under the control of an SV40 promoter. In order to achieve transient expression in COS cells a fragment of DNA containing the SV40 early promoter (Figure 3.1) was incorporated into the plasmid pGS4 encoding the 1-100RD1-CAT chimera.

The plasmid pSV.SPORT used as a source of the SV40 early promoter is a eukaryotic expression vector capable of transiently expressing cloned genes in COS cells. Sequential digestion of pSV.SPORT by the restriction enzymes *Xba1* and *Cla1* resulted in the release of a 433bp DNA fragment (Figure 3.4) which contained not only the SV40 early promoter but also the bacterial SP6 promoter which was selected to allow expression of chimeric species in a cell-

free coupled transcription/translation system. In order to facilitate subcloning of this fragment into pGS4 the plasmid was first digested sequentially by the restriction enzymes *Xba1* and *Nar1* (which generated a complementary sticky end to Cla1) to release a 172bp 'stuffer' fragment under conditions described in materials and methods. The 4452bp digestion product of these reactions was gel purified to remove the 172bp fragment and thus prevent recircularization upon subsequent ligation. Both *Cla1* and *Nar1* sites were lost upon ligation of the two fragments to generate pGS7 (Fig. 3.1). The successful generation of this construct was confirmed by the release of a 500bp DNA fragment upon digestion the plasmid DNA with the restriction enzymes *Nde1* and *Xba1* (Figure 3.5, lane 4). By contrast, plasmids devoid of the SV40/SP6 promoter fragment release a 300 bp fragment upon incubation with *Nde1* and *Xba1* (Figure 3.5, lanes 2,6 and 10).

3.2.3 Generation of pGS8

In order to assess the effect of fusing N-terminal fragments of RD1 to CAT upon membrane localisation it was necessary to generate a control plasmid which expressed native CAT only. This was achieved by digestion of pGS7 with the restriction enzymes *Sal1* and *Xho1* to release a 330bp fragment containing the RD1 specific sequence. Following gel purification, the 4.54kb digestion product was recircularized by ligation due to the complementary nature of the sticky ends generated by *Sal1* and *Xho1*. Following transformation and DNA purification the successful generation of pGS8 was confirmed by failure to linearise plasmid DNA by digestion with *Xho1*.

3.2.4 Generation of pGS11

As had been previously demonstrated (Shakur et al. 1993) full-length RD1 was observed to be predominantly membrane associated when expressed in COS cells. In contrast a construct where the extreme N-terminal 25 amino acids had been deleted (met^{26} RD1) was entirely cytosolic when expressed in COS cells (Shakur et al. 1993). The plasmid pGS11 was designed to generate

an RD1-CAT chimera which was analogous to met^{26}RD1. A 241bp DNA fragment encoding amino acids 26-100RD1was generated by PCR using the primer

GSOL24, 5'- GCTTATCTAGAATGCTGAACCGTGAGCTC-3' when paired with the primer GSOL15 used in the generation previously of pGS7. This fragment was digested with the restriction enzymes *Xba1* and *Xho1*, gel purified (figure 3.6), and substituted for the 'wild-type' 1-100RD1 fragment in pGS7. Following purification of plasmid DNA from overnight cultures, Taq cycle sequencing confirmed the generation of the construct.

3.2.5 Generation of pGS13

Shakur et al (Shakur et al. 1993) have previously suggested that the membrane targeting of RD1 is a function of information contained within its extreme N-terminus. In order to test the hypothesis, primers

GSOL12, 5'-GCGAGGGAATTCTAGAATGCCTCTGGTT-3' and

GSOL13, GCATATCTCGAGCGCCTTTTGAACTGGT-3'

were used to generate a 79bp DNA fragment encoding amino acid residues 1-25 0f RD1 which used full-length RD1 as a template (Figure 3.7). Following digestion by the restriction enzymes *Xba1* and *Xho1*, the fragment was purified by electrophoresis through a 1.75% LMP agarose gel and substituted for the 1-100 amino acid RD1 fragment in the plasmid pGS7 to generate a 1-25 amino acid RD1-CAT fusion. Confirmation of the successful generation of this construct was obtained by sequencing miniprep DNA purified from overnight cultures.

3.3 Expression of pGS7, pGS8, pGS11 and pGS13 in COS Cells

3.3.1 Assay of CAT Activity

Plasmids pGS7, pGS8, pGS11 and pGS13 encoding 1-100RD1-CAT, native CAT, 26-100RD1-CAT and 1-25RD1-CAT respectively were transiently expressed upon transfection in COS-7 cells by the DEAE-dextran method. 48

hours post transfection, cells were harvested and assayed for CAT activity as described in materials and methods. The results of these experiments are given in Table 3.1 .

In typical transfection experiments CAT activity of the order of 1-10U per µl cell homogenate (1.5-2.0 c.p.m.x10^{-4}; range n= 5 separate transfections) was observed. For native CAT, expressed using the plasmid pGS8, the majority of CAT activity was clearly found in the high speed supernatant, cytosol fraction (Table 3.1). This is consistent with data reported by Seed & Sheen (Seed and Sheen 1988) using a different CAT plasmid. In marked contrast to this, when COS cells were transfected with the plasmid pGS7, to produce the chimera 1-100RD1-CAT, then a profound shift in the distribution of activity was seen such that the major fraction of CAT activity now occurred in the membrane fraction (Table 3.1). This distribution reflects that of RD1 where, in various transfection experiments, around 85% of this enzyme was found to be membrane associated (Shakur et al. 1993). However, transfection of COS cells with the plasmid pGS11, which encoded the chimera 26-100RD1-CAT, demonstrated that the distribution of CAT activity now paralleled that of native CAT itself with CAT activity now predominately in the cytosol fraction (table 3.1). Such data indicates that fusion of the N-terminal 100 amino acids of RD1 to CAT can confer membrane association on a normally soluble enzyme, in this instance the bacterial protein CAT. In marked contrast to this 26-100RD1-CAT demonstrated an identical distribution to soluble native CAT. The chimeric species 1-25RD1-CAT was also expressed transiently in COS cells transfected with the plasmid pGS13. Analysis of CAT activity showed, as with 1-100RD1-CAT, a marked increase in membrane association when compared to experiments done using either native CAT or the 26-100RD1-CAT chimera (Table 3.1). However, from these analyses where distribution was assessed using CAT activity, this 25 amino acid segment of RD1 appeared to be less efficient in conferring membrane association on CAT than was seen for the

longer extension found in the 1-100RD1-CAT chimera (Table 3.1).This is consistent with previous studies (Shakur et al. 1993) done on the analogous truncation for RD1 (met^{26}RD1) and also shows that merely generating a chimeric CAT does not lead to membrane association. Such experiments are entirely consistent with the notion that the first 25 amino acids of RD1 appears to provide the information necessary for conferring membrane association on a soluble protein.

3.3.2 Triton X-100 Solubilization of pGS7 Transfected COS Cells

COS cells transfected with pGS7 expressing the 1-100RD1-CAT chimera were harvested and a high-speed pellet (P2) fraction isolated from the post nuclear supernatant as described previously Samples of the P2 pellet were incubated on ice in the presence of either 0.05%, 0.1%, 0.2%, 0.5% or 1.0% Triton X-100 for 30 minutes followed by centrifugation at 150,000g for 20 minutes to separate membrane pellet and supernatant fractions. Duplicate samples of each fraction were assayed for CAT activity and compared to a sample incubated in the absence of Triton X-100. The results were plotted as the percentage of Triton solubilized CAT activity against [Triton X-100]. Figure 3.8 represents the data typical of experiments done three times using membranes from three separate transfections. The results of these experiments demonstrate that the 1-100RD1-CAT chimera can be stripped from COS cell membranes by incubation in the presence of the non-ionic detergent Triton X-100. Indeed, in this respect, the chimeric form appears to behave in an analogous fashion to full-length RD1 subjected to the same treatment with Triton X-100 (Shakur et al. 1993; Shakur et al. 1995). 1-100RD1-CAT demonstrated that a release of membrane associated CAT activity of ~40% when incubated with 0.1% Triton X-100 and that increasing the detergent concentration to 1.0% resulted in a loss of >80% of the membrane associated CAT activity. The dose-dependent solubilization of this chimeric species by

Triton X-100 closely followed the detergent release of native RD1 from cerebellum derived membranes (Shakur et al. 1995) and was similar to that seen for the solubilization of RD1 from transfected COS cells. Approximately 20% of CAT activity remained in the pellet fraction after incubation in the presence of 2.0% detergent. This might indicate that a fraction of 1-100RD1-CAT, like full-length RD1 observed in brain is associated with detergent-insoluble membrane remnants, which could include associated cytoskeletal components. Nevertheless, that the non-ionic detergent Triton-X-100 can release the majority from membranes suggests that the association is hydrophobic in nature.

This hypothesis was given further credence by the observation made previously my Shakur et al (Shakur et al. 1993) that 2M NaCl could not remove the 1-100RD1-CAT chimera from membranes thereby suggesting that electrostatic interactions were not important in localisation of this chimeric protein.

3.3.3 Subcellular Fractionation of COS Cells Expressing Chimeras

Experiments were done so as to assess whether RD1-CAT chimeras showed a similar subcellular distribution to full-length RD1 in transfected COS cells. To do this, COS cells were grown to ~75% confluency on 24.5cm x 24.5cm tissue culture plates and then transfected by essentially the same method as used for the 10cm plates as described previously. Forty eight hours post transfection the COS-7 cells were harvested, resuspended in a 1 ml final volume of TEN buffer and disrupted by forty strokes of the pestle in a Dounce homogeniser. A post nuclear supernatant was prepared as described previously (Shakur et al. 1993). This was layered on top of a non-linear 0.5 M/1.6 M sucrose step gradient and centrifuged at 50,000gav for 1 hr. Membranes were harvested from the interface and solid sucrose added to yield a concentration of 1.6M. These were layered under a 0.6-1.6 M continuous sucrose gradient prior to centrifugation at 50,000g for 18 hr. After this the gradient was

separated into fractions of 180µl which were taken for immediate analysis of CAT activity and the plasma membrane marker enzyme 5'- nucleotidase.

The result of such an experiment is shown in Figure 3.9. Two peaks of CAT activity were identified, a small peak was detected in the fractions corresponding to 0.6M-0.8M, sucrose and a larger peak of activity was observed in that fraction of the gradient corresponding to 1.06M sucrose (Figure 3.9). The small peak of CAT activity was located at the top of the gradient, whereas the larger peak was detected in an area of the gradient corresponding to the plasma membrane. That this fraction did indeed contain plasma membranes was confirmed by assaying for the presence of the plasma membrane marker enzyme 5'- nucleotidase. As can be seen from Figure 3.9, 5'- nucleotidase activity was detected in the sucrose gradient, moreover, this activity was concentrated in a single peak which co-migrated through the gradient at the same position as the major peak of CAT activity. This demonstrates that the chimeric 1-100RD1-CAT was indeed associated with the plasma membrane when expressed in COS cells as found for native RD1 (Shakur et al. 1993)

3.3.4 Western-Blot Analysis of Chimeras Expressed in COS Cells

In order to gain an independent assessment of the distribution of CAT and the various chimeras between the cytosol and membrane fractions, Western blotting experiments were carried out. COS cells were transfected with either pGS7, pGS8, pGS11 or pGS13 and then harvested 48 hours post-transfection. Pellet and high speed supernatant fractions were prepared and proteins were resolved by SDS-polyacrylamide gel electrophoresis. These were subsequently transferred to nitro-cellulose membrane by means of electrophoretic elution essentially according to the methodology of Harlow and Lane (Harlowe and Lane 1988). Following blocking, blots were then probed with a commercially available polyclonal rabbit anti-CAT first antibody (5-Prime 3-Prime). These were then challenged with an HRP conjugated goat anti-rabbit second antibody

and developed by the ECL western blotting method (Amersham). The result of a typical experiment is shown in Figure 3.10. Lanes 1-2 represent cells transfected by pGS8, lanes 3-4 pGS7, lanes 5-6 pGS11 and lanes 7-8 pGS13 corresponding to cells expressing native CAT, 1-100RD-CAT, 26-100RD-CAT and 1-25RD-CAT respectively. Lanes 1,3,5 and 7 represent pellet fractions whereas lanes 2,4,6 and 8 contain the high-speed supernatants. In the cells transfected with pGS8 the presence of an immunoreactive species of ~26kDa was observed which corresponds to the size expected for native CAT. It was found predominantly, (85-91%, range; n=3 separate experiments) in the supernatant fraction. This indicated that CAT was expressed essentially as a soluble protein when transiently transfected into COS cells . Lanes 3 and 4 (Fig. 3.10) correspond to the pellet and supernatant fractions of cells transfected with pGS7 encoding 1-100RD-CAT, however use of this antibody failed to detect any immunoreactive species despite the ability to detect CAT enzymatically. A similar observation was made on cells expressing the 26-100RD-CAT chimera where, again, no significant immunoreactive species was noted in either pellet or supernatant (Figure 3.10, lanes 5 &6). Failure to detect chimeras between CAT and either the 1-100 amino acid or the 26-100 amino acid RD1 fragments might occur as a result of a conformational change upon folding of the chimeric protein which prevented the antibody, which was raised against the N-terminus of CAT, recognising the CAT moeity. In marked contrast, using cells transfected so as to express the 1-25RD-CAT, two major protein species of ~30kDa and ~26kDa were detected. Whilst the ~30kDa component is of the size predicted for the chimera 1-25RD1-CAT, the species at ~26kDa is as expected for native CAT. This suggests that both the 1-25RD1-CAT chimera and native CAT were simultaneously expressed in COS cells transfected with this plasmid. Consistent with the ~26kDa band being native CAT, it was observed that it migrated identically to the single species found in COS cells transfected with pGS8 and was primarily associated with the cytosol

fraction (Figure 3.10, lane 8). In profound contrast to this, it was observed that the ~30kDa band (fig. 3.10; lane 7), corresponding to 1-25RD1-CAT, was exclusively associated with the membrane fraction. This is entirely consistent with fusion of the N-terminal 25 amino acid splice region of RD1 being able to confer membrane association on CAT and, therefore, by inference, upon the 'core' PDE4A protein as observed with RD1.

3.3.5 Cell-Free Expression of RD1-CAT Chimeras

To determine whether chimeras of 1-100RD-CAT and 1-25RD-CAT would be capable of subsequently associating with membranes in the absence of any mechanism for post translational modification, a cell-free, coupled transcription/translation system was utilised to generate mature chimeric proteins. The advantage of using a coupled system lies in the fact that plasmid DNA is used as a template for protein production which circumvents the need to generate RNA transcripts to programme a separate translation reaction. Plasmids were subjected to the reaction conditions as described in materials and methods, protein products were resolved by SDS-PAGE and subsequently visualised by autoradiography.

Fig. 3.11 shows a typical experiment where, in lane 1, the plasmid pGS8 containing the native CAT gene was used. This produced a single species of ~26kDa, corresponding to that expected for native CAT (25.5kDa; (Seed and Sheen 1988)). Subsequent incubation of this product with COS cell membranes, followed by separation of pellet (Fig. 3.11; lane 2) and supernatant (Fig. 3.11; lane 3) fractions, failed to show any membrane association of native CAT. When the plasmid pGS7 was expressed in the *in vitro* system, then three predominant bands were observed of ~38kDa, ~35kDa and ~33kDa (Fig. 3.11, lane 4). These are of the calculated sizes for initiation events occurring not only at the first Met start codon (38.6kDa) but also at Met^{26} (35.5kDa) and Met^{37} (34.2kDa) which provide the next two available met residues. Initiation at these three sites would be expected to yield the full length 1-100RD1-CAT

chimera together with 26-100RD1-CAT and 37-100RD-CAT. Additionally, several other minor bands were noted which might reflect other initiation events in the reaction (Fig. 3.11, lane 4). Using such a mixture of mature chimeric CAT species formed in this synthetic system, it was noted that only the largest chimera, presumably 1-100RD1-CAT became membrane associated (Fig. 3.11, lane 5) and thus depleted from the soluble fraction (fig. 3.11, lane 6). In marked contrast to this, the intensity of the ~35kDa and ~33kDa bands (Fig. 3.11 lanes 4 and 6), due to 26-100RD1-CAT and 37-100RD1-CAT, respectively, remained identical in both the initial synthesis mix and the supernatant fraction from membrane incubation. Thus these, presumably, N-terminally truncated species showed no evidence for membrane association. In confirmation that these two truncated species did not associate with membranes, the plasmid pGS11 was used, which not only produced in this system a ~35kDa species reflecting 26-100RD-CAT but also a ~33kDa species that was presumed to be the chimera 37-100RD1-CAT, and also native CAT itself at ~26kDa (Fig. 3.11, lane 7). None of these species became associated with the membrane fraction (Fig. 3.11, lane 8) with both remaining exclusively in the supernatant fraction (Fig. 3.11; lane 9). The failure of both the 26-100RD1-CAT chimera and the 37-100RD1-CAT chimera to become membrane associated also indicates that merely adding additional amino acid residues to the N-terminus of CAT does not confer upon this protein the ability to become membrane associated. Such species also act as internal 'book-keeping' controls showing that these species are entirely recovered in cytosol whereas 1-100RD1-CAT associates with membranes.

