An investigation into the regulation of nPKCs by phosphorylation

Davey. B. Parekh

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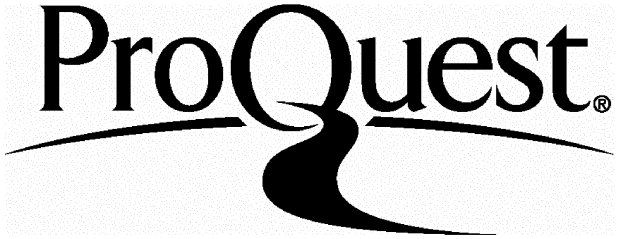
44 Lincoln's Inn Fields
London
WC2A 3PX



Abstract of Thesis

Three phosphorylation sites in the catalytic domains of çPKC isoforms have been shown to have profound effects on activity. These are the 'T loop', 'TP' and 'hydrophobic' sites. Little is known of the regulation of phosphorylation at these sites. The investigations here elucidate some of the controls that act upon the equivalent sites in nPKCs.

To establish a system for these studies, polyclonal antisera selective for the phosphorylated forms of these sites in two nPKC isoforms, nPKCδ and nPKCε, were characterised. These tools were then used to investigate which cell culture condition is most suitable for monitoring the signalling inputs to these proteins. All three phosphorylation events were blocked by the C1-domain inhibitor calphostin-C. Initial studies established that T loop phosphorylation was under the influence of PI3-kinase, based upon sensitivity to the inhibitor LY294002. It was also found that the TP sites in nPKCδ and nPKCε were sensitive to the PKC-catalytic site inhibitor bis-indolylmaleimide I, consistent with an autophosphorylation process. The hydrophobic site did not appear to be autophosphorylated, but was influenced by phosphorylation at the T loop site. Phosphorylation at the hydrophobic site was shown to be under the control of mTOR, and also sensitive to amino acid deprivation, consistent with mTOR control. Intact cell studies demonstrated the potential role of PKCζ as a PKC-hydrophobic site kinase.

Signalling initiated by both growth factors and β1-integrin activation played a role in the phosphorylation of these PKC sites. Cells had to be chronically deprived of both stimuli to reach a low basal phosphorylation at the T loop and hydrophobic sites in these nPKC isoforms. Interestingly, the tumour suppresser, PTEN, controlled the suspension-dependent behaviour of these phosphorylation sites. The complex behaviour of these PKC phosphorylations, their mutual interactions, effects upon activity and the implications for cell behaviour are discussed.

Acknowledgements

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Abbreviations

aPKC	atypical PKC
ATP	Adenosine 5'-triphosphate
BIM-I	Bisindolylmaleimide-I
bp	base pairs
BSA	Bovine serum albumin
cPKC	classical PKC
cpm	counts per minute
$CaCl_2$	Calcium chloride
DAG	Diacylglycerol
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra-acetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)N, N. N'N' tetra-acetic acid
FBS	Foetal bovine serum
GST	Glutathione S-transferase
GTP	Guanosine 5'-triphosphate
HEK	Human embryonic kidney (cells)
IPTG	Isopropyl-β-D-thiogalactopyranoside
KLH	Keyhole limpet hemocyanin
kDa	kilodalton
LPA	lysophosphatidic acid
LY 294002	2-(4-morpholinyl)-8-phenyl-4H-1 benzopyran-4-one
M	Molar
MAPK	Mitogen activated protein kinase
MEFs	Mouse embryonic fibroblasts

MBP	Myelin basic protein
mTOR	mammalian TOR
nPKC	novel PKC
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PC	Phosphatidyl choline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PDK1	PIP_3-dependent kinase-1
PH	Pleckstrin homology (domain)
PtdIns(3,4)P_2	Phosphatidylinositol (3,4) bisphosphate
PtdIns(3,4,5)P_3	Phosphatidylinositol (3,4,5) trisphosphate
PI 3-kinase	Phosphatidylinositol 3-kinase
PKA	Protein kinase A (cAMP-dependent kinase)
PKB	Protein kinase B
PKC	Protein kinase C
PKD	Protein kinase D
PLC	Phospholipase C
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
PRK	Protein kinase C-related kinase
PS	Phosphatidyl serine
PTEN/MMAC	Phosphatase and tensin homologue or mutated in multiple advanced cancers
SDS	Sodium dodecyl suphate
SH2/3	Src homology 2/3 (domain)
TOR	Target of Rapamycin
TPA	12-O-tetradecanoylphorbol-13-acetate

1.0 Introduction

1.1 General principles of signal transduction

In order for the cells to adapt, or respond to their changing environment, they rely on signals being received and interpreted from one cell to another. These communication signals come from their surroundings in various forms, such as hormones, neurotransmitters, or components of the extracellular matrix. In general, the signalling ligands bind to a specific receptor on, or within, a target cell which then transduces the signal through the plasma membrane and into the cell. The main purpose of this is to relay the information from the extracellular environment into the cell, while excluding the processes within the cell from its hostile environment. The receptor permits a cell to communicate with its environment by initiating a cascade of signalling pathways within the cell. This communicates the information to various parts of the cell to initiate an appropriate response. The consequence of all these processes is to allow cells to communicate with one-another over distances so they can change, where and when necessary, to adapt and evolve to their dynamic environment.

1.2 Signalling through cell surface receptors

The binding of an extracellular signal (ligand) to a receptor is the first stage in the process of signal transduction. There are many different types of receptor to accommodate the variety of signals that occur. The response of a cell, or tissue, is largely dependent on the type of receptors present and the intracellular signalling pathways activated. Different cell types may have different receptors for the same ligand, where each initiates a different response within the cell. Alternatively, a cell-type-specific response may occur where the same receptor may exist on different

cell-types, and ligand binding may elicit a different response in the different cells. Hence, receptors have a defined binding specificity for a given ligand, and there is an effector-specific response induced by the ligand-receptor complex.

A large variety of receptors exist in higher eukaryotic multicellular organisms. They can be categorised into two groups; intracellular receptors for hydrophobic membrane-permeable signals (such as sex hormones), and cell-surface receptors for hydrophilic signals. There are different types of cell-surface receptors, and they include the following:

1. G-protein coupled receptor
2. Ion-channel receptor (e.g. acetylcholine receptors)
3. Receptor with intrinsic kinase activity:

3a. Tyrosine kinase-linked receptor

3b. Serine/Threonine kinase-linked receptor (e.g. receptors for the TGFβ family)

4. Receptors with associated catalytic activity:

4a. Non-kinase receptors associated with protein kinases (e.g. cytokine receptors which associate with the JAK-protein tyrosine kinase family)

4b Receptors with associated cyclase activity. For example, the antinatriuretic peptide (ANP) receptors (type-A, B, or C), which bind natriuretic peptides (ANP, BNP, or CNP) to influence cardiovascular and body fluid homeostasis. ANP-receptors have a variable ligand-binding domain a transmembrane region followed by a conserved, intracellular regulatory and guanylyl cyclase catalytic domain.

In many cases, almost as soon as the signalling cascade is initiated, the ligand-receptor complex is inhibited or downregulated. This is the last stage of signalling, and ensures that cells are not over-stimulated. Signal transduction mediated by G-protein coupled receptors and receptor tyrosine kinases are discussed more fully here because PKC activation through these receptors has been well documented and this is the subject matter of this thesis.

G-protein coupled receptors have 7 transmembrane spanning helices, and are associated with heterotrimeric G proteins on the cytosolic side (Dohlman et al., 1987). Ligand binding on the extracellular side of the receptor, leads to an exchange of GDP for GTP on the α-subunit of the G protein, associated on the intracellular side of the receptor (Lambright et al., 1994; Lambright et al., 1996; Sondek et al., 1996; Sondek et al., 1994). The GTP-bound α-subunit undergoes a conformational change. This modification releases the GTP-bound α-subunit from the inhibitory βγ-subunits, so it can interact with an effector. However, the βγ-subunits of G proteins can also activate certain effectors such as p21ras, to activate the MAP kinase pathway (Crespo and Leon, 2000; Koch et al., 1994). An example of a GTP-bound α-subunit which activates an effector is the GTP-bound Gαq-subunit. This G-protein activates one of the PLCβ proteins (a family of four mammalian genes), which hydrolyse PtdIns(4,5)P_2 at the plasma membrane to release Ins(1,4,5)P_3 in the cytosol and diacylglycerol (DAG) in the membrane. Both these second messengers activate other processes within the cell, such as PKC. This has been reviewed by Gutkind and co-workers (Gutkind, 1998; Murga et al., 2000).

Receptor tyrosine kinases are the second largest group of cell-surface receptor (Yarden et al., 1986). The ligands may be monomeric (e.g. EGF) or oligomeric (e.g. PDGF-AA/AB/BB). Ligand-bound receptors dimerise or oligomerise following a conformational change. By juxtaposing the cytosolic-receptor tyrosine kinase domains from two or more receptors, trans-autophosphorylation takes place on tyrosine residues (Gullick et al., 1985). These phospho-tyrosines serve as docking sites for cytosolic proteins containing domains such as the Src homology 2 (SH2) or Phospho-tyrosine binding (PTB) domains (Anderson et al., 1990; Koch et al., 1991; Moran et al., 1990). For example, PLCγ, ras-GAP, Grb2, and the p85 subunit of PI 3-kinase all bind to phospho-tyrosines within a defined motif on the receptor via their SH2 domains. This has been thoroughly reviewed by Schlessinger, and Pawson (Lemmon and Schlessinger, 1994; Lemmon and Schlessinger, 1998; Pawson, 1995; Schlessinger, 1993; Schlessinger, 1994). The motif surrounding the phospho-tyrosine determines specificity (see below). Other intracellular signalling

intermediates, such as Shc, have both SH2 and PTB domains. These domains serve not only to recruit the effectors to the activated receptor for phosphorylation, but also induce their activation by conformational change. Specificity in the binding of these domains to their targets is dependent upon the immediate surrounding sequences. For example, the SH2 domain in the p85 subunit binds phospho-tyrosines preferentially within the YXXM or YMXM motif (where the letters refer to the single letter nomenclature for amino acids).

1.2.1 Signal transduction in multicellular organisms

The activation of effectors, following receptor engagement, leads to the generation of second messengers which recruit and activate other effectors to establish a signalling cascade. For example, PDGF-receptor mediated PI 3-kinase activation, leads to the generation of 3-OH-phosphorylated phosphoinositides such as $PI(3,4,5)P_3$. This lipid forms a binding site for certain Pleckstrin-homology (PH) domain-containing proteins and recruits these signalling intermediates to the plasma membrane for either their activation or to bring them in close proximity to their substrates, or both (Ferguson et al., 1995; Lemmon and Ferguson, 2000; Lemmon et al., 1995; Lemmon et al., 1996). Examples of this are, PLCγ, $PtdIns(3,4,5)P_3$-dependent kinase-1 (PDK-1), and protein kinase B (PKB) (Alessi et al., 1997a; Anderson et al., 1998; Ginger and Parker, 1992; Katan and Parker, 1987; Parker et al., 1994).

These interactions between downstream effectors and common second messengers permit broad signal integration and networking. For example, PDGF-receptor activation leads to the activation of PI 3-kinase and ras. GTP-bound ras can activate PI 3-kinase through association, while ras-GAP (GTPase activating protein) can terminate ras signalling (Lockyer et al., 1999; Marte et al., 1997; Rodriguez-Viciana et al., 1996). Another example is PI 3-kinase and phospholipase-C, where second

messengers from both proteins are important for activating members of the PKC family (see below) (Batty et al., 1997; Parker et al., 1994).

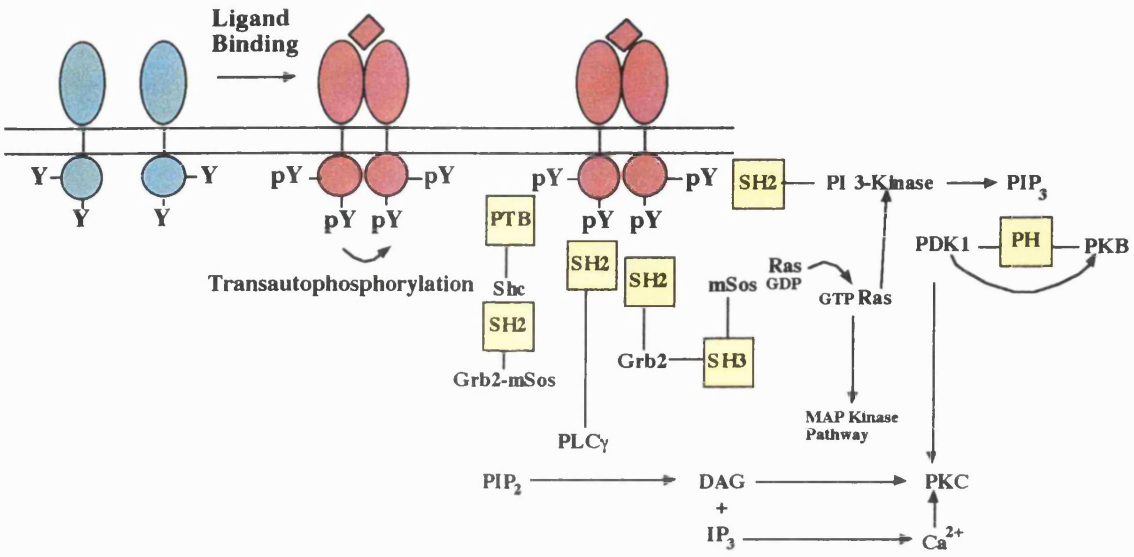


Figure 1. Schematic representation of growth factor-induced signalling.
Examples of proteins recruited to the tyrosine-phosphorylated receptor are included.

These independently folding domains which mediate protein-protein or protein-lipid interacting domains, are fundamental in signal transduction. Many other interacting domains exist and have been extensively reviewed by Pawson and co-workers (Parker et al., 1995; Parker et al., 1994; Pawson, 1994; Pawson, 1995; Pawson and Gish, 1992; Pawson and Nash, 2000; Pawson et al., 1993; Scott and Pawson, 2000).

1.3 Signalling through integrin receptors

Signalling also exists at focal adhesions. Cells adhere to extracellular matrix, such as fibronectin or collagen through specialised cell-surface receptors called integrins

(Tamkun et al., 1986). They not only bind ligands in the extracellular matrix but also soluble ligands such as fibrinogen and intercellular adhesion molecules (ICAM). This is thought to be important for not only physically attaching calls to the extracellular matrix, but also for survival and proliferation of various cell types (Frisch and Ruoslahti, 1997).

β-Chain	α-Chain
β1	α(1-9, V)
β2	α(L, M, X)
β3	α(V, IIb)
β4	α6
β5	αV
β6	αV
β7	α(4, E)
β8	αV

Table 1. The different combinations of α and β chains of integrin receptors.

Integrin receptors are heterodimeric complexes of α and β chains (Ruoslahti, 1991). They possess an amino-terminal extracellular domain which recognises, in many cases, an RGD sequence on the integrin-binding site in the extracellular matrix (Hautanen et al., 1989; Ruoslahti, 1996; Ruoslahti and Pierschbacher, 1987). This is followed by a short trans-membrane region, and finally a cytoplasmic tail of variable length. A total of 17α and 8β chains are known in mammalian cells. They form approximately 21 different types of integrin receptor. Some redundancy in signalling does exist between the various types of receptor, but most intergrins have specific functions.

When integrins bind their ligands in the extracellular matrix, they cluster together to generate focal adhesions at the cell membrane. These structures permit integrins to communicate into and out of the cell to regulate cell adhesion, spreading and migration (Burridge et al., 1988). The focal adhesion comprises a large number of structural and signalling proteins. Actin-binding proteins associate with the actin

cytoskeleton at the focal adhesion, and include α-actinin, filamin, talin, tensin, paxillin, vinculin and tensin. Signalling proteins which colocalise with these structures include $p125^{FAK}$, $p130^{CAS}$, c-src, PI 3-kinase, PKCs, ras, the tyrosine phosphatases LAR and PTP1B, the serine/threonine phosphatase PP1, and the dual specificity phosphatase PTEN. This is reviewed by (Craig and Johnson, 1996; Dedhar and Hannigan, 1996; Yamada and Miyamoto, 1995).

An early event following integrin engagement is the tyrosine phosphorylation of a number of proteins such as $p125^{FAK}$, $p130^{CAS}$, paxillin, tensin and src. Integrin receptors do not possess kinase activity and it is not clear how these proteins are tyrosine-phosphorylated. One possible mechanism is that $p125^{FAK}$ undergoes a conformational change and activation following association with a ligand-bound integrin receptor. Then $p125^{FAK}$ oligomerises and trans-autophosphosphorylates at tyrosine 297, providing docking sites for other SH2-containing proteins such as members of the Src family of tyrosine kinases, which go on to phosphorylate other proteins.

More phospho-tyrosine sites are generated to interact with other SH2 domain-containing proteins, and result in propagating the signal further downstream (Chen et al., 1995; Hildebrand et al., 1995; Schaller et al., 1992; Schaller et al., 1999; Schaller et al., 1994; Schaller and Parsons, 1994; Schaller and Parsons, 1995; Vuori et al., 1996). For example, phosphorylation of $p125^{FAK}$ by src leads to the PTB or SH2-mediated association of Shc, where it is also phosphorylated at tyrosine residues. The phospho-tyrosine residues on Shc act as docking sites for the SH2 domain in Grb2, in the Grb2-mSos complex. This leads to the activation of the ras-MAP kinase pathway. GTP-bound ras also activates the PI 3-kinase pathway (Chen et al., 1996; Schlaepfer et al., 1994; Schlaepfer and Hunter, 1997; Schlaepfer and Hunter, 1998; Schlaepfer et al., 1998).

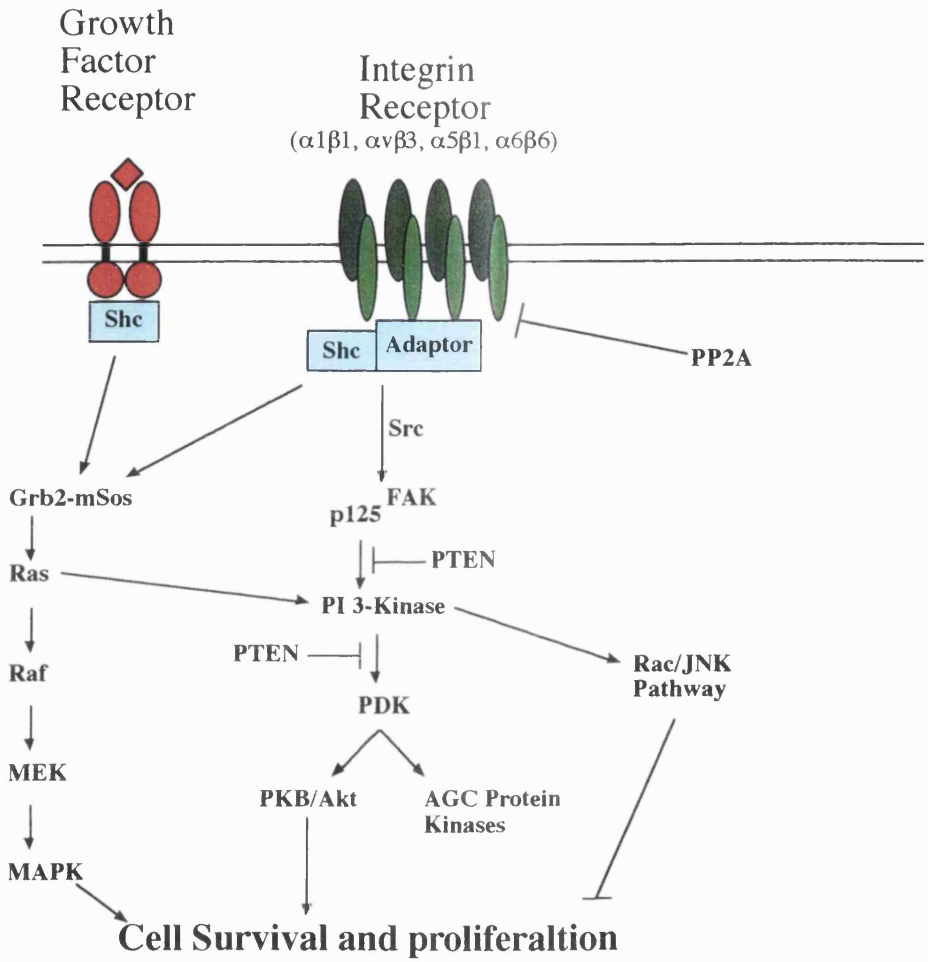


Figure 2. Scheme to depict signal integration between the activation of two different types of receptors.
The SH2/PTB-containing adaptor protein, Shc is provided as one example of a protein which interacts with both types of receptor, to communicate with two different receptor signalling pathways.

The activation of the PI 3-kinase pathway in suspension cells has been shown to be crucial in avoiding cell-detachment dependant apoptosis (anoikis). PI 3-kinase activation leads to the production of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 (Khwaja et al., 1997). This stimulates a PH domain-mediated recruitment of PDK-1 and

PKB/Akt. PDK-1 activates PKB/Akt by phosphorylating it at the activation/T loop (T308). PKB/Akt is then phosphorylated at the hydrophobic site (S473) by a mechanism that is still unclear. This allows PKB/Akt to catalyse the phosphorylation of Bad, the apoptotic protein, to promote cell survival. Downward and coworkers demonstrated that MDCK cells were protected from anoikis when expressing constitutively active ras. This was due to the activation of PI 3-kinase from ras association (Downward, 1998; Kauffmann-Zeh et al., 1997; Khwaja et al., 1997).

Though integrin signalling is regulated, the precise mechanism for each specific integrin activation is unresolved. However, there is evidence suggesting that β1 subunits are regulated though phosphorylation in the cytoplasmic tail at tyrosine, threonine, and serine residues. For example, phosphorylation at S785 promotes the loss of the β1 subunit from focal adhesion. It seems that the protein phosphatase PP2A is important in regulating this process (Mulrooney et al., 2000). Clearly, there are many details to be resolved on this issue and that concerning other integrin pathways. However, it is attractive to propose that 'cross-talk' between growth factor-activated and integrin-activated signalling pathways provide a mechanism for regulation of the signalling intermediates, as suggested earlier (Giancotti and Ruoslahti, 1999; Schlaepfer et al., 1994).

Below I will discuss some of the proteins that mediate signalling processes within the cell, with particular emphasis on those which are relevant to this study (chapters 3, 4, 5, and 6). The transducers reviewed are the PI 3-kinase family, PDK-1, mTOR, PTEN/MMAC1 and finally the PKC family.

1.4 PI 3-kinase

The PI 3-kinase pathway is very important in the activation of many different pathways. Some of these pathways activate members of the AGC superfamily of protein kinases. This will be discussed further with reference to the novel class of

PKC isoforms in subsequent chapters. As described above, a variety of signalling pathways, stimulated by either growth factors or integrin engagement, lead to the activation of this family of lipid kinases. The lipid products of a PI 3-kinase reaction depend upon which member of the PI 3-kinase family is catalysing the reaction. Signalling intermediates downstream of this step are activated, or recruited through a PH or FYVE-domain based interaction with the 3-phosphorylated inositol head group of phosphoinositides.

Since the products of the PI 3-kinase reaction (PtdIns(3)P, PtdIns(3,4)P_2, or PtdIns(3,4,5)P_3), are not cleaved by any of the PtdIns-specific PLC isoforms, this PI 3-kinase-activated signalling pathway is distinct from that which is induced by a rise in plasma membrane DAG or cytosolic calcium. The regulation of the products of PI 3-kinases is mediated largely by 5-OH and 3-OH-specific phosphoinositide phosphatases such as SHIP and PTEN, respectively (Bolland et al., 1998; Maehama and Dixon, 1998; Maehama and Dixon, 1999).

1.4.1 Classes of PI 3-kinase

The different isoforms of the PI 3-kinase family are divided into four classes, I-IV (Leevers et al., 1999; Vanhaesebroeck et al., 1997; Vanhaesebroeck and Waterfield, 1999). All the isoforms possess a catalytic domain linked to a PI kinase (PIK) domain of unknown function. Members of Class I are heterodimeric, with a p110 catalytic subunit and a regulatory/adapter subunit. Under in vivo conditions they catalyse the phosphorylation of PtdIns(4,5)P_2 to generate PtdIns(3,4,5)P_3. This leads to the subsequent rise of PtdIns(3,4)P_2 by the action of 5-OH-specific phosphoinositide phosphatases (Hinchliffe and Irvine, 1997; Woscholski and Parker, 1997). Class I-type PI 3-kinases are further divided into two groups, class IA and IB. Members of the former class are recruited to a tyrosine phosphorylated receptor, via an SH2 domain-mediated interaction, and activated by conformational change and phosphorylation of the p85 subunit. Class IB-type PI 3-kinases require the

activation of heterotrimeric G-protein coupled receptors since it was shown that the βγ-subunits can directly activate the p110γ catalytic subunit (Krugmann et al., 1999; Luttrell et al., 1997; Maier et al., 1999). Both classes are further regulated by GTP-bound ras.

Class II-type PI 3-kinases are larger molecules, and possess a C-terminal C2 domain, but are not regulated via this domain by calcium since these C2 domains lack important aspartate residues needed for coordination of calcium ions (Arcaro et al., 1998). This class can utilise two different substrates, with a preference in the order: PtdIns > PtdIns(4)P (Domin et al., 1997). PtdIns(4,5)P_2 is not a substrate. Members of class III PI 3-kinase isoforms are homologous to the yeast vesicular protein-sorting lipid kinase (vps34) (Volinia et al., 1995).

Class	Features/domains	Subunits Catalytic	Adaptor	Regulation	Substrates *In vitro*	*in vivo*
IA	Adaptor and ras-binding domain	p110 α, β, δ	p85 α, β, γ	Tyr Kinase & ras	PI PI(4)P PI(4,5)P_2	P(4,5)P_2
IB	ras-binding domain	p110γ	p101	G-protein & ras		
II	C2 domain	C2α, β, γ	?	?	PI PI(4)P	?
III	Only the PIK and kinase core domain	Vps34p homologue	p150	Constitutive?	PI	PI
IV	Variable	PIK-domain+ Related PI kinase domain	Protein-specific	Variable	Serine/ Threonin residues	Serine/ Threonin residues

Table 2. The different classes of the PI 3-kinase family.
Adapted from (Vanhaesebroeck and Waterfield, 1999).This table includes information on their domains, regulation, and preferred substrates.