Using the plasmid pGS13, which encodes a chimera formed from the first 25 amino acids of RD1 fused to CAT, two major bands were observed. These were a species of ~30kDa, which would be predicted to reflect the chimera 1-25RD1-CAT, and a further band of ~26kDa, which would be predicted to be native CAT, as the next available Met codon would be expected to produce this

(Fig.3.11, lane 10). Lane 11 (Fig. 3.11) shows that only the 1-25RD1-CAT chimera became associated with the membrane pellet fraction and depleted in the soluble fraction (Fig. 3.11, lane 12) whereas the presumed native CAT species remained exclusively in the soluble fraction (Fig. 3.11, lane 12).

Multiple translation can occur in the combined transcription/translation system (Kozak 1986; Promega 1993) and it is believed that such an event provides the most plausible explanation for the production of multiple products by this system. Certainly the species observed upon SDS-PAGE migrate with sizes consistent with the start of translation occurring at the indicated methionine residues. This is exemplified by, for example, the observed co-migration of the first product (Fig. 3.11; track 7) from the plasmid pGS11, namely 26-100RD1-CAT, with the second product (Fig. 3.11, track 4) from the 1-100RD1-CAT containing plasmid pGS7, which would be predicted to produce 26-100RD1-CAT due to a second start occurring at met26. Indeed, analysis of the nucleotide sequence surrounding these methionine residues indicates that they do in fact provide sub-optimal Kozak sequences (Kozak 1986). Thus, for the initiation of translation, Kozak (Kozak 1986) has identified an optimal sequence of ACCATGG, with sub optimal sequences being those with a purine at position -3 to the ATG codon. From inspection of the sequence of the RD1 fragment in pGS7 it was noted that the initiating methionine was located within a region which obeys the consensus for a sub-optimal Kozak sequence AGAATGC (Kozak 1986). However, sub-optimal Kozak sequences (Kozak 1986) are also evident at both Met26 (AGGATGC) and at Met37 (GAAATGA)which would be expected to heighten the chances of occurrence of translation products occurring at these points in the *in vitro* transcription/translation system used here. Additionally, it may also explain why multiple species were seen in the COS cell expression studies using the RD1-CAT chimera (see Fig. 3.10). For example, analysis of the products of the plasmid pGS13, showed that in both COS cells (Fig. 3.10) and the *in vitro*

transcription/translation system (Fig. 3.11, track 10), two products were observed, namely the 1-25RD1-CAT chimera and also native CAT.

It is thought extremely unlikely that the presence of multiple bands observed in this system are generated as a result of proteolysis. However, in order to try and address the question of possible proteolysis various strategies were employed. These included employing a cocktail of protease inhibitors, used routinely when isolating PDEs isoforms [see for example (Shakur et al. 1993)] in the reaction using the plasmid pGS7. However, these compounds prevented product formation by the *in vitro* transcription/translation system (data not shown). In contrast, addition of soybean trypsin inhibitor, at concentrations of up to 1mg/ml, allowed for a pattern of product formation (data not shown) which was identical to that seen in its absence (Fig. 3.11 track 4). However, as the targets for action of trypsin inhibitor are proscribed this observation cannot be used to eliminate protease action. If, however, the smaller species found using, for example, the plasmid pGS7 were due to proteolysis then a time course of the production of these species might be expected to follow a product precursor relationship, with 1-100RD1-CAT being produced initially. In contrast, it was observed (Fig. 3.12) that the time course of production of the three products examined showed great similarity in both the rate of production and the time at which a maximum yield was produced. Routinely, analyses were performed after reactions were incubated for 90min (see Methods). However, here it was noted (Fig. 3.12) that the absolute levels and the proportions of all three products reached maximal expression 70 minutes after incubation and these levels remained stable even after some 240min. Additionally, it is evident that the addition of the crude membrane fraction did not alter the relative proportions of the species produced in the *in vitro* transcription/translation system. For example, the relative amounts of three products of plasmid pGS11, presumed to be 26-100RD1-CAT, 37-100RD1-CAT and CAT itself appear identical upon analysis of both the

products of the *in vitro* transcription/translation system (Fig. 3.11, track 7) and also in the supernatant fraction after incubation with membranes (Fig. 3.11, track 9).

It is believed that such time course data, together with the observations that met26 and met37 in RD1 provide sub-optimal Kozak sequences (Kozak 1986) and that the *in vitro* transcription/translation system is susceptible to multiple initiation events (Kozak 1986; Promega 1993), make multiple initiation the most probable explanation for the multiple products produced by the *in vitro* transcription/translation system in this study (Fig. 3.11). Moreover, it is possible to exploit this promiscuity to provide internal controls given that when presented with a mixture of protein products transcribed from the same gene, only those species possessing the unique N-terminal splice region will associate with membranes.

3.4 Conclusions

In summary, a eukaryotic expression vector was constructed to allow investigation of the role of the unique N-terminal splice domain of the PDE4A RD1 in determination of membrane association by creating in-frame fusions between various regions of RD1 and the N-terminus of the soluble bacterial enzyme chloramphenicol acetyltransferase (CAT). Expression of these plasmids in COS cells demonstrated chimeric proteins containing the unique N-terminal splice region of RD1 could be detected enzymatically and by immunoblotting in the membrane fraction whilst native CAT was exclusively cytosolic. Furthermore, expression of those same plasmids in a cell-free expression system demonstrated that it was possible to generate mature chimeric proteins which would associate with added membranes.

Figure 3.2. Generation of a PCR Fragment encoding the N-terminal 1-100 Amino Acids of RD1.

A 321bp DNA fragment was successfully generated by PCR using synthetic oligonucleotide primers and the plasmid pSVL-RD1 (Shakur et al. 1993) as a template. pSVL-RD1 contains a DNA insert encoding the open reading frame (ORF) corresponding to the PDE4A isoform RD1 (Davis et al. 1989).

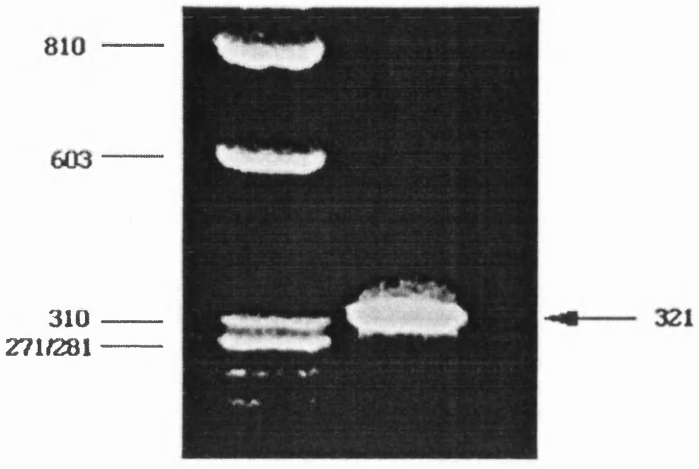

810
603
310
271/281
321

Figure 3.3: Generation of the 1-100RD1-CAT Fusion Plasmid pGS4.

Cloning of a PCR fragment encoding the N-terminal 100 amino acids of RD1 into the plasmid pBLCAT2 (Luckow and Schultz. 1987) resulted in the loss of the unique *BamH1* restriction enzyme recognition site. Lanes 1 & 2 correspond to native pBLCAT2 digested in the absence (lane 1) and presence (lane 2) of *BamH1*. The appearance of a single band in lane 2 denotes the presence of the recognition site for this enzyme. In contrast, digestion of pGS4 with BamH1 (lanes 3 &4) shows no difference when the plasmid was incubated in the absence (lane 3) of presence (lane 4) of the enzyme thus indicating the loss of the *BamH1* site.

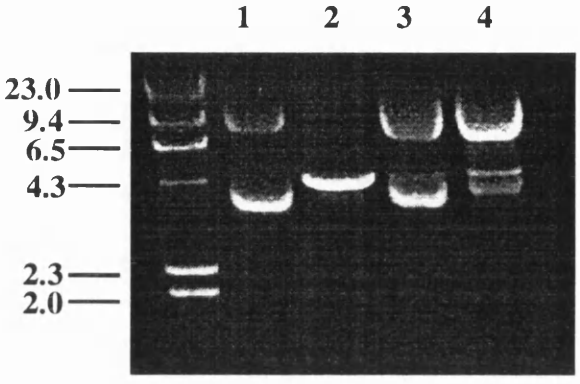

1
2
3
4
23.0
9.4
6.5
4.3
2.3
2.0

Figure 3.4: Generation of a DNA Fragment Containing the SV40 Early Promoter.

The plasmid pSV.SPORT1 (Life Technologies Ltd, UK) was digested with the restriction enzymes *Cla1* and *Xba1* according to the manufacturers instructions. The resultant 433bp DNA fragment, which contained the SV40 early promoter and SP6 bacterial promoter, was isolated by agarose gel electrophoresis as described in Methods (section 2.6). A 564bp band generated by digestion of bacteriophage Φ X174 DNA with the restriction enzyme *HaeIII* is also shown.

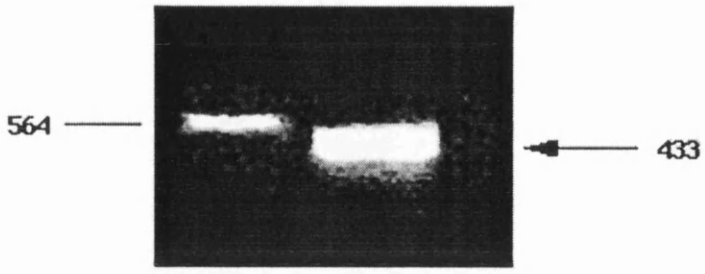

564
433

Figure 3.5: Generation of the Eukaryotic Expression Vector pGS7.

Plasmid DNA was isolated from five colonies and incubated in the absence (lanes 1,3,5,7 & 9) and presence (lanes 2,4,6,8 & 10) of the restriction enzymes *Nde1* and *Xba1*. Lane 4 shows the presence of the 500bp DNA fragment predicted upon digestion of pGS7 with *Nde1* and *Xba1*. Lanes 2, 6 and 10 show a 300bp fragment predicted to occur upon incubation of pGS4 with the same enzymes. The 2.3kb, 2.0kb and 0.5kb fragments of bacteriophage λDNA digested with the restriction enzyme HindIII were used as molecular weight markers. This gel was overexposed to allow visualization of the 300bp and 500bp digestion products.

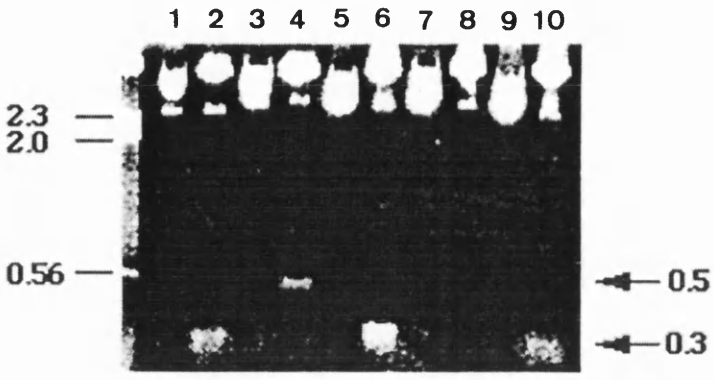

1 2 3 4 5 6 7 8 9 10
2.3
2.0
0.56
0.5
0.3

Figure 3.6: Generation of a PCR Fragment Corresponding to Amino Acids 26-100 of RD1.

This figure shows the successful generation of a 241bp PCR fragment generated when synthetic oligonucleotide primers designed to correspond to amino acids 26-100 of RD1 were used in a PCR reaction utilizing the plasmid pSVL-RD1 (Shakur et al. 1993) as a template.

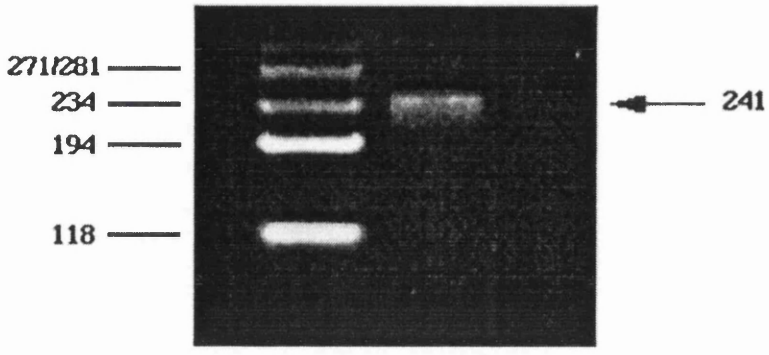

271/281
234
194
118
241

Figure 3.7: Generation of a PCR Fragment Corresponding to Amino Acids 1-25 of RD1.

This figure shows the successful generation of a 79bp PCR fragment generated when synthetic oligonucleotide primers designed to correspond to amino acids 1-25 of RD1 were used in a PCR reaction utilizing the plasmid pSVL-RD1 (Shakur et al. 1993) as a template. *HaeIII* digested bacteriophage Φ X174 DNA is shown as molecular standards.

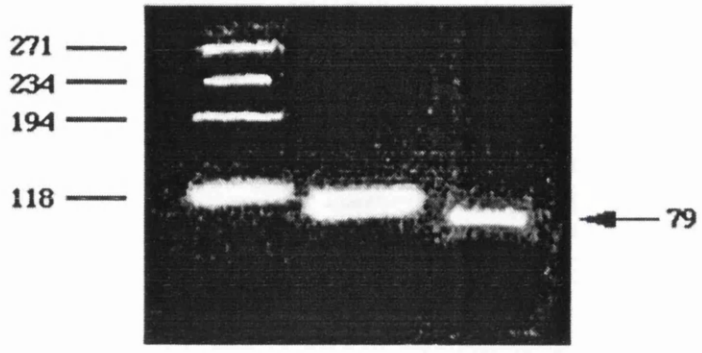

271
234
194
118
79

Table 3.1. Distribution of CAT activity in Native- and Chimeric- CAT Transfected COS Cells.

COS cells were transfected with the indicated plasmid and then, after 48h, membrane and high speed supernatant fractions isolated as described in Methods. All CAT assays were performed in duplicate as described in the Methods section. The distribution between the membrane (pellet) and cytosol (high speed supernatant) fractions are given as percentages. This data provides the mean of three separate transfection experiments done on different occasions with errors as SEM. No CAT activity was evident in COS cells which were mock transfected with either the plasmids pSVL or pSPORT.

plasmid	Construct	Membrane (%)	Cytosol(%)
pGS8	CAT	13 ± 2	87 ± 2
pGS7	1-100RD CAT	64 ± 3	36 ± 3
pGS11	26-100RD CAT	17 ± 2	83 ± 2
pGS13	1-25RD CAT	51 ± 3	49 ± 3

Figure 3.8: The Effect of Triton X-100 Concentration on Membrane Association of 1-100RD1-CAT

COS cells were transfected with pGS7 to express the chimera 1-100RD1-CAT. Following transfection, membranes were prepared and replicate samples incubated at 0°C for 30 minutes with varying concentrations of the non-ionic detergent Triton X-100 as indicated. High-speed pellet and supernatant fractions were prepared by centrifugation at 100,000g for 30 minutes. CAT activity was determined as reported in Methods (section 2.9). Results are given as percentage CAT activity in the membrane fraction versus detergent concentration. The data shown are typical of one experiment done three times using membranes from separate transfection experiments.