These isoforms exist as heterodimeric complexes with an amino-terminally myristoylated serine/threonine protein kinase. However, it is not clear how these

isoforms are regulated. Their exclusive substrate is PtdIns, and these proteins are thought to generate the bulk of the cellular PtdIns(3)P (Herman et al., 1991a; Herman et al., 1991b; Stack et al., 1993).

Members of class IV PI 3-kinase isoforms include all other protein kinases that have a catalytic domain with significant homology to that in class I, II or III-type PI 3-kinases (Vanhaesebroeck and Waterfield, 1999). Most notably, they include the TOR proteins (Tor1p, Tor2p and mTOR), DNA-dependent protein kinase, ataxia telangiectesia mutated (ATM) and the ATM-related protein, designated ATR. Members of this group function as protein kinases only and have no detectable lipid kinase can activity. Interestingly, the catalytic domains of class I and II-type PI 3-kinases also phosphorylate their regulatory subunits as part of their regulatory process, indicating that protein kinase activity is a conserved feature of this class of proteins.

The sensitivity of the catalytic domains in the PI 3-kinase members towards the same type of inhibitors, LY294002, and the structurally distinct compound Wortmanin further demonstrates the close structural and functional homology in the catalytic domain between the members of this family (Domin et al., 1997; Vanhaesebroeck et al., 1997). LY294002 is often used to determine the involvement of PI 3-kinase signalling. This compound inhibits the catalytic domain of PI 3-kinases as a competitive inhibitor of the ATP-binding site. It tends to be used at between 5-20μM. LY294002 is often used in preference to Wortmanin as it is more stable in aqueous solutions.

1.4.2 Signalling by PI 3-kinases

Proteins containing either PH or FYVE (Fab1p, YOTB, Vac1p, and Early Endosomal Antigen 1 (EEA1)) domains bind the products of PI 3-kinases. FYVE domains bind PI(3)P, and are thought to be the principle sensors of this lipid in the

cell (Driscoll and Vuidepot, 1999; Fruman et al., 1998; Fruman et al., 1999). Proteins with this domain tend to be involved in membrane traffic and include class III-type PIP-kinases. Proteins with a PH domain are frequently involved in signal transduction (Bottomley et al., 1998; Rameh et al., 1997; Rameh and Cantley, 1999; Rameh et al., 1995; Rameh et al., 1998). For example, PDK-1, PKB/Akt, Tec family kinases (e.g. Btk), PLCγ2, ras^{GEF} and ras^{GAP}, Grb2 and IRS-1. This has been discussed in the following reviews: (Pawson, 1994; Pawson, 1995; Pawson and Gish, 1992; Pawson and Nash, 2000; Pawson et al., 1993; Schlessinger, 1994)

Signalling involving PH domain-containing proteins provide yet another example of signal intergration and cross-talk. The PtdIns(3,4,5)P_3-dependent recruitment of PLCγ2 via its PH domain, promotes the hydrolysis PtdIns(4,5)P_2 leading to a rise in DAG and release of Ins(1,4,5)P_3. This stimulates the recruitment and necessary conformational change for the activation of the classical and novel PKC isotypes. This will be examined further in chapters 3 and 4. In general, the different products of the PI 3-kinase family have an important influence in both intracellular signalling and regulating membrane trafficking events. The capacity to phosphorylate phosphoinositides is an important distinction between members of class I-III and class IV of PI 3-kinases, such as mTOR.

1.5 Phosphatidylinositol(3,4,5)trisphosphate-dependent kinase (PDK-1)

An effector of the PI 3-kinase signalling pathway is PDK-1. This was originally identified by two groups as a phosphatidylinositol(3,4,5)trisphosphate-dependent kinase, which phosphorylates the activation/T loop (T308) of PKB/Akt (Alessi et al., 1997a; Stephens et al., 1998). PDK-1 is a 63KDa protein with an amino-terminal catalytic domain (within the AGC superfamily of protein kinases) and a carboxy-terminal PtdIns(3,4,5)P_3-specific PH domain, and ubiquitously expressed in human

tissues (Vanhaesebroeck and Alessi, 2000). Activation of class I-type PI 3-kinases leads a rise in PtdIns(3,4,5)P_3 in the plasma membrane. The increase in PtdIns(3,4,5)P_3 levels promotes the PH domain-dependent recruitment of PDK-1 to the membrane to phosphorylate its substrates, which include members of the AGC superfamily of protein kinases, PKB/Akt, $p70^{S6K}$ and $p90^{RSK}$ (Alessi et al., 1997b; Dutil et al., 1998; Jensen et al., 1999; Le Good et al., 1998; Pullen et al., 1998; Stephens et al., 1998). This function of PDK-1 activation of PKCs is also conserved in yeast, where the PDK-1 yeast homologue activates Pkc1p (Inagaki et al., 1999). The PtdIns(3,4,5)P_3-dependent recruitment of PDK-1 is thought to be the primary mechanism of regulation, although recently, it has been shown that PDK-1 must also be phosphorylated at serine-241 to reach full activity (Casamayor et al., 1999).

It seems that the substrates to be phosphorylated at the activation/T loop sites are regulated themselves by a mechanism which controls accessibility to PDK-1. For example, the PDK-1-dependent phosphorylation of the activation/T loop site in PKB/Akt (T308) only takes place once the PH domain in PKB/Akt has also bound to either PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2, and undergone the necessary conformational change (Franke et al., 1997; Stokoe et al., 1997; Walker et al., 1998). Another example is that phosphorylation of the activation/T loop site in $p70^{S6K}$ is thought to be regulated by essential multi-site, proline-directed, phosphorylation in the autoinhibitory region of the carboxy-terminal tail, and by phosphorylation in the hydrophobic site (T412) (Balendran et al., 1999b; Pullen et al., 1998). Some of the phosphorylations in $p70^{S6K}$ are also thought to be directly under the control of mTOR (Brown et al., 1995)(see part 1.6 also).

Similar to $p70^{S6K}$, $p90^{RSK}$ is also under the control of other kinase pathways as well as PDK-1. In this case, it involves the ERK/MAP Kinase pathway and autophosphorylation. ERK phosphorylates two residues, one in the activation loop within the C-terminal kinase domain (T577), and another in the linker region (S369). The C-terminal kinase becomes activated and autophosphorylates at another site (which resembles the hydrophobic motif in PKB/Akt and PKCs) in the linker region

(S386). This dual phosphorylation in the linker region activates the PKC-like N-terminal kinase domain. However, full activity is only achieved following PDK-1 mediated phosphorylation at the activation/T loop in the N-terminal kinase domain (S227) (Dalby et al., 1998; Fisher and Blenis, 1996; Jensen et al., 1999; Sutherland et al., 1993; Vik and Ryder, 1997). Another example is the activation of the serum- and glucorticoid-induced protein kinase (SGK) by insulin or IGF-1 stimulation. Insulin or IGF-1 stimulation activates PDK-1 which phosphorylates the Tloop in SGK to activate it (Park et al., 1999; Perrotti et al., 2000). Hence, PDK-1 is critical in the activation process of numerous PI 3-kinase-dependent downstream signalling pathways, which integrates signalling pathways activated from the stimulation of different types of cellular receptors.

1.6 mTOR signalling

mTOR is a 290KDa-mammalian homologue of the yeast Tor1p and Tor2p proteins, which were originally characterised as the target of the immunosuppressant rapamycin-FK506 binding protein-12 inhibitory complex (Brown et al., 1994; Heitman et al., 1991). As described earlier, it is a member of the PI kinase related kinases (PIK) (Keith and Schreiber, 1995; Kunz et al., 1993). The primary structure of mTOR closely resembles the Tor1p and Tor2p proteins, suggesting it has a similar function in mammalian cells (Brown et al., 1994; Chiu et al., 1994; Sabatini et al., 1994; Sabers et al., 1995).

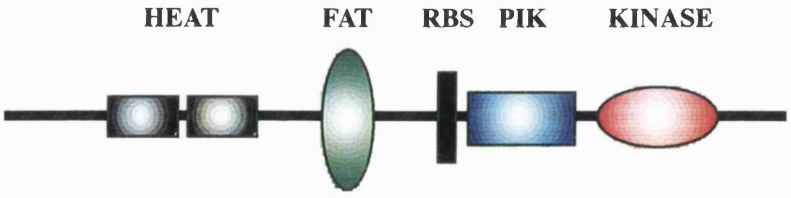


Figure 3. Domain structure of Tor1p, Tor2p and mTOR
The HEAT repeats and the FAT domain are thought to mediate protein-protein interactions (Kunz et al., 2000). RBS is the rapamycin/FKBP12 binding site.

Much of the present understanding of mTOR function and behaviour comes from investigations on the Tor1p and Tor2p proteins in yeast. For example, the control of translational initiation and nutrient-dependant G1-phase progression (Barbet et al., 1996; Di Como and Arndt, 1996). The control of translational initiation operates through the yeast homologue of type 2A protein phosphatase (SIT4), and the type 2A protein phosphatase-associated protein Tap42p (Di Como and Arndt, 1996). In the presence of abundant nutrient conditions TOR initiates translation by stimulating the type 2A protein phosphatase to associate with Tap42p. This rapamycin-sensitive step is thought to control the phosphorylation and activity of Npr1p (serine/threonine kinase), and stability of Tat2p (tryptophan permease), which controls G-phase progression (Beck et al., 1999; Schmidt et al., 1998; Schmidt et al., 1997). Tor2p has a rapamycin-insensitive role also, which controls the actin cytoskeleton via a Rho-type mechanism (Rho1p and 2, Rom2p (a GEF) and Sac7p (a GAP)). In general, Tor2p activates Rom2p which promotes GTP/GDP exchange on Rho1p and 2. GTP-bound Rho1p activates Pkc1p, which then activates the MAP kinase cascade to control the actin cytoskeleton and gene transcription (Schmidt et al., 1997; Schmidt et al., 1996).

During periods of nutrient deprivation (or rapamycin treatment) in yeast cells, the translational initiation complex scaffold protein, eIF-4G and Tat2p are degraded. This is under the control of the Tor1p and Tor2p proteins, and blocks translation. Furthermore, the interaction of Tor1p and Tor2p proteins with Tap42p (which promotes the type 2A protein phosphatases), negatively regulates RNA Polymerase (Pol) I and III, reduces Tat2p and the activity of Npr1p by dephosphorylation. Hence, TOR inhibition by rapamycin treatment or nutrient deprivation instructs the cellular machinery to terminate, or reduce all biosynthetic processes, to promote autophagy, and subsequently arrest in G1 phase of the cell cycle (Beck and Hall, 1999; Beck et al., 1999).

In mammalian cells, mTOR drives translational initiation by the combined action on $p70^{S6Kinase}$ and the inhibition of the translational initiation factor binding protein 4E-BP1, or PHAS-I (Hara et al., 1997; Thomas and Hall, 1997). The mTOR-dependent phosphorylation of PHAS-I prevents it from binding to and inhibiting the translational initiation factor eIF4E. This leads to the initiation of translation. However, it should be noted that the rapamycin-sensitive phosphorylation sites in PHAS-I are distinct from a number of other sites phosphorylated within a serine/threonine-proline motif, hence PHAS-I may also be regulated by the phosphorylation of multiple protein kinases. $p70^{S6Kinase}$ has an important role in this regulation, and is the convergence point of two signalling pathways. The activation of $p70^{S6Kinase}$ is controlled by mTOR and PDK-1. Growth factor stimulation of cells leads to the activation of the PI 3-kinase pathway. The products of this pathway induce the recruit of PDK-1 to the plasma membrane which then phosphorylates $p70^{S6Kinase}$ at the activation/T loop. Activated $p70^{S6Kinase}$ then phosphorylates the 40S ribosomal S6 protein, which drives translation of 5'TOP (terminal oligopyrimidine tract) mRNA transcripts (Beretta et al., 1996; Jefferies et al., 1997). These abundant transcripts encode some of the ribosomal proteins leading to an increase in availability of translational machinery. However, if amino acids are lacking, mTOR is inhibited and no longer inhibits PHAS-I. The unphosphorylated PHAS-I protein remains associated with eIF4E and stops translation.

Under certain conditions, mTOR also controls transcription (Beck and Hall, 1999; Yokogami et al., 2000). It has been suggested that this is direct because immunopurified mTOR was shown to phosphorylate Ser727 in a STAT3-derived peptide under *in vitro* conditions. However, it was unclear whether this was mediated by mTOR or a co-immunopurified protein. The transcriptional activator STAT3 (which regulates RNA Polymerase II) may integrate signalling from different cellular inputs involving mTOR and cytokine signalling. Following cytokine stimulation of cells, the JAK kinase family and mTOR phosphorylate STAT3 at tyrosine and serine/threonine residues, respectively. The mTOR protein also promotes RNA Polymerase I and III-mediated transcription by inhibiting the

retinoblastoma protein (pRb), through phosphorylation. The loss of function of mTOR, which leads to G1 cell cycle arrest, parallels the effect of inhibiting Tor1p or Tor2p in yeast. Hence, TOR function appears to be conserved from yeast to man. However, all the effectors downstream of mTOR remain to be determined.

It seems that much of TOR function in yeast is mediated through the regulation of a Tap42p-type 2A protein phosphatase complex. The inhibition of type 2A protein phosphatases by constitutively active TOR, activates downstream signalling (Beck and Hall, 1999; Schmelzle and Hall, 2000). A similar mechanism has been proposed for mammalian cells (Nanahoshi et al., 1998; Peterson et al., 1999). However, others argue that not only do amino acids regulate mTOR directly, but there is a growth factor-dependant input to mTOR through PI 3-kinase and PKB/Akt (Nave et al., 1999; Scott et al., 1998). This remains controversial as other studies do not detect a change in either mTOR autophosphorylation or an mTOR-dependant phosphorylation of PHAS-I following amino acid stimulation or insulin signalling (Peterson et al., 2000). This has led to the suggestion that mTOR is constitutively active and that mTOR-mediated phosphorylation of 4EBP1 is required before there is an observed growth factor-induced PKB/Akt influence (Gingras et al., 1999). Many questions concerning the regulation of mTOR under different physiological conditions remain to be answered. What does seem apparent is that mTOR influences a wide spectrum of pathways which control the growth of cells. This has been reviewed by several groups (Dennis et al., 1999; Schmelzle and Hall, 2000; Thomas and Hall, 1997). The nature of these growth-controlling pathways leads mTOR to interact with many different signalling proteins which are critical for cell survival and growth.

1.7 PTEN/MMAC1

Prior to 1997, many proto-oncogenes had been identified as mutated tyrosine kinases, but very few phospho-tyrosine phosphatases were identified as tumour

suppressors. In 1997, two groups reported that the tumour suppressor PTEN/MMAC1 (phosphatase and tensin homologue or mutated in multiple advanced cancers) was a phospho-tyrosine phosphatase, which mapped to chromosome 10q23 (Li et al., 1997a; Steck et al., 1997). PTEN/MMAC1 was found to be deleted or mutated in many tumours (Li et al., 1997a). It was also discovered that the inherited disorders of Cowden's disease and Bannayan-Zonana Syndrome were due to the loss of PTEN/MMAC1 function (Liaw et al., 1997; Marsh et al., 1997).

PTEN/MMAC1 was initially implicated in the negative control of tyrosine phosphorylated proteins associated with focal adhesions such as $p125^{FAK}$ (Gu et al., 1998; Tamura et al., 1998). However, other groups revealed that PTEN/MMAC1 also affects 3-OH-phosphoinositides, without affecting insulin-induced PI 3-kinase activity. Furthermore, a phosphatase inactive mutant of PTEN/MMAC1 (C124S) led to a persistent rise in 3-OH-phosphoinositides, even in the absence of physiological stimuli. These studies revealed that PTEN/MMAC is a dual specificity phosphatase, which dephosphorylates proteins at tyrosine residues and phosphoinositides, specifically at the 3 position of the inositol ring (Maehama and Dixon, 1998; Maehama and Dixon, 1999; Myers et al., 1998). It was also discovered that by using a lipid phosphatase defective mutant, which retained its protein phospho-tyrosine phosphatase activity (G129E), the 3-phosphoinositide phosphatase activity of PTEN was absolutely critical for tumour suppression (Myers et al., 1998).

There is little information on the regulation of PTEN/MMAC1. However, it seems that PTEN/MMAC1 is constitutively active and is regulated by a C2 domain-dependent association with its lipid substrates, as revealed by structural analysis (Lee et al., 1999). The lipid-dependent regulation of PTEN/MMAC1 is an attractive idea as both growth factor and integrin-signalling pathways could lead to the co-recruitment of PI 3-kinases and PTEN/MMAC1 to acutely regulate 3-OH phosphoinositide-dependent signalling. A protein kinase family that may be under

the dual control of PI 3-kinases and PTEN/MMAC1 is PKC. This is examined further in chapter 5.

1.8 PKC family and signalling

1.8.1 PKC domain structure and function

Protein Kinase C (PKC) was originally identified as a protein kinase from rat brains which displayed activity towards a histone substrate. Later, it was determined that activation was dependent on binding phosphatidylserine (PS), DAG, or tumour-promoting phorbol esters, and calcium ions (Inoue et al., 1977; Nishizuka, 1984; Takai et al., 1977).

The protein kinase was chromatographically separated into three distinct forms, α, β, and γ, and represented the first isotypes of PKC to be identified and cloned, and identified some of their regulatory properties (Coussens et al., 1986; Huang et al., 1986a; Huang et al., 1986b; Parker et al., 1986). Screening a rat-brain cDNA library for sequences with a similar catalytic domain as the previously identified PKCs, identified a different sub group of PKC isoforms.

The three new isoforms were PKCδ, ε and ζ (Ono et al., 1987a; Ono et al., 1987b). Low stringency screening and PCR methods led to the identification of PKCη, θ, ι (human λ) and also a group of PKC-related kinases (PRKs) (Mukai and Ono, 1994; Osada et al., 1990; Osada et al., 1992; Palmer et al., 1995).

Since then, a further related protein kinase has been identified from human cell lines called PKCμ, while the rat homologue has been identified as PKD (Johannes et al., 1994; Valverde et al., 1994). Recently, two other relatives of this enzyme have been identified, PKCν and PKD-2 (Hayashi et al., 1999; Sturany et al., 2000). PKCν has

about 77% sequence homology with PKD and PKCμ. PKD-2 shares a similar degree of sequence with PKD-1, particularly within the C1 and PH domains.

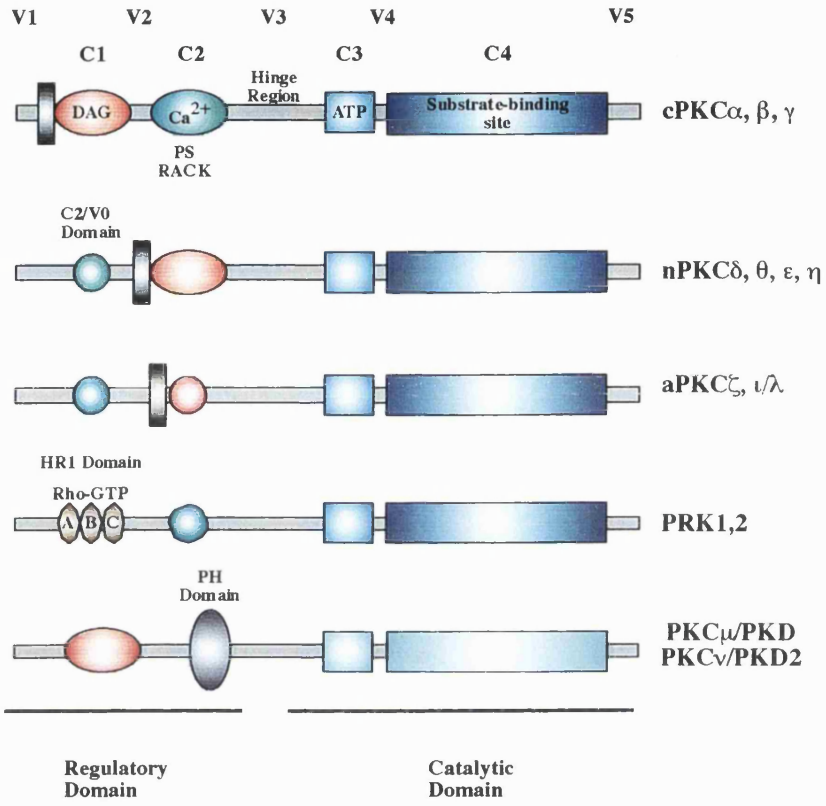


Figure 4. Domain structure representation of the PKC family
The members that have been included are on the basis of homology in the catalytic domain. PKCμ and PKCν share some key regulatory features (C1 domain) but have distinctive kinase domains.

The PKC family has been divided into three groups based primarily on their requirement for binding certain cofactors for activation. They have an amino-terminal regulatory domain, and a carboxy-terminal catalytic domain. A flexible hinge region, termed V3 separates the regulatory and catalytic domains. They are

composed of variable (V) and conserved (C) regions, some of which have defined roles.

1.8.2 The C1 domain and the Pseudosubstrate motif

Amino terminal to the C1 domain is the pseudosubstrate/autoinhibition motif, which blocks entry of substrates into the catalytic site of PKCs. This suppresses the catalytic activity of PKC unless bound to the necessary cofactors, and if mutated, it can lead to persistent activation of the PKC isoform (Dutil and Newton, 2000; Keranen and Newton, 1997; Pears et al., 1990). Cofactor binding leads to a change in conformation of the protein such that the pseudosubstrate motif is no longer in the active site. There is a PS-like domain adjacent to the HR1a and HR1b domains in PRKs. However, this motif is absent in PKCμ/PKD. The C1 domain in classical and novel PKC isoforms has two cysteine-rich zinc finger motifs (C1a and C1b), where each motif has the following pattern of Cys and His residues:

$H\text{-}X_{12}\text{-}C\text{-}X_2\text{-}C\text{-}X_{13/14}\text{-}C\text{-}X_2\text{-}C\text{-}X_2\text{-}C\text{-}X_4\text{-}H\text{-}X_2\text{-}C\text{-}X_7\text{-}C$

where X refers to any amino acid.

This domain is responsible for binding DAG or phorbol esters. Both DAG and phorbol esters compete for binding at the C1 domain, binding to one of the two repeated elements with high affinity, as demonstrated with the C1 domain in PKCδ (Bogi et al., 1998). Only one zinc finger exists in the C1 domain of atypical PKCs, but this is not able to bind DAG. A C1 domain is entirely missing in PRKs. As a result, DAG does not regulate members of either the aPKC or PRK subgroups. To illustrate the independent function of a C1 domain, a hybrid protein was generated with the C1 domain from PKCδ and the catalytic domain of PKCζ. This PKCδ/ζ hybrid was able to translocate to the plasma membrane in response to phorbol ester treatment of cells, and be downregulated in a manner similar to wild-type PKCδ (Goode and Parker, 1994). This illustrates that the C1 domain can function

independently of the rest of the protein, and impart new properties on a protein that are absent in the wild-type. Similar chimera have also been made for PKB/Akt (Andjelkovic et al., 1999). Many other classes of C1-containing phorbol binding protein have been identified, for example β2-chimaerin and Munc-13 (Caloca et al., 1999; Orita et al., 1997). In fact, 54 single and 34 doublet C1 domain-containing proteins have been identified, this is reviewed by (Hurley et al., 1997).

1.8.3 C2 and the HR1 domains.

A calcium-binding C2 domain is only found in the cPKCs. This is the site of calcium binding. A calcium binding-C2 domain also exists in a number of other proteins such as synaptotagamin (p65), rabphilin 3A, Doc2, cPLA2, Ras-GAP, and PLCγ. The C2 domains in these proteins confer a calcium-dependent phospholipid binding. In cPKCs, calcium binding at the C2 domain promotes PS binding and activation.

The calcium binding-C2 domain is absent in novel and atypical PKC isoforms, however, it has been revealed that both these groups possess a C2-like fold amino terminal to the C1 domain (originally referred to as the V0 domain). Sequence analysis of the V0 domain in novel and atypical PKC isoforms reveal close homology between the following isoforms:

PKCε and PKCη
PKCδ and PKCθ
PKCι/λ and PKCζ

Although the V0 domain does not confer a calcium-dependent PS binding, it has been postulated that it mediates protein-protein interactions, or protein targeting. This is based on observations in which the two C2 domains in synaptotagamin (C2A and C2B) mediate binding to syntaxin and clathrin AP2, respectively. Furthermore,

the binding site for RACKs (receptors for activated C kinases), has been mapped to the C2 domain in cPKCs and is thought to influence targeting. The C2 domain in PTEN/MMAC1 is also thought to target it to the plasma membrane. To further support this role of the V0 domain in protein targeting, if the V0 domain is blocked using a peptide which inhibits RACK-dependent binding at the C2 domain in PKCβ, there is no longer a phorbol ester-dependent translocation of PKCε to the plasma membrane. Recently, the binding site of growth associated protein-43 (GAP43) was mapped to the V0 domain in PKCδ, and further demonstrated that the V0 domain has a C2-like fold, but lacks critical acidic residues needed for co-ordinating with the calcium ion (Dekker and Parker, 1997; Pappa et al., 1998a; Pappa et al., 1998b).

The HR1 domain in PRKs and yeast Pkc1p, is composed of three repeated motifs of 55 residues; HR1a, b, c. HR1a and b mediate GTP-bound RhoA binding in a co-operative fashion. Furthermore, the HR1 domain has also been shown to be capable of binding RhoB, and influences activation of the kinase. The HR1 domain displays sequence homology with a repeated sequence in the yeast PKC isoform, Pkc1, suggesting an ancestral link between these two protein kinases. In support of this contention, Pkc1 is also under Rho1 control in yeast (see 1.6).