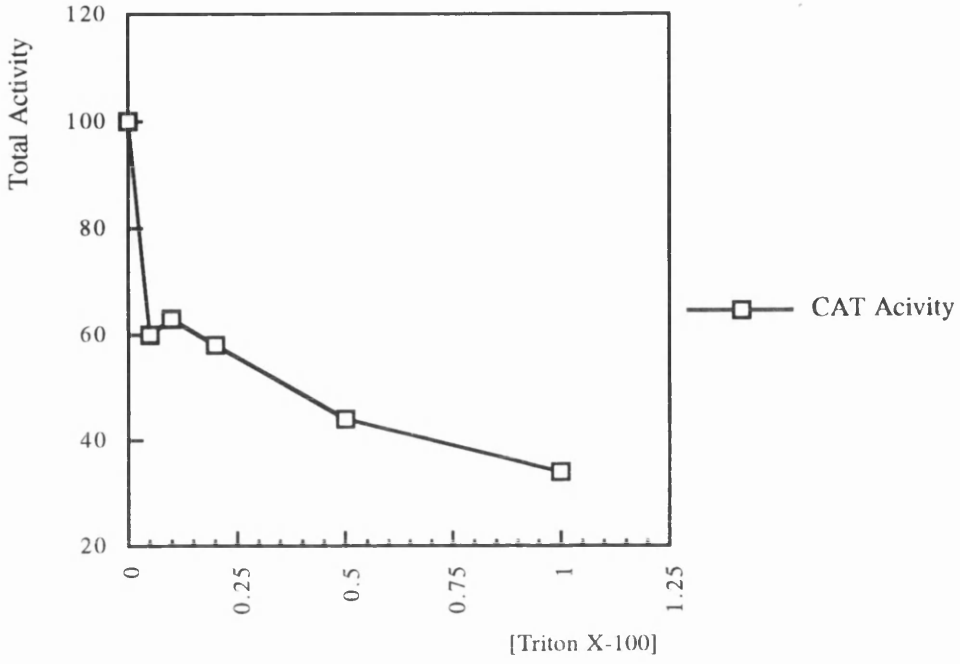

Total Activity
120
100
80
60
40
20
0
0.25
0.5
0.75
1
1.25
[Triton X-100]
CAT Acivity

Figure 3.9: Subcellular Distribution of CAT Activity in COS cells Transfected with pGS13

COS cells were transfected with pGS13 to express the chimera 1-25RD1-CAT as described in Methods (section 2.8.1). Following transfection, a post-nuclear membrane preparation was subjected to centrifugation on a continuous sucrose density gradient (0.5M-1.6M) as described in Methods (section 2.10.1). Fractions were taken and assayed for CAT activity and 5'-nucleotidase activity as described (Methods, sections 2.9 and 2.10.2 respectively). Results are given as percentage enzyme activity versus sucrose concentration. These data are tyipical of one experiment done twice with membranes from separate transfections.

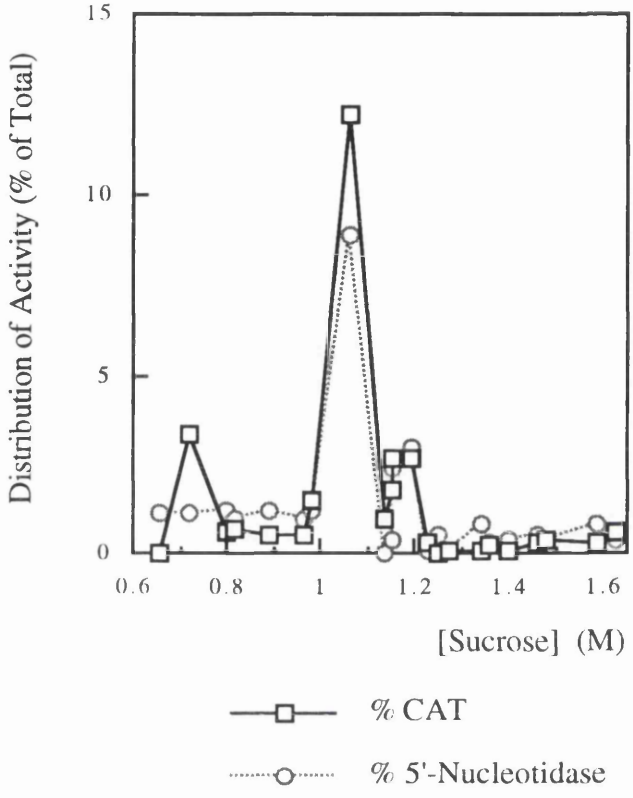

15
10
5
0
Distribution of Activity (% of Total)
0.6
0.8
1
1.2
1.4
1.6
[Sucrose] (M)
% CAT
% 5'-Nucleotidase

Figure 3.10: Western Blot Analysis of COS Cells Transfected with RD1-CAT Chimeric Plasmids.

COS cells were transfected with pGS8 (lanes 1 & 2), pGS7 (lanes 3 & 4), pGS11 (lanes 5 &6) or pGS13 (lanes 7 &8) as described in Methods (section 2.8.1). High-speed supernatant and pellet fractions were electrophoresed on denaturing acrylamide gels (Methods, section 2.11.2) prior to transfer to nitrocellulose as described (Methods, section 2.11.3). Membranes were probed with an anti-CAT polyclonal antibody (5'-Prime 3'-Prime) before visualization by enhanced chemiluminescence. Lanes 1,3,5 and7 represent pellet fractions whereas lanes 2,4,6 & 8 correspond to supernatant fractions. These results are typical of one experiment repeated three times.

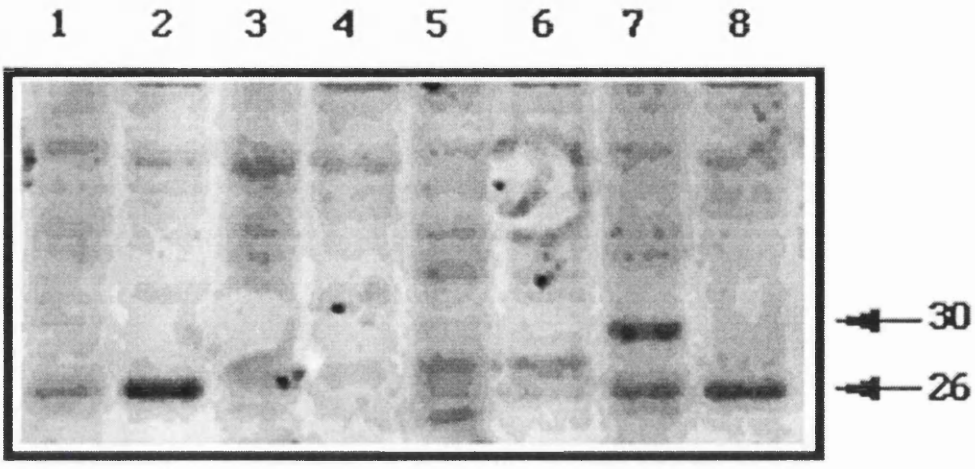

1
2
3
4
5
6
7
8
30
26

Figure 3.11: Cell-Free Expression of RD1-CAT Chimeras.

Plasmids encoding various RD1-CAT chimeras were used to prime a coupled transcription/translation system to produce mature chimeric CAT species in an *in vitro* system. This was done in the presence of ^{35}S-methionine to produce radioactive products as described (Methods, section 2.12). Following transcription/translation duplicate samples were incubated in the absence and presence of COS membranes. After incubation a high speed supernatant (soluble) fraction and a membrane fraction were isolated. These two fractions, and that of the original product of the transcription/translation reaction, were subjected to SDS-PAGE. Identification of the radioactive species was done using a phosphorimager. All fractions were normalized for volume and concentration. Lanes 1-3 represent native CAT provided by the plasmid pGS8; lanes 4-6 represent 1-100RD1-CAT provided by the plasmid pGS7; lanes 7-9 represent 26-100RD1-CAT provided by the plasmid pGS11; lanes 10-12 represent 1-25RD1-CAT provided by the plasmid pGS13. The lanes are in sets of three, where the first track shows the original products of the transcription/translation reaction, the second the membrane (high speed pellet) fraction and the third the soluble (high speed supernatant) fraction. Thus lanes 1, 4, 7 &10 show the original material emanating from the transcription/translation experiment; lanes 2, 5, 8 & 11 the high speed pellets after incubation in the presence of COS membranes and lanes 3, 6, 9 & 12 the high speed supernatants obtained after centrifugation of the incubation mixture done in the presence of COS membranes. The size of the chimeric proteins are indicated in kilodaltons These data are typical of an experiment done three times.

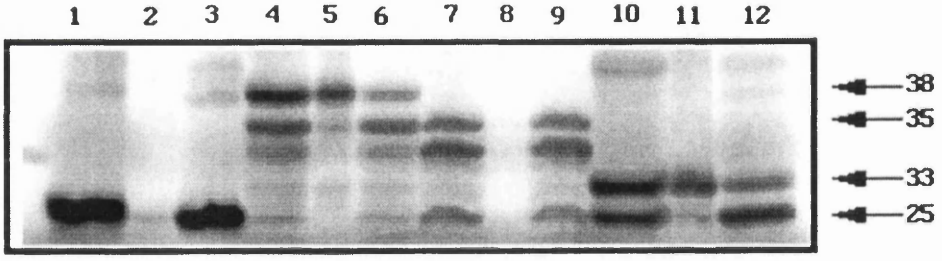
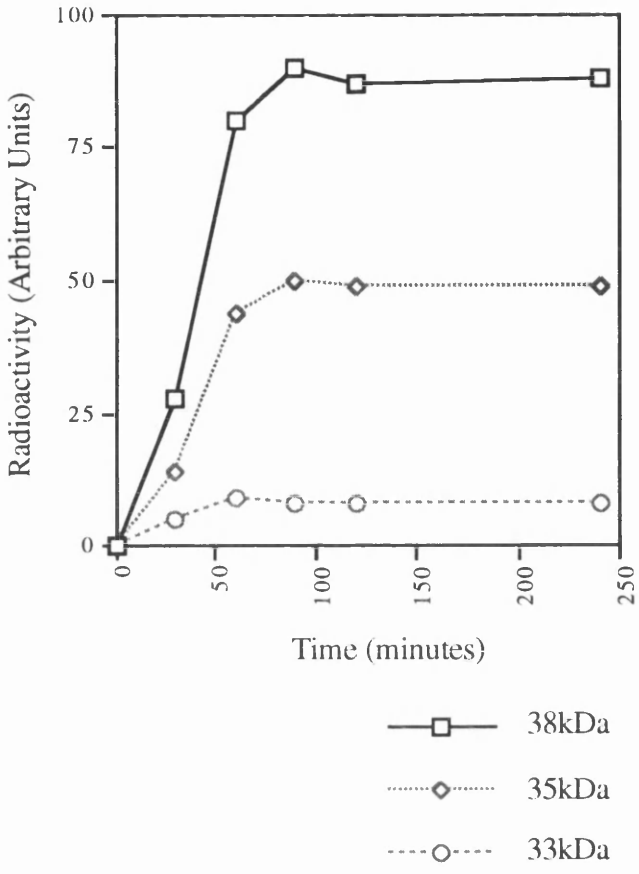

1
2
3
4
5
6
7
8
9
10
11
12
38
35
33
25

Figure 3.12: Time Course of Cell-Free Expression of RD1-CAT Chimeras.

Transcription/translation reactions were performed for the various indicated times using the plasmid pGS7 (1-100RD1-CAT) as a template. After the designated time, aliquots of the reaction mixture were immediately boiled in Laemmli sample buffer. These were then subjected to SDS-PAGE and the radioactive species identified using a phosphorimager. Gel lanes were loaded such that equal amounts of the transcription/translation reaction mixture were analysed. Shown are levels of radioactive associated with the three predominant bands which were observed (see fig. 3.11; lane 4). These species were at ~38kDa (B), presumed to be 1-100RD1-CAT, ~35kDa (E), presumed to be 26-100RD1-CAT and ~33kDa, presumed to be 37-100RD1-CAT (H). These data are typical of an experiment done twice.


Radioactivity (Arbitrary Units)
100
75
50
25
0
0
50
100
150
200
250
Time (minutes)
38kDa
35kDa
33kDa

CHAPTER 4

Determination of the Role of N-Terminal Cysteine Residues in the Membrane Association of RD1

4.1 Introduction

Previous studies have shown that upon transient transfection of the cDNA encoding the type 4A cyclic AMP specific phosphodiesterase RD1 (RNPDE4A1) in COS cells the major fraction of activity is found associated with the plasma membrane (Shakur et al. 1993). Furthermore, imunoblotting studies have detected that *in vivo*, RD1 is the only PDE4A species expressed in rat brain cerebellum (McPhee et al. 1995; Shakur et al. 1995) where it was found associated with synaptosomal membranes. In contrast met^{26}RD1 created by engineered deletion of the extreme N-terminal 25 amino acid splice region of RD1 and which forms a 'core' enzyme comon to all type 4A PDEs was detected exclusively in the cytosolic fraction of transfected COS cells. Furthermore, met^{26}RD1 was also observed to display increased activity and decreased thermostablity when compared to full-length RD1. This has led to the suggestion that this unique N-terminal 25 amino acid region may be implicated in the subcellular localization of RD1 and play some role in regulation of the enzyme possibly through interaction with another protein or lipid membrane component (Shakur et al. 1993).

I have demonstrated that an in-frame fusion of the unique N-terminal 25 amino acids of RD1 to the reporter gene CAT will result in targeting of this normally soluble enzyme to plasma membranes when expressed in COS cells (Scotland and Houslay 1995). However, the mechanism by which this interaction occurs remains to be determined. One method of targeting of proteins to the plasma membrane involves acylation which involves the covalent attachment of one or more fatty acid molecules at sites located within either the N- or C- terminus of the protein. The addition of lipids to proteins has been well documented (Chapman et al. 1992; Gaudin et al. 1991; Grand 1989; O'Brien and Zatz 1984; Okubo et al. 1991; Perez et al. 1991; Schlesinger et al. 1980; Soly and Meighen 1991; Spiegel et al. 1991b) and normally occurs

through one of two mutually exclusive pathways involving palmitic acid or myristic acid. Myristoylation which has been observed for numerous proteins including G protein α subunits (Linder et al. 1991; Spiegel et al. 1991a; Wildman et al. 1993) and ADP-ribosylation factor (Haun et al. 1993; Kahn et al. 1992) where it is linked through an amide bond to a highly conserved amino terminal glycine residue (Han and Martinage 1992; Linder et al. 1991; Spiegel et al. 1991a). Analysis of the amino acid sequence of RD1 revealed the presence of a proline at position 2 rather than the required glycine so it was therefore deemed unlikely that myristoylation was the mechanism by which the subcellular localization of RD1 was determined. Palmitoylation is a modification which has also been observed for many proteins (Beranger et al. 1991; Glomset et al. 1990; Hancock et al. 1989; Kloc et al. 1991; Leevers et al. 1994; O'Dowd et al. 1989; Okubo et al. 1991). Such modified proteins containing covalently bound palmitate are strongly anchored in the plasma membrane (Grand 1989). In most examples the palmitate moiety is attached via a thioester bond to a cysteine which is located within the consensus sequence CAAX found only at the carboxy terminus (Beranger et al. 1991; Cox and Der 1992; Glomset et al. 1990; Grand 1989; Hancock et al. 1989; Kloc et al. 1991; Leevers et al. 1994). However, in recent years proteins have been identified which are palmitoylated on cysteine located close to their amino terminus (Parenti et al. 1993; Sudo et al. 1992; Widmer and Caroni 1993). Indeed it has been reported that the neuronal protein GAP-43, which is palmitoylated on two N-terminal cysteines, becomes only partially membrane-associated upon prevention of acylation of either cysteine (Zuber et al. 1989).

Analysis of the amino acid sequence of RD1 revealed the presence of two cysteines at positions 8 and 11 within the N-terminal 25 amino acids (Figure 4.1). I have shown previously that mature chimeras containing the unique splice region of RD1 fused to the N-terminus of CAT generated in a cell-free expression system (ch. 3, section 3.2.5 and (Scotland and Houslay

1995)Scotland and Houslay 1995) can associate with added membranes. Thus it seems unlikely that post-translational modification in the form of palmitoylation is the mechanism through which the chimera and, therefore by inference RD1, becomes membrane associated. Furthermore, it has been suggested that palmitoyl transferase itself is membrane associated (Degtyarev et al. 1994) and as such would be absent from the cell-free system. As well as serving as sites for the attachment of palmitc acid, cysteines are also important for the formation of di-sulphide bridges. It has been demonstrated that cathepsin E, an intracellular aspartic protease, is retained in the endoplasmic reticulum through a mechanism involving covalent dimer formation via a disulphide bridge (Finley and Kornfeld 1994). More recently Shimada etal (Shimada et al. 1996) demonstrated that rat endothelin-converting-enzyme 1 (ECE1) exists as a plasma membrane associated disulphide linked dimer when expressed in COS cells. Mutation analysis revealed that dimer formation was disrupted by mutation of a specific cysteine residue and, although monomers showed no difference in subcellular localization, they demonstrated markedly different kinetic characteristics from the dimer form. Moreover, a disulphide bridge between the two SH2 domains of the signalling protein SH-PTP2 is thought to be critical for enzymatic activation (Eck et al. 1996). Such observations clearly demonstrate the importance of cysteine in a structural and functional context, therefore site-specific PCR mutagenesis was employed to synthesise DNA fragments in which cysteines 8 and 11 of RD1 were replaced with serines to determine whether these residues were critical in the conferrment of membrane association by the unique splice region of the PDE4a RD1 upon the soluble bacterial enzyme chloramphenicol acetyltransferase (CAT).

4.2 Site-Specific Mutagenesis of Cysteine Residues in the N-Terminus of RD1

4.2.1 Overlap Extension Mutagenesis

The method of mutagenesis described by Ho *etal* (Ho et al. 1989) and outlined in Figure 4.2, was used to substitute serines for cysteines at positions 8 and 11 in the RD1 specific sequence of the expression vector pGS7 (Scotland and Houslay 1995). The method made use of a pair of complementary primers (Figure 4.2, B & C) which contained the mutation of interest. Primer C, when paired with a sense PCR primer A, was used to generate a PCR fragment with complementary ends to a second fragment generated by pairing primer B (Figure 4.2) with an anti-sense primer D. By combining the reaction products of the two reactions in a further round of PCR, this time using only primers A & D, a hybrid fragment was generated by virtue of the complementary nature of the 3' ends of fragments synthesised in the first two reactions. The result is the generation of an almost 100% mutagenic PCR product which was cloned into pGS7 by the inclusion of *Xba1* and *Xho1* sites in the A and D primers respectively.

4.2.2 Generation of pGS9

The synthetic oligonucleotide primers

GSOL 10, 5'-CCAGTCACGACGTTGTAA-3' and

GSOL 6, 5'-GGTCTCGCTGAAGAAGT-3',

were used in a PCR reaction to generate a 96bp DNA fragment using pGS4 (Scotland and Houslay 1995) as the template (Figure 4.3, lane 1).

Simultaneously in a second reaction, the primers

GSOL 1, 5'-ACTTCTTCAGCGAGACC-3' and

GSOL 15, 5'-GGCTCCTCGAGCTTCCAGTGTGT-3'were used to generate a 293bp fragment, again with pGS4 as the template DNA (Figure 4.3, lane 2).

Post PCR, the reaction products were separated from the pGS4 template DNA by electrophoresis through a 1.75% LGT agarose gel as described in Materials and Methods. Both bands were excised from the gel and a fragment of each band was combined in a third PCR reaction this time in the presence only of primers GSOL 10 and GSOL 15. A hybrid 389bp DNA fragment was successfully generated by virtue of the complementary nature of the 3'-ends of each primary PCR product (Figure 4.4). After purification from the PCR reaction mixture this fragment was digested overnight with the restriction enzymes *Xba1* and *Xho1* as described in Materials and Methods. This resulted in the generation of two DNA fragments of 307bp and 72bp (Fig.4.5, lane 2). Following purification by gel electrophoresis to remove the minor digestion products the 307bp DNA fragment was substituted for the wild-type RD1 fragment in the plasmid pGS4 (Scotland and Houslay 1995) resulting in the generation of an intermediate plasmid named pGS5 (not shown). That pGS5 contained the Cys8Ser mutation was confirmed by Taq cycle sequencing (Figure 4.6) as described previously. pGS5 was digested with the restriction enzymes *Xba1* and *Xho1* to release the mutagenic 307bp RD1 fragment which was purified by gel electrophoresis and finally subcloned into pGS7 to replace the 'wild-type RD1 fragment. This vector, capable of transient expression in COS cells was designated pGS9.

4.2.3 Generation of pGS12

The same strategy was used in generating a plasmid containing the double Cys8,11Ser mutation by using pGS5 as template DNA and primers

GSOL 10, 5'-CCAGTCACGACGTTGTAA-3' and

GSOL 16, 5'-CTTGGAGCTGGTCTCGC-3' to generate the 96bp fragment, and

GSOL 2, 5'-GCGAGACCAGCTCCAAG-3' paired with

GSOL 15, 5'-GGCTCCTCGAGCTTCCAGTGTGT-3'

in the reaction to synthesise the 293bp fragment. Following digestion and purification of the mutagenic fragment as above, the presence of the Cys11 mutation was confirmed by sequencing (Figure 4.7) and the plasmid designated pGS12.

4.2.4 Generation of pGS14

Plasmid pGS14 containing the Cys11Ser mutation was constructed using the same oligonucleotides;

GSOL 10, 5'-CCAGTCACGACGTTGTAA-3' and

GSOL 16, 5'-CTTGGAGCTGGTCTCGC-3' to generate the 96bp fragment, and

GSOL 2, 5'-GCGAGACCAGCTCCAAG-3' paired with

GSOL 15, 5'-GGCTCCTCGAGCTTCCAGTGTGT-3'

but in this instance pGS4 (Scotland and Houslay 1995) served as the PCR template. As before, the integrity of the mutation was confirmed by DNA sequencing (data not shown).

Plasmids constructed, primers used and mutations generated are summarised in Table 4.1.

4.2.5 COS cell Expression of Plasmids Containing Cysteine Mutations

Plasmids pGS9, pGS12 and pGS14 containing the point mutations Cys8Ser, Cys8,11Ser and Cys11Ser respectively were transfected in COS cells as described in Materials and Methods. Post transfection, cells were harvested and high-speed pellet (P2) and supernatant (S2) fractions prepared as before. Each fraction was assayed for CAT activity and the distribution compared to CAT activity for cells transfected with the vector pGS7 expressing the 'wild-type' RD1 fragment. The distribution of CAT activity between the high-speed P2 pellet and S2 supernatant fractions was expressed as a percentage of total CAT activity.

The results of these experiments are summarised in Table 4.2 When COS cells were transfected with pGS7, CAT activity was detected predominantly (64%± 3) in the high-speed P2 pellet fraction reflecting previous results obtained for cells expressing this construct (Ch. 3 section 3.2.1) Upon transfection of COS cells with the plasmid pGS9 containing the Cys8Ser mutation, the distribution of CAT activity was observed to be almost identical to that observed for the wild-type chimera with the majority of CAT activity (63% ± 4) found in the P2 pellet, suggesting that this cysteine residue was not critical in determining membrane association of the RD1-CAT chimeric protein.

When COS cells were transfected with the plasmid pGS12 which contained the double cysteine to serine mutations at positions 8 and 11, a marked difference in the distribution of CAT activity was observed (Table 4). In cells expressing this construct, 88% ± 3 of the total enzyme activity was observed in the P2 pellet fraction with a concomitant decrease in the high-speed S2 supernatant. That this increase in membrane associated CAT activity was due to the double mutation substituting cysteines at positions 8 and 11 (Figure 4.1), was demonstrated when COS cells were transfected with the plasmid pGS14. This plasmid encoded a mature chimeric protein species in which only the cysteine at position 11 was mutated to serine and it was noted that CAT activity data for this construct displayed the same distribution of enzyme activity previously observed for both pGS7 and pGS9 with 63% ± 3 associated with the P2 pellet fraction. It was therefore concluded that substitution of individual cysteine residues at positions 8 and 11 of RD1 by serine residues, did not hinder the ability of the RD1-CAT chimera to associate with membranes upon transient expression in COS cells, and suggested that neither residue was post-translationally modified by the addition of palmitic acid. Subsequent replacement of both cysteines by serines however, caused a dramatic increase in the percentage of CAT activity bound to membranes in the P2 pellet fraction.

4.2.6 Cell-free Expression of Chimeric RD1-CAT Protein Species Containing Cysteine Mutations

Previously it has been demonstrated that plasmids encoding RD1 CAT chimeras can generate mature protein species in a cell-free enviroment which could associate with membranes which were added posttranslationally (Scotland and Houslay 1995). The method used in cell-free generation of proteins and subsequent membrane association assay as described in a previous chapter (Ch.3, section 3.2.5) was utilized to determine whether RD1-CAT chimeras carrying cysteine mutations within their N-terminal regions, could associate with membranes. Plasmids pGS9, pGS12 and pGS14 containing Cys8Ser, Cys8/11Ser and Cys11Ser respectively, were used to programme protein synthesis in a coupled transcription/translation system in the presence of [^{35}S] methionine. Postranslation, duplicate samples of the reaction mix were incubated in the absence (-membranes) and presence (+membranes) of COS cell P2 membranes. Following separation of membrane pellet and supernatant fractions by centrifugation, samples, including those to which no membranes hade been added, were loaded onto denaturing SDS acrylamide gels and the proteins separated by electrophoresis. The result of one a typical experiment is shown in Figure 4.8.

It has previously been shown that when pGS7 encoding the 'wild-type' RD1-CAT chimera was expressed in the cell-free system, three predominant protein species were observed of ~ 38kDa, ~35kDa and ~ 33kDa (Ch.3, fig. 3.11, lanes 4-6) which corresponded to full-length 1-100RD1-CAT, 26-100RD1-CAT and 37-100RD1-CAT respectively and as was observed previously, only the 38kDa 1-100RD1-CAT protein species was able to associate whilst the membrane pellet fraction (Ch.3, fig. 3.11, lane 5) while the truncated 35kDa and 33kDa species were exclusively cytosolic (Ch.3, fig. 3.11, lane 6). When the reactions were programmed by pGS9 encoding Cys8Ser (Figure 4.8; lanes 1-3), pGS12 (Figure 4.8; lanes 4-6) and pGS14

encoding cys11ser (Figure 4.8; lane7-9) the 38kDa full-length chimera was able to associate with added membranes in the same manner as 38kDa protein corresponding to 'wild-type' 1-100RD1-CAT (Ch.3, fig. 3.11, lane 5). The smaller 35kDa and 33kDa protein species generated by pGS9 (Figure 4.8; lane 3) and pGS14 (Figure 4.8; lane 9) remained wholely cytosolic as observed for those same chimeric species encoded by pGS7 (Ch.3, fig. 3.11, lane 6). When the plasmid pGS12 containing the double mutation of cysteines to serines at positions 8 and 11 was expressed in the coupled transcription/translation system, a band corresponding to ~38kDa was observed (Figure 4.8, lane 4). This, upon the post-translational addition of COS cell membranes, was found to associate with the membrane pellet fraction (Figure 4.8, lane 5) in the same manner as the 38kDa species generated by pGS9 and pGS14 (Figure 4.8, lanes 2 & 8 respectively). However, in marked contrast to the plasmids expressing the cys8ser and cys11ser mutations, it was noted that there was an almost complete absence of both the 35kDa and 33kDa chimeric protein species (Figure 4.8, lanes 4& 5). Moreover, the observation that the 38kDa band for each plasmid used in this experiment demonstrated a similar level of intensity, suggests that the the lack of the 35kDA and 33kDa protein species in the lanes corresponding to reactions programmed with pGS12 (Figure 4.8, lanes 4-6) was not the result of a non-specific reduction in the level of protein synthesis but was, in some way, related to the substitution of serines for cysteines at positions 8 and 11.

4.3 Conclusions

The PDE4A phosphodiesterase RD1 (RNPDE4A1) is found associated with the plasma membrane upon expression in transfected COS cells and also, for the native enzyme, in brain (McPhee et al. 1995; Shakur et al. 1995). This occurs by virtue of information contained within the first 25 amino acids at the extreme N-terminus (McPhee et al. 1995; Scotland and Houslay 1995; Shakur et al. 1993). Analysis of the amino acid sequence

revealed the presence of two cysteine residues at positions 8 and 11. In order to ascertain whether post-translational or structural modification such as the formation of a disulphide bridge was necessary for membrane association of the enzyme, a series of plasmids based on the eukaryotic expression vector pGS7 (Scotland and Houslay 1995) were generated in which both cysteines were substituted either sequentially or simultaneously with serines. Transfection of these plasmids in COS cells demonstrated that cysteine at either position 8 or position 11 made no difference to the ability of the 38kDa chimeric RD1-CAT protein species to associate with the plasma membrane. Conversely however, mutation of both cysteines resulted in an apparent increase in the amount of the 38kDa chimera protein associated with the plasma membrane fraction of transfected COS cells.

Subsequent expression of the same plasmids in a cell-free coupled transcription/translation system resulted in the generation of a mixture of full-length and truncated protein species due to multiple initiation events from internal methionine codons contained within sub optimal Kozak sequences (Craig et al. 1992; Kozak 1986; Promega 1993) and which has been reported previously for this system (Scotland and Houslay 1995; Smith et al. 1996). The post-translational addition of membranes to proteins synthesised in the cell-free system revealed that those protein species containing single cysteine/serine substitutions were able to associate with membranes in a manner identical to the 'wild-type ' protein. The protein species containing mutations of cysteines at both positions 8 and 11 also demonstrated the same level of binding to membranes as the 'wild-type' chimera and those with single cysteine/serine substitutions. However, in marked contrast, it was evident upon expression of this plasmid that the level of synthesis of truncated protein species was greatly reduced. This observation suggests that the increased percentage of membrane CAT activity seen upon transfection and expression of pGS12 in COS cells was not due to an increased membrane association but likely to result from a

decrease in expression of soluble truncated proteins located in the cytosolic fraction of the cells. Unfortunately it was not possible to obtain independent confirmation of this observation by immunoblotting as the anti-CAT antisera failed to identify the 1-100RD1-CAT chimera (ch. 3, section 3.2.4). Such a reduction in the expression of N-terminally truncated species is unlikely to reflect an artifact since this observation was made using two different expression systems, COS cells in which expression is driven by an SV40 promoter and rabbit reticulocyte lysate where expression is directed by the SP6 promoter. It is possible that a common explanation may account for the changes seen in both systems. Since transcription is initiated in each expression system from different promoters it is suggested that the reduction in the level of soluble truncated RD1-CAT chimeras may reflect a change at the level of translation. One factor which can affect translation efficiency is the presence of secondary structure in the mRNA (Kozak 1994). Previous experiments with the RD1-CAT chimera expressing plasmids have shown the presence of multiple protein species thought to arise as a result of translation from internal methionine residues contained within sub-optimal Kozak sequences (Scotland and Houslay 1995) by means of leaky ribosome scanning (Kozak 1986; Kozak 1990; Kozak 1994). It has been demonstrated that the presence of secondary structure in RNA can alter translation (Brierley et al. 1989; Jacks et al. 1988; Zinoni et al. 1990) and Kozak (Kozak 1990) has previously shown that recognition of an AUG codon in a suboptimal context improves with the introduction of a hairpin loop near the begining of the protein-coding sequence. Analysis of the DNA sequence encoding the N-terminal 25 amino acids of RD1 does not reveal any significant propensity for secondary structure formation. However, mutation of the cysteines at positions 8 and 11 was achieved by substituting A for T (Figure 4.8, boxed residues) which forms the first base of the triplet codon corresponding to cysteine and it is therefore tentatively suggested that these substitutions are more favourable to the formation of a

hairpin loop (Figure 4.8) beginning approximately 14 nucleotides downstream from the initiating methionine. This is in exellent agreement with the observations of Kozak (Kozak 1990). The presence of any such structure might be expected to cause the 40S ribosomal subunit to slow down resulting in more efficient translation from the upstream ATG codon.

In summary, a series of plasmids were generated containing mutations of cysteines located in the extreme N-terminal RD1 specific fragment in an attempt to determine whether these residues were post-translationally or structurally modified and thus confer plasma membrane association upon full-length RD1 and N-terminal RD1-CAT fusions. Expression of plasmids containing single cysteine mutations in both COS cells and a cell-free system displayed the same pattern of membrane association as the wild-type construct. Expression of the plasmid containing the double cysteine mutation revealed an apparent increase in membrane association in COS cells. This was shown however, in the cell-free system to be caused by a decrease in the amount of truncated soluble protein chimeras produced rather than a genuine increase in membrane association. An explanation for this observation is suggested which involves the formation of a secondary structural hairpin loop upstream of the internal methionine at position 26, due to the nucleotide substitutions involved in the cys8/11ser double mutation which may result in an increased fidelity of translation from the initiating methionine.

Figure 4.1: Amino Acid Sequence 1-25 of RD1

This figure shows the primary amino acid sequence (single letter code) for the unique N-terminal splice domain of RD1. Cysteines 8 and 11 are shown underlined.

NH_2-MPLVDFFCETCSKPWLVGWWDQFKR-COOH

Figure 4.2: Schematic Diagram of Overlap-Extension Mutagenesis

Complementary oligonucleotide primers (B & C) designed to contain the mutation of interest are combined with a sense (A) and anti-sense (D) primer to generate short DNA fragments in two separate PCR reactions. DNA fragments generated during the first round of PCR are combined in a second PCR reaction with the sense and anti-sense primers A and D but without the mutagenic primers. A full-length fragment containing the mutation of interest is then generated by virtue of the complementary nature of the two primary reaction products. Mutagenic fragments can be subcloned by designing restriction enzyme recognition sites into primers A and D.