The C1 and C2 domains regulate activation of PKCs. Both calcium and DAG binding is required in cPKCs while nPKCs are only dependent upon binding DAG, to relieve autoinhibition. Although PKCs are dependent to varying degrees on binding cofactors, to reach full catalytic potential, they must also be phosphorylated at specific residues (especially within the catalytic domain).

1.8.4 The catalytic domain.

The catalytic domains of the PKCs share a high level of sequence homology. This homology forms the basis of the criteria for inclusion into this family of enzymes. It extends from domains C3-to-V5. The C3 domain is the ATP binding pocket, and

very similar to that in other protein kinases. In some PKC members (PKCγ), a short V4 domain separates the C3 and C4 (substrate-binding) domain. Since the catalytic domain in PKCμ/PKD is too dissimilar from that in other members of the PKC family, this enzyme is not considered by many as a member of the PKC family.

The V5 region consists of approximately 50 residues, but is thought to have a number of different functions such as mediating protein-protein interactions, and regulating catalytic activity. There are two phosphorylation sites identified in this region in cPKCs. Phosphorylation of these sites is thought to permit the V5 region to interact with the upper and lower lobes of the kinase domain to generate a closed/stable conformation which is insensitive to phosphatases and proteases (Bornancin and Parker, 1996; Bornancin and Parker, 1997; Pears and Parker, 1991b). This structural configuration is postulated from the behaviour of the equivalent region in a member of the AGC protein kinase superfamily, PKA. The most carboxy-terminal phosphorylation site in classical and novel PKCs (the hydrophobic or 'FSF/Y' site) is absent in PKA, although it does possess part of the consensus site motif: FXXF, instead of; FXXFS/TF/Y. In aPKCs and PRKs, a glutamate and aspartate respectively, replaces the phosphorylatable Ser or Thr residues of classical and novel PKCs. These acidic residues could partially mimic the negative charge on phosphorylated Ser or Thr residues at the hydrophobic site.

1.8.5 Regulatory phosphorylations in PKCs.

PKCδ is phosphorylated at tyrosine, serine and threonine residues. EGF-treated keratinocytes appear to stimulate src-mediated tyrosine phosphorylation of PKCδ. EGF-stimulated-tyrosine phosphorylation of PKCδ has been reported to increase when cells are co-stimulated with phorbol esters, implying that an activated protein is required. However, uncertainty still surrounds the function of these phospho-tyrosines as both increases and decreases in activity have been reported depending on which substrate is used in the assay (Haleem-Smith et al., ; Kronfeld et al., 2000;

Li et al., 1996a). Tyrosine phosphorylation in distinct regions of PKCδ are thought to regulate specific functions. Kronfeld and coworkers demonstrated that tyrosine phosphorylation in the regulatory domain (at residues 187 and 311) reduces catalytic activity, while an increase is observed if tyrosine residues are phosphorylated in the catalytic domain. However, uncertainty still surrounds these findings for several reasons. For example, there is no mention of the influence of phosphorylation at the activation/T loop or the hydrophobic site on the activity, or the effects of tyrosine phosphorylation on conformation of PKC, or their effects on interaction with other proteins, perhaps via an SH2 or PTB mechanism. These observations clearly have some importance, but it is difficult to assess whether they are directly influencing activity or another process.

The activation/T loop site (Thr497) in PKCα was identified as essential for catalytic activity. This was found to be conserved in all members of the AGC family of protein kinases, including PKB/Akt, $p70^{S6K}$, and $p90^{RSK}$. Structural analysis of the T loop site in PKA (Thr197) was found to align critical residues within the catalytic site for efficient catalysis. This site was found to be equally important and critical in PKCα and PKCβ (Orr and Newton, 1994; Srinivasan et al., 1996). The 'TP' site in PKCβI (2) (Thr642) was described as autophosphorylated (Flint et al., 1990), and this was also shown to be the case for the equivalent site in PKCδ (Li et al., 1997b). However, unphosphorylated PKCα was found to be thermally unstable, and very sensitive to both protein phosphatases and oxidation (Bornancin and Parker, 1996).

Once the T loop, TP and hydrophobic sites are all phosphorylated in PKCα, a closed conformation develops, which is no longer sensitive to phosphates or oxidation. All three sites are thought to 'prime' the kinases for maximal catalytic activity, and do not represent an 'all or nothing' requirement for the protein kinase since it is still dependent upon cofactor binding. Above is a table of the effects of T loop, TP, and hydrophobic sites on the activity in PKCs.

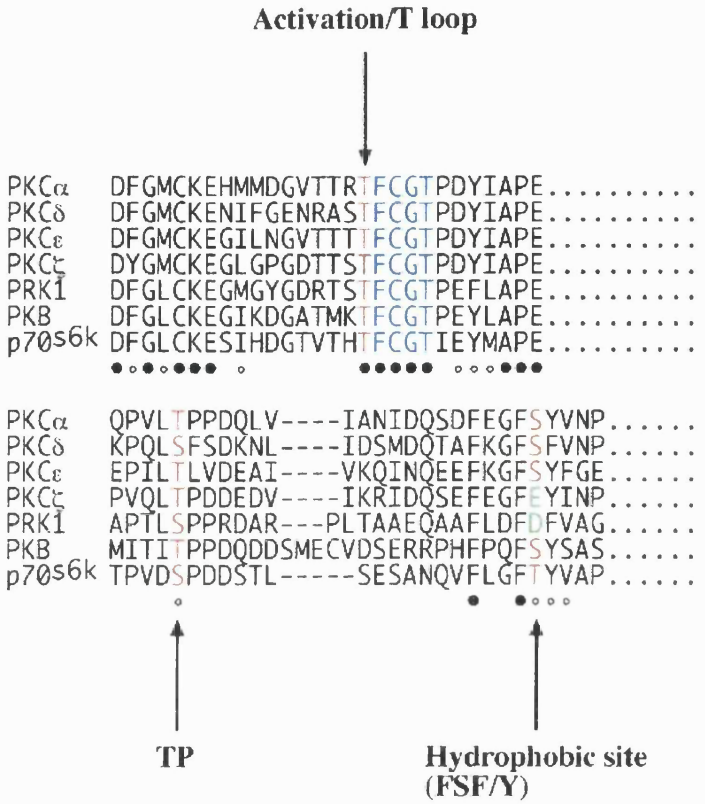


Figure 5. Sequence alignment of part of the catalytic domain illustrating conservation of the three sites amongst different members of the PKC family, PKB/Akt, and p70S6K.
The black circles indicate conservation of particular residues around the phosphorylation site, which define the motif. The two stretches of amino acids are separated by approximately 125 residues.

A possible model of phosphorylation at the T loop, TP and hydrophobic site in PKC is that following ligand binding at the plasma membrane, the T loop and hydrophobic sites become phosphorylated to generate an active kinase, which then autophosphorylates at the TP site. If DAG levels drop below a 'threshold' amount, the protein retains its phosphorylation at these sites, but switches to 'an active latent form' in which the PKC cannot phosphorylate its substrates due to autoinhibition. Once DAG levels rise again, it binds at the C1 domain to relieve autoinhibition and

allow access of substrates into the active site of the PKC. This final stage in the 'life' of PKCs is their downregulation.

PKC Isotypes	Activation Loop	Effect of No phosphate/ Ala Mutation	C-terminal Autophosphorylation	Effect of No phosphate/ Ala Mutation	C-terminal Hydrophobic	Effect of No phosphate/ Ala Mutation
Classical						
α	T497 TFCGT	Inactive	T638 TPPDQ	Inactivation-Sensitive	S657 FSYVN	Inactivation-Sensitive
β1(II)	T500 TFCGT	Inactive	T641 TPPDQ	*Inactive	S660 FSFVN	Lower relative Ca2+ Sensitivity
β2(I)	T500 TFCGT		T642 TPTDK	Inactive (Insoluble?)	S661 FSYTN	
γ	T514 TFCGT		T655 TPPDR		T674 FTYVN	
Novel						
δ	T505 TFCGT	Low Activity	S643 SFSDK	Low Activity	S662 FSFVN	Low Activity
ε	T566 TFCGT		T710 TLVDE	Low Activity	S729 FSYFG	Low Activity
η	T513 TFCGT		T655 TPIDE		S674 FSYVS	
θ	T538 TFCGT		S676 SFADR		S695 FSFIN	
Atypical						
ζ	T410 TFCGT	Low Activity	T560 TPDDE		E579 FEFIN	
ι	T403 TFCGT		T574 TPDDD		E555 FEYIN	

Table 3. Priming phosphorylation sites in the PKC superfamily
The amino acid number of the sites listed vary by one or two residues between different species. The available information on the effect of a lack of phosphate, or an alanine mutation at the priming phosphorylation site, on the catalytic activity is included.

*Residues flanking the T641 site in PKCβ2(I) can still be autophosphorylated, and compensate for the lack of phosphate at this site. When the flanking autophosphorylation sites are also mutated to alanine residues, the lack of phosphate at T641 results in an inactive protein.

1.8.6 Downregulation of PKCs

Persistently high DAG levels lead to eventual degradation of PKCs. If exposed to phorbol esters, a much less metaboliseable C1 domain-binding effector, certain PKC isoforms undergo rapid degradation, referred to as downregulation. Since levels of PKC in the cell are dynamically regulated, for a net loss of PKC to be observed, the downregulation pathway must exceed the synthetic pathway.

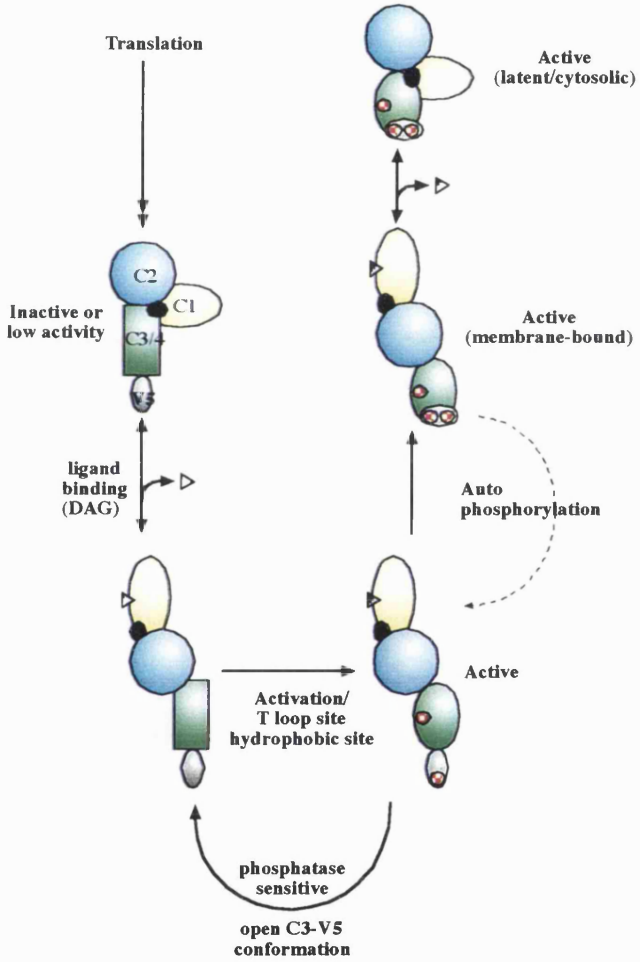


Figure 6. Illustrating a view of the PKC life cycle.
This incorporates findings from several sources including studies from this thesis. The three red-hatched circles depict the three phosphorylation sites and how, together with DAG binding can form an active PKC.

The PKC isoforms maybe degraded through a ubiquitin-proteosome mechanism if in the cytosol, or through a lysosomal-mediated process (Pryer et al., 1992). However, there does appear to be an isoform-specific mechanism for downregulation, and this may be attributed to the length of time these isoforms are at the plasma membrane, or bound to a particular structure which influences their probability of downregulation (Kiley et al., 1995; Olivier and Parker, 1992). Although cofactor binding, and hence kinase activity seem to be important for downregulation, catalytically inactive PKCα and PKCγ can still be downregulated, indicating that autophosphorylation is not critical for this mechanism (Pears and Parker, 1991a).

Studies on overexpressed PKC isoforms in yeast revealed that PKCε, which was not downregulated in a TPA-dependent manner, did become downregulated when coexpressed wih PKCγ or PKCδ. It seemed that catalytic activity of PKCγ or PKCδ promoted vesicle traffic and downregulation (Goode et al., 1995; Parker et al., 1995; Smart and Goode, 1998). The degradation of an activated membrane-bound form of PKCα, induced by vesicle trafficking from the plasma membrane to the endosomes was shown to proceed through a caveolin-dependent internalisation mechanism.

It was also demonstrated that PKCα retains the phosphorylation at the T loop and hydrophobic sites almost until it is degraded in a perinuclear vesicular compartment (Hansra et al., 1999; Prevostel et al., 2000). Furthermore, the dephosphorylation step, is mediated through the heterotrimeric type 2A protein phosphatases (Hansra et al., 1996; Hansra et al., 1999)

1.9 Aims and Objectives of this study

At the start of these investigations, classical and novel PKCs were already shown to be controlled by cofactor binding. However, it had become evident that PKCs are regulated by phosphorylation within the polypeptide. Despite this realisation, there was almost no understanding of the mechanisms involved in these events. The

objective of this thesis was to provide an understanding of these processes. To achieve this, I sought first to develop reagents (phospho-site-specific antisera) and a system suitable for analysis (chapter 3). These were then developed and used to analyse multiple regulatory inputs into one particular phosphorylation site (chapter 4). Subsequent studies investigated the model itself, providing insight into further regulatory inputs (chapter 5). Finally, I employed an expression system to assess the influence of these phosphorylations on cellular behaviour.

2.0 Materials and Methods

2.1 Materials

Chemical and radiochemicals

National Diagnostic	Acrylagel (ultra pure), Bisacrylagel (ultra pure)
BDH Laboratory supplies	Glycerol, Ethanol, methanol, Triton-X-100, Tween-20, microcystin, silver nitrate,
Calbiochem	Keyhole limpet hemocyanin,
Amersham Pharmacia Biotech	Radiochemicals, Horseradish peroxidase-coupled donkey anti-rabbit, anti-mouse IgG secondary antiserum
Schleicher & Schuell	Nitrocellulose membranes
Gibco BRL	G418/Neomycin, prestained protein markers, 1kb and 123bp ladder
Stratagene	FuGene transfection reagent

Sources of reagents used for pharmacological treatments are indicated in the box below. All other reagents were obtained from Sigma-Aldrich Ltd. Cell culture reagents were supplied by Gibco BRL and I.C.R.F media production services. Restriction enzymes and other DNA modifying enzymes were purchased from New England Biolabs, Promega, or Stratagene.

2.2 Methods

2.2.1 Generation of antisera

Peptides were coupled to keyhole limpet hemocyanin (KLH) beads to immunise rabbits. KLH beads (10 mg) were dialysed in 500ml of PBS buffer overnight. This was then mixed with 10mg of immunising peptide and 980 µl of PBS, and followed by a 15 minute incubation at room temperature, after a 10 and 5 µl addition of glutaraldehyde. PBS (18 mls) containing 200 µl of 1 M glycine was added and then aliquoted into 6 tubes. These were sent a rabbit immunising laboratory to be injected into rabbits for sera production.

2.2.2 Phosphorylation-site specific antisera

Site specific polyclonal antiserum was raised against the following phospho-peptides:

Isoform	Sequence	Motif
cPKCα	FEGFS(P)YVNP	FSY / S(P)657
nPKCδ	RAST(P)FCGT	T Loop / T (P)505
	PQLS(P)FSDK	TP / S (P)643
	FKGFS(P)FVNP	FSF / S(P)662
nPKCε	TTTT(P)FCGT	T Loop / T (P)566
	PILT(P)LVDE	TP / T710
	FKGFS(P)YFGE	FSY / S(P)729

All the sera were tested against phosphorylated and dephosphorylated forms of the immunising peptides. The sera showed some cross-reactivity for the

unphosphorylated peptide. Therefore all Western blotting analyses were performed in the presence of the cognate dephosphorylated peptide (dephospho-peptide) at 1 µg/ml

2.2.3 Total cell lysates

Transiently transfected cells were lysed in 4X SDS-sample buffer (See buffer list part 2.5), and briefly sonicated to shear genomic DNA.

2.2.4 Polyacrylamide Gel Electrophoresis

The protein samples were separated by molecular weight on a 10% SDS-PAGE using a 6% stack. Pre-stained protein standard markers were run in parallel.

2.2.5 Western Blotting

Proteins were transferred to nitrocellulose membranes for 1 hour at 200 millivolts and 0.4 amps. The membranes were blocked for 1 hour in Blotting Buffer buffer (See buffer list part 2.5) and then incubated with primary antibodies, diluted at 1/3500 in PBS-Tween-20, for 1 hour at room temperature, or for 16 hours at 4°C. They were washed for three 5-minute intervals, in PBS-Tween-20, and then incubated for 1 hour at room temperature with horseradish peroxidase-coupled donkey anti-rabbit IgG secondary antiserum, diluted at 1/5000 in PBS-Tween-20. Finally, the membranes were washed again as described above. The Western blots were developed using ECL™

2.2.6 Transfection and cell culture

2.2.6.1 HEK/293 cells

HEK/293 cells were grown on 90 mm dishes to around 70% confluency in 10% foetal bovine serum (FBS) in DMEM in a humidified 10% CO_2 incubator at 37°C. For each transfection, 5 µg of DNA was mixed with 500 µl of 0.4 µm filtered 0.25 M $CaCl_2$ and 500 µl of 2XBBS solution and then incubated at room temperature for 20 minutes. After the incubation, the calcium chloride-DNA-BBS solution was added drop-wise to the culture medium and the cells left in a humidified 5% CO_2 incubator at 37°C for between 16-to-24 hours. Following this, the cells were washed twice with phosphate buffered saline (PBS) and maintained in 10% FBS in DMEM, for a further day. After this, the cells were trypsinised, washed with PBS and then resuspended in DMEM to give a cell suspension of around a $1X10^6$ cells/ml. The cells were maintained in suspension for 24 hours in a polypropylene tube at approximately $1X10^6$ cells/ml in a humidified 10% CO_2 incubator at 37°C for serum starvation.

2.2.6.2 Nutrient deprivation of HEK/293 Cells

Transfected HEK/293 cells were serum starved for 24 hours in suspension, as before, and then the cells were washed once in PBS followed by incubation in an amino acid-free media for 2.5 hours. Amino acid deprived cells were then stimulated with dialysed 10% FBS either in the presence or absence of amino acids or leucine alone as indicated.

2.2.6.3 UM-UC-3 cells

Wild type UM-UC-3 cells or the UM-UC-3 PTEN-stable transfectants were grown on a 90mm dish to around 70% confluency in 10% FBS in DMEM in a humidified 10% CO_2 incubator at 37°C. The UM-UC-3 PTEN-stable transfectants were grown additionally in the presence of 400 µg/ml of G418 (Neomycin). For transfections, the medium was removed from the dish and replaced with 5 mls of Optimem and then left for 30 minutes in a humidified 10% CO_2 incubator at 37°C. For each transfection, 600 µl of Optimem was mixed with 18 µl of FuGene reagent and left for 20 minutes. Following this period, 20 µg of DNA was added to it and left for a further 10 minutes, before adding to the cells. The dish was then left in a humidified 10% CO_2 incubator at 37°C for 16 hours. Following this, the medium was replaced with 10% foetal bovine serum in DMEM and the dish was put back into the incubator for 24 hours. Following this period, the cells were either, trypsinised and maintained in suspension in DMEM without serum in suspension at approximately $1X10^6$ cells/ml, or maintained adherent in the presence or absence of serum or inhibitors as indicated.

2.2.6.4 Generating stably transfected epsilon mutant cell lines in epsilon knock out mouse embryo fibroblast (MEF) cells.

PKCε knock-out MEF cells ($PKC\varepsilon^{-/-}$ MEFs) were grown on a 90 mm dish to around 70% confluency in 10% FBS in DMEM in a humidified 10% CO_2 incubator at 37°C. The cells were transfected as described for UM-UC-3 cells. Selection was performed by maintaining the cells in 10% FBS in the presence of 200 µg/ml of hygromycin B. The media was replaced every two days, until clones were isolated.

2.2.7 Activation of integrin signalling

Integrin signalling was stimulated by either A, replating the suspension cell cultures on specific substrates, or B, through the cross-linking of selective β1-integrin-bound, anti β1-integrin monoclonal antisera.

A, poly-D-lysine and collagen were used as non-specific and specific extracellular matrices respectively. An aliquot of 3 mls, from a 50 μg/ml (in PBS) solution of either matrix, was added to 2 cm dishes and left overnight at 4°C. Following this, the medium was removed and the plate was gently washed with PBS before an aliquot of 2 mls of serum starved cells in suspension was added to it. At the times indicated, the cells were treated and then harvested in 4X SDS-sample buffer.

B, An aliquot of 500 μl from a serum starved cell suspension culture was incubated with 20 μg/ml of anti-β1 integrin antiserum for 20 minutes at 4°C. The cells were gently washed once with warm DMEM (37°C), and then incubated with warm DMEM containing 10 μg/ml of anti-mouse IgG (Fc-specific) cross-linking antisera for the times indicated. The whole cell lysates were then analysed by immunoblot as described earlier.

2.2.8 Immunoprecipitation and kinase activity determination

2.2.8.1 Immunoprecipitation

Myc-immunocomplexes were prepared by lysing the cells on ice with 400 μl of ice-cold lysis buffer (See buffer list part 2.5). Insoluble material was removed after incubation with 40 μl of Protein-A Sepharose for 10 minutes at 4°C and centrifuged for 5 minutes (12,000g). The supernatants were incubated with 4 μg of anti-myc

antibody at 4°C for 20 minutes, followed by 20 µl of Protein-G Sepharose beads at 4° C for 60 minutes. The beads were washed twice with lysis buffer, then once with lysis buffer containing 0.1% Triton-X 100.

2.2.8.2 Determination of nPKC catalytic activity

Myc-immunoprecipitated PKCδ or PKCε (10 µl bead slurry) was incubated with 25 µl of a Master mix reaction mixture (See buffer list part 2.5). This was incubated with 5 µl of ATP Mix (See buffer list part 2.5) for the time period indicated at 25° C. The reaction was stopped with 4 µl of 4X Sample buffer (See buffer list part 2.5), and the proteins were separated on a 12.5% SDS-PAGE gel. Following this, the gel was stained with Coomassie-Brillant Blue buffer (See buffer list part 2.5), and the MBP activity quantified by cutting out gel pieces and Cerenkov counting. Specific activities were determined by scanning stained PAGE gels to quantify PKCδ and expressing kinase activity as a function of this quantity, in arbitrary units.

2.2.8.3 Coomassie Staining of SDS-polyacrylamide gels

The gels were incubated with Coomassie-Brillant Blue buffer (See buffer list part 2.5) for 30 minutes and then destained in Destain (See buffer list part 2.5) over night to visualise the myelin basic protein and immunoprecipitated PKCδ or PKCε.

2.2.9 Pharmacological Treatment

A 1ml aliquot of a serum starved cell suspension was incubated with one of the agents listed below, for either 30 minutes, or the time indicated in the legends. The cells were then stimulated for 30 minutes with 100 µl FBS and then harvested using

4X sample buffer and analysed for PKCδ, or ε, protein load and phosphorylation by Western blotting.

Agents	Source	Concentration
LY294002	Calbiochem	10 μM
Rapamycin	Calbiochem	20 nM
Bisindolylmaleimide-I	Alexis	10 μM
Go6983	Sigma	1 μM
Okadaic acid	Alexis	1 μM
Calyculin	Calbiochem	10 nM
Phorbol 12-myristate 13-acetate	Sigma	100 nM
PDGF-BB	R&D Systems	30 ng/ml
LPA	Sigma	10 μM
Calphostin-C	Calbiochem	200 nM
Compound 48/80	Sigma	5 μg/ml

2.3 FACS Sorting for cell cycle and Sub G1 DNA content determination

This was achieved by propidum iodide staining. The cells were washed with PBS, treated to trypsin, and pelleted. They were resuspended in cold 70% ethanol and then incubated at 4^0C for 30 minutes to fix. The cells were washed three times in PBS, treated with 100 μg/ml RNase for 15 minutes at room temperature and then washed once in PBS. Finally, the cells were resuspended in 50 μg/ml of propidium iodide and then analysed by flow cytometry. The propidium iodide fluorescence was measured above 600 nm.

2.4 Molecular biological techniques

2.4.1 Generation of chemically competent DH5α for transformation

A single colony of DH5α cells was inoculated in 2.5 mls of LB media and incubated over night at 37°C. This was then used to inoculate 250 mls of LB to grow to an OD_{600} of 0.4-0.6. The cells were harvested by centrifugation at 4500g for 5 minutes, and then resuspended in filtered, ice-cold, TFB-I (See buffer list part 2.5). Harvesting the cells again by centrifugation, and then resuspending them in filtered, ice-cold, TFB-II (See buffer list part 2.5) followed this. The cells were aliquoted in 500 µl, and rapidly frozen in a dry ice and ethanol mix.

2.4.2 Polymerase chain reactions and agarose gel electrophoresis

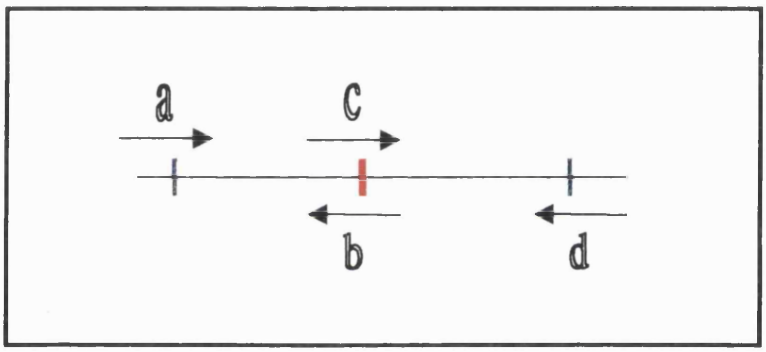
The reactions were performed using 50 µg of DNA template, 100 pmoles of each of the primers, 5 µl of 10X Pfu buffer, 0.25 mM of each of the four dNTP's, and sterile water to a total volume of 50 µl. Finally, 2.5 units (1 µl) of Pfu DNA polymerase was added to the reaction mix, and a drop of mineral oil put on top. The tubes were placed in a Biometra thermal cycler and the reaction cycled as follow:

Cycle 1.	5 minutes at 95°C
Cycle 2.	30 seconds at 95°C
	30 seconds at 55°C
	3 minutes at 68°C
	Repeated 35 times
Cycle 3.	30 minutes at 68°C

The reaction was stopped with 5 μl of 10X DNA gel-loading buffer, and separated on a 1.2% agarose gel in 1X TAE buffer.