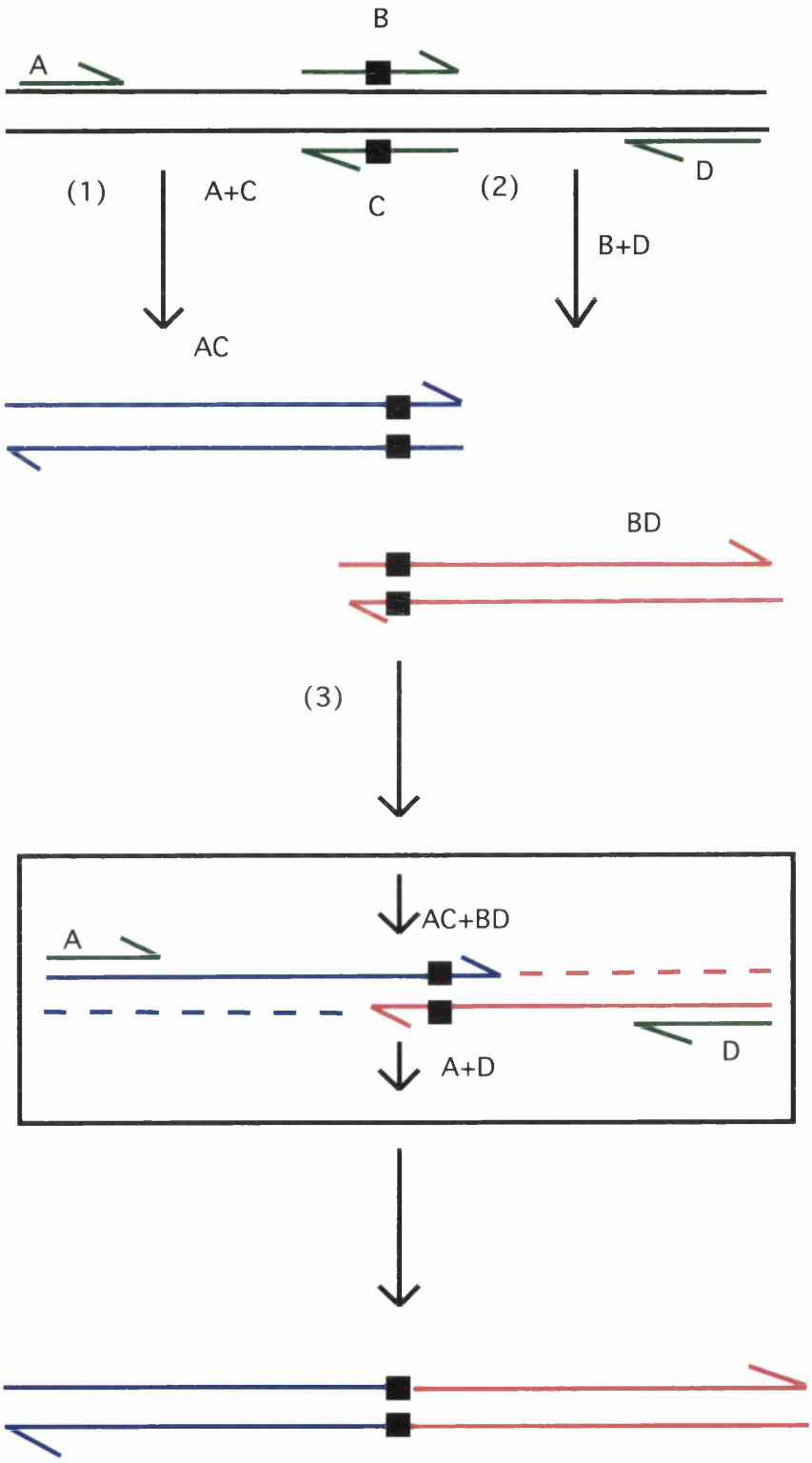

B
A
D
(1)
A+C
C
(2)
B+D
AC
BD
(3)
AC+BD
A
D
A+D

Figure 4.3: Generation of Primary Mutagenic PCR Products

The results of two PCR reactions visualized by ethidium bromide staining of an agarose gel are shown. Lane 1 shows the presence of a 96bp band which is the size predicted for a band generated using primers GSOL10 and GSOL6 when the plasmid pSG4 was used as a template (ch. 3, section 3.1.1). Lane 2 contains a band of 293bp corresponding to the size predicted for a band synthesised when primer GSOL1 was combined with GSOL15. *HaeIII* digested bacteriophage Φ X174 DNA markers are indicated to the left of the gel. All sizes given are nucleotide base pairs.

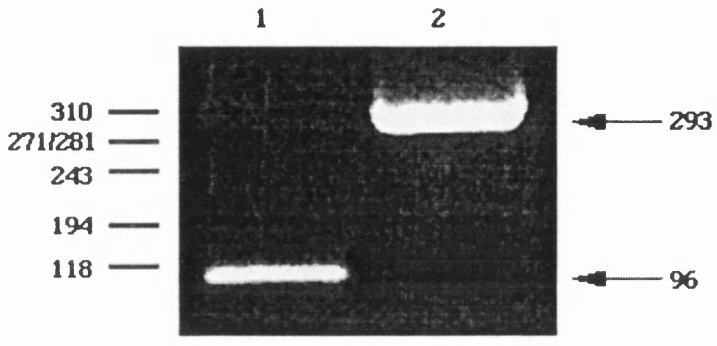

1
2
310
271/281
243
194
118
293
96

Figure 4.4: Generation of a Secondary Mutagenic PCR Product

The primary mutagenic PCR products shown in Fig.4.3 were combine in a second PCR reaction but in the absence of the mutagenic primers GSL1 and GSOL6. By virtue of the complementary nature of the 3' ends of both fragments a 389bp full-length DNA fragment was generated using primers GSOL10 and GSOL15. *HaeIII* digested bacteriophage Φ X174 DNA was used as molecular weight markers as indicated on the left side of the gel.

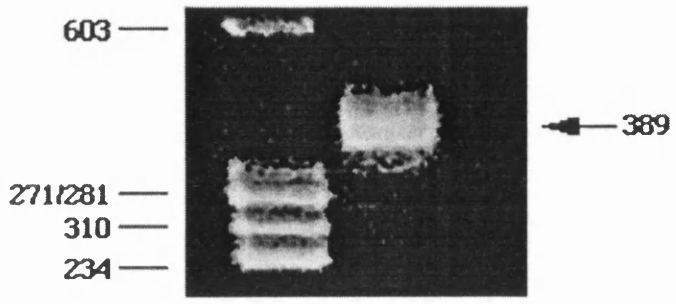

603
271/281
310
234
389

Figure 4.5: Digestion of the Mutagenic 389bp PCR Fragment prior to Subcloning

The 389bp PCR product shown in Fig. 4.4 was digested by incubation at 37°C in the presence of the restriction enzymes *Xba1* and *Xho1*. This figure shows the successful generation of two bands of 307bp and 72bp as expected upon digestion of the 389bp fragment by these two enzymes.

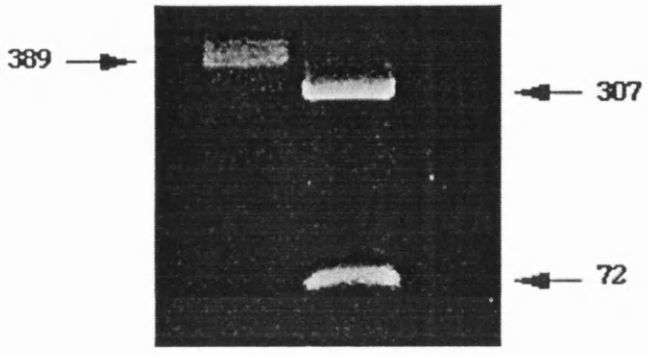

389
307
72

Figure 4.6: Confirmation of the Generation of the Cys8Ser Mutation

A mutagenic DNA fragment was generated in which the cysteine at position 8 of the RD1 specific sequence was mutated to serine. Shown here is a printout from an Applied Biosystems Model 373 automated DNA sequencer. The nucleotide substitution encoding the mutation is underlined.

Applied Biosystems

Model 373A
Version 1.1f

Sample 05
DyeTerminator{AnyPrimer}
Lane 5
Signal: G:92 A:44 T:27 C:18

Points 844 to 7414 Base 1: 844
9206653 MATRIX FILE
GS3
M19 DNA M1/1

Tue, Mar 16, 1993 18:05
X: 0 to 7063 Y: 0 to 1200

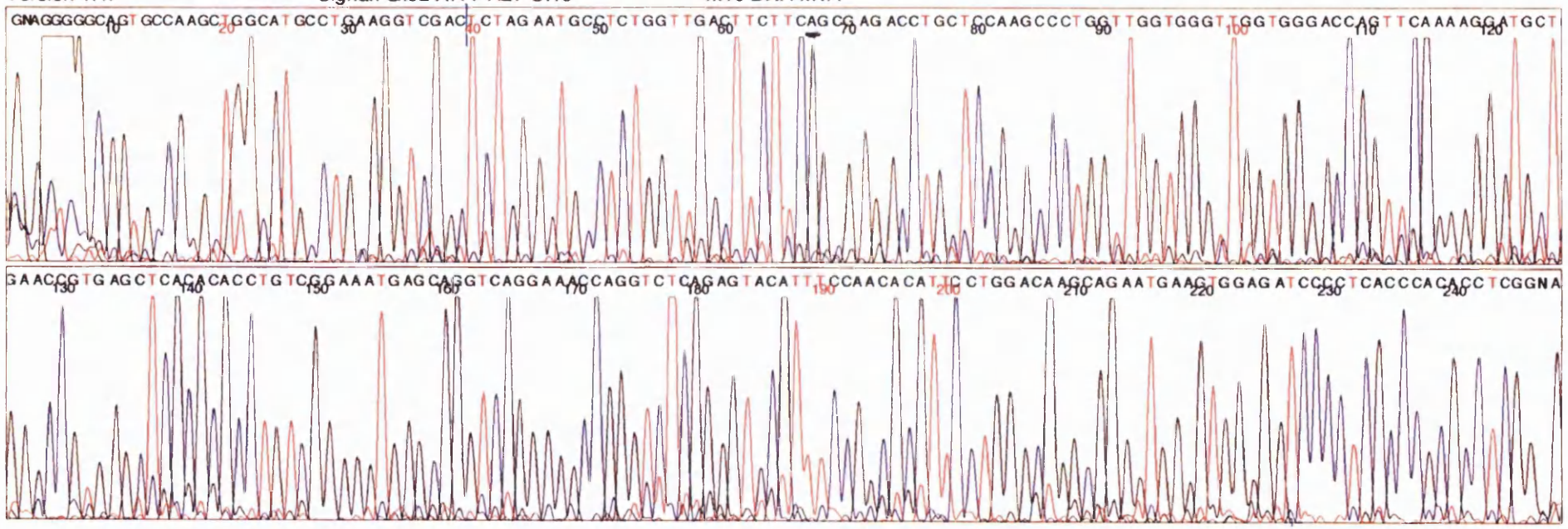


Figure 4.7: Confirmation of the Generation of the Cys8/11Ser Mutation

Using plasmid pGS9 as template in a PCR reaction cysteine 11 was mutated to serine Shown here is a printout from an Applied Biosystems Model 373 automated DNA sequencer. The nucleotide substitutions encoding both mutations are indicated by arrows.

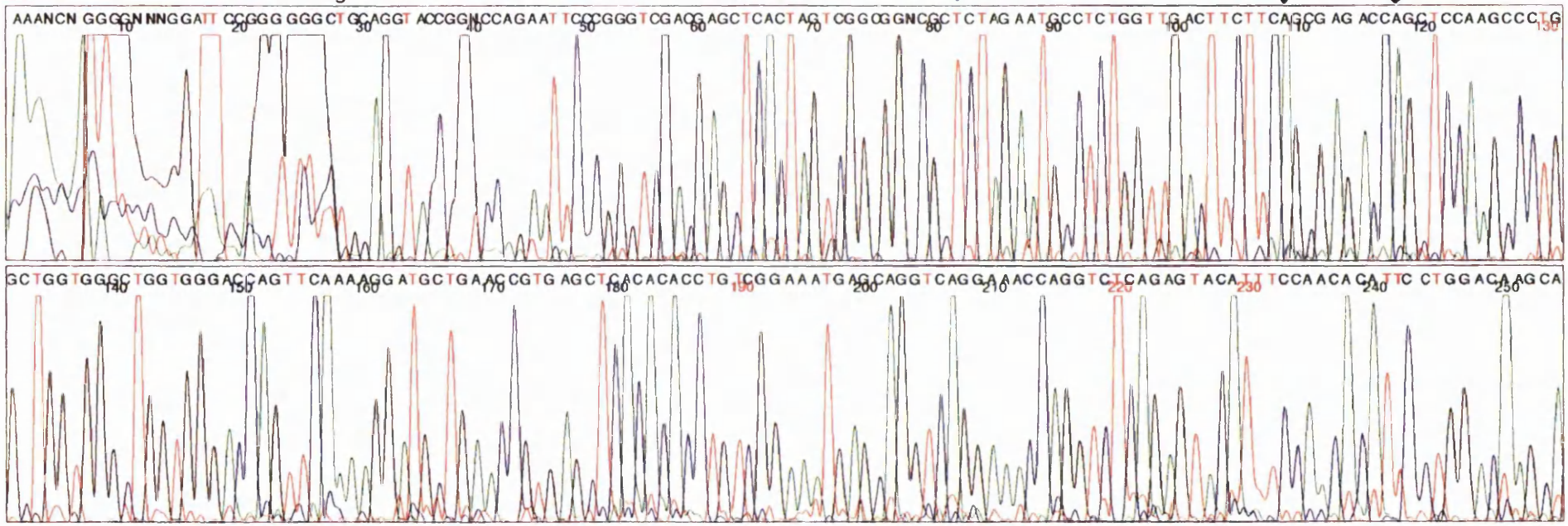

AAANCN GGGGN NNGGATT CCGGGG GGGCTGCAGGT ACCGGNCCAGAAT TCCCGGGTCGACGAGCTCACTAGTCGGCGGNCGCTCTAGAATGCCTCTGGTTGACTTCTTCAGCG AG ACCAGCTCCAAGCCCTG
GCTGGTGGGCTGGTGGGACCAGTTCAAAAGGATGCTGAACCGTGAGCTCACACACCTGTCGGAAATGAGCAGGTCAGGAAACCAGGTCTCAGAGTACATTTCCAACACATTC CTGGACAAGCA

Table 4.1 Summary of Plasmid Constructs Containing N-Terminal Cysteine Mutations

Site-specific mutagenesis was carried out essentially according to the method of overlap extension as described by Ho *et al* (Ho et al. 1989). Mutagenic fragments were subcloned into pGS7 following removal of the wild-type RD1 fragment. Successful generation of each mutation was confirmed by sequencing.

plasmid	Primers	Construct
pGS9	GSOL 1, 5'-ACTTCTTC**A**GCGAGACC-3'	Cys8Ser
	GSOL 6, 5'-GGTCTCGC**T**GAAGAAGT-3'	
pGS12	GSOL 16, 5'-CTTGGAGC**T**GGTCTCGC-3'	Cys8,11Ser
	GSOL 2, 5'-GCGAGACC**A**GCTCCAAG-3'	
pGS14	GSOL 16, 5'-CTTGGAGC**T**GGTCTCGC-3'	Cys11Ser
	GSOL 2, 5'-GCGAGACC**A**GCTCCAAG-3'	

Table 4.2 Distribution of CAT activity in COS Cells Transfected With Chimeras Containing N-Terminal Cysteine Mutations

COS cells were transfected with the indicated plasmid and then, after 48h, membrane and high speed supernatant fractions isolated as described in Methods. All CAT assays were performed in duplicate as described in the Methods section. The distribution between the membrane (pellet) and cytosol (high speed supernatant) fractions are given as percentages. These data provides the mean of three separate transfection experiments done on different occasions with errors as SEM. No CAT activity was evident in COS cells which were mock transfected with either the plasmids pSVL or pSPORT.

plasmid	Construct	Membrane (%)	Cytosol (%)
pGS7	1-100RD CAT	64 ± 3	36 ± 3
pGS9	Cys8SerRD CAT	63 ± 4	37 ± 4
pGS12	Cys8,11SerRD CAT	88 ± 3	17 ± 3
pGS13	Cys11Ser CAT	63 ± 2	37 ± 2

Figure 4.8: Cell-Free Expression of Plasmids Encoding Cysteine Mutations

Plasmids pGS9, pGS12 and pGS14 encoding cys8ser, cys8/11ser and cys11ser respectively were expressed in the presence of radiolabelled methionine in a cell-free coupled transcription/translation system and used in a membrane association assay as described in Methods (section 2.12). Mature chimeric proteins generated from these plasmids were resolved by SDS-PAGE and analysed by phosphorimager. Lanes 1-3 represent pGS9, lanes 4-6 represent pGS12 and lanes 7-9 correspond to pGS14. All volumes and concentrations were normalized to allow direct comparison. The lanes are in sets of three where the first track shows the original product of the transcription/translation reaction, the second the high-speed membrane pellet and the third the soluble high-speed supernatant fraction. Thus lanes 1, 4 & 7 show the original material emanating from the transcription/translation experiment; lanes 2, 5 & 8 the high speed pellets after incubation in the presence of COS membranes and lanes 3, 6, & 9 the high speed supernatants obtained after centrifugation of the incubation mixture done in the presence of COS membranes. These data are typical of three separate experiments.