2.4.3 Generating the mutant PKCε

The strategy for generating the mutations was developed from work described by .Ho,S,N and co-workers (Ho et al., 1989). The red line indicates the site to be mutated, and while the two blue lines are unique restriction sites to allow cloning back into the cDNA.



Three PCR reactions were performed, firstly between primers a+b, and primers c+d. The product of these reactions were resolved on a 1.2% agarose gel by electrophoresis, purified using the Qiagen QIAquick gel extraction kit, and mixed together for a final PCR reaction. The product of this was ligated into EcoRV-cut pBluescript™ and identified by the blue/white colour assay (see below).

The primers used for the alanine and glutamate mutants of the FSY site are as follows:

Sense FEY primer (c):

GAATTCAAAGGCTTCGAATACTTTGGTGAAGACCTGATGCCC

Antisense FEY primer (b):

GGGCATCAGGTCTTCACCAAAGTATTCGAAGCCTTTGAATTC

Sense FAY primer (c):

AACCAGGAAGAATTTAAAGGCTTCGCATACTTTGGTGAA

Antisense FAY primer (b):

TTCACCAAAGTATGCGAAGCCTTTAAATTCTTCCTGGTT

5' primer (a) using a unique *Xba1* site:

AACATCCTTCTAGATGCAGAAGGC

3' primer (d) using a unique *Xho1* site:

ACTCGAGCGGCCGCCACT

For the T / activation loop glutamate mutant the following primers were used:

Sense T loop primer (c):

AATGGTGTGACAACTACCGAATTCTGTGGGACTCCTGACTAC

Antisense T loop primer (b):

GTAGTCAGGAGTCCCACAGAATTCGGTAGTTGTCACACCATT

5' primer (a) using a unique *Bst B1* site:

GACGACTTGTTCGAATCCATC

3' primer (d) using a unique *Xba1* site:

GCCTTCTGCATCTAGAAGGATGTT

2.4.4 Ligation reactions

Ligation reactions were performed in 10 µl at 16°C over night, using T4 DNA ligase and T4 DNA ligase buffer. A 3:1 molar ratio of insert to vector was calculated using the following expression:

$$\frac{3X(50ng \text{ of template } X \text{ kb size of insert})}{\text{kb size of vector}}$$

For blue/white colour selection assays, the pBluescript™ SK (+) vector was used.

2.4.5 Transformation of DH5α for blue/white colour selection

An aliquot of 50 µl of competent DH5α cells were mixed with 4 µl of the ligation reaction mix, and incubated in ice for 30 minutes. The cells were 'heat shocked' at 42°C for 40 seconds, placed back on ice for 5 minutes, and then mixed with 1 ml of

LB media and incubated at 37°C for 1 hour. A 200 µl aliquot of this bacterial inoculant was spread on to an LB plate containing 100 µg/ml of ampicillin, 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and 40 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), then incubated at 37°C over night.

White colonies (recombinant) were picked using a sterile tip and grown in 1 ml LB media over night to be checked by miniprep analysis. The extracted DNA was checked for the presence of the insert that was cloned into the pBluescript™ vector. When the clone, containing the recombinant pBluescript™ vector was identified, it was mini-prepped, using the Qiagen mini prep kit, from an over night 5 mls culture grown in LB media, and then sequenced (see below) to confirm the presence of the mutated site.

2.4.6 Sequencing the mutant DNA in pBluescript™ vector

For each reaction 0.5 µg of DNA template was mixed with 3.2 picomoles of sequencing primer 8 µl of Terminator Ready Reaction Mix and made up to 20 µl with sterile distilled water. The PCR products were purified by means of ethanol precipitation for ABI sequencing by adding 80 µl of sterile distilled water, 300 µl of 95% ethanol (BDH), and 7.5 µl of 2M sodium acetate (pH4.5) to it. This was incubated at 21°C for 15 minutes, and then centrifuged at maximum setting for 20 minutes. After removing the supernatent, the pellet washed with 300 µl of 70% ethanol, and then centrifuged again for 5 minutes. The supernatant was removed and the pellet dried. This was then sequenced using the ABI sequencer.

2.4.7 Cloning the mutant DNA into the wild type PKCε cDNA

Both the recombinant pBluescript™ vector and the PKCε cDNA, in the pcDNA3.1/Hygro+ vector (Invitrogen), were cut with the same pair of restriction endonucleases. They were resolved on a 1% agarose gel by electrophoresis, cut out and purified as described earlier. Subsequently, a ligation reaction was set up between the PKCε cDNA in the pcDNA 3.1/Hygro+ vector, and the mutated insert from the pBluescript™ vector. This was later transformed into DH5α bacterial cells and plated on to ampicillin-containing LB plates, as described earlier. Colonies were picked the next day for mini prep analysis to identify the recombinant PKCε cDNA in the pcDNA 3.1/Hygro+ vector clones. These were maxi-prepped by using the Qiagen™ maxiprep kit and checked for purity by dividing the absorbance measured at 260 nm by 280 nm. Subsequently, the concentration was determined from the product of the absorbance at 260 nm and 50 μg/ml.

2.4.8 Colony growth in soft agar

A feature of transformed cells is their capacity to grow in soft agar and form colonies. We used this approach to determine whether the PKCε knock-out MEF cells (PKCε$^{-/-}$ MEFs) alone, or when stably transfected with either the wild-type PKCε or the phosphorylation-site specific mutants, display this transformation phenotype. PKCε$^{-/-}$ MEF cells, with or without the stable expression of PKCε (or a mutant of PKCε), were grown on a 90 mm dish to around 70% confluency in 10% FBS in DMEM (with 200μg/ml hygromycin) in a humidified 10% CO_2 incubator at 37°C. The cells were trypsinised and then resuspended in fresh media to a concentration of 1X10^4 cells/ml. A 1ml aliquot of this cell suspension was mixed with 5mls of a 0.17% agarose solution (0.17% agarose melted in PBS), to give a

final cell suspension of $1.7X10^3$ cells/ml, and an agarose concentration of 0.142%. The soft agar-cell suspension mixture was transferred into a 15ml polypropylene tube, placed on ice for 5 minutes and finally incubated in a humidified 10% CO_2 incubator at 37°C for 3 weeks. To detect the presence of colonies in the soft agar media, the agar solution was gently pressed flat between two cell culture dishes and examined under the microscope at X10 magnification. Colonies were defined by the presence of a group of approximately 50-100 cells when examined under the microscope.

2.5 Buffers

4X SDS-Sample Buffer

0.25 M Tris base, 8% SDS, 10% glycerol, 1.54% DTT, 12%,urea, and 10mM EDTA.

Lysis buffer

20 mM Tris-HCl pH 7.5, 2 mM EDTA, 10 mM benzamidine, 0.2 mM PMSF, 15 µg/ml leupeptin, 100 µg/ml aprotinin, 100 nM okadaic acid, and 1% Triton-X 100

Blotting Buffer

2% BSA (w/v), dissolved in 0.1% (v/v) Tween-20 in PBS (PBS-Tween-20)

2XBBS

50 mM *N, N,-bis* (2-hydroxyethyl)-2-aminoehtanesulfonic acid, 280 mM NaCl,1.5mM Na_2HPO_4, pH6.95

Master mix reaction mixture

0.2 mg/ml myelin basic protein, 10 mM $MgCl_2$, 20 mM Tris HCl pH 7.5, and 2.5 µl of lipids (0.5 mg phosphatidylserine, 0.5 µg TPA, dried down and resuspended in 100 µl of Tris HCl pH7.5, and 1% Triton X-100

ATP Mix

200 µM ATP, 10 µCi/ml of [γ-^{32}P]

Coomassie-Brillant Blue buffer

0.1% w/v Coomassie-Brillant Blue, 10% acetic acid, and 50% methanol

Destain Buffer

10% acetic acid, and 50% methanol

TFB-I

30 mM potassium acetate,10 mM calcium chloride, 50 mM manganese chloride,100mM rubidium chloride, and 15% glycerol, adjusted to pH 5.8 with glacial acetic acid

TFB-II

10 mM PIPES, 10 mM calcium chloride, 100 mM rubidium chloride, and 15% glycerol, adjusted to pH 6.5 with 1 M potassium hydroxide

10X DNA gel loading buffer

0.4% Bromophenol blue (w/v), 25% Ficoll [Type-400] (w/v), 1 mM EDTA

1X TAE buffer

0.48% Tris- Base (w/v), 0.1% glacial acetic acid, 50mM EDTA

3.0 Characterisation and preliminary use of phospho-site directed antisera to PKCδ and PKCε.

3.1 Introduction

Investigations aimed at studying the three phosphorylation sites in the catalytic domain of the classical PKC isotypes, PKCα and PKCβ2, established some key principles concerning their regulation and function, (Bornancin and Parker, 1996; Bornancin and Parker, 1997; Cazaubon et al., 1994; Cazaubon and Parker, 1993; Filipuzzi et al., 1993; Garcia-Paramio et al., 1998; Gysin and Imber, 1996; Hansra et al., 1996; Hansra et al., 1999; Keranen et al., 1995; Pears et al., 1992). Based on these studies, predictions were made on the equivalent sites present in the novel class of PKC isoforms, but these predictions still had to be tested. Several options were available to try and investigate the phosphorylation sites in PKCδ and PKCε, two members of the novel class of PKC isoforms. They include:

mass spectroscopy;
in vivo ^{32}P labelling of PKC-expressing cells (wild type and mutants), and attempting to identify the presence or absence of spots on an autoradiograph;
using phosphorylated serine or threonine-specific antisera

The use of phospho-specific antisera was most practical for a number of reasons. It did not require the production of microgram quantities of protein, the use of large quantities of ^{32}P isotope, or phosphopeptide mapping.

Having to generate large quantities of protein for each experiment can be cumbersome, time consuming, and can lead to the introduction of many additional variables due to the possibility of degradation. Phosphopeptide mapping can be difficult to interpret, especially if the effects of reagents are subtle. Using antisera

has many advantages, such as better reproducibility, a relatively simple technique, i.e. Western Blotting, to analyse samples, and it provides a reagent for further studies, i.e. direct in situ analysis.

Antisera were raised to the three predicted phosphorylated sites to examine occupancy, and which signalling pathways might be responsible for inducing them *in vivo*. Initially, the objective was to establish the optimum conditions for using these antisera so that only the phosphorylated sites are detected during Western Blotting. The questions that are addressed in this chapter relate to characterisation of antisera, defining a system, and determining whether diacylglycerol or PI 3-kinase influences the phosphorylation of these sites. The general issues are:

Characterisation of antisera

Can an excess of the phospho-peptide, to which the antiserum was raised, i.e. the immunogenic peptide, compete out the signal from the protein, phosphorylated at that site? Will the presence of dephospho-peptide, mixed with the phospho-specific antiserum affect the signals?

Defining the system

What are the optimum, and most reproducible, conditions for achieving low basal phosphorylations at the observed phosphorylation sites when the protein is over-expressed transiently in cells? Does serum deprivation in suspension, or cell density in suspension, have an effect? Furthermore, what is the minimum time requirement for serum deprivation? From a low basal state, can we detect sites becoming phosphorylated following stimulation of the cells with an appropriate agonist, and how quickly does this occur? Are the phosphorylated sites, detected following stimulation, a consequence of newly synthesised protein and not the unphosphorylated sites becoming rephosphorylated?

Does diacylglycerol (DAG) affect the phosphorylation of these sites?

A competitive inhibitor to the DAG-binding site, Calphostin-C (Kobayashi et al., 1989), was used to define the affect of DAG binding, at the C1-domain and the subsequent conformational changes, on these sites.

How do the effects/phosphorylations observed relate to the characterised DAG input?

Does a PI 3-kinase pathway influence the phosphorylation of these sites?

Ann Le Good had demonstrated earlier that PDK-1 would phosphorylate the T loop site in a number of PKC isoforms. The question of whether PDK-1 phosphorylates the T loop site in nPKC isoforms under *in vivo* conditions was addressed.

3.2 Results

3.2.1 Characterisation of antisera

Initial Western Blots of the PKCδ and PKCε hydrophobic site phosphorylation were probed using antisera raised to peptide sequences corresponding to the phosphorylated hydrophobic site in PKCα. This was possible due to high sequence homology that exists between the isoforms at these sites (Figure 1). Antisera were later raised to the phosphorylated forms of the hydrophobic sites designed specifically for the two isoforms.

An initial experiment was performed comparing the three predicted sites in PKCδ, using protein derived from three different sources. A bacterially expressed, GST-PKCδ purified fusion protein, and two whole cell lysates from transiently transfected HEK/293 cells. One of the whole cell lysates was from cells deprived of serum for 24 hours; the other was maintained in 10% FBS in DMEM throughout this

period (Figure 2). The bacterially expressed GST-PKCδ fusion protein appears only to be phosphorylated at Ser 643 (the TP site), but not at Thr 505 (the activation/T loop site), or Ser 662 (the hydrophobic site). There is also no change in the phosphorylation at Ser 643 in the samples comparing the two whole cell extract samples.

Isoform	T / Activation loop site	Residue number
PKCα	**TTRTFCGTP**	Thr 497
PKCδ	**RASTFCGTP**	Thr 505
PKCε	**TTTTFCGTP**	Thr 566
	TP site	
PKCα	**PVLTPPDQL**	Thr 638
PKCδ	**PQLSFSDKN**	Thr 643
PKCε	**PILTLVDEA**	Thr 710
	Hydrophobic site	
PKCα	**FEGFSYVNPQ**	Ser 657
PKCδ	**FKGFSFVNPQ**	Ser 662
PKCε	**FKGFSYFGED**	Ser 729

Figure 1. Comparison of the three sites in three different PKC isoforms in rat

The Thr 505 and Ser 662 sites are only clearly phosphorylated when lysates are obtained from cells maintained in 10% FBS in DMEM. Serum deprivation of HEK/293 cells, expressing PKC δ for 24 hours, resulted in a reduction of phosphorylation at these sites. The faint bands that appear in the lanes for GST-PKC δ fusion protein from bacteria, and from serum deprived HEK/293 whole cell lysates, at the Thr 505 and Ser 662 sites could represent a number of possibilities. They could be a result of detection of an unphosphorylated site due to the presence of dephospho-specific antibodies in the antisera, or non-specific detection, perhaps due to over exposed Western Blots.

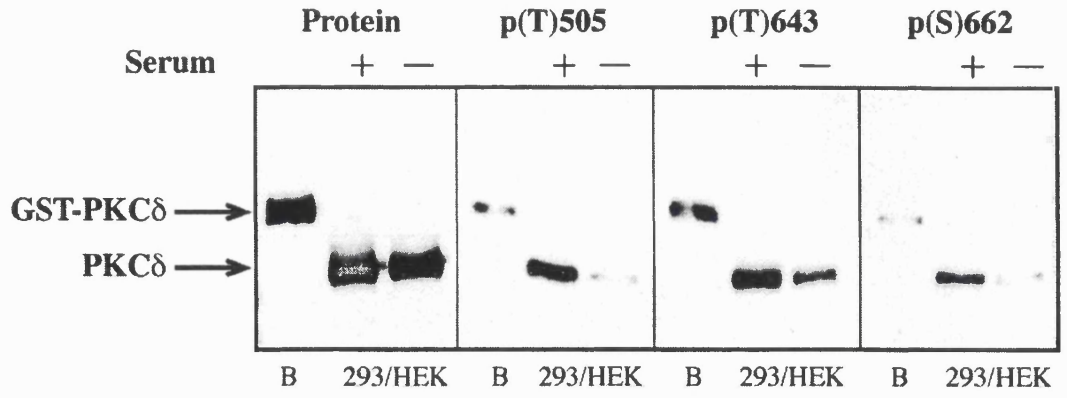


Figure 2. Serum-dependant phosphorylation of the T505 and S662 sites, but not T643 site.
PKCδ was transfected into 293/HEK cells (293/HEK) and either maintained in serum (+), or serum deprived (-) for 24 hours. PKCδ was also transfected into BL21 (DE3) bacterial cells as a GST-linked fusion protein (B) as a negative control. Extracts were analysed by Western Blotting for PKCδ protein expression and for phosphorylation at the T505, T643 and S662 sites as described in Chapter 2.

The T505 and S662 sites in PKCδ are only clearly phosphorylated when expressed in 293/HEK cells maintained in serum. These sites are not phosphorylated when expressed in bacterial cells. Dr Ben Webb verified this in the laboratory. Analysis of GST-PKCδ, expressed in bacteria by mass spectroscopy, revealed the presence of the phosphorylated S643 site.

The T643 site of PKCδ appears to be phosphorylated in 293/HEK cells in the absence of serum and when expressed in bacteria as a GST-PKCδ fusion protein. It was found that the anti-phospho Ser 657 antisera (PKCα hydrophobic site, PPA 168 antisera) were competed when incubated in the presence of 1μg/ml, or more, of PPA168 immunising phospho-peptide. This was true for the hydrophobic sites in both PKCδ and PKCε (Figure 3).

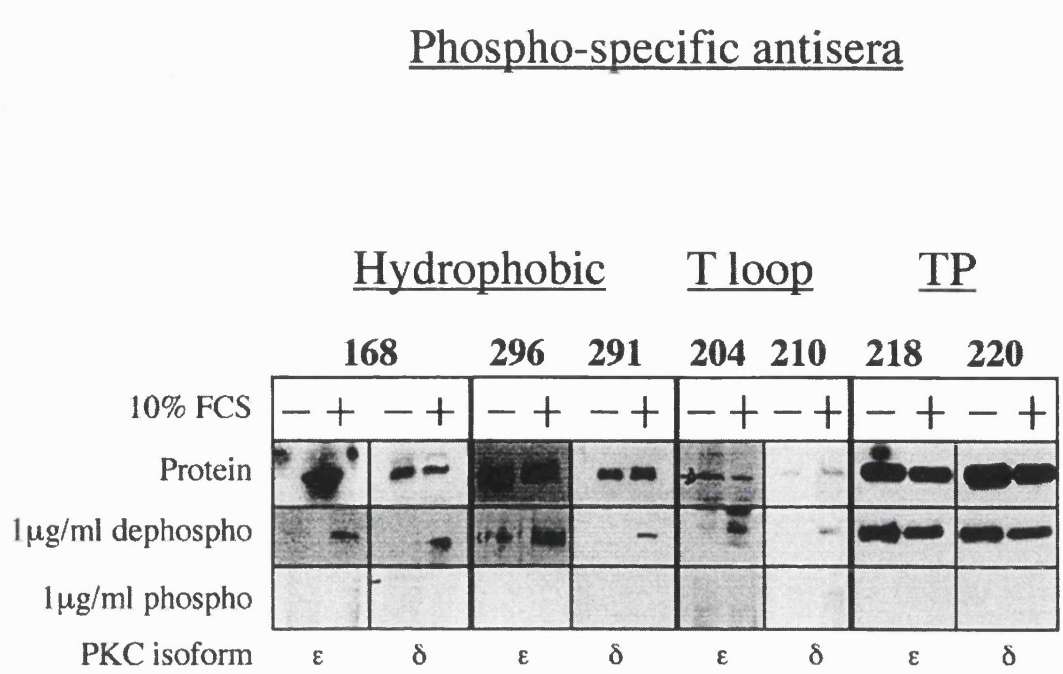


Figure 3. Competition analysis of phospho-specific antisera against the hydrophobic, T loop and TP sites in PKCδ and PKCε.
Polyclonal antisera were raised to phosphorylated peptides that corresponded to each of the predicted phosphorylation sites in PKCδ and PKCε. They were tested against samples extracted from 293/HEK whole cell lysates which were either maintained in serum (+10% FCS), or deprived of serum for 24 hours (-10% FCS), as positive or negative controls respectively. The Western blots were incubated with primary antisera in the presence of 1μg/ml of either the phospho-peptide or dephospho-peptide for determination of competition.

Western blots probed with the PPA168 antiserum alone often resulted in a high basal. It was predicted that the high basal signals could be due to either the binding of excess antibodies to the dephospho-epitope, or the presence of antibody specific for the dephospho-epitope, as discussed earlier. To overcome this, membranes were probed with antisera in the presence of an excess of the dephosphorylated derivative of the immunising PPA168-phospho-peptide, i.e. the PPA168-dephospho-peptide. At 1μg/ml of dephospho-peptide, the high basal was reduced further while the signals resulting from the positive controls remained high.

Identical studies were also carried out with the anti-phospho Ser 662 (PPA291) and anti-phospho Ser 729 (PPA291) antisera (specifically relating to the hydrophobic sites in PKCδ and ε respectively). The signal from the serum-stimulated sample, using the PPA 291 or 296, was competed fully by their respective immunising phospho-peptides at 1μg/ml. It appeared that the non-specific signal was reduced when the blots were incubated with anti sera in the presence of 1μg/ml of the dephospho-peptide.

The anti-phospho activation/T loop antisera for PKCδ and PKCε (PPA 210 and 204 respectively) were also fully competed in the presence of 1μg/ml of the immunising phospho-peptide. The dephospho-peptides, at a concentration of 1μg/ml, were able to reduce much of the signal suspected to be originating from the detection of unphosphorylated epitope on the Western blot (Figure 3). This was consistent with the properties determined for the anti-phospho TP site antisera for both PKCδ and PKCε (PPA 220 and 218 respectively) also (Figure 3). Therefore all subsequent Western blots were probed with antisera in the presence of 1μg/ml of the respective cognate dephospho peptides. These findings are representative of several experiments performed.

3.2.2 Defining the system

Characterising the specific antisera against their respective phosphorylated and dephosphorylated cognate peptides allowed their specificity to be optimised. In order to maximise the signal from the phosphorylated site being examined, it was also important to investigate which cell culture treatment would give the most reproducible phosphorylation signal. Though much of the data has been illustrated for PKCδ, similar findings were also made for PKCε.

There was considerable variability observed in achieving a low basal level of phosphorylation, at the T loop and hydrophobic sites, when cells were deprived of

serum while adherent. Several other members of the laboratory had also noticed this. This variability was not thoroughly investigated because it was observed that cells deprived of serum for 24 hours, while in suspension, always led to a low basal level of phosphorylation at the T loop and hydrophobic sites. Interestingly, the phosphorylation at the TP site did not change after any of these treatments. However, if the serum-starved cells are treated with the PKC catalytic inhibitor (BIM-1), the TP site does become dephosphorylated, suggesting that it is autophosphorylated (see chapter 4).

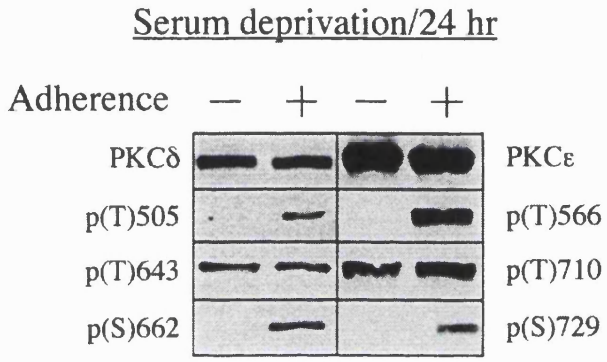


Figure 4. Effect of adherence on serum deprivation of the three sites in nPKC HEK/293 cells were transfected with either PKCδ or PKCε in duplicate. One each of the duplicates were deprived of serum in suspension, the other was deprived while still adherent, for a total of 24 hours. Western Blotting monitored expression and phosphorylation of the T loop, TP and hydrophobic sites of two isoforms.

It was important to determine the minimum time needed to deprive the suspension cells of serum to consistently achieve low basal phosphorylation levels at these sites. Both the phosphorylated Thr 505 and Ser 662 sites in PKCδ needed a minimum of 24 hours of serum deprivation, while in suspension, before an essentially complete loss of phosphorylation at the T loop and hydrophobic sites was observed (Figure 5a). The treatment that would give the most robust phosphorylation signal was then investigated. Serum gave a modest phosphorylation at the Thr 505 and Ser 662 sites after 20 minutes treatment, which was most obvious by 30 minutes.

Figure 5a

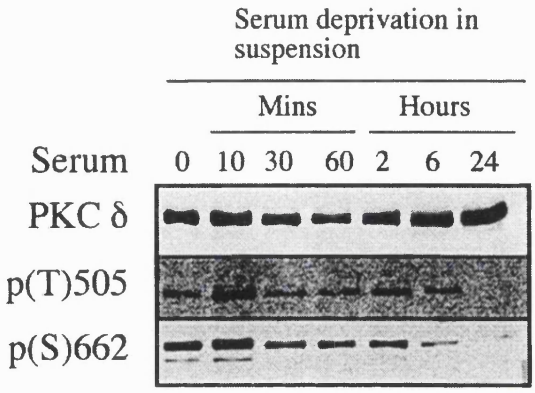


Figure 5b

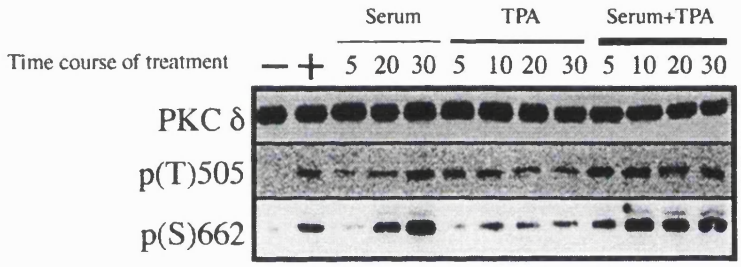


Figure 5. Time course of serum deprivation and stimulation

a. Time course of serum deprivation of 293/HEK cells.
b. Time course of stimulation with TPA and serum.

a. HEK.293 cells were transfected with PKCδ and deprived of serum while in suspension. Aliquots were removed at various times and analysed as described below.

b. The effect of combining serum with TPA on the time-dependent phosphorylation of PKCδ at T505 and S662 was then examined. Cultures of serum deprived HEK/293 cells were either stimulated with serum (10%), TPA (100nM), or both, for the time (minutes) indicated. Also included is a sample from an untreated cell culture and serum-stimulated cell culture, as a negative control (-) and a positive control (+), respectively. The extracts were analysed for PKCδ protein expression and for phosphorylation at the T505 and S662 sites by Western blotting.