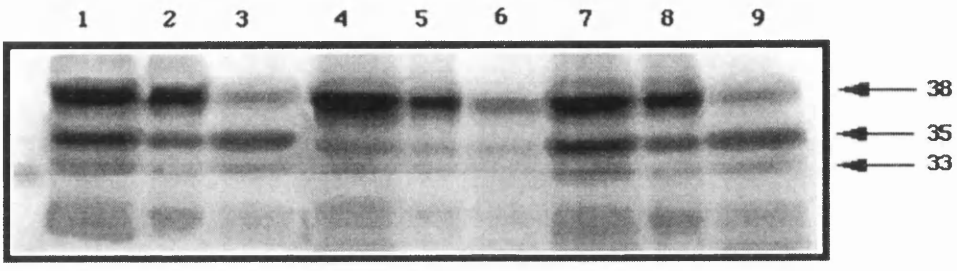
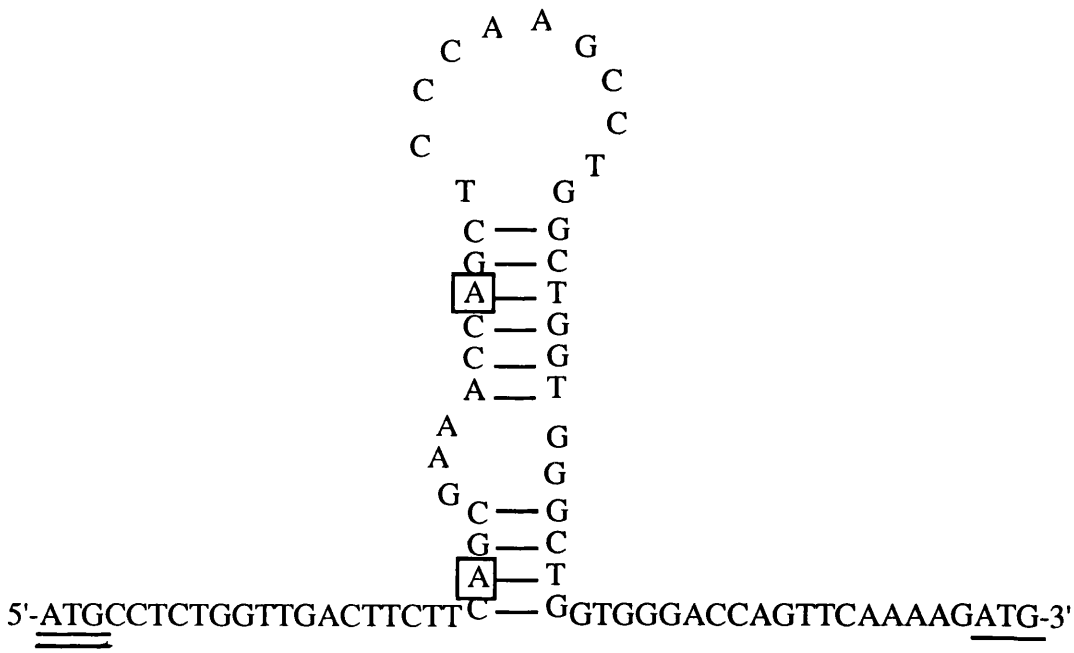

1
2
3
4
5
6
7
8
9
38
35
33

Figure 4.9: Proposed Secondary Structure of the 5' Leader Sequence of Cys8/11Ser RD1 mRNA

Shown here is the putative hairpin loop structure that may be adopted by RD1 mRNA encoding the double cys8/11ser mutation. It is proposed that the base substitutions (boxed residues) favour the formation of this structure thus causing the ribosome to pause at the first methionine (double underlined) thus preventing translation from the second methinonine (single underlined).


5'-ATGCCTCTGGTTGACTTCTTCGGTGGGACCAGTTCAAAAGATG-3'

CHAPTER 5

Identification of Specific Amino Acid Residues Responsible for the Membrane Association of the PDE4 RD1

5.1 Introduction

The cyclic AMP specific phosphodiesterase RD1 (RNPDE4A1) was found to be associated with the synaptosomal plasma membrane in rat brain (McPhee et al. 1995; Shakur et al. 1995) and also upon transient expression in transfected COS cells (Shakur et al. 1993). By means of engineered deletions of the cDNA for RD1, Shakur et al (Shakur et al. 1993) have demonstrated that the extreme N-terminal may be involved in determining the subcellular localization of the enzyme. More recently, Scotland et al (Scotland and Houslay 1995) were able to demonstrate that chimeric constructs containing the at least the first 25 amino acids of RD1 were sufficient to direct the normally soluble bacterial protein chloramphenicol acetyltransferase (CAT) to the plasma membrane upon tranfection in COS cells and also upon addition the addition of membranes to mature chimeric proteins generated in a cell-free rabbit reticulocyte lysate system. In contrast, chimeric protein species in which this first 25 amino acids were deleted exhibited an exclusively cytosolic CAT activity identical to native CAT (Scotland and Houslay 1995). Thus it was confirmed that the extreme N-terminal 25 amino acids were involved in determining membrane association upon the PDE4A RD1.

Shakur et al have previously shown that hydroxylamine was unable to cause the release of RD1 from either cerebellum or COS cell membranes and that [^{3}H] palmitate was not incorporated into the RD1 protein immunoprecipitated from COS cells transfected with RD1 cDNA (Shakur et al. 1995). Furthermore, site-specific mutagenesis of N-terminal cysteines identified as potential sites for the addition of palmitate, failed to prevent association of the chimeric 1-100RD-CAT protein with membranes when expressed in COS cells or upon their addition to full-length chimeras generated in a cell-free expression system (Ch. 4, section 4.1.5 & 4.1.6). Therefore it is suggested that the unique N-terminal splice region of the PDE4A RD1 contains discrete domains or linear amino acid

sequences which are responsible for directing the soluble 'core' met26 RD1 and the bacterial protein CAT to membranes. In order to investigate this contention it was necessary to determine whether this splice region possessed the ability to adopt a structural conformation in the absence of the 'core' met26 RD1 enzyme. By means of a computer analysis program based on the algorithm of Chou and Fasman (Chou and Fasman 1978) for the determination of super-secondary structure from primary amino acid sequence, a putative structure was obtained which suggested that the unique splice region of the PDE4A RD1 consisted of a number of independently folded structural elements namely an N-terminal α-helix followed by a mobile hinge region and α second a-helix (Fig.5.1). Furthermore, in a collaboration with Dr. J. Smith and Prof. I. Trayer (University of Birmingham) and Dr. J. Beattie (Hannah Research Institute, Ayr) a 25 amino acid peptide corresponding to the unique splice region of the PDE4A RD1 was synthesised and its structure determined by ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) spectroscopy. Analysis of this peptide provided confirmation of the structure observed by computer analysis, that the splice region was comprised of two independently folding helical regions. The first helical region was formed by an N-terminal amphipathic α-helix with an apolar section (residues 2-7), followed by a highly mobile 'hinge' region (residues 8-13), and a second helix consisting of multiple overlapping turns and containing a distinct compact, hydrophobic, tryptophan-rich domain (residues 14-20) followed by a polar region encompassing residues 21-25 (Fig. 5.2 and Smith et al (Smith et al. 1996)). Based on the fact that two independent lines of inquiry provided essentially identical well defined structures I generated a series of deletion mutations and amino acid substitutions within this unique splice region in order to determine the role of individual structural features in conferrment of membrane association upon the PDE4A RD1 and the soluble bacterial enzyme chloramphenicol acteyltransferase (CAT).

5.2 Generation and Expression of N-Terminal Deletion Mutation RD1-CAT Chimeras

5.2.1 Construction of Deletion Mutations

A modification of the method of overlapping extension mutagenesis described by Ho et al (Ho et al. 1989) and in some detail in the preceding chapter was used to successfully engineer various deletions within the 1-25 amino acid RD1 N-terminal region. The PCR primers used and the mutations generated are summerised below

Primer pairs

GSOL17, 5'-GGTCGACTCTAGAATGTGCGAGACCTGC-3' and

GSOL18, 5'-GCAGGTCTCG CACATTCTAGAGTCGACC-3'

were used to delete the hydrophobic residues P2-F7 (inclusive);

GSOL27, 5'-GACTTCTTCC CCTGGCTGG-3' and

GSOL28, 5'-CCAGCCAGGGGAAGAAGTC-3'

were used to delete the polar region of the first α-helix along with the hinge region C8-K13 (inclusive);

GSOL36, 5'-CCTGCTCCAAGGACCAGTTCAAAAGG-3' and

GSOL37, 5'-CCTTTTGAAC TGGTCCTTGGAGCAGG-3'

were used to delete the unique tryptophan-rich domain P14-W20 (inclusive);

GSOL38, 5'-GCTGGTGGAT GCTGAACC-3' and

GSOL39, 5'-GGTTCAGCATCCACCAGC-3'

were used in the generation of the polar amino acids D21-R25 (inclusive).

Following restriction enzyme digestion with *Xba1* and *Xho1* as before, PCR fragments were recovered from 1.75% low gelling temperature agarose gels and substituted for the 'wild type' RD1 N-terminal fragment of pGS7

(Scotland and Houslay 1995) to create the plasmids pGS10, pGS15, pGS17 and pGS18 containing the deletions ΔP2-F7, ΔC8-K13, ΔP14-W20, ΔD21-R25. Confirmation of each deletion was obtained by DNA sequencing as described previously (Ch. 3).

5.2.2 Generation of the Plasmid pGS22

The method of overlap extension mutagenesis described previously by Ho etal (Ho et al. 1989) was used to insert a cassette of seven alanine residues (ala_7) between amino acid residues K13 and D14 of the tryptophan-rich deletion containing plasmid pGS17 by using the following primers,

GSOL61, 5'-CCTGCTCCAAGGCAGCAGCAGCAGCAGCA GACCAGTTCAAAAGG-3'

GSOL62, 5'-CCTTTTGAACTGGTCTGCTGCTGCTGCTGC TGCTGCCTTGGAGCAGG-3'

Subsequent confirmation of the successful generation of the insertion was obtained by Taq cycle sequencing as before and the resultant plasmid designated pGS22.

5.2.3 Expression of Deletion Mutations in COS Cells

Deletion mutation containing plasmids were transfected into COS cells as described in materials and methods (ch. 2, section 2.8.1) and propagated for 48 hours before harvesting and subsequent preparation of pellet and supernatant fractions. Samples of pellet and supernatant were assayed for CAT activity essentially by the method of Seed and Sheen (Seed and Sheen 1988) and described previously (ch. 2, section 2.9). As in the previous experiments to evaluate the effect of cysteine mutations upon membrane association of RD1-CAT chimeric protein species, the plasmid pGS7 was used as a positive control. The results of the individual deletion mutations generated are summarised in table 5.1.

When pellet and soluble fractions were prepared from COS cells transfected with pGS10 expressing the P2-F7 deletion, it was observed that

almost 53% of CAT activity was associated with the membrane fraction (Table 5.1). While this represents a decrease in membrane associated CAT activity when compared to cells transfected with the wild-type pGS7 (table 5.1) it is evident that deletion of the hydrophobic region of the first amphipathic α helix failed to abolish membrane association of the major proportion of RD1-CAT chimera.

The plasmid pGS15 was generated by replacing the DNA encoding for the wild-type 1-100 amino acid RD1 fragment by a PCR fragment containing an 18bp deletion corresponding to the polar region of the first amphipathic α helix combined with the mobile hinge region (residues C8-K13) identified by computer predictions and NMR analysis (Figs. 5.1; & 5.2; (Smith et al. 1996)). When pGS15 was transfected in COS cells a significant amount of CAT activity was detected (Table 5.1). Separation of membrane and soluble fractions showed that almost 56% of this CAT activity was associated with membranes (Table 5.1). Interestingly, that the deletion encoded by pGS15, which encompasses the cysteine residues at positions 8 and 11, failed to prevent membrane association of the 1-100RD1-CAT chimeric protein confirms the contention investigated in chapter 4 (sections 4.1.5 & 4.1.6) that these residues are not directly responsible for the conferrment of membrane association upon RD1 by acting as sites for the covalent attachment of palmitate, nor through the formation of intra- or inter-molecular di-sulphide bridges. In marked contrast to the above results, when COS cells were transfected by the plasmid pGS17, in which the 1-100 amino acid RD1 fragment contained an engineered 21 bp deletion corresponding to the tryptophan-rich region from amino acids P14-W20, only 16% of the total CAT activity was found associated with the membrane fraction compared to almost 70% observed for cells transfected with pGS7 expressing the wild-type 1-100RD1-CAT chimera. This marked shift in the distribution of CAT activity from the membrane to the soluble fraction of COS cells transfected with pGS17 mirrors earlier results observed previously

for cells transfected with both pGS8 expressing native CAT, and pGS11 encoding 26-100RD1-CAT which displayed 13% and 16% membrane associated CAT activity respectivly (results ch. 3, table 3.1 and Scotland & Houslay (Scotland and Houslay 1995)). The final polar region identified by computer analysis and NMR studies of the unique splice region of the type IVA PDE RD1 encompassed amino acid residues D21-R25 and therefore deletion of this region was engineered to generate the plasmid pGS18. As observed for the other deletion containing plasmids, pGS18 expressed CAT activity when transfected into COS cells (Table 5.1). However, analysis of the distribution of CAT activity between the membrane and cytosolic fractions of pGS18 transfected COS cells showed a decrease in membrane associated CAT activity from 66% seen for full-length RD1-CAT to 32% for the deletion D21-R25 (Table 5.1). A possible explanation for this observation, while by no means conclusive proof, is offered. Smith et al (Smith et al. 1996) through NMR studies on a peptide corresponding to the unique splice region of the type IVA PDE RD1 demonstrated that the while the tryptophan-rich region was located at the NH_2-terminus of an amphipathic α-helix it adopted a somewhat loose conformation due to the presence of amino acids containing large aromatic side-chains. Perhaps by adopting this conformation a hydrophobic patch is provided through which membrane association takes place, indeed such a phenomenon has been described for the association of the 'loose-binding form' of rat liver cytochromeb_5 with liposomes (Vergeres et al. 1995). It may be the case then that deletion of the amino acids directly downstream of the tryptophan-rich region of RD1 causes it to lose this unusual conformation and thereby decrease its ability to efficiently direct membrane association. It is also interesting to note that this deletion encompassed the positively charged residues lysine and arginine (K24-R25) which have been implicated as stop transfer sequences in a number of membrane associated proteins such as H^+,K^+-ATPase (Bamberg and Sachs 1994) and deiodinase (Toyoda et al. 1995). Whether an analogous

role is served by these residues in RD1 is undetermined although it is unlikely as the N-terminus of RD1 does not appear to contain an α-helix large enough to span the lipid bilayer.

5.2.4 COS cell Expression of Alanine Cassette Containing Plasmid pGS22

Previous results have demonstrated that deletion of the hydrophobic tryptophan-rich region P14-W20 in the unique N-terminal splice region of the type IVA PDE RD1 abolished membrane association of mature chimeric RD1-CAT protein species when expressed in transfected COS cells. In order to ascertain whether membrane association was conferred by hydrophobic interaction alone or whether it was due to the presence of specific amino acid residues, amino acids P14-W20 were substituted by an equally hydrophobic 'cassette' comprised of 7 consecutive alanines which was inserted between residues K13 and D14 in the RD1 specific sequence of the plasmid pGS17 by means of overlap extension mutagenesis (Ho et al. 1989). The resultant plasmid, which was confirmed to contain the alanine cassette was designated pGS22. The use of alanine substitutions and scanning alanine mutagenesis has been well documented in the literature in, for example determining the role played by di-leucine motifs in lysosomal targeting (Haft et al. 1994; Hunziker and Fumey 1994; Leteourneur and Klausner 1992; Ogata and Fukuda 1994; Pond et al. 1995) and endoplasmic reticulum targeting (Vergeres et al. 1995).

Upon transfection and subsequent expression of pGS22 in COS cells it was noted that approximately 23% of CAT activity was associated with the membrane fraction compared to 66% for full-length 1-100RD1-CAT encoded by pGS7 (Table 5.1) thus leading to the conclusion that substitution of amino acids P14-W20 by a stretch of similarly hydophobic amino acids was not not sufficient to reconstitute membrane association lost by deletion of this region. Although membrane association was not completely abolished it was reduced to a level similar to that previously observed for the 26-100RD1-CAT protein

chimera expressed by pGS11 (results ch. 3, table 3.1 and Scotland & Houslay (Scotland and Houslay 1995)).

5.2.5 Cell-free Expression of Deletion Mutation Containing Plasmids

Plasmids pGS10, pGS15, pGS17 and pGS18 containing the mutations ΔP2-F7, ΔC8-K13, ΔP14-W20, ΔD21-R25 respectively, were used to programme sysnthesis of mature chimeric protein species in a cell-free coupled transcription/translation system and used in the membrane association assay described previously in some detail (Scotland and Houslay 1995) and Ch. 3, section 3.2.5. Briefly, duplicate samples of each reaction were incubated with either buffer (minus membranes) or buffer containing COS membranes (plus membranes) followed by separation of the membrane pellet and supernatant fractions. Following SDS-PAGE, gels were visualized by phosphorimager. When the reaction was programmed with either pGS10 (Fig. 5.3, lanes 4-6) or pGS15 (Fig. 5.3, lanes 7-9) it was noted that the major chimeric protein species of ~38kDa was found in the membrane pellet fraction in a manner identical to the reaction programmed by pGS7 expressing the wild-type plasmid (Fig. 5.3, lanes 1-3). In marked contrast to this, the mutant chimera expressing the deletion ΔP14-W20 encoded by the plasmid pGS17 was unable to become associated with membranes and remained completely cytosolic (Fig. 5.3, lanes 10-12) thus mirroring the results obtained when this construct was transiently expressed in COS cells. When pGS18 encoding the deletion D21-R25 was expressed in the cell-free system it was noticed that whilst the mutation was still able to associate with membranes it did so with a much lower affinity (Fig. 5.3, lanes 13-15) than either wild-type 1-100RD1-CAT, ΔP2-F7 or ΔC8-K13 (Fig.5.3, lanes 1-3, 4-6 and 7-9 respectively). This result was also observed to mirror the result obtained for transient expression of pGS18 in COS cells.

5.2.6 Cell-free Expression of the Alanine Cassette Containing Plasmid pGS22

In order to investigate whether plasma membrane association of RD1 was determined by specific amino acids located within its amino terminal 25 amino acids, or if this was merely a function of the extremely hydrophobic nature of this region, a plasmid was constructed in which residues 14-20 (inclusive) were replaced by an alanine cassette (ala_7) resulting in the generation of pGS22. Utilising the same strategy as for the deletion mutation containing plasmids pGS10, pGS15, pGS17 and pGS18, mature chimeric proteins were synthesized by means of cell-free coupled transcription/translation system in the presence of radiolabelled [^{35}S] methionine according to Materials and methods (Ch.2, section 2.11.1) and evaluated for their ability to associate with added membranes. The result of one such experiment is shown in figure 5.4. As in previous experiments, the plasmid pGS7 encoding the wild-type 1-100RD1-CAT was employed as a positive control for membrane association (Fig.5.4, lanes 1-3). Lanes 4-6 (Fig.5.4) represent pGS17 encoding the P14-W20 deletion and as before this construct does not associate with added membranes (Fig. 5.4, lane 5) but remains associated with the supernatant fraction (Fig. 5.4, lane 6). Substitution of the P14-W20 region with the alanine cassette did not restore the ability of the mature chimeric protein to associate with membranes (Fig. 5.4, lanes 7-9).

Conclusions

Computer based analyses and NMR spectroscopy determined that the unique N-terminal splice domain of RD1 was comprised of a series of distinct structural elements. Deletion mutagenesis was employed to generate a series of plasmids containing deletions encompassing the entire splice domain expressed as a fusion with CAT. Expression of mutagenic plasmids in both COS cells and a cell-free system revealed that membrane association of RD1-CAT chimeras was

determined by a unique tryptophan-rich motif contained within amino acids 14-20.

Figure 5.1: Computer Prediction of the Secondary Structure of the N-Terminal 1-25 Amino Acids of RD1

The supersecondary structure of the N-terminal splice domain of RD1 as predicted by DNASTAR software based upon the algorithm of Chou and Fasman (Chou and Fasman. 1978). These indicate two regions of putative α helix (depicted in red), the first extending from Leu^3 to Thr^{10} and the second extending from Gln^{22} to Arg^{25}. Sandwiched between these two putative helical domains are a putative region of turn (depicted in blue) and a putative region of β-sheet (depicted in green).

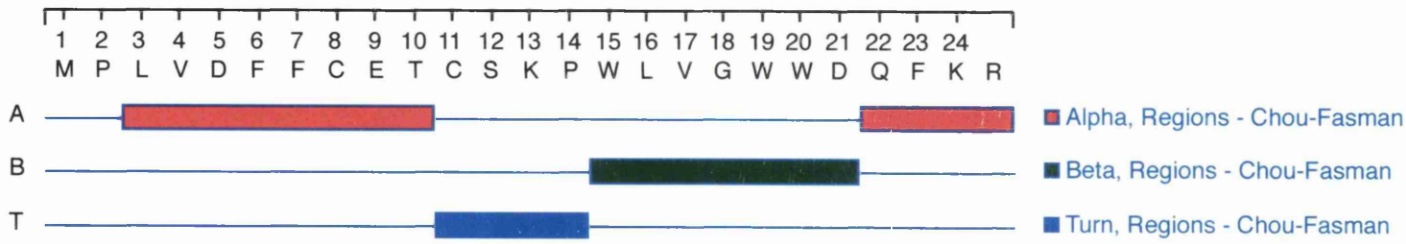

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
M P L V D F F C E T C S K P W L V G W W D Q F K R
A
B
T
Alpha, Regions - Chou-Fasman
Beta, Regions - Chou-Fasman
Turn, Regions - Chou-Fasman

Figure 5.2: Structure of the N-Terminus of RD1 as Determined by H^1-NMR

The figure shows the two orientations of the mean calculated structure for a peptide corresponding to 1-25 RD1. On the *left-hand side*, both backbone and side chains atoms are shown (no hydrogen atoms) for all residues. The helical path of the backbone is emphasised by the *white ribbon.* Certain residues are labelled. On the *right-hand side*, molecular surface maps are shown in the corresponding orientations. The surface is coloured according to charge (*red*, negative; *blue*, positive). Hydrophobic residues are shown in *white*. This figure was reproduced by kind permission of Dr. J. Smith (Univ. of Birmingham)

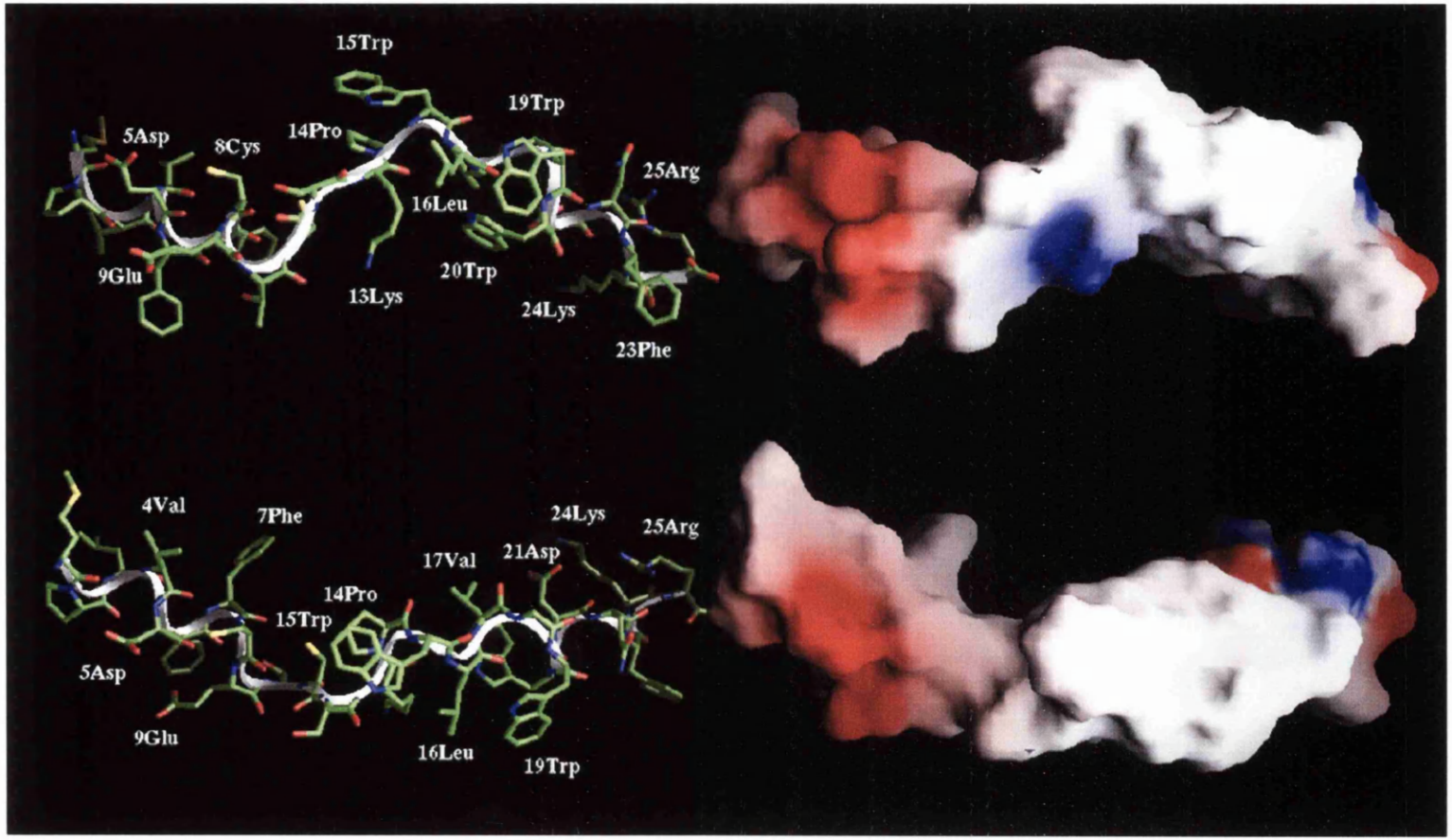

15Trp
19Trp
5Asp
8Cys
14Pro
25Arg
16Leu
9Glu
20Trp
13Lys
24Lys
23Phe
4Val
7Phe
24Lys
25Arg
17Val
21Asp
14Pro
15Trp
5Asp
9Glu
16Leu
19Trp

Table 5.1 Distribution of CAT activity in COS Cells Transfected With Chimeras Containing N-Terminal Deletion Mutations

COS cells were transfected with the indicated plasmid and then, after 48h, membrane and high speed supernatant fractions isolated as described in Methods. All CAT assays were performed in duplicate as described in the Methods section. The distribution between the membrane (pellet) and cytosol (high speed supernatant) fractions are given as percentages. These data provides the mean of three separate transfection experiments done on different occasions with errors as SEM.

plasmid	Construct	Membrane (%)	Cytosol (%)
pGS7	1-100RD CAT	65.0 ± 1.5	34.0 ± 1.5
pGS10	ΔP2-F7RD CAT	52.5 ± 2.5	47.5 ± 2.5
pGS15	ΔC8-K13RD CAT	55.5 ± 1.5	44.5 ± 1.5
pGS17	ΔP14-W20RD CAT	16.0 ± 2.6	84.0 ± 2.6
pGS18	ΔD21-R25RD CAT	32.0 ± 2.4	68.0 ± 2.4
pGS22	ala_7(14-20)	22.6 ± 1.8	77.4 ± 1.8

Figure 5.3: Cell-Free Expression of Plasmids Encoding RD1 Deletion Mutations

Plasmids pGS7, pGS10, pGS15, pGS17 and pGS18 encoding 1-100RD1-CAT and deletion mutations Δ P2-F7, ΔC8-K13, ΔP14-W20 and Δ D21-R25 respectively, were used to prime a coupled transcription/translation system to produce mature chimeric CAT species in an *in vitro* system. This was done in the presence of ^{35}S-methionine to produce radioactive products. Following transcription/translation duplicate sample were incubated in the absence and presence of COS membranes. After incubation a high speed supernatant (soluble) fraction and a membrane fraction were isolated. These two fractions, and that of the original product of the transcription/translation reaction, were subjected to SDS-PAGE. Identification of the radioactive species was done using a phosphorimager. All fraction were normalized for volume and concentration. Lanes 1-3 represent pGS7, lanes 4-6 represent pGS10, lanes 7-9 correspond to pGS15, lanes 10-12 represent pGS17 and lanes 13-15 correspond to pGS18. The lanes are in sets of three, where the first track shows the original products of the transcription/translation reaction, the second the membrane (high speed pellet) fraction and the third the soluble (high speed supernatant) fraction. Thus lanes 1, 4, 7, 10 & 13 show the original material emanating from the transcription/translation experiment; lanes 2, 5, 8, 11 and 14 the high speed pellets after incubation in the presence of COS membranes and lanes 3, 6, 9, 12 and 15 the high speed supernatants obtained after centrifugation of the incubation mixture done in the presence of COS membranes. These data are typical of an experiment done three times.

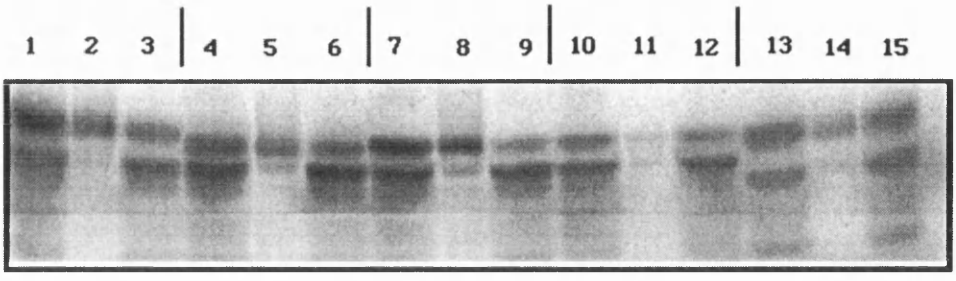
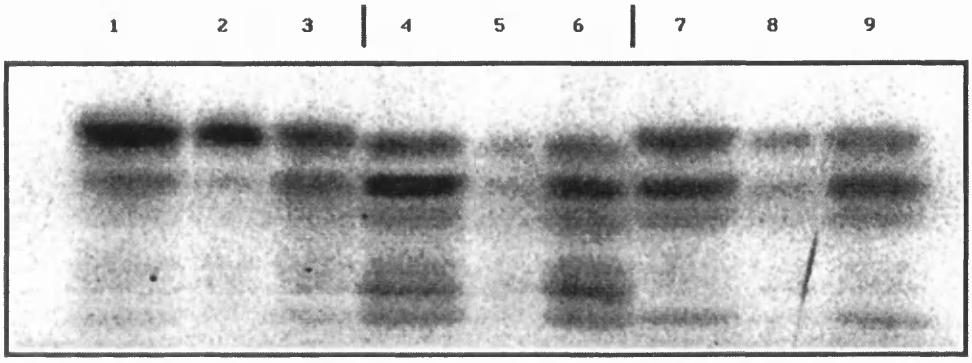

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 5.4: Cell-Free Expression of PlasmidpGS22 Encoding the mutation (*ala*$_7$(14-20))

Plasmids pGS7 pGS17 and pGS22 encoding wild-type 1-100RD1-CAT, ΔP14-W20 and *ala*$_7$(14-20) respectively, were used to prime a coupled transcription/translation system to produce mature chimeric CAT species in an *in vitro* system. This was done in the presence of ^{35}S-methionine to produce radioactive products as described (Methods, section 2.12). Following transcription/translation duplicate samples were incubated in the absence and presence of COS membranes. After incubation a high speed supernatant (soluble) fraction and a membrane fraction were isolated. These two fractions, and that of the original product of the transcription/translation reaction, were subjected to SDS-PAGE. Identification of the radioactive species was done using a phosphorimager. All fraction were normalized for volume and concentration. Lanes 1-3 represent pGS7, lanes 4-6 represent pGS17 and lanes 7-9 correspond to pGS22. The lanes are in sets of three, where the first track shows the original products of the transcription/translation reaction, the second the membrane (high speed pellet) fraction and the third the soluble (high speed supernatant) fraction. Thus lanes 1, 4 & 7 show the original material emanating from the transcription/translation experiment; lanes 2, 5 & 8 the high speed pellets after incubation in the presence of COS membranes and lanes 3, 6 & 9 the high speed supernatants obtained after centrifugation of the incubation mixture done in the presence of COS membranes. These data are typical of an experiment done three times.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CHAPTER 6

Conclusions & Perspectives

cAMP phosphodiesterases are a family of enzymes encoded by four distinct genes that display an additional level of complexity through the existence of multiple splice variants (Beavo 1995). They show high homology within a central domain of approximately 270 amino acids that is believed to encompass their active site. This degree of homology is particularly high within specific sub families. Although the C-termini are conserved in a gene specific fashion, the N-termini demonstrate little or no sequence homology. It has been suggested that, in the case of the type-IVA PDE, RD1 (Shakur et al. 1993) and in the case of the type-IVB enzyme, PDE-4 (Lobban et al. 1994) that their respective N-terminal domains may be involved in regulation in addition to conferring membrane association.

The extreme N-terminal domain of RD1 comprises a sequence of 25 amino acids that is unique amongst all PDEs that have been cloned to date. Deletion of this domain by engineered truncation has been shown to render RD1 as a fully soluble enzyme that is found as an active species in the cytosol of transfected COS cells (Lobban et al. 1994; Shakur et al. 1993). The contention that this domain can confer membrane association upon an essentially soluble protein (Shakur et al. 1993) has been investigated here. A plasmid vector was designed and constructed which facilitated the generation of in-frame fusions between the amino terminus of the soluble bacterial reporter enzyme chloramphenicol acetyltransferase and various N-terminal RD1 fragments that could be transiently expressed in COS cells under control of the SV40 early promoter. Moreover, by including the SP6 promoter in the plasmid it was possible to generate mature RD1-CAT chimeras in a cell-free environment using an *in vitro* transcription/translation system. This, in turn, led to the development of a novel assay where mature chimeras were assessed for their ability to associate with added membranes.