TPA had little effect on the phosphorylation at the T loop and hydrophobic sites, even after 30 minutes. However, after only 5 minutes treatment with both serum and TPA, there was clear phosphorylation at the Thr 505 and Ser 662 sites, which

seemed to be maximal by 10 minutes. A further 20 minutes was required during serum alone treatment to reach the same level (Figure 5b)

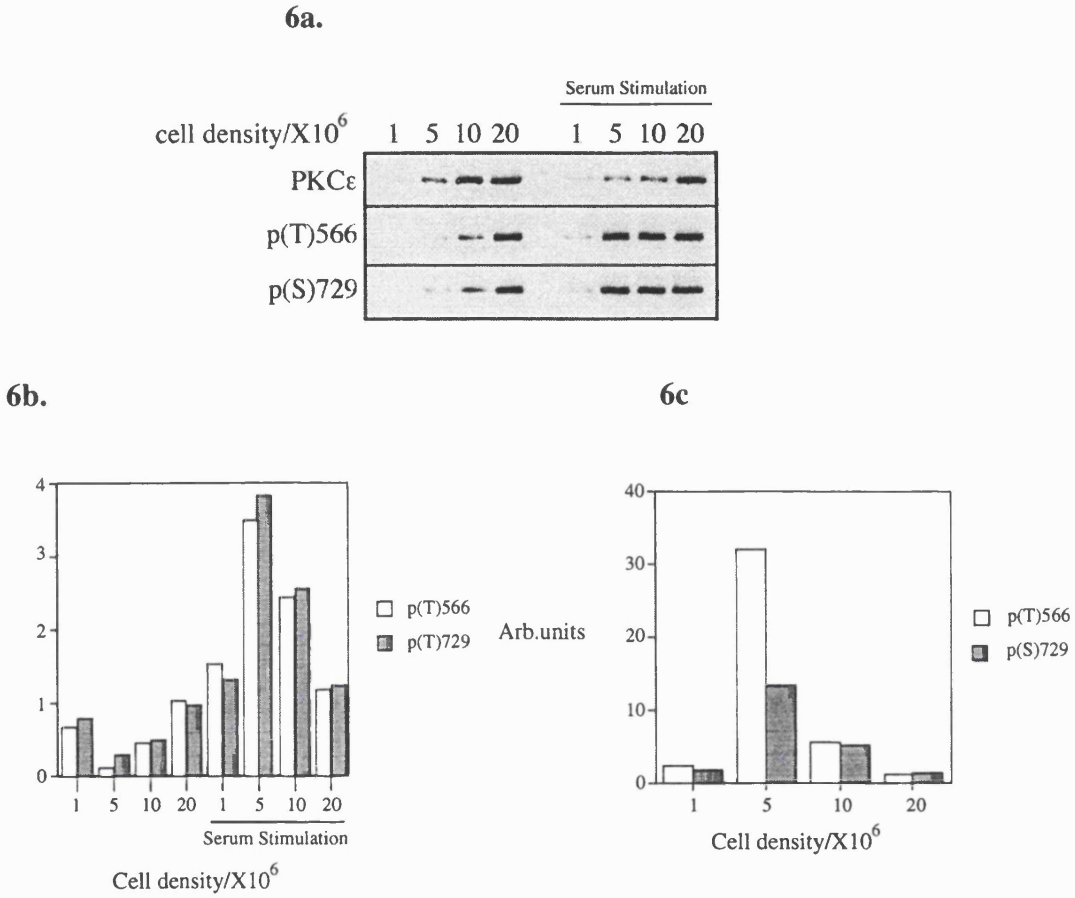


Figure 6. Detection of serum-induced phosphorlyation of PKCε is dependent on cell density while in suspension

HEK/293 cells transfected with PKCε, were counted to establish four different cell density suspension cultures. They were deprived of serum as described in Chapter 2. The effect of varying cell number on both the efficiency of serum deprivation in suspension and serum-induced phosphorylation of the T566 and S729 sites was then determined by Western blotting, 6a. The bands representing the serum-induced phosphorylation of the T566 and S729 sites were expressed as a function of the respective protein load, 6b, and also as a function of the respective unstimulation site (6c), to determine which cell density leads to the greatest increase in serum-induced phosphorylation.

Another factor investigated was the cell number in suspension. We consistently observed a strong signal from a cell culture, deprived of serum while in suspension,

at a density of 10 million cells per ml of media ($10X10^6$ cells/ml). Following serum stimulation, there was not as big an increase in the intensity of the phospho-specific bands as that observed for a cell density of $5 X 10^6$ cells/ml (Figure 6a, 6b).

Since the cognate dephospho-peptide was included during Western blotting, it was suspected that the observed signal was due to the detection of the phosphorylated site. In the cultures containing high cell densities, clumps of cells could be observed. These cell-cell interactions may have been sufficient to trigger a signalling pathway leading to the phosphorylation of PKCδ. This is consistent with the notion of the initiation of some signalling pathways following cell-cell interactions (Dunant and Ballmer-Hofer, 1997; Lampugnani and Dejana, 1997).

There appeared to be many fewer cell clumps in the cell culture with $5X10^6$ cells/ml, which maybe reflected in the low basal level of phosphorylation detected. However, if the suspension cell density was less than $1 X 10^6$ cells/ml, there was an insufficient amount of material for reliable detection from these cultures. This was true for both the anti-phospho Thr 556 and Ser 729 sites, using PPA 204 and 291 respectively (Figure 6a).

If a greater volume was harvested from the cell culture containing the lowest cell density, it is possible that this would have the greatest serum-induced increase in phosphorylation at the sites being examined. This is because there is likely to be fewer cell-cell interactions in this culture. However, the larger volume of cell culture that would have to be handled, would be impractical and inefficient. A density of $5X10^6$ cells/ml clearly gave the greatest increase in phosphorylation following stimulation, as a proportion of the unstimulated sample, and as a function of the protein load (Figure 6c).

Since the phosphorylation at the Thr 505 and Ser 662 sites can be detected within 5 minutes following treatment with serum and TPA, it suggests that these phosphorylations are not a consequence of new protein being synthesised following

stimulation. To clarify this, the serum deprived cells were pre-incubated for 30 minutes with the protein synthesis inhibitor, cyclohexamide, and then stimulated with serum for 30 minutes (Figure 7). The Ser 662 site became phosphorylated following cyclohexamide treatment.

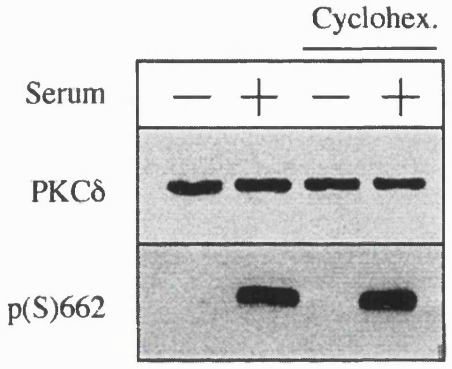


Figure 7. Cyclohexamide pre-treatment does not affect the serum-induced phosphorylation of the hydrophobic site.
PKCδ was transiently transfected into 293/HEK cells. The cells were deprived of serum for 24 hours while in suspension, as described in Chapter 2. Cells were either stimulated with serum (10% final), for 30 minutes, or treated with cyclohexamide (100μM) for 30 minutes before stimulation with serum. The extracts were analysed for PKCδ protein expression and for phosphorylation at the S662 sites by Western blotting.

In order to employ these conditions routinely, it was necessary to assess the state of the cells, to ensure they were not compromised for viability. To determine if the cells were undergoing apoptosis due to serum deprivation while in suspension, FACS analysis was performed to detect to presence of DNA in the sub-G1 phase of the cell cycle. The absence of sub-G1 content DNA clearly illustrates that the loss of phosphate, induced by serum deprivation in suspension, was not a consequence of cells undergoing apoptosis (Figure 8). Dr K. Procyk, in the laboratory, determined that there was no detectable caspase activation under these conditions, confirming these observations.

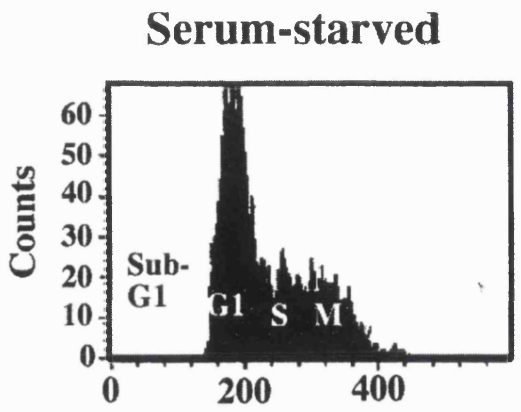


Figure 8. Propidium Iodide Staining revealing the absence of apoptotic (Sub G1-content) DNA.
PKCδ was transiently transfected into 293/HEK cells. The cells were deprived of serum for 24 hours while in suspension, as described earlier in Chapter 2. They were then prepared for propidium iodide staining,, as described in Chapter 2, to determine the proportion of DNA at defined stages of the cell cycle.

3.2.3 Determining DAG-mediated control

To determine the requirements for serum-induced phosphorylation, we investigated the necessity of DAG binding at the C1 domain for PKC δ and ε phosphorylation. In the absence of serum treatment, there was a very low basal phosphorylation at the two sites, and no observable effect of Calphostin-C treatment (Figure 9). Calphostin-C pre-treatment prior to serum addition, suppressed phosphorylation of the T loop and hydrophobic sites in both novel isoforms tested. This suggests that DAG binding at the C1 domain is absolutely necessary for these sites to become phosphorylated.

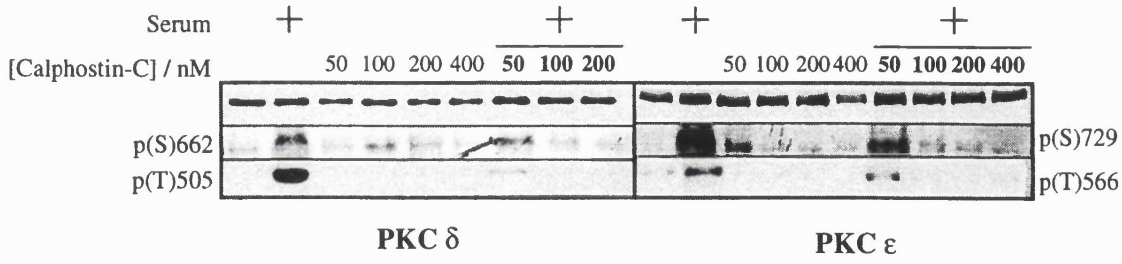


Figure 9. DAG binding at the C1 domain is critical for phosphorylation at the T loop and hydrophobic sites in nPKCs.
PKCδ or PKCε was transiently transfected into 293/HEK cells. The cells were deprived of serum for 24 hours while in suspension, as described earlier in Chapter 2. They were stimulated with serum (+) for 30 minutes with or without pretreatment with Calphostin-C for 30 minutes at the concentrations indicated. The extracts were analysed for PKCδ or PKCε protein expression and for phosphorylation at the S662 or S729 sites (hydrophobic site in PKCδ or PKCε, respectively) and at the T505 or T566 sites (T loop site in PKCδ or PKCε, respectively) by Western blotting.

This suggested that a C1-domain dependent activation/conformational change of nPKC is required for effective serum induced phosphorylation. This is supported by the effect of TPA on these phosphorylations, as discussed earlier, where serum and TPA together, led to a rapid phosphorylation that was optimum within 5 (Thr 505), or 10 (Ser 662) minutes. This appears to be consistent with *in vitro* observations from Dr A. Le Good in our laboratory, showing TPA acts co-operatively with PI $(3,4,5)P_3$ to support PDK-1 phosphorylation of PKCδ at the Thr 505 site *in vitro*.

3.2.4 Determining PI 3-kinase influence on the phosphorylation of these sites

As noted above, the earlier in vitro experiments demonstrated that PDK-1 would phosphorylate the T loop site in a number of the PKC isoforms. As a PtdIns(3,4,5)P_3-dependent enzyme, we could exploit this to determine the physiological role of PDK-1 on the phosphorylation of the T loop site in these isoforms. It was then determined if pharmacological reagents, which have been previously shown to affect specific proteins, would influence the phosphorylation of these sites. Transiently transfected HEK/293 cells with PKCδ or PKCε, were serum starved and then treated with the PI 3-kinase inhibitor, LY294002 before serum stimulation. This resulted in complete inhibition of the serum-induced phosphorylation of the T loop and hydrophobic sites in PKCδ and PKCε (Figure 10). This was shown also for the hydrophobic site in both the isoforms (see in chapter 4).

The co-expression of PKCδ or PKCε with the recently characterised PI (3,4,5) P3-dependant kinase-1 (PDK 1) (Alessi et al., 1997b; Anderson et al., 1998; Stephens et al., 1998; Stokoe et al., 1997), consistently gave a high basal level of phosphorylation following serum starvation (Figure 10). Following serum treatment, the Thr 566 site (T loop in PKCε) was phosphorylated 2.5 times above the sample from serum-stimulated cells without exogenous PDK1. The Thr 505 site (T loop in PKCδ) was more highly phosphorylated in the presence of exogenous PDK-1. Furthermore, when PDK1 is coexpressed with either PKC isoform, LY294002 treatment effectively blocked the increment of serum-induced increase.

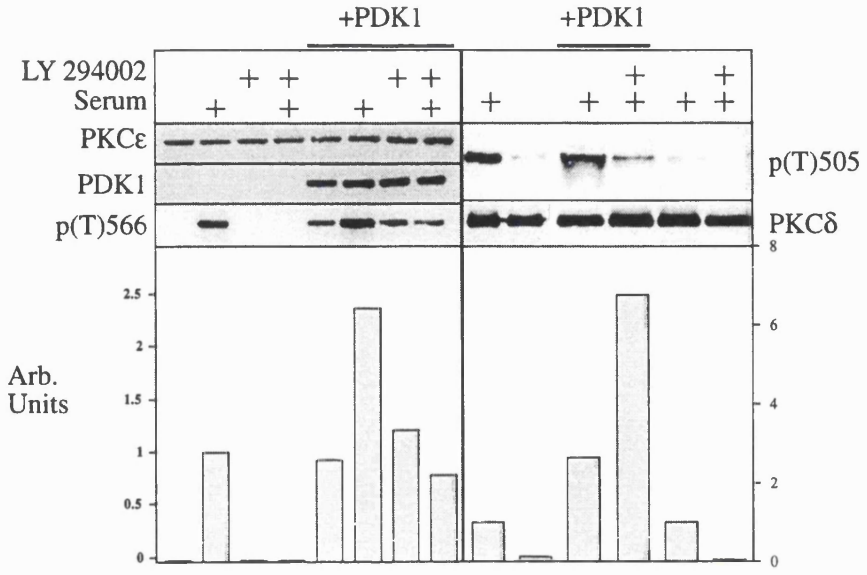


Figure 10. Effect of PDK-1 co-expression with nPKC on the serum-induced phosphorylations
PKCδ or PKCε was transiently transfected into 293/HEK cells. The cells were deprived of serum for 24 hours while in suspension, as described earlier in Chapter 2. They were either stimulated with serum (10% final) for 30 minutes, or pre-treated with LY294002 (10μM) for 30minutes. The extracts were analysed for PKCδ or PKCε protein expression and for phosphorylation at the T505 or T566 site (T loop site in PKCδ or PKCε, respectively) by Western blotting. These observations are consistent with three independent experiments that had been performed, though not in the same format as in figure 10.

3.3 Discussion

There appear to be a number of critical factors that seem to influence the efficiency of phospho-specific detection. They are the amount of protein loaded on the gel, and the concentration of both antibody and dephospho-peptide in the primary incubation of the Western blot. The presence of dephospho-peptide, in the primary antibody incubation led to a reduction in the non-specific signal. However, the phospho-specific signal was competed out by the phosphopeptide, which the antisera were raised against. This illustrated the presence of phospho-specific, and

dephospho-specific, antibody in the antiserum.

To identify conditions for a low basal phosphorylation at the T loop and hydrophobic site on a consistent basis, it was important to deprive the cells of serum while in suspension for a minimum of 24 hours. However, it seemed that the number of cells in suspension was also critical. A low basal level of phosphorylation at the T loop and hydrophobic site was observed for cells deprived of serum while adherent, but this was very inconsistent. This variability was not examined in more depth at the time, but it was suspected that a possible cause for the phosphorylation, detected at the T loop and hydrophobic sites, could be the influence of cell-cell and cell-matrix interactions. This is examined further in chapter 5.

Attempts were made at affinity purifying the phospho-specific antibodies from the sera. However, the eluted protein was too unstable, even in the presence of a cocktail of commercially available protease inhibitors. It seems that certain factors were purified out of the serum that stabilised the antibodies in the sera. For reasons of stability, cost and convenience, the Western blots were routinely probed with unpurified antisera in the presence of the cognate dephosphopeptide.

The absence of a phospho signal at the Thr 505 and Ser 662 sites in the bacterially expressed GST-PKCδ fusion protein, suggested that these sites are not autophosphorylated. This paralleled earlier findings for the equivalent sites in PKCα (Bornancin and Parker, 1996; Bornancin and Parker, 1997; Cazaubon et al., 1994). However other possibilities could also explain this finding. For example, the lack of certain cofactors present in mammalian cells following stimulation (e.g. DAG, PS, and other anionic or neutral lipids) may prevent the PKC from autophosphorylating at those sites. Alternatively, the interaction between the GST components of each fusion molecule may impose steric hindrance, and prevent two PKCδ molecules dimerising in the necessary orientation to permit trans-autophosphorylation.

The absence of sub G1 content DNA, from serum starved cells in suspension for 24 hours, suggested that the observed responses were not a feature of cells undergoing anoikis. A colleague in the laboratory using an assay to detect the activity of caspases, verified this (Parekh et al., 2000a). The viability of these cells under these conditions of minimal nPKC phosphorylation provides a useful context for the studies subsequently undertaken.

The absence of phosphate at the T loop and hydrophobic sites in the two isoforms following serum deprivation in suspension, suggested that they are more sensitive to phosphatase action under these conditions, compared to the TP site (Ser 643 and Thr 710, PKCδ or ε respectively). The lack of a serum-induced phosphorylation at Ser 643 and Thr 710 (the TP site in PKCδ and ε respectively) and no apparent effect of serum deprivation for 24 hours suggested the antisera was not detecting the presence of a phosphate at these sites. However, the phospho-specific antisera were competed out by their respective phospho-peptides (Figure 2d). Alternatively, it could also imply that the rate of dephosphorylation for the TP site in vivo is less than the rate of phosphorylation. Being in close proximity to the active site of the same kinase would support a model of autophosphorylation of this site. Later experiments, particularly using the Bis-indolylmaleimide-1 compound (BIM-1), support the notion that this site is autophosphorylated. This suggested that the PKC isoform had some activity, even though it was not phosphorylated at the T loop and hydrophobic sites. However, the catalytic activity determined was very low.

Calphostin-C inhibited the serum-induced phosphorylation of the T loop and hydrophobic sites. Therefore, binding DAG at the membrane must be important for activation of the PKC isoform but also effective phosphorylation.

The rapid phosphorylation response observed, following stimulation of the serum-starved cells with the combination of TPA and serum, at the T loop and hydrophobic

sites, suggested that other factors are required to have this effect, in addition to DAG. Even though the rate of DAG accumulation in the membrane maybe a limiting factor in the response induced by serum alone, other signalling pathways induced by serum must also be important. One example is the PI 3-kinase pathway, which is poorly activated by TPA treatment. The inhibition of the serum-induced phosphorylation of the T loop by the PI 3-kinase inhibitor, LY294002, suggested that PI 3-kinase is indeed having a role. PI 3-kinase appears to influence the phosphorylation of the T loop by generating PI (3,4,5) P3 at the plasma membrane. It has been proposed that the PH domain, in PDK1, binds to this lipid product, and it then phosphorylates the T loop site, as described for PKB/Akt, (Alessi et al., 1997b; Stephens et al., 1998; Stokoe et al., 1997), p70 ^{S6K}, (Alessi et al., 1998) , and p90 RSK ADDIN ENRfu (Jensen et al., 1999) . The effect on PKC δ was supported by in vitro studies. In fact, it was shown that PDK1 was likely to be responsible for the phosphorylation of the equivalent site in all the other members of the PKC family (Dutil et al., 1998; Le Good et al., 1998) .

These conditions were established to define an inducible system for the in vivo phosphorylation of the T loop and the hydrophobic sites in PKCδ and PKCε. It was also demonstrated that at least two signalling pathways (PI 3-kinase and PLC) are required for this to occur. Characterising the phospho-specific antisera has made it possible to examine further the occupation of all three sites under differing in vivo conditions. It was now important to know what other signalling pathways are required to control these events, particularly with reference to the phosphorylation of the hydrophobic site.

4.0 Regulatory inputs to the phosphorylation of the hydrophobic site.

4.1 Introduction

Uncertainty surrounds what is known about the regulation of the hydrophobic site in the novel PKC isoforms. Studies on the equivalent site in cPKCβ1 (II) had concluded that it is autophosphorylated. However, there are still several unanswered questions remaining with this investigation such as the effects of co-precipitating kinases and phosphatases, and the stability of the kinases under *in vitro* conditions (Behn-Krappa and Newton, 1999). Many of these questions stem from the investigations on the hydrophobic site in cPKCα (Bornancin and Parker, 1997; Gysin and Imber, 1996). These had revealed that the phosphorylation of the hydrophobic site in cPKCα is acutely controlled, sensitive to protein phosphatases and oxidants, and influenced by thermal stability. Together with the T loop and the TP sites, it plays an essential role in not only 'priming' the protein kinase for maximum catalytic activity, but also in its desensitisiation (Gysin and Imber, 1996; Hansra et al., 1996; Hansra et al., 1999; Parekh et al., 2000b).

It had been shown previously that the phosphorylation of the hydrophobic site in $p70^{S6kinase}$ is important for catalytic activity. However, this phosphorylation event is sensitive to the inhibitory effects of the potent immunosuppressant drug rapamycin (Ferrari et al., 1993). To assess the role of the upstream kinases or phosphatases, in controlling the phosphorylation of the hydrophobic site in the nPKC group, investigations were aimed at determining what control mechanism these enzymes were under. The phosphorylation of the hydrophobic site in PKCδ and PKCε was examined to determine if it is regulated by a similar mechanism to the equivalent site in $p70^{S6kinase}$, which would implicate an involvement of mTOR

(mammalian Target Of Rapamycin) (Chiu et al., 1994; Ferrari et al., 1993; Sabatini et al., 1994) and PI 3-kinase in the regulation of the hydrophobic site.

Investigations were also aimed at identifying the kinase responsible for phosphorylating the hydrophobic site in nPKCs. This was the principle focus of Dr W.H Ziegler, who purified a large multi-protein complex, displaying activity towards the hydrophobic site in PKCα and PKCδ. Further analysis of the protein complex revealed PKCζ/ι as a constituent. I complemented these *in vitro* studies with *in vivo* investigations to assess the effect of wild-type and mutant forms of PKCζ on the serum-induced, and rapamycin-sensitive phosphorylation of the hydrophobic site in PKCδ and PKCε.

4.2 Results

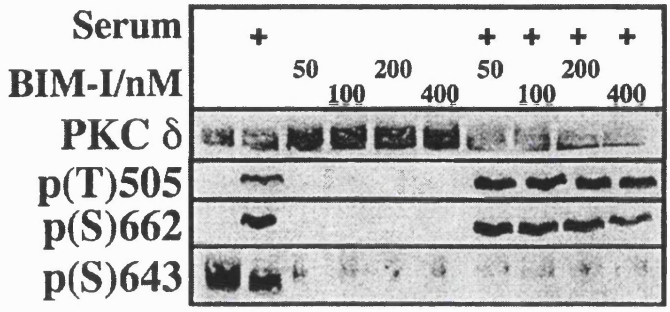
4.2.1 Is the hydrophobic site in nPKC isoforms autophosphorylated?

To assess the requirement of catalytic activity for the phosphorylation of the hydrophobic site in PKCδ and PKCε, a PKC catalytic inhibitor, Bis-indolylmaleimide-I (BIM-I), was used. The response of the hydrophobic site to BIM-I treatment was compared to the TP site in PKCδ and PKCε (S643 and S710, respectively), since the S643 site in PKCδ had previously been described as an autophosphorylation site (Le Good et al., 1998; Li et al., 1997b; Parekh et al., 1999).

The serum-induced phosphorylation of the hydrophobic site in PKCδ and PKCε was unaffected by BIM-I treatment. However, BIM-I treatment did appear to inhibit the phosphorylation of the S643 site and the S710 site in PKCδ and PKCε, respectively (Figure 1). This suggested that the serum-induced phosphorylation at the

hydrophobic site in PKCδ and PKCε was not dependent upon a BIM-I sensitive catalytic activity.

a. PKCδ



b. PKCε

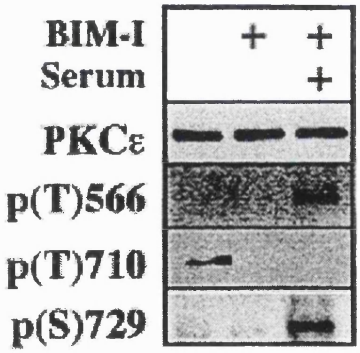


Figure 1. BIM-I treatment only affects the phosphorylation at the TP site in both PKCδ and PKCε.