Expression of RD1-CAT chimeras in COS cells showed that only those protein species that contained the extreme amino terminal 1-25 fragment of RD1, 1-100RD1CAT and 1-25RD1-CAT were associated with the plasma membrane. In contrast, chimeras missing this region, namely 26-100RD1-CAT, were shown to be were exclusively cytosolic in a manner identical to that observed for native CAT. Furthermore, independent confirmation of the ability of the 1-100RD1CAT and 1-25RD1-CAT chimera to associate with membranes was obtained following synthesis in the cell-free system. As observed for the COS cell expression studies, chimeric RD1-CAT species devoid of the unique amino terminal region were found in the soluble fraction in a manner reminiscent of native CAT. Thus it can be concluded that the N-terminal 25 amino acids of RD1 contain structural information that allows for membrane association even after the biosynthesis of the mature protein.

The original observation made by Shakur et al (Shakur et al. 1993) suggested that when COS cells were transiently transfected with the cDNA for RD1, approximately 85% of the total PDE activity was found associated with the P2 pellet fraction while the remainder was observed in the soluble S2 cell fraction. This was in contrast to the distribution observed for native RD1 expressed in cerebellum which was exclusively associated with the membrane fraction (Shakur et al. 1995). This suggested to me that the soluble fraction observed in transfected COS cells may be a feature of the transient expression and test system employed here. Expression of RD1-CAT chimeras in the cell-free system demonstrated that in addition to full-length species, a series of truncated proteins are also synthesised. It is thought that the most likely explanation for the generation of such soluble truncated species may be as a result of multiple translation initiation events from internal methionines when the first initiating codon fell within a suboptimal Kozak sequence (Kozak 1986; Kozak 1991; Kozak 1995). This gives rise to the production of catalytically active N-terminally truncated RD1-CAT chimeras which, lacking the extreme 1-

25 amino acids of RD1, were found exclusively in the cytosol. On this basis it would seem that the generation of such soluble truncated RD1-CAT chimeric protein species in transfected COS cells could account for the CAT activity associated with the S2 high-speed supernatant fraction. Indeed, immunoblotting data obtained previously for the 1-25RD1-CAT chimera (chapter 3, section 3.2.4, Fig. 3.10) has shown that two immunoreactive protein species were identified by an anti-CAT antibody but that only the larger species corresponding to 1-25RD1CAT was found in the membrane fraction. In contrast, the smaller species corresponding to native CAT was completely cytosolic. Subsequent to this, McPhee et al (McPhee et al. 1995) have shown that the soluble, truncated met26RD1 could also be generated in COS cells transfected with the full-length cDNA for RD1, in a similar fashion to that observed here for RD1-CAT chimera transfection. The realisation that soluble "RD1" in RD1 transfected COS cells was, in fact, the N-terminally truncated met26RD1 provided further evidence that membrane association of RD1 is a function of information contained within its first 25 amino acid residues.

How then might this domain confer membrane association upon a soluble protein? Certainly, in the case of RD1, it is unlikely that this region serves as a cleavable N-terminal signal sequence. There are a number of reasons for this which include (i) analysis of the amino acid sequence fails to reveal a stretch of amino acids that is sufficiently hydrophobic that it could function as a transmembrane domain, (ii) that the engineered deletion of amino acids 1 to 25 to produce met26RD1 results in the synthesis and expression of a fully active, and therefore correctly folded, soluble enzyme (Shakur et al. 1993). Indeed, its activity is some 2-fold greater than native RD1 (Shakur et al. 1993). If RD1 was a transmembrane integral protein with a cleaved signal sequence, then one might have expected to generate an inactive enzyme due to incorrect folding, (iii), in addition, membrane-associated RD1 in both transfected COS cells (Shakur et al. 1993) and in brain (Lobban et al. 1994) are

greater in size than met^{26}RD1 implying that they are indeed distinct species, (iv) that the membrane-associating 1-25RD1-CAT and 1-100RD1-CAT chimera are of the predicted size and (v) that these two chimera can be synthesised *in vitro* to yield mature products which demonstrate selective association with membranes.

However, while the presence of a conventional signal peptide can be effectively ruled out as the method by which RD1 associates with membranes, it should be noted that some proteins do possess N-terminal signal sequences that are not cleaved but can themselves function as membrane anchors by insertion across the lipid bilayer (see von Heijne (1990) and section 1.7.1). Moreover, it is worth noting that amongst proteins associated with membranes by means of membrane anchors, there is a low degree of amino acid sequence conservation between signal sequences (von Heijne 1990). However, certain features of secondary structure appear to be common. These take the form of three distinct domains; at the extreme N-terminus is a positively charged region of 1-5 amino acids, followed by a central hydrophobic domain of 7-15 amino acids and then a more polar carboxy terminal domain of 3-7 amino acids which, if applicable, contains the site for cleavage by the signal peptidase. Application of the above criteria to the 1-25 amino acid N-terminal domain of RD1 shows an absence of any such features. Missing, in particular, is the characteristic positive charged polar sequence at the extreme N-terminus required for a signal peptide. Furthermore, although there are distinct hydrophobic regions within this domain, such as from leu3 to phe7 and from trp15 to trp20, it should be noted that neither of these regions is contains ~19 hydrophobic amino acids required to constitute a membrane spanning anchor.

One means of attachment of essentially soluble proteins to membranes is through hydrophobic interaction, via N-terminal acylation using either myristoyl (McIlhinney et al. 1987) or palmitoyl (Spiegel et al. 1991b) residues. The theory that such a modification may serve to anchor RD1 to the plasma

membrane can be regarded as untenable since analysis of the amino acid sequence confirms that there is no putative myristoylation site (glycine) to be found at position 2 within this N-terminal region. Thus the only other possibility is palmitoylation of cysteine residues located at positions 8 and 11. While most investigators have regarded palmitoylation as a C-terminal phenomenon (Grand 1989; Peter et al. 1992) several groups have published evidence that N-terminal cysteines may also be modified by the addition of palmitate. Skene and Virag (Skene and Virag 1989) and Zuber and colleagues (Zuber et al. 1989) have demonstrated that the neuronal growth-cone protein GAP-43 is membrane associated by palmitoylation of its N-terminal cysteines, and more recently it has been shown that some G protein α subunits palmitoylated on N-terminal cysteines (Grassie et al. 1994; McCallum et al. 1995a; McCallum et al. 1995b; Parenti et al. 1993). Analysis of the amino terminus of RD1 show that there are two cysteines at positions 8 and 11 that may serve as sites for palmitoylation. However, investigations made during the course of this study tend to suggest that this mode of attachment is extremely unlikely to determine membrane association upon RD1. Firstly, efficient membrane association has been observed for both the 1-25RD1-CAT and 1-100RD1-CAT chimeras that had been synthesised in a cell-free system. Secondly, site specific mutagenesis was employed to substitute cys8 and cys11 for serines. Upon expression in transfected COS cells the majority of CAT activity of such mutant forms, as determined by CAT assay, was associated with the P2 pellet fraction in a manner similar to that previously observed for the wild-type 1-100RD1-CAT chimera

Furthermore, during the course of this research, Shakur et al (Shakur et al. 1995) published results which showed that not only was COS cell expressed RD1 unable to incorporate [^{35}S] labelled palmitate, but incubation in the presence of hydroxylamine, which has been shown to cause the release of covalently associated palmitate, was unable to cause the release of RD1 from

isolated membranes of either cerebellum or transiently transfected COS cells (Lobban et al. 1994).

While mutation of neither cys8 or cys11 affected membrane association, it was observed that this was not the case for a chimera expressing mutations of both cysteines, rather it appeared that this mutation caused an increase in the fraction of CAT activity found to be associated with the P2 membrane fraction. In this instance, approximately 90% of the CAT activity, as determined by enzyme assay, was found associated with the P2 pellet fraction of transiently transfected COS cells. This was in contrast to the 70% level of association observed for the wild-type 1-100RD1-CAT chimera.

An explanation for this apparent increase in membrane localisation of the RD1-CAT chimera came from analysis of the cell-free coupled transcription/translation data. It was noted that when plasmids containing the individually mutated cysteines were used to direct protein synthesis, these gave rise to a mixture of membrane associated and soluble chimeric protein species in an identical manner to that previously observed for the wild-type 1-100RD1-CAT chimera (Ch. 3, fig. 3.11). However, only the full-length chimera was found to associate with added membranes whereas the truncated chimeras remained in the soluble fraction. When cell-free protein expression was driven by the plasmid containing the double cys8/11 mutation it became apparent that using the double cysteine mutation the levels of the truncated soluble protein species were much lower than those observed using a plasmid encoding either the individual cysteine mutations or the non-mutated 1-100RD1-CAT chimera. This suggests that the distribution of CAT activity observed in transfected COS cells expressing the double cysteine mutation does not involve an actual increase in the amount of enzyme associated with the membrane fraction, rather this is directly related to the reduced expression of non-membrane associating, truncated chimera. These necessarily will result in a decrease in the amount of soluble CAT activity that can be measured in the cytosol.

The reason why such a unique pattern of protein expression should be solely confined to the plasmid construct encoding the double cysteine mutation remains elusive. However, there are certain factors which rule out the possibility that this is due to an experimental artefact. Since the same pattern of expression has been observed in two entirely separate expression systems, where protein synthesis is directed by two unrelated promoters, it is highly improbable that the effect is modulated at the level of transcription. This would seem to implicate that the reduced level of soluble proteins synthesised is controlled at the level of translation. One factor that has been shown to affect translation is the presence of secondary structure in the mRNA (Brierley et al. 1989; Zinoni et al. 1990). Formation of a hairpin loop near the beginning of a protein-coding sequence has been demonstrated to cause the ribosome to pause for longer at an AUG codon presented in a suboptimal context, thus allowing more efficient translation than would normally be expected (Kozak 1990). Mutation of cysteine to serine involves changing the first nucleotide of the triplet codon (TGC) from T to A. It is suggested that when the RD1-CAT gene fusion was engineered in which cys8 and cys11 were both mutated to serines, the substitution of T for A in both triplet codons altered the nucleotide composition sufficiently to favour the formation of a hairpin loop structure. It is highly unlikely that such a structure could form if only one triplet codon was modified. Furthermore, it is postulated that the hairpin loop would form approximately 14 nucleotides downstream of the initiating AUG codon and thus corresponds to the optimal distance between the leading edge of the ribosome and its AUG-recognition centre (Kozak 1990). The functional consequence of this would be to slow ribosomal scanning of the mRNA, thus allowing more time for recognition of the AUG codon. However, it should be emphasised that while the hairpin loop structure may provide an interesting explanation as to why the double cysteine mutation containing RD1-CAT chimera gives rise to less of the soluble truncated proteins species than the non-mutated form, it must be pointed

out that, at this stage, it is purely conjecture and would require extensive experimental investigation currently outwith the scope of this study to provide confirmation.

Since the available evidence obtained during the course of this study effectively rules out the possibility that RD1 is membrane associated through a mechanism involving palmitoylation of N-terminal cysteines then it stands to reason that membrane association and, therefore subcellular localisation, is conferred by some other information contained within this unique region. Several proteins are targeted or retained in specific intracellular compartments by means of linear peptide sequences, examples of which have previously been discussed at length (Introduction, section 1.7). A computer program (Protean, DNASTAR) based upon the algorithm of Chou and Fasman (Chou and Fasman 1978) which can be employed to predict a protein super-secondary structure from its primary amino acid sequence was used in an attempt to determine whether the N-terminal 1-25 amino acids of RD1 could adopt a specific structural conformation when considered in an independent context to the full-length protein. By using this approach a putative structure was obtained suggesting that the N-terminal region of RD1 may contain a number of independently folded structural motifs comprised of two α helices separated by a mobile hinge region (Ch. 5, fig. 5.1). While computer programs may provide valuable information about protein structures, the very multitude of existence of such methods tends to suggest that no single algorithm can be regarded as completely satisfactory. As such an accurate determination of protein structure can only be obtained by the experimental methodology such as X-ray diffraction crystallography or Nuclear Magnetic Resonance spectroscopy (NMR). In collaboration with Dr. James Beattie from the Hannah Research Institute, Ayr and Dr. John Smith at University of Birmingham, a 25 amino acid peptide was synthesised to correspond to extreme N-terminus of RD1. This peptide was subjected to investigation by (^{1}H-NMR) as an alternative strategy to provide an

independent structural determination. Adoption of this approach confirmed the previously generated computer prediction that the N-terminus of RD1 is composed of two distinct helical structures separated by a mobile 'hinge' region (Smith et al. 1996). This gives support to the contention that this region has distinct functional properties (Houslay et al. 1995; McPhee et al. 1995; Shakur et al. 1993; Shakur et al. 1995), identified as an ability to attenuate the V_{max} of RD1 and to locate this enzyme exclusively to membranes. The first helical domain was found to be comprised a well formed amphipathic α-helix encompassing amino acid residues 2-8, whereas the second helical region contains a distinct, compact, highly hydrophobic domain (residues 14-20) followed by a polar region (residues 21-25). The structure obtained by NMR spectroscopy suggested that residues 14-20 favoured the formation of a distorted helical structure. While the presence of so many hydrophobic amino acids containing large bulky side chains in an α helical conformation is unusual, nevertheless, such a structure has been observed previously for the cytoskeletal protein actin (Kabsh et al. 1990).

In order to address whether membrane association was attributable to one of these distinct structural domains, overlap extension mutagenesis (Ho et al. 1989) was employed to engineer deletions in the unique splice region of RD1. Four truncations were selected to probe functional attributes. These were (i) deletion of the hydrophobic region of the N-terminal α-helical domain (residues 2-7), (ii) deletion of the polar region of this α-helical domain together with the 'hinge' region (residues 8-13), (iii) deletion of the distinct hydrophobic, tryptophan-rich domain formed within the second helical region (residues 14-20) and (iv) deletion of the polar end of the second helical region (residues 21-25). Expression of these constructs in both COS cells and the cell-free system demonstrated that mature chimeric proteins containing the deletions ΔP2-F7, ΔC8-K13 and ΔD21-R25 all became membrane associated. In marked contrast to this, the mutant chimera expressing the deletion ΔP14-W20 was

unable to become associated with membranes in the cell-free expression system and showed a marked reduction in membrane association when expressed in COS cells. This suggests that the information which is critical for membrane association lies within this distinct, strongly hydrophobic, tryptophan-rich domain. ^{1}H-NMR analysis showed that the stretch of amino acids given by ^{14}pro-^{19}trp forms a discrete domain which is stabilised by hydrogen bonding and Van der Waals interactions (Smith et al. 1996). This is an intensely hydrophobic domain. Thus, in order to investigate whether membrane association was a function of the hydrophobicity of this domain *per se* , residues ^{14}pro-^{20}trp were replaced with a 'cassette' of seven alanine residues. Expression of this mutant chimera showed that it failed to become membrane associated. This further strengthens the case against the N-terminal domain adopting a transmembrane helical structure, since such a string of alanines would be expected to favour the adoption of a more rigid helical structure than that observed for the wild type domain (Vergeres et al. 1995). Thus the ability of RD1 to become membrane-associated is not due to a non-specific hydrophobic interaction at this point within the splice region. Rather, it would appear to be determined by the unique structural properties of this tryptophan rich domain bounded by ^{14}pro-^{20}trp.

The N-terminal 1-25 amino acid domain is an amphipathic structure and it is possible that it could fold to yield a hydrophobic loop able to partition into the lipid bilayer in a similar fashion to that proposed (Houslay et al. 1995) for the initial insertion of the hydrophobic domain of anchoring signal peptides and for the cytochrome b5 as suggested by the observations of Vergeres et al (Vergeres et al. 1995). Certainly such a mode of attachment would be consistent with our observations that membrane bound RD1-CAT chimeras, and RD1 itself (Lobban et al. 1994; Shakur et al. 1993), are solubilized only with Triton X-100 and not by high ionic strength, implying that ionic interactions do not solely, if at all, determine the anchoring reaction. However,

that both RD1 (Lobban et al. 1994; Shakur et al. 1993) and the RD1-CAT chimeras appear to show specificity as regards membrane association, with preferential association with the plasma membrane, militates against a simple partition of this anchor into the lipid milieu as either part of or the sole means of membrane association unless it interacted with a specific lipid species showing a highly restricted subcellular distribution.

The PDE4A isoform, RD1 appears then to possess information contained within the unique domain formed from the first 25 amino acids of the protein that allows it to become membrane-associated. That deletion of this domain from RD1 produces a fully active soluble enzyme (Lobban et al. 1994; Shakur et al. 1993) and that this domain can confer membrane association upon CAT suggests that RD1 is formed from a core soluble protein together with an N-terminal anchoring domain. It is my contention that RD1, through this N-terminal domain, becomes membrane-anchored by interacting with a membrane-associated protein via an interaction which is predominantly hydrophobic in nature. Such a specific targeting may be intimately related to the biological functioning of this particular type-IVA PDE splice variant, one likely consequence of which relates to the conferment of specific intracellular targeting.

Further studies are needed however, to define (i) the precise residues involved in targeting, (ii) the components of the targeting mechanism and (iii) the biological consequences of targeting of RD1.

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