HEK/293 cells were transfected with either PKCδ (1a) or PKCε (1b), and then deprived of serum while in suspension, for 24 hours. The cells were stimulated with serum (10%) for 30 minutes (+) before or after treatment with the PKC catalytic inhibitor BIM-I for 30 minutes. Cells transfected with PKCδ were treated with a range of different BIM-I concentrations (nM), while cells transfected with PKCε were treated with BIM-I at 10μM for 30 minutes (1b). The cells were harvested by centrifugation, and then lysed in 4X SDS sample buffer. Extracts were analysed for protein expression, and for phosphorylation at the T loop site (505/566), the hydrophobic site (662/729) and the TP site (643/710) in PKCδ and PKCε, by Western blotting.

However, BIM-I did affect the phosphorylation of the TP site in the concentration range that would inhibit the catalytic activity of nPKCs. The acute effect of BIM-I on the phosphorylated S643 site in PKCδ was to cause its dephosphorylation.

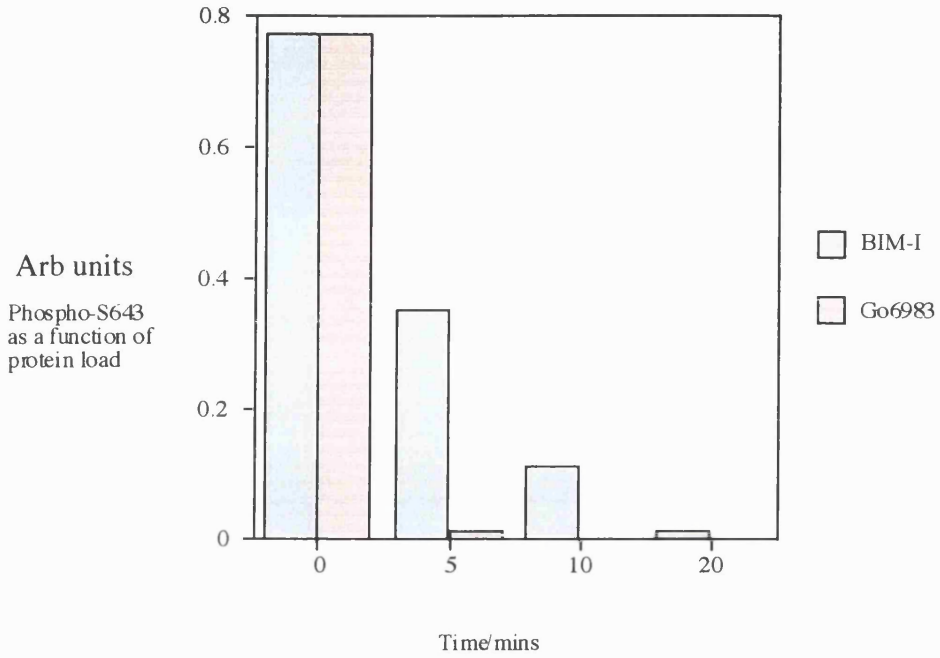


Figure 2 Time-dependant dephosphorylation of the TP site in response to either BIM-I or Go6983 treatment.
HEK/293 cells were transfected with PKCδ and then deprived of serum while in suspension for 24 hours. The cells were treated with either BIM-I (200μM) or Go6983 (1μM), two different PKC catalytic inhibitors. Samples were removed at various times and lysed. The extracts were analysed for PKCδ protein expression and phosphorylation at S643, the TP site, by Western blotting. The phosphorylated S643 bands were quantified by densitometry, and expressed as a function of the respective protein load. This is one of two independent experiments, which gave similar results.

The same effect was observed for another catalytic site inhibitor Go6983. The half-life for dephosphorylation of the S643 site was between 5 and 10 minutes if using BIM-I, and less than 5 minutes if using Go 6983 (Figure 2). This suggests that the autophosphorylation site could be more sensitive to inhibition by Go6983 than BIM-

I. However, it is not clear whether the rate of entry into cells differs between the two compounds. Neither of the reagents affected the serum-induced phosphorylation of the T loop site or hydrophobic site in the nPKC isoforms studied.

4.2.2 The relationship between the phosphorylation of the T loop and the hydrophobic site in nPKC isoforms.

Bornancin and coworkers suggested that loss of phosphate at the T loop site would sensitise the hydrophobic site to dephosphorylation by generating a more 'open' catalytic domain. This would provide greater access for phosphatase(s) to dephosphorylate the hydrophobic site (Bornancin and Parker, 1997). To assess whether there is a requirement for phosphorylation at the T loop site to stabilise the phosphorylation at the hydrophobic site in nPKCs, a T loop alanine substitution mutant was employed. Interestingly, there was no detectable level of serum-induced phosphorylation at the hydrophobic site in PKCδ when the alanine 505 substitution mutant (T505A-PKCδ) was expressed in HEK/293 cells (Figure 3); this was also true for the equivalent mutant in PKCε. This suggested that the T loop site might need to be phosphorylated before the hydrophobic site. However, previous studies with cPKCα revealed that there is a mutually protective effect between these two phosphorylations, which reduced their susceptibility to phosphatases (Bornancin and Parker, 1997). By extrapolating from this, it could be proposed that there might be no obligatory order of phosphorylation in PKCδ and PKCε.

To investigate this further, HEK/293 cells expressing the alanine 505 substitution mutant, were stimulated with serum, in the presence of the PP1 and PP2A-protein phosphatase inhibitor, okadaic acid (Figure 3). In the presence of this phosphatase inhibitor, there was a serum-induced phosphorylation at the hydrophobic site in the T loop alanine substitution mutants of both PKCδ and PKCε. Thus, there is no obligatory order of phosphorylation between the T loop and the hydrophobic site in the presence of PP1 and PP2A protein phosphatase inhibitors. However, in the

absence of these protein phosphatase inhibitors, there does seem to be a dependence upon the phosphorylation at the T loop site for retaining a phosphate at the hydrophobic site.

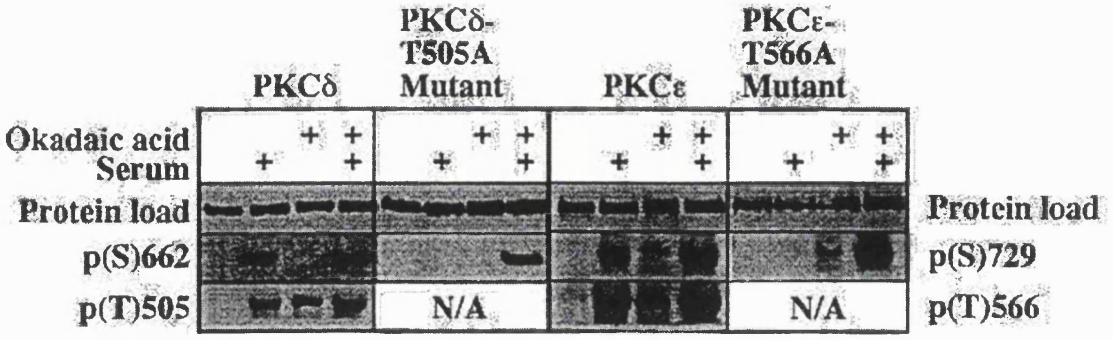


Figure 3. The hydrophobic site in nPKC isoforms is more sensitive to phosphatase action in the T loop Ala-mutant.
HEK/293 cells were transfected with either PKCδ, PKCε, or the T loop site alanine substitution mutants of these isoforms, and then deprived of serum while in suspension for 24 hours. The cells were treated with either serum alone (10%), okadaic acid alone (1μM), or both serum and okadaic acid together, for 30 minutes. Cells were harvested by centrifugation, and lysed with 4X SDS sample buffer. The lysates were analysed by Western blotting for protein expression, phosphorylation at the hydrophobic site in PKCδ and PKCε, and at the T loop site in the wild type nPKC isoforms. The phospho-specific T loop site antiserum does not immunoreact with the alanine residue in the T loop site alanine substitution mutants of PKCδ and PKCε, hence N/A, not applicable.

By treating cells, over-expressing the T loop alanine substitution mutants of nPKC isoforms, with okadaic acid, it was possible to assess the activity of the nPKCδ in the absence of the T loop phosphorylation, but with the two C-terminal sites occupied. This analysis indicated that when cells are treated with both serum and okadaic acid, the T loop is not essential for catalytic activity when the TP and hydrophobic sites are phosphorylated (Figure 4). Although it seems that phosphorylation at the T loop is not essential for activity, it has not been ruled out that other sites might become phosphorylated in response to okadaic acid and serum treatment, in addition to the hydrophobic and TP sites. It is evident that the activity

of PKCδ was higher following serum and okadaic acid compared to serum alone. This would support the notion that other events may be triggered by okadaic acid.

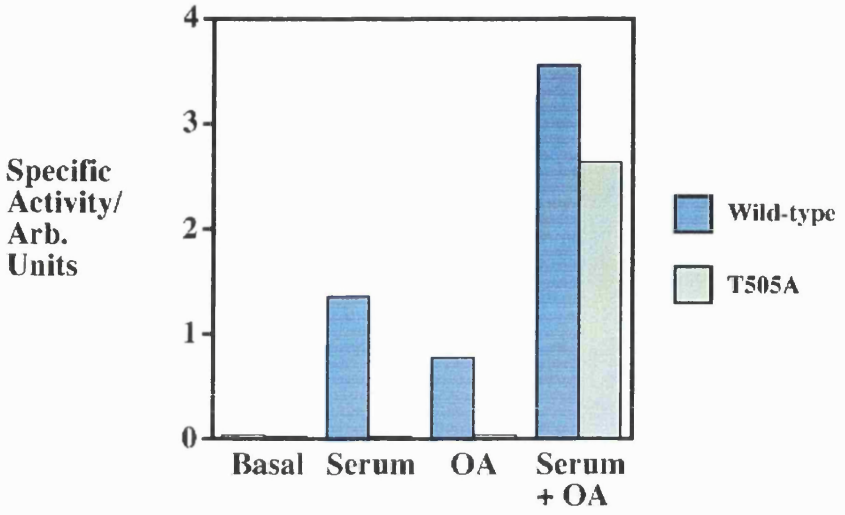


Figure 4. The specific activity of the T505A substitution mutant of PKCδ is high if purified from okadaic acid and serum treated cells.
HEK/293cells were transfected with either PKCδ or the T loop site alanine substitution mutant of this isoform (T505A), and then deprived of serum while in suspension for 24 hours (basal). The cells were treated with serum alone (10%), 1μM okadaic acid alone (OA), or both serum and okadaic acid together (serum+OA), for 30 minutes. PKCδ and the T loop site alanine substitution mutant were immunoprecipitated from the cells, using an anti-PKCδ antiserum, and assayed for catalytic activity using myelin basic protein as a substrate. Cerenkov counting of the SDS-PAGE purified myelin basic protein quantified catalytic activity. The relative protein load for each sample was determined from scanning densitometric analysis of Coomassie stained gels. Specific kinase activities were determined as a function of the respective PKCδ protein load. This was one of three similar experiments which gave similar results.

The conclusion that the T loop phosphorylation may not be essential is supported by that of Stempka and co-workers (Stempka et al., 1997) who suggested that initially, phosphorylation of the T loop site is critical for activation of the protein kinase, but that once the TP and hydrophobic sites were phosphorylated, the loss of phosphate at the T loop site did not lead to an inactivation of the protein kinase. However, it is

still possible that other sites were being phosphorylated to support the role initially provided by the phosphate at the T loop site under these circumstances.

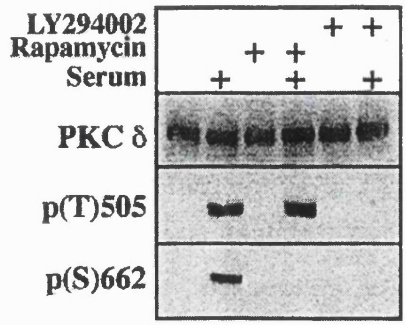
As noted above, stimulation of cells with serum and okadaic acid led to the phosphorylation at the hydrophobic site in the T505A-PKCδ mutant protein. This response did not appear to be due to a pathway induced by okadaic acid treatment alone. However, an okadaic acid-induced phosphorylation was observed at the T loop site in the wild-type PKCδ and PKCε. This response led to a significant rise in the activity, indicating that the hydrophobic site phosphorylation was not essential for activity, although the activity was lower than that of serum stimulated wt-PKCδ.

4.2.3 mTOR controls one pathway of nPKC phosphorylation.

Efforts were directed at identifying the regulation of the hydrophobic site in nPKC isoforms. Attempts were made to determine whether the phosphorylation of this site is regulated by mTOR in a similar manner to that previously described for the equivalent site in $p70^{S6kinase}$. HEK/293 cells were transfected with PKCδ and then deprived of serum in suspension as described earlier.

The cells were treated with the mTOR-specific inhibitor, rapamycin for 30 minutes, and then stimulated with serum. It appeared that rapamycin treatment inhibited the serum-induced phosphorylation of the hydrophobic site while not affecting the T loop site.

a. PKCδ



b. PKCε

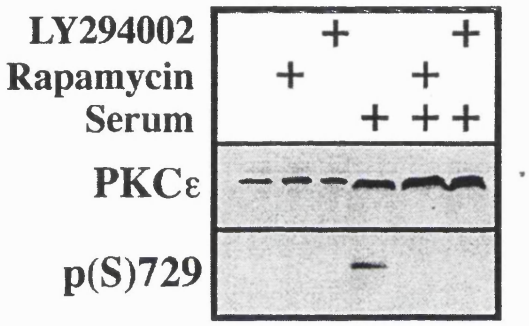


Figure 5. Rapamycin treatment inhibits the serum induced phosphorylation of the hydrophobic site.
HEK/293 cells were transfected with either PKCδ (a) or PKCε (b), and then deprived of serum for 24 hours while in suspension. The cells were treated either rapamycin (20nM) or LY294002 (10μM) for 30 minutes, or stimulated with serum (10%) following pre-trreatment with these inhibitors. Cells were harvested by centrifugation and then lysed with 4X-SDS sample buffer. Extracts were analysed by Western blotting for PKC protein expression and phosphorylation at the T loop and hydrophobic site (T505 and S662 respectively) in PKCδ (5a), or the hydrophobic site (S729) in PKCε (5b). This was one of five similar experiments.

This was consistent for the hydrophobic site in both nPKC isoforms (Figure 5a, 5b). It appeared that two distinct pathways influence the phosphorylation of the T loop and hydrophobic site in nPKC isoforms.

The effects of rapamycin treatment on the phosphorylation sites also appeared to influence the catalytic activity of the protein kinase (Figure 6). When PKCδ is immunoprecipitated from HEK/293 cells deprived of serum for 24 hours while in suspension (no T505 or S662 phosphorylation), the catalytic activity is very low. If the cells are stimulated with serum, then there is greater than a 50-fold increase in the catalytic activity of PKCδ.

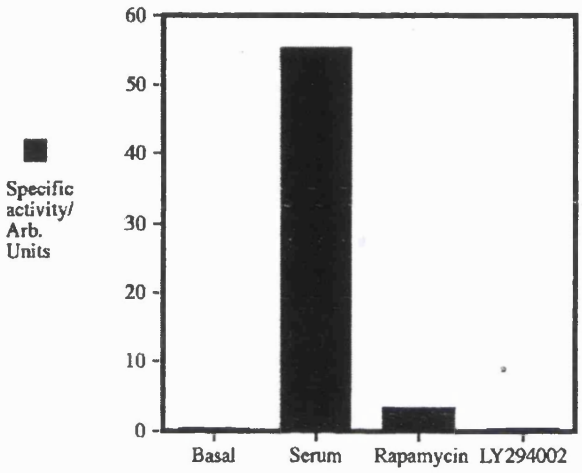


Figure 6. Rapamycin and LY294002 pre-treatment inhibit the serum-induced activity of PKCδ.
HEK/293 cells were transfected with amino-terminal, myc-tagged PKCδ. The cells were deprived of serum while in suspension for 24 hours. Cells were treated with serum (10%) for 30 minutes, or pre-treated with rapamycin (20nM) or LY294002 (10μM) for 30 minutes, and then stimulated for 30 minutes with serum. Myc-tagged PKCδ was immunoprecipitated from the cells using an anti-myc antiserum, and assayed for catalytic activity using myelin basic protein as a substrate. Cerenkov counting, of the SDS-PAGE purified myelin basic protein, was used to quantify activity. The relative protein load for each sample was determined from scanning densitometric analysis of Coomassie stained gels. Specific kinase activities were determined as a function of the respective PKCδ load. This was one four similar experiments.

However, if the cells are treated with rapamycin (no S662 phosphorylation), before serum stimulation, there was much reduced PKCδ activity, approximately 10% of maximum. However, there is an inconsistency here. The experiments above show

that the catalytic activity of wt-PKCδ, immunopurified from cells treated with okadaic acid (where the T loop and TP site are phosphorylated, but not the hydrophobic site), is 50% of the maximum. This may imply that okadaic acid is triggering another site such that this is now almost fully active without the hydrophobic site.

Another explanation is that there is a slower, okadaic acid-induced, phosphorylation at the hydrophobic site. This may not be detected by the phospho-specific antisera as there might be insufficient epitope. Treatment of HEK/293 cells deprived of serum for 24 hours while in suspension, with okadaic acid for 60 minutes did led to the detection of a phosphorylated hydrophobic site (data not shown). However, it is unclear how this treatment, for this period of time, would effect the cell. The situation following rapamycin treatment may require the phosphorylation at the hydrophobic site because there is no okadaic acid-induced phosphorylation detected at other site(s). Furthermore, it is not clear what effect, if any, rapamycin treatment has on other sites phosphorylated in response to okadaic acid treatment.

To determine whether mTOR has a role in the serum-induced phosphorylation of the hydrophobic site, HEK/293 cells were co-transfected with PKCδ or PKCε with wild-type mTOR ($mTOR^{wt}$), or a rapamycin-resistant form of mTOR ($mTOR^{rap\text{-}res}$). The aim of this was to assess whether exogenous mTOR protein, with modified rapamycin sensitivity, affects the serum-induced phosphorylation of the rapamycin-sensitive hydrophobic site. The aim was to establish whether or not mTOR really was involved in the phosphorylation of the hydrophobic site (Figure 7).

HEK/293 cells, over-expressing either nPKC isotype with $mTOR^{wt}$, were deprived of serum as described earlier, and then stimulated with serum. Western blot analysis, with phospho-specific antisera, revealed phosphorylation at both the T loop and hydrophobic sites following serum treatment. Also consistent with earlier observations, rapamycin or LY294002 treatment of the HEK/293 cells led to an inhibition of serum-induced phosphorylation at the hydrophobic site. The presence

of exogenous mTOR^{wt} expression did not alter the inhibitor effect of LY294002 treatment on the serum-induced phosphorylation at the T loop site. This supported the earlier observation that regulation of T loop site phosphorylation is not via an mTOR-dependant pathway.

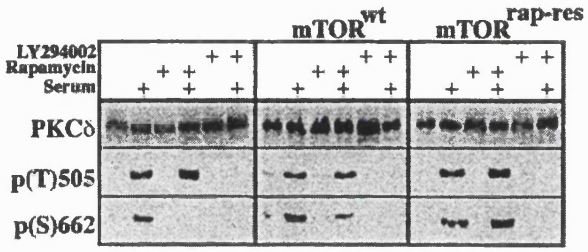


Figure 7. Co-expression of PKCδ with mTOR$^{rap-res}$ leads to rapamycin insensitive, serum-induced phosphorylation at the hydrophobic site.
HEK/293 cells were transfected with PKCδ alone or together with either wild-type mTOR (mTOR^{wy}) or a rapamycin-resistant form of mTOR (mTOR$^{rap-res}$). The cells were deprived of serum for 24 hours while in suspension, and then treated with serum (10%) for 30 minutes, or following treatment with rapamycin (20nM) or LY294002 (10μM) for 30 minutes. Cells were harvested by centrifugation and then lysed with 4X-SDS sample buffer. Extracts were analysed by Western blotting for PKCδ and PKCε protein expression and phosphorylation at the T loop and hydrophobic site (T505 and S662 respectively) in PKCδ, and S729 in PKCε. This was one of two similar experiments.

The presence of mTOR$^{rap-res}$ seemed to overcome the inhibitory effects of rapamycin treatment on the serum-induced phosphorylation of the hydrophobic site in PKCδ and PKCε. LY294002 treatment still inhibited the serum-induced phosphorylation of the T loop and hydrophobic sites in cells expressing the nPKCδ isoform and mTOR$^{rap-res}$ (Figure 7). LY294002 inhibits at the ATP-binding site in the catalytic domain of PI 3-kinase. It is still not clear whether LY294002 mediates its inhibitory effects on the phosphorylation at the hydrophobic site by inhibiting the PI 3-kinase or mTOR, or both, since mTOR also has a PI 3-kinase-like catalytic domain. This is distinct from the binding site of the rapamycin-FKBP-12 complex in mTOR.

4.2.4 Effects of amino acid deprivation on the serum-induced phosphorylation in nPKCs.

The effect of $mTOR^{rap\text{-}res}$ in overcoming the inhibitory effects of rapamycin treatment on the serum-induced phosphorylation of the hydrophobic site in PKCδ and PKCε, clearly supports the role of mTOR in this pathway. Hara, K and co-workers, had previously reported that mTOR plays a role in sensitising mammalian cells to nutrient deprivation (Hara et al., 1998).

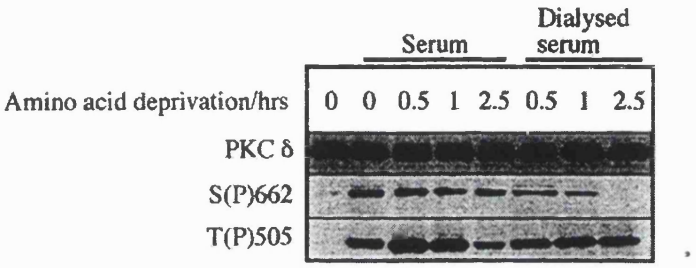
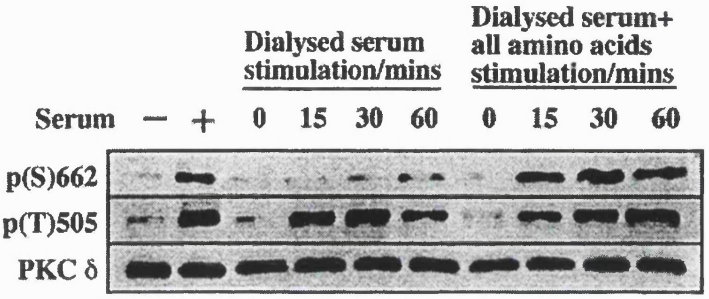


Figure 8. Amino acid deprivation of cells inhibits the serum-induced phosphorylation at the hydrophobic site in a time-dependant manner.
HEK/293 cells were transfected with PKCδ and deprived of serum for 24 hours while in suspension. The cells were washed once in PBS and then incubated in amino acid-free DMEM for the times shown. Cells were stimulated for 30 minutes with either serum or dialysed serum (10%). The cells were harvested by centrifugation and then lysed with 4X-SDS sample buffer. Extracts were analysed by Western blotting for PKCδ protein expression and phosphorylation at the T loop and hydrophobic site (T505 and S662 respectively) in PKCδ. This was one of two similar experiments.

To determine whether amino acid deprivation influenced the serum-induced phosphorylation of the hydrophobic site in nPKC isoforms, HEK/293 cells were deprived of both serum and amino acids and then stimulated with dialysed serum. Since dialysed serum was deficient in amino acids, the effect of stimulation in the absence of these nutrients could be determined. There appeared to be a time-dependant reduction in the capacity of dialysed serum to stimulate the phosphorylation of the hydrophobic site in PKCδ (Figure 8). Incubation of

HEK/293 cells in amino acid-free DMEM for 150 minutes was sufficient to block dialysed serum-induced phosphorylation of the hydrophobic site.

a. all amino acids



b. leucine.

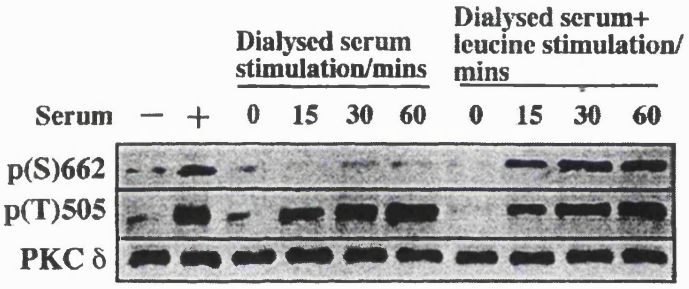


Figure 9. PKCδ from amino acid deprived cells become phosphorylated in response to dialysed serum treatment in the presence of either a, amino acids, or b, only leucine.

HEK/293 cells were transfected with PKCδ and deprived of serum for 24 hours while in suspension. The cells were washed once in PBS and then incubated in amino acid-free DMEM for 2.5 hours while in suspension. Cells were stimulated for 30 minutes with either serum for 30 minutes (+), or dialysed serum (10%) for the times (minutes) shown. The serum and amino acid deprived-cells were stimulated with dialysed serum alone, or in the presence of a solution containing either a, all amino acids, or b, only leucine. The addition of amino acid(s) from the stock solutions replenished the amino acid-deficient DMEM, where the final concentration was equal to that in standard DMEM. The cells were harvested by centrifugation and then lysed with 4X-SDS sample buffer. Extracts were analysed by Western blotting for PKCδ protein expression and phosphorylation at the T loop and hydrophobic site (T505 and S662 respectively) in PKCδ. This is one of two similar experiments.

A similar reduction was not observed at the T loop site, suggesting a specific effect on the hydrophobic site, and therefore, an independent pathway. This appeared to support the earlier findings of rapamycin-mediated mTOR inhibition, and hence, an independent pathway for the serum-induced phosphorylation of the hydrophobic site. The hydrophobic site was only phosphorylated when the cells, deprived of serum and amino acids, were stimulated with dialysed serum together with a solution containing all the amino acids (Figure 9).

Hara, K and co-workers, had also reported that cells were able to overcome the inhibitory effects of amino acid deprivation on $p70^{s6kinase}$ phosphorylation when supplemented with only the amino acid leucine (Hara et al., 1998). To determine whether the phosphorylation of the hydrophobic site in PKCδ was also acutely sensitive to the absence, or presence of leucine, HEK/293 cells (deprived of serum and amino acids) were stimulated with dialysed serum in the presence of leucine (Figure 9). The combination of leucine and dialysed serum appeared to stimulate the phosphorylation of the hydrophobic site, while apparently not affecting the T loop site. This seemed to verify the earlier findings of an involvement of mTOR in the phosphorylation of only the hydrophobic site, in nPKC isoforms.

4.2.5 Characterising a component of the V5α-kinase complex.

Efforts were then directed towards identifying the kinase that phosphorylates the hydrophobic site. Dr W. H Ziegler in the laboratory purified a membrane-associated kinase complex from rat brains by a factor of approximately 10, 000-fold. The purified kinase displayed high specific activity towards the hydrophobic site in the V5 domain in PKCα and PKCδ, hence had the characteristics of a V5α-kinase (Ziegler et al., 1999). The hydrophobic site in proteins expressed in bacteria were used as substrates, since it had been demonstrated earlier that the S662 site in bacterial expressed PKCδ was not phosphorylated (see Chapter 3). PKCδ was

expressed in bacteria as a GST-linked fusion protein. PKCα was expressed in bacteria as a GST-linked V5-domain fusion protein, since the entire protein would not fold correctly (Bornancin and Parker, 1996).

In attempts to identify the active kinase, mass spectrometry and sequencing of tryptic peptides failed to yield useful information. However, Coomassie staining of a PAGE-gel from the final preparation did reveal a band resolving at approximately 80,000 kDa. Western blotting the V5α-kinase complex using a range of different commercially available antisera revealed the presence of an atypical PKC isotype. Unfortunately, the antisera recognised both PKCζ and PKCι, so it was difficult to identify which aPKC isotype might be involved.

Since it appeared that an atypical PKC isoform might be a component in the V5α-kinase complex, *in vivo* experiments were performed to determine whether expression of exogenous PKCζ, or mutant forms of it would affect the serum-induced phosphorylation at the hydrophobic site in PKCδ. Also investigated was the issue of whether the presence of exogenous PKCζ, or mutant forms of it, have any influence on the inhibition imposed by rapamycin or LY294002 treatment.

The following mutants of PKCζ were used:

PKCζ Mutant	Phenotype
PKCζ T410E/T560E	Activated?
PKCζ T410E	Activated?
PKCζ T410A	Dominant negative?

(The catalytic activity of the different PKCζ mutants have not been characterised under the conditions in which the experiments were performed.)

In the presence of the PKCζ T410E/T560E mutant there was a high basal phosphorylation at the hydrophobic site in PKCδ. Since the basal phosphorylation was high from the beginning, a robust increase in serum-induced phosphorylation was not observed at the hydrophobic site. Furthermore, in the presence the PKCζ T410E/T560E mutant, the serum-induced phosphorylation of the hydrophobic site in PKCδ appeared to be insensitive to not only rapamycin treatment but also to LY294002 treatment (Figure 10).

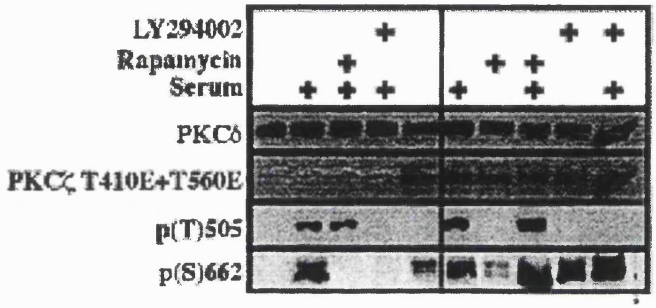


Figure 10. PKCζ T410E/T560E mutant expression leads to an LY294002 and rapamycin-insensitive phosphorylation of the hydrophobic site in PKCδ. HEK/293 cells were transfected with PKCδ alone, or together with a constitutively active PKCζ mutant (PKCζ T410E+T560E) and then deprived of serum for 24 hours while in suspension. The cells were stimulated with serum (10%) with or with out pre-treatment with rapamycin (20nM) or LY294002 (10μM). The cells were harvested by centrifugation and then lysed with 4X-SDS sample buffer. Extracts were analysed by Western blotting for PKCδ and PKCζ protein expression and phosphorylation at the T loop and hydrophobic site (T505 and S662 respectively) in PKCδ. This was one of two independent experiments.

To determine whether this effect was specific for the hydrophobic site in PKCδ, the serum-induced phosphorylation at the T loop site was also monitored. The T loop site appeared to be largely unaffected by the overexpression of the PKCζ T410E/T560E mutant. The serum-induced phosphorylation of the T loop site was still sensitive to LY294002 pre-treatment. In the presence of exogenous PKCζ T410E mutant, the hydrophobic site was insensitive to the inhibitory effects of rapamycin and LY294002 treatment. There was only a modest rise in the serum-

induced phosphorylation at the hydrophobic site, perhaps since the basal phosphorylation was already high. However, when the cells were treated with rapamycin, a robust serum-induced phosphorylation at the hydrophobic site was observed.

A possible explanation of why there was not a serum-induced increase in phosphorylation at the hydrophobic site following LY294002 treatment, but only following rapamycin treatment, could be that LY294002 treatment would lead to the absence of phosphate at the T loop site. This would sensitise the phosphate at the hydrophobic site to dephosphorylation. This would be consistent with the observations in figure 3. It seems that the lack of sensitivity of the hydrophobic site in PKCδ to LY294002 treatment in the presence of the PKCζ mutant suggests that the sensitivity normally occurs through the T loop phosphorylation of PKC ζ/ι (which is by-passed).

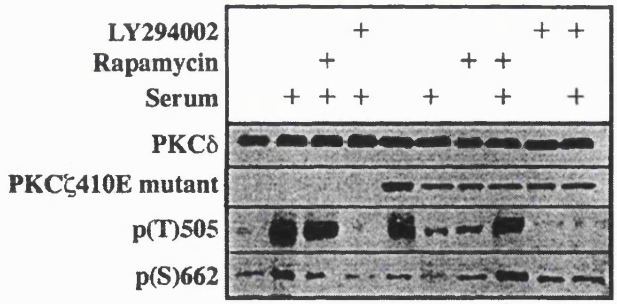


Figure 11. PKCζ T410E mutant leads to LY294002 and rapamycin-insensitive phosphorylation of the hydrophobic site in PKCδ.
HEK/293 cells were transfected with PKCδ alone, or together with an activated PKCζ mutant (PKCζ T410E) and then deprived of serum for 24 hours while in suspension. The cells were stimulated with serum (10%) with or with out pre-treatment with rapamycin (20nM) or LY294002 (10μM). The cells were harvested by centrifugation and then lysed with 4X-SDS sample buffer. Extracts were analysed by Western blotting for PKCδ and PKCζ protein expression and phosphorylation at the T loop and hydrophobic site (T505 and S662 respectively) in PKCδ. This is one of two similar experiments.

The co-expression of PKCδ with the PKCζ T410A mutant appeared to block the serum-induced phosphorylation of the hydrophobic site (Figure 12). This suggests that active PKCζ is important in the pathway for phosphorylating the hydrophobic site, and that the contribution PKCζ has may be more than structural. However, it is possible that the low activity form of PKCζ (T410A) may not be autophosphorylated at certain key residues. The lack of these phosphorylations could affect the structure of the protein kinase whereby it is no longer able to function as a scaffold protein in the V5α-kinase complex. Hence, it is difficult finally to distinguish the various contributions PKCζ could have in the V5 kinase complex from the above observations.

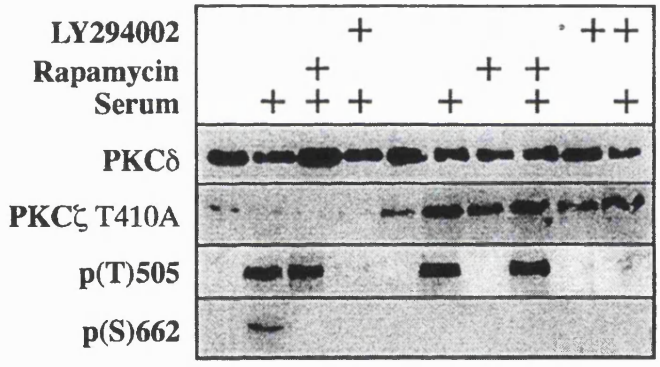


Figure 12. Dominant negative PKCζ inhibits serum-induced phosphorylation of PKCδ at the hydrophobic site.
HEK/293 cells were transfected with PKCδ alone, or together with a dominant negative PKCζ mutant (PKCζ T410A) and then deprived of serum for 24 hours while in suspension. The cells were stimulated with serum (10%) with or with out pre-treatment with rapamycin (20nM) or LY294002 (10μM). The cells were harvested by centrifugation and then lysed with 4X-SDS sample buffer. Extracts were analysed by Western blotting for PKCδ and PKCζ protein expression and phosphorylation at the T loop and hydrophobic site (T505 and S662 respectively) in PKCδ. This is one of two similar experiments.

To try and investigate this further, wild-type PKCζ was co-expressed with PKCδ and the cells were treated as before. It was thought that wild-type PKCζ would have sufficient activity to become autophosphorylated at key residues and adopt a native conformation. If PKCζ is behaving only as a scaffold protein, it is possible that under these conditions, PKCζ would assemble more of the active components of the V5α-kinase, leading to a more robust serum-induced phosphorylation of the hydrophobic site in PKCδ. The serum-induced phosphorylation at the hydrophobic site in PKCδ does appear to have increased when co-expressed with wt-PKCζ.

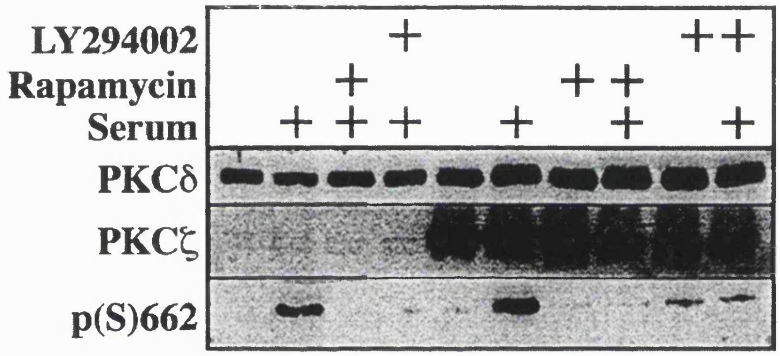


Figure 13. Wild-type PKCζ does not protect the serum-induced phosphorylation of the hydrophobic site in PKCδ from inhibitor treatment. HEK/293 cells were transfected with PKCδ alone, or together with the wild-type PKCζ mutant and then deprived of serum for 24 hours while in suspension. The cells were stimulated with serum (10%) with or with out pre-treatment with rapamycin (20nM) or LY294002 (10μM). The cells were harvested by centrifugation and then lysed with 4X-SDS sample buffer. Extracts were analysed by Western blotting for PKCδ and PKCζ protein expression and phosphorylation at the T loop and hydrophobic site (T505 and S662 respectively) in PKCδ. This is one of two similar experiments.

The protective effect of PKCζ, against rapamycin or LY294002 treatment towards the serum-induced phosphorylation at the hydrophobic site in PKCδ was not observed. It seems that PKCζ may be on the pathway which phosphorylates the

hydrophobic site, and that the catalytic activity of this atypical PKC isotype is a requirement. The presence of the PKCζ T410A mutant did not affect the serum-induced phosphorylation of the T loop site in PKCδ, suggesting that it is a selective effect on the hydrophobic site.

4.3. Discussion

It appears that there are three distinct pathways that control the phosphorylation in the nPKC isotypes examined. The TP site seems be autophosphorylated, while the T loop site and the hydrophobic site are not. While the phosphorylation of the T loop site has been characterised and shown to require a PDK-1 input (Chou et al., 1998; Dutil et al., 1998; Le Good et al., 1998), evidence for the hydrophobic site appears much less clear. It seems that the hydrophobic site in PKCδ and PKCε is under the control of mTOR (Figure 7). This is very similar to the control mechanism described earlier for the equivalent site in $p70^{S6kinase}$ (Ferrari et al., 1993). An additional component required in the phosphorylation of the hydrophobic site in PKCδ, is PKCζ. Since the protein kinase responsible for phosphorylating the hydrophobic site in $p70^{S6kinase}$ has not been identified, it is not possible to state whether or not PKCζ also has a role in the phosphorylation at the equivalent site in this enzyme.

Evidence from studies on the equivalent hydrophobic site in the classical PKC isotype PKCβII(1), suggested that it is autophosphorylated (Behn-Krappa and Newton, 1999). However, treatment of cells with the catalytic domain inhibitors, BIM-I or Go6983, indicated that the hydrophobic site in PKCδ or PKCε are not autophosphorylated (Figure 1). Under conditions of serum deprivation the TP site in PKCδ (S643), which had been previously characterized as an autophosphorylation site (Li et al., 1997b), did become dephosphorylated in response to treatment with the PKC catalytic inhibitors, BIM-I and Go6983; this occurred in a time-dependent

manner. This indicates that the nPKC isoform does have some activity in the serum-deprived, suspension cells which maintains a high level of phosphorylation at the S643 (TP) site, under these conditions. BIM-I treatment inhibits the PKC catalytic activity which might lead to a greater dephosphorylation (off) rate then a phosphorylation (on) rate.

Serum stimulation leads to an accumulation of DAG, and a recruitment of the PKC isoforms to the plasma membrane (Huang, 1989; Orr et al., 1992). The nPKC isoform binds DAG via the C1-domain to generate an activated conformation (Mosior and Newton, 1995). It is not clear whether the T loop or the hydrophobic site become phosphorylated first. However, it does seem that a phosphate at the hydrophobic site is more sensitive to phosphatase action than at the T loop, as suggested by the effect of an alanine substitution at the T loop. The T505A mutant only accumulates a phosphate at the hydrophobic site if the cells are stimulated with serum in the presence of the PP1 and PP2A protein phosphatase inhibitor, okadaic acid. A possible explanation for this is that a phosphate is more labile at the hydrophobic site if the T loop site is unphosphorylated. Once the T loop site becomes phosphorylated, a change in conformation could occur to the polypeptide at the C-terminus, leading to the phosphate at the hydrophobic site no longer being exposed.

This behavior of alanine substitution mutants of the T loop site in PKCδ and PKCε is consistent with that observed for PKCα, (Bornancin and Parker, 1996; Bornancin and Parker, 1997; Gysin and Imber, 1996). These papers describe how the absence of a phosphate at either the T loop, TP, or hydrophobic site can sensitize the remaining sites to dephosphorylation. However, it seems that the absence of a phosphate at the T loop site can still produce a fully active protein kinase. This is supported by findings from Stempka and co-workers (Stempka et al., 1997). It appears there is no absolute order of phosphorylation between the three sites, but there does appear to be an inter-dependence, or preferred order between the T loop and the hydrophobic sites. This is consistent with the response to rapamycin and

LY294002 treatment. Rapamycin treatment affects only the hydrophobic site. The absence of a phosphate at the hydrophobic site does not significantly increase the sensitivity of the T loop site to phosphatase action. However, after LY294002 treatment, the absence of phosphate at the T loop site probably affects the sensitivity of the hydrophobic site to phosphatases. Nevertheless, mTOR, which also appears to regulate the phosphorylation of the hydrophobic site (Figure 7), is also sensitive to inhibition by the PI 3-kinase inhibitors, LY294002 and wortmannin (Brunn et al., 1996). Therefore, inhibition of mTOR with either rapamycin or the PI 3-kinase inhibitors, would prevent the accumulation of phosphate at the hydrophobic site in PKCδ and PKCε.

To put the observations of the phosphorylation at the hydrophobic site in a physiological context, the effects of nutrient deprivation was examined. Depriving cells of amino acids inhibits the serum-induced phosphorylation of the hydrophobic site in both PKCδ and PKCε. This did not affect the serum-induced phosphorylation of the T loop site, suggesting that the PI 3-kinase/PDK-1 pathway is independent of the pathway which leads to the phosphorylation of the hydrophobic site. Though it is possible that the nutrient regulation of the hydrophobic site in PKCδ is via the control of PKCζ in the V5α-kinase complex, it is more likely to operate through the regulation of mTOR, as described in yeast (Cardenas et al., 1999; Di Como and Arndt, 1996), or the mammalian homologue of the yeast Ure2p protein (Beck and Hall, 1999; Beck et al., 1999). However, this mechanism has not been clearly defined. The nutrient-dependent control mechanism appears to resemble that operating for p70 S6 kinase (Ferrari et al., 1993). Some groups have reported that mTOR directly phosphorylates this site in p70 S6 kinase (Burnett et al., 1998; Hara et al., 1998). It has also been reported that there is a functional interaction between mTOR and PKCδ (Kumar et al., 2000). However, numerous groups have proposed that mTOR operates by negatively regulating protein phosphatases, this has been supported primarily by epigenetic studies on the Tor1p and Tor2p proteins in yeast (Chen et al., 1998; Chung et al., 1999; Di Como and Arndt, 1996; Hara et al., 1998;

Harris et al., 1999; Jiang and Broach, 1999; Nanahoshi et al., 1998; Nanahoshi et al., 1999).

Dr W. H Ziegler and co-workers determined whether the V5α-kinase would also phosphorylate the hydrophobic site in PKCδ. Li, W and co-workers had demonstrated earlier that a GST-linked PKCδ fusion protein expressed bacteria, had about one-tenth the activity of the wild-type protein. They also revealed that the TP site in GST-linked PKCδ is phosphorylated under conditions of bacterial expression. This was verified by examining the TP site in GST-linked PKCδ under similar conditions of bacterial expression, using phospho-S643 specific antiserum (chapter 3, figure 2). Dr W. H Ziegler and co-workers demonstrated that GST-linked PKCδ autophosphorylates at a site distinct from the hydrophobic site. Furthermore, the hydrophobic site is only phosphorylated if the GST-linked PKCδ fusion protein is incubated in the presence of the purified V5α-kinase protein preparation, as detected using the phospho-S657 specific antisera (Ziegler et al., 1999). V5α-kinase protein was extracted from U937 cells, treated with either rapamycin or LY294002 to determine whether the serum-induced phosphorylation of the T loop and hydrophobic site in PKCδ would be affected. The activity of the V5α-kinase was inhibited by more then 75% when extracted from cells pre-treated with either rapamycin or LY294002 (Ziegler et al., 1999). Since a complete inhibition in activity of V5α-kinase was not observed following treatment with the inhibitors, it suggested that under *in vivo* conditions other rapamycin or LY294002-sensitive processes, such as recruitment to a particular cellular compartment, could also be involved in the phosphorylation of the hydrophobic site.

The identification of PKCζ as a possible component of the V5α-kinase led to a series of *in vivo* studies to clarify the role it might play. It seems that PKCζ is a required component of the V5α-kinase complex, however, it still remains unclear in what context it is involved. The PKCζ T410E/T560E mutant seemed to by-pass the inhibition of rapamycin and LY294002 treatment on the serum-induced phosphorylation of the hydrophobic site in PKCδ. A possible explanation of why

the hydrophobic site accumulates in a phosphorylated form in PKCδ, even in the absence of a phosphate at the T loop site, could be that the PKCζ T410E/T560E mutant is more active than the endogenous wt-PKCζ, such that the rate of phosphorylation exceeds the rate of dephosphorylation.

Overexpressing wt-PKCζ with PKCδ could have had many possible implications. For example, if wt-PKCζ was behaving as a scaffold protein in the V5α-kinase complex and supporting the components that associated with it, transiently over-expressing PKCζ could titre out the other components. This would lead to a reduction in the serum-induced phosphorylation observed at the hydrophobic site. This was not observed (figure 12). It is also possible that expressing large amounts of PKCζ, did not titre out the components and this resulted in the generation of more active V5α-kinase. This could lead to a greater serum-induced phosphorylation at the hydrophobic site, even in the absence of a phosphate at the T loop site (Figure 12). Another possibility is that if PKCζ is the active V5 kinase, or the limiting kinase in the V5 kinase complex, transiently over-expressing PKCζ with PKCδ could lead to an abundance of active V5 kinase. Consequently, there ought to be a more robust serum-induced phosphorylation at the hydrophobic site.

There was a rapamycin and LY294002-mediated inhibition on the serum-induced phosphorylation of the hydrophobic site in PKCδ, in the presence of exogenous wild-type PKCζ (Figure 15). This is distinct from the effect of the PKCζ T410E mutant or the PKCζ T410E/T560E mutant on the hydrophobic site in PKCδ (Figures 10 and 13). It is possible that in order for PKCζ to by-pass the inhibitory effects of rapamycin or LY294002 on the hydrophobic site in PKCδ, PKCζ has to be much more catalytically active. Under conditions of wild-type PKCζ over-expression, it is possible that endogenous mTOR has more influence on the phosphorylation state of the hydrophobic site in PKCδ. Treatment with LY294002 would have the combined effect of inhibiting the serum-induced phosphorylation of the T loop site in PKCδ and wild-type PKCζ as well as inhibiting the catalytic activity of mTOR. All these

factors probably contribute to the inhibition of the serum-induced phosphorylation of the hydrophobic site in PKCδ. While rapamycin pre-treatment would inhibit mTOR, it would not inhibit the serum-induced phosphorylation of the T loop site in PKCδ. However, the effect of mTOR inhibition still appears to have led to the inhibition of the hydrophobic site in PKCδ. This suggests that mTOR has a greater influence on the phosphorylation state at the hydrophobic site than PKCζ perhaps via a phosphatase that acts preferentially on this site

There remains the possibility that the V5α-kinase may be regulated by a different mechanism, such as recruitment to the plasma membrane, or targeting to a defined cellular compartment. It could be the controlled recruitment of the V5α-kinase that was sensitive to treatment with either rapamycin or LY294002. Under such conditions, the protein phosphatases would maintain the protein in a dephosphorylated state in the absence of a local increase in the V5α-kinase. We were unable to test this as the components of the V5α-kinase complex were not fully identified. Since the membrane-associated PKCζ, recovered from cells treated with either rapamycin or LY294002 was inhibited by less than 50%, it could support the possibility that treatment with these inhibitors does not significantly affect the activity of the V5α-kinase directly, but instead regulates the mechanism of recruitment of these proteins to a given intracellular compartment.

The regulatory mechanism of the phosphorylation at the hydrophobic site in PKB/Akt has been examined by several groups (Alessi et al., 1997b; Balendran et al., 1999a; Toker and Newton, 2000). However, there does not appear to be a generally accepted mechanism. These mechanisms, and their possible relevance on the regulation of the hydrophobic site in PKCδ and PKCε will be discussed further in the final discussion chapter, chapter 7.

5.0 Adherence-mediated control of the phosphorylation of nPKCs

5.1 Introduction

Early observations showed that depriving adherent cells of serum for 24 hours did not lead to a consistent reduction in phosphorylation at the T loop, TP, or hydrophobic sites in either PKCδ or PKCε. This suggested that a signalling pathway was activated which functioned in the absence of stimuli from serum treatment. An integrin-mediated signalling pathway had already been demonstrated by several groups in providing proliferative and cell survival signals (Boudreau et al., 1995; Bourdoulous et al., 1998; Crouch et al., 1996; Frisch and Francis, 1994; Frisch and Ruoslahti, 1997; Frisch et al., 1996a; Frisch et al., 1996b; Malik and Parsons, 1996; Meredith et al., 1993; Ng et al., 1999a; Ng et al., 1999b; Udagawa and McIntyre, 1996). PKC has also been implicated as a key player of integrin-mediated signalling (Disatnik and Rando, 1999; Ng et al., 1999a; Ng et al., 1999b; Wang et al., 2000; Woods and Couchman, 1992). These studies relating integrin signalling with PKC, supported the hypothesis of an integrin-mediated signalling pathway influencing the phosphorylation of the T loop and hydrophobic sites in PKCδ and PKCε. The aim of these investigations was to determine whether integrin engagement, or signalling, influenced the phosphorylation of these sites in PKCδ and PKCε, and what control mechanisms are involved in regulating this form of signalling.

One protein that will be examined in more depth is the tumour suppresser protein PTEN. PTEN is a well-characterised product of a tumour suppresser gene (Cantley and Neel, 1999; Dahia, 2000; Di Cristofano and Pandolfi, 2000; Podsypanina et al., 1999). It has been directly implicated in the control of the both the PI 3-kinase signalling pathway and the integrin activation-mediated signalling pathway (Gu et al., 1998; Tamura et al., 1999a; Tamura et al., 1999b; Tamura et al., 1999c; Vazquez

and Sellers, 2000; Wu et al., 1998). PTEN is a dual phosphatase, with specificity for the phosphate on tyrosine residues in proteins and the phosphate specifically at the third position of the inositol ring in poly-phosphorylated phosphatidyl inositides such as PI (3,4,5)P_3 and PI (3,4) P_2 producing PI (4,5)P_2 and PI (4)P, respectively (Lee et al., 1999; Maehama and Dixon, 1998; Tamura et al., 1998). In this way, PTEN reverses the effects of PI 3-kinase signalling (Vazquez and Sellers, 2000). The effects of PTEN on both modulating the PI 3-kinase pathway and the adherence-dependant signalling pathway (Lee et al., 1999; Maehama and Dixon, 1998; Tamura et al., 1999a; Tamura et al., 1999b) will be examined with respect to the phosphorylation at the T loop or hydrophobic site in the nPKC isoforms.

5.2 Results

5.2.1 The effect of integrin engagement on the phosphorylation of the T loop and hydrophobic site in nPKC isoforms.

Early observations revealed that the T loop and hydrophobic sites remained highly phosphorylated when the cells were deprived of serum while adherent (Figure 1a). Treatment with serum did not appear to lead to an increase in the phosphorylation of the T loop or hydrophobic sites in PKCδ. Furthermore, treatment with the potent PI 3-kinase inhibitor, LY294002, for 30 minutes did not seem to affect these sites either. It appeared that this was in complete contrast to the observations described earlier in chapters 3 and 4, in which treatment of cells deprived of serum in suspension with LY294002, led to an inhibition of the serum-induced phosphorylation of the T loop or hydrophobic sites in the nPKC isoforms. This implied that disruption of the interactions between the cells and their extracelluar matrix was required before a low basal level of phosphorylation is observed.

Figure 1a

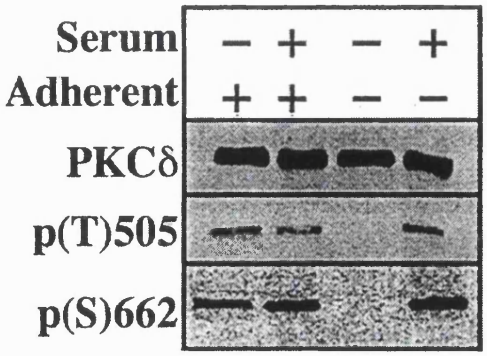


Figure 1b

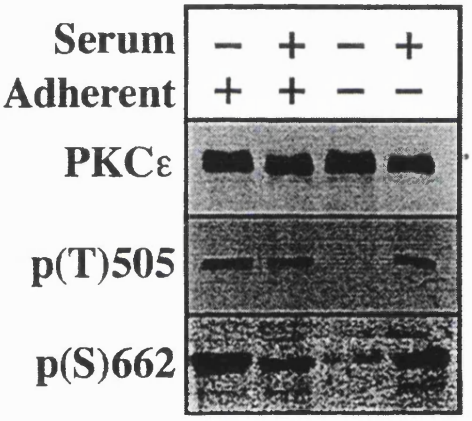


Figure 1a and 1b. Effect of adherence on phosphorylation at the T loop and hydrophobic site in nPKC isoforms following serum deprivation of HEK/293 cells.
HEK/293 cells were transiently transfected with either PKCδ (1a) or PKCε (1b), deprived of serum for 24 hours while either in adherent or in suspension (adherent-), and then treated with serum (10%-final) for 30 minutes. PKCδ and PKCε protein expression and phosphorylation at the T loop site (T505 and T566, PKCδ and PKCε, respectively) and at the hydrophobic site (S662 and S729, PKCδ and PKCε, respectively) was monitored by Western blotting.

Unless the T loop and hydrophobic sites are unphosphorylated following serum deprivation, the effects of acute LY294002 treatment would not be observed. Furthermore, a serum-induced phosphorylation at the T loop and hydrophobic sites

was only observed when HEK/293 cells were deprived of serum while in suspension for 24 hours and then treated with serum. Similar observations were also made for PKCε, suggesting that these responses were not peculiar to one PKC isoform (Figure 1b).

The requirement of depriving HEK/293 cells of stimulation from both serum and integrin engagement for 24 hours before a low basal phosphorylation is detected at the T loop and hydrophobic sites in the nPKC isoforms suggests that the 'turn over' of these phosphorylations is very low. Once the sites are phosphorylated, they do not become dephosphorylated unless chronically deprived of all stimuli. Furthermore, it implies that signalling from integrin engagement is sufficient to maintain the phosphorylation at these two sites in the absence of serum.

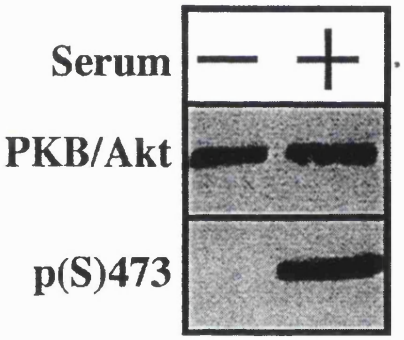


Figure 2. Effect of serum deprivation of adherent HEK/293 cells on phosphorylation at the hydrophobic site in PKB/Akt.
Adherent HEK/293 cells were deprived of serum for 24 hours. Whole cells extracts were analysed by Western blotting for protein expression and phosphorylation at the hydrophobic site (S473).

This is consistent with observations on the equivalent sites in classical PKCα (Bornancin and Parker, 1996; Bornancin and Parker, 1997; Hansra et al., 1996; Hansra et al., 1999). However, this is in contrast to the behaviour of the hydrophobic site (S473) in PKB/Akt, which will become unphosphorylated in serum-deprived adherent HEK/293 cells (Figure 2). This suggests that the

hydrophobic site in PKB/Akt is not regulated in quite the same way as the equivalent site appears to be in nPKCs by these stimuli.

5.2.2 The influence of the adherence-mediated phosphorylation on the catalytic activity of PKCδ.

To determine whether adherence also influenced the catalytic activity of the PKC isoforms, PKCδ was immunopurified from HEK/293 cells deprived of serum for 24 hours while adherent. This was compared to the catalytic activity of PKCδ extracted from HEK/293 cells deprived of serum while in suspension (Figure 3). The effect of serum stimulation of cells under both situations was also noted.

By expressing the specific activity of PKCδ from a non-treated cell, as a function of that from a serum stimulated cell, the influence of adherence on the catalytic activity could be assessed.

Phosphorylation at the T loop and hydrophobic sites seem to have a direct effect on the catalytic activity of the protein kinase. Although adherent HEK/293 cells were cultured in the absence of serum for 24 hours, PKCδ still appeared to have almost maximum activity. Infact, there did not seem to be an increase in catalytic activity following serum treatment of adherent cells. To observe a serum-induced increase in catalytic activity, the HEK/293 cells had to be deprived of serum while in suspension for a minimum of 24 hours. This is consistent with observations in chapters 3 and 4 where catalytic activity paralleled the phosphorylation at the T loop and hydrophobic sites.

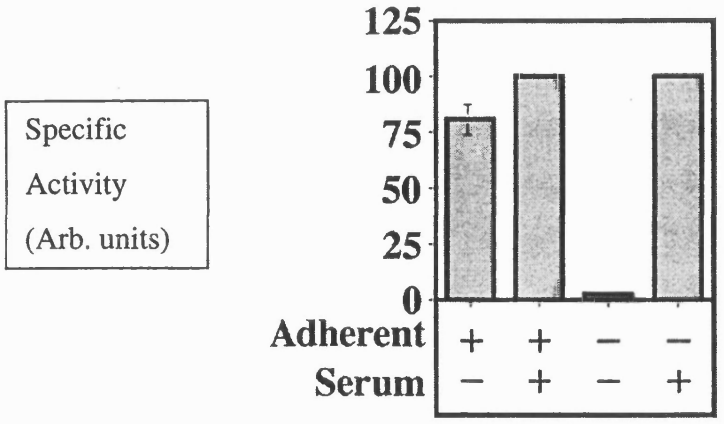


Figure 3. The influence of adherence on catalytic activity of PKCδ.
HEK/293 cells, transiently over expressing myc-tagged PKCδ, were deprived of serum for 24 hours while either adherent or in suspension (adherent -) and then treated for 30 minutes with serum. Myc-tagged PKCδ was immunopurified using an anti-myc antiserum and then assayed for catalytic activity using myelin basic protein (MBP) as a substrate. The activity was quantified by Cerenkov counting of SDS-PAGE-purified MBP. Relative PKCδ concentrations were determined by densitometry of SDS-PAGE-purified PKCδ bands. Specific activities were calculated as kinase activity/PKCδ concentration. These represent the means of duplicate determinations from three independent experiments.

5.2.3 Activation of the β1-integrin pathway affects the phosphorylation of the T loop and hydrophobic sites in PKCδ.

Although it seemed likely that adherence could mediate the phosphorylation of the T loop and hydrophobic sites in the PKC isoforms, the next step was to determine whether integrin activation alone would have the same effect. As discussed in part 5.1 of this chapter, several groups had previously established that integrin signalling could affect PKC function/translocation. Furthermore, since PI 3-kinase has been shown to be under the control of integrin signalling in certain conditions (Khwaja et al., 1997), it seemed a strong possibility that this pathway also mediated the phosphorylation of the T loop and hydrophobic sites in these nPKC isoforms.

Two approaches were used to determine whether β1-integrin activation influenced the phosphorylation of the T loop and hydrophobic sites in nPKC isoforms. The first required the use of an anti-β1-integrin antiserum. Antisera would bind to the extra-cellular epitope of the β1-integrin polypeptide and lock it into an activated/ligand-bound conformation. The next step was to cross-link the antibodies, to mimic the clustering of β1-integrin chains in the event of binding to an extra-cellular matrix. Two different anti-β1-integrin antisera were used; 12G10 (obtained from Prof. Martin Humphries) and TS2/16 (obtained from I.C.R.F monoclonal antibody production). The second approach was to note the effect of plating serum-deprived HEK/293 cells, for a limited time, on to a collagen or poly-D-lysine matrix.

When serum-deprived HEK/293 cells were treated with either the anti-β1-integrin antiserum alone or the cross-linking anti-anti-β1-integrin antiserum alone, no effect on the phosphorylation of the T loop and hydrophobic sites in PKCδ was observed. However, when these cells were treated first with the anti-β1-integrin antiserum and then with the cross-linking antibody, the T loop and hydrophobic sites in PKCδ were affected (Figure 4a). It seems that integrin engagement by 12G10 and cross-linking at the cell surface of serum-starved cells was sufficient to induce the phosphorylation at the T loop and hydrophobic sites.

To determine whether this effect was unique to the action of 12G10 treatment, the equivalent experiment was performed using a different anti-β1-integrin antiserum, but which detected the same epitope as 12G10, called TS2/16. Integrin engagement and subsequent cross-linking on the surface of serum-deprived HEK/293 cells in suspension was also sufficient to induce the phosphorylation at the T loop and hydrophobic sites in PKCδ (Figure 4b). The β1-integrin-induced phosphorylation of the T loop and hydrophobic sites in PKCδ after 60 minutes, was not significantly increased with subsequent treatment of the cells with serum.

Figure 4a

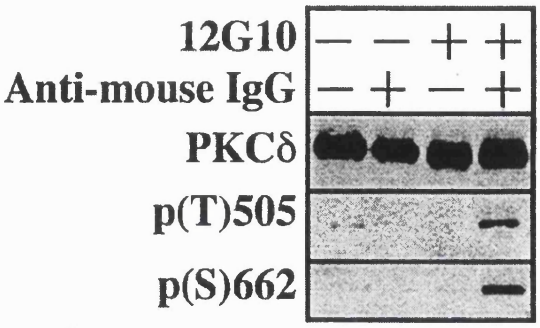


Figure 4b

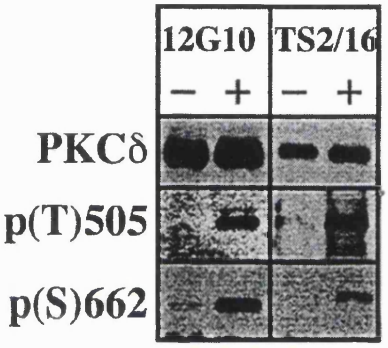


Figure 4a and 4b. Effects of β1-integrin activation on the surface of HEK/293 cells in suspension.
HEK/293 cells, transiently transfected with PKCδ, were serum-staved while in suspension for 24 hours. In 4a, cells were incubated with either 12G10 (anti- β1-integrin activating antisera) alone for 30 minutes, anti-mouse IgG antibody (cross-linking antibody) alone for 30 minutes, or first 12G10 followed by the cross-linking antibody. In 4b, the serum-deprived suspension culture of transfected HEK/293 cells were incubated first with either 12G10 or TS2/16 and then with the cross-linking antibody. PKCδ protein expression and phosphorylation at the T loop and hydrophobic sites were monitored by Western blotting of cell extracts.

As described earlier (chapters 3 and 4), treatment of serum-deprived HEK/293 cells with the potent mTOR inhibitor rapamycin, inhibits the serum-induced phosphorylation of only the hydrophobic site. However, treatment with the PI 3-

kinase inhibitor LY294002, inhibits the serum-induced phosphorylation of both the T loop and hydrophobic sites. This was true for both PKCδ and PKCε. Interestingly, following serum treatment of the cells, the phosphorylated T loop and hydrophobic sites were no longer sensitive to the effects of acute inhibitor treatment.

The next step was to determine if the β1-integrin-induced phosphorylation of the T loop and hydrophobic sites behaved in the same way. One of the questions this addressed is whether the signalling pathway initiated following the engagement of the β1-integrins was the same as that which led to the serum-induced phosphorylation of the T loop and hydrophobic sites in nPKC isoforms (Figure 5).

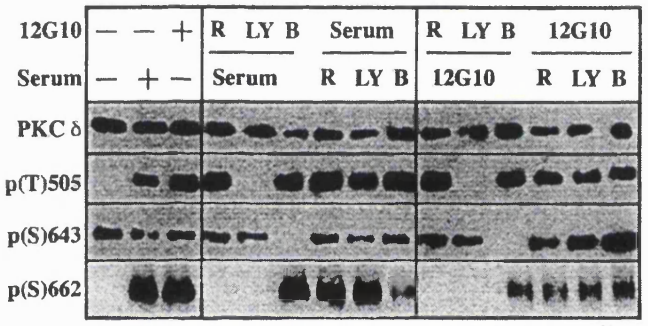


Figure 5. The effect of rapamycin, LY294002, and BIM-I on the β1-integrin activation-induced phosphorylation of PKCδ.
HEK/293 cells, transiently transfected with PKCδ, were serum-staved while in suspension for 24 hours. The cells were treated for 30 minutes with either 20nM rapamycin (R), 10μM LY294002 (LY), or 10μM Bisindolylmaleimide-I (B) before stimulation (above serum or 12G10) or after stimulation (below serum or 12G10). PKCδ protein expression and phosphorylation at the T loop (T505), TP (S643) and hydrophobic sites ((S729), was determined by Western blotting of cell extracts. This is one of three similar experiments.

Activation of the β1-integrin signalling pathway in serum-starved HEK/293 suspension cells led to the same pattern of sensitivity to rapamycin and LY294002 treatment as previously observed following serum treatment. Rapamycin treatment before 12G10-mediated β1-integrin activation led to an inhibition of the

phosphorylation at the hydrophobic site in PKCδ. Pre-treatment with LY294002 resulted in an inhibition of the β1-integrin-induced phosphorylation of both the T loop and hydrophobic sites.

Consistent with previous observations (chapter 4), acute treatment of serum deprived HEK/293 cells in suspension with rapamycin or LY294002 did not affect the phosphorylation at the TP site in PKCδ (S643). However, treatment of these serum-starved cells with the PKC catalytic inhibitor BIM-I prior to 12G10-mediated β1-integrin activation did lead to the loss of phosphate at the TP site. However BIM-I treatment, did not affect the β1-integrin-induced phosphorylation of either the T loop or hydrophobic sites. Furthermore, if the serum-starved HEK/293 cells were treated with inhibitors after 12G10-mediated β1-integrin activation, there was no detectable effect of the inhibitors on the phosphorylation at the T loop, TP, or hydrophobic sites in PKCδ. The apparent reduced phosphorylation at the hydrophobic site in PKCδ following 12G10-mediated β1-integrin activation and then LY294002 treatment, was not consistently observed.

These experiments suggest that the signalling pathways activated following serum stimulation of HEK/293 cells (deprived of serum for 24 hours while in suspension), are also activated following 12G10-mediated β1-integrin activation. Both acute serum treatment and β1-integrin activation lead to the phosphorylation of the T loop and hydrophobic sites within 30 minutes of stimulation. Furthermore, the two forms of stimulation lead the T loop and hydrophobic sites to be similarly affected by treatment with inhibitors. The TP site seemed to be unaffected by the effects of β1-integrin activation as it was already phosphorylated. This was consistent with the behaviour of this site following serum treatment, and therefore supports the earlier hypothesis.

It was possible that the signalling pathway(s) activated following integrin engagement differed between suspension and adherent cells. Therefore, HEK/293 cells, deprived of serum for 24 hours while in suspension, were replated on to a cell

culture dish, pre-coated with either collagen or poly-D-lysine, for one hour (Figure 6). It seems that only when the serum-starved cells are plated on to collagen for one hour is there a phosphorylation at the T loop or hydrophobic site. Furthermore, if these cells are then treated with the PI 3-kinase inhibitor, LY294002, for 30 minutes there is no apparent change in the phosphorylation at either the T loop or hydrophobic site in the nPKC isoforms.

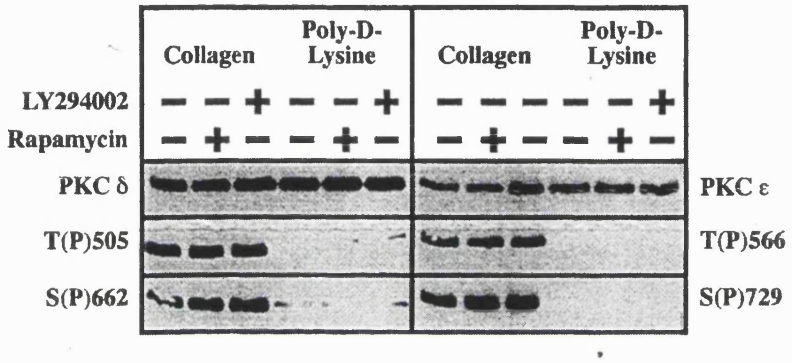


Figure 6. Effect of plating HEK/293 cells onto a β1-integrin substrate (collagen) or onto (poly-D-lysine) on the phosphorylation at the T loop and hydrophobic site in two nPKC isoforms.
HEK/293 cells transiently over-expressing either PKCδ or PKCε, were deprived of serum for 24 hours while in suspension. They were plated onto either collagen or poly-D-lysine for one hour. PKCδ and PKCε protein expression and phosphorylation at the T loop site (T505 and T566, PKCδ and PKCε, respectively) and at the hydrophobic site (S662 and S729, PKCδ and PKCε, respectively) was monitored by Western blotting.

This is consistent with earlier observations, in which the phosphorylated forms of the T loop and hydrophobic sites, following 12G10-mediated β1-integrin activation of serum-deprived suspension HEK/293 cells, displayed little sensitivity to the effects of acute inhibitor treatment. Therefore, it seems that the β1-integrin signalling pathway(s) activated when serum-starved HEK/293 cells bind to a collagen-based extracellular matrix is similar to the pathway initiated following β1-integrin engagement and cross-linking at the surface of suspension cells.

Since extracellular matrix factors, such as fibronectin and collagen, also exist in serum, the serum-induced phosphorylation observed at the T loop or hydrophobic site in the nPKC isoforms could be due to the activation of signalling pathway(s) following β1-integrin engagement and cross-linking at the surface of suspension cells alone. This raised the question of whether phosphorylation at these two sites in nPKC isoforms can be induced by growth factor stimulation of HEK/293 cells. To test this, HEK/293 cells (deprived of serum for 24 hours while in suspension) were treated with two purified growth factors that are predominant in serum; platelet derived growth factor B-chain homodimer (PDGF-BB) and lysophosphatidic acid (LPA), for 30 minutes (Figure 7).

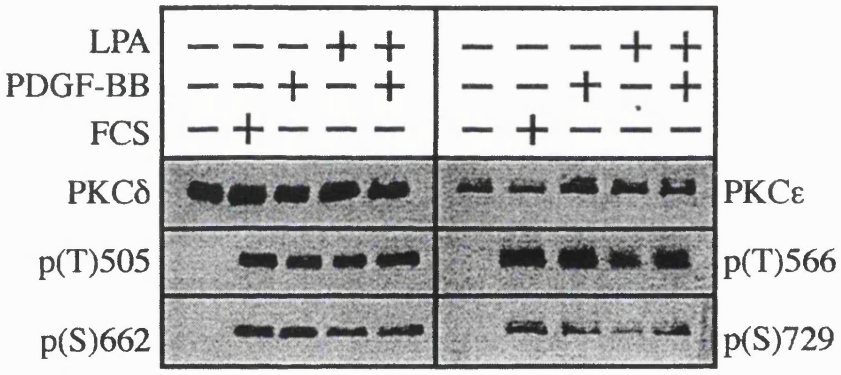


Figure 7. The effect of treating HEK/293 cells with purified growth factors. HEK/293 cells transiently over-expressing either PKCδ or PKCε, were deprived of serum for 24 hours while in suspension. The cells were treated with either 30ng/ml PDGF-B-chain homodimer (PDGF-BB), 10μM LPA or both PDGF-BB and LPA together for 30 minutes. PKCδ and PKCε protein expression and phosphorylation at the T loop site (T505 and T566, PKCδ and PKCε, respectively) and at the hydrophobic site (S662 and S729, PKCδ and PKCε, respectively) was monitored by Western blotting.

It seems that treatment of serum-deprived HEK/293 cells with either PDGF-BB or LPA can induce the phosphorylation at the T loop or hydrophobic site in PKCδ and PKCε. Therefore, both β1-integrin engagement and growth factor stimulation can elicit the phosphorylation at these two sites in the nPKC isoforms.

5.2.4 The role of PTEN in the phosphorylation of nPKC isoforms at the T loop and hydrophobic site.

PTEN has been implicated in modulating the integrin-induced signalling pathway by dephosphorylating phospho-tyrosine residues in focal adhesion associated proteins such as $p125^{FAK}$ which influences the PI 3-kinase pathway (Lee et al., 1999; Maehama and Dixon, 1998; Tamura et al., 1999a; Tamura et al., 1999b). To determine whether PTEN is involved in the regulation of the phosphorylation at the T loop or hydrophobic site in the nPKC isoforms, a $PTEN^{-/-}$ cell line (UM-UC-3) was used.

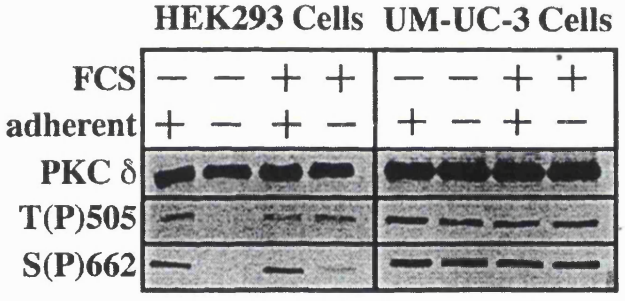


Figure 8. The influence of PTEN on the phosphorylation of PKCδ in suspension.
HEK/293 cells (expressing endogenous PTEN), and UM-UC-3 cells ($PTEN^{-/-}$), were transiently transfected with PKCδ, and then deprived of serum for 24 hours while adherent or in suspension (adherent -). PKCδ protein expression ad phosphorylation at the T loop (T505) and hydrophobic sites (S729), was determined by Western blotting of cell extracts. This was similar to three previous experiments.

UM-UC-3 cells originate from a bladder carcinoma lacking the PTEN gene (Hamilton et al., 2000). PKCδ was transiently transfected into both HEK/293 cells

(expressing PTEN) and UM-UC-3 cell (lacking PTEN) and then deprived of serum for 24 hours while either adherent or in suspension (Figure 8).

Interestingly, both PKCδ and PKCε remained fully phosphorylated at the T loop and hydrophobic site when expressed in UM-UC-3 cells deprived of serum for 24 hours while in suspension (Figure 9). This was in contrast to the phosphorylation at these sites when expressed in HEK/293 cells under identical conditions. Furthermore, when the suspension culture of serum deprived UM-UC-3 cells expressing PKCδ, were treated with serum for 30 minutes, there did not appear to be an increase in the phosphorylation at the T loop or hydrophobic site. Again, this is in contrast to the phosphorylation at these sites in nPKC isoforms when expressed in HEK/293 cells under the same conditions.

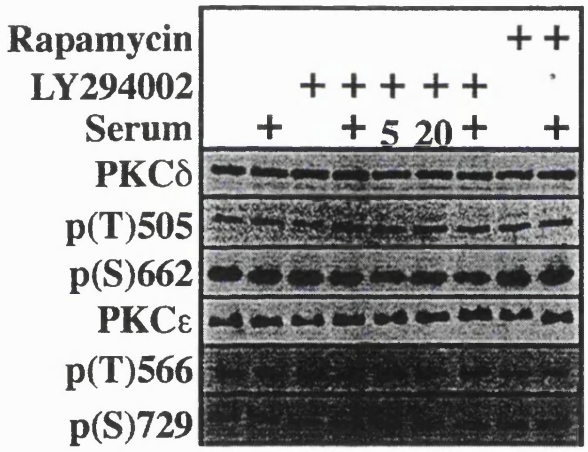


Figure 9. The effect of rapamycin or LY294002 treatment on the serum-induced phosphorylation of two nPKC isoforms in UM-UC-3 cells.
UM-UC-3 cells were transiently transfected with either PKCδ or PKCε deprived of serum for 24 hours while in suspension. The cells were treated with either 20nM rapamycin or 10μM LY294002, and then stimulated with serum for either 5 minutes (5), 20 minutes (20) or 30 minutes (+). PKCδ and PKCε protein expression and phosphorylation at the T loop site (T505 and T566, PKCδ and PKCε, respectively) and at the hydrophobic site (S662 and S729, PKCδ and PKCε, respectively) was monitored by Western blotting. This is one of four similar experiments.

Consistent with earlier observations, acute treatment of a serum deprived UM-UC-3 suspension cell culture with either rapamycin or LY294002 for 30 minutes, did not affect the phosphorylation at either the T loop or hydrophobic sites in PKCδ (Figure 9). These responses were also observed for PKCε (Figure 9) implying that these effects were not a distinction between PKCδ and other members of the nPKC family.

The absence of PTEN in the UM-UC-3 cells seems to have had an affect on the phosphorylation at the T loop and hydrophobic site following serum deprivation for 24 hours in suspension. Interestingly, whether UM-UC-3 cells were deprived of serum under adherent or suspension conditions, or whether treated with serum or left untreated, the phosphorylation of the T loop and hydrophobic sites seemed to have remained unchanged. This could reflect the possibility of a greater 'on rate' then 'off rate' of phosphorylation at these sites, under these conditions. The effect of an absence of PTEN on the phosphorylation at the T loop and hydrophobic sites in UM-UC-3 cells under the various conditions, appears to be very similar to the effect of depriving HEK/293 cells of serum for 24 hours while adherent.

To further clarify the role of PTEN in the pathway for phosphorylating the T loop and hydrophobic sites in nPKC isoforms, UM-UC-3 cells stably expressing PTEN (UM-UC-3+PTEN) were obtained from Dr Roy Katso at the Ludwig Institute of Cancer Research in London. The aim was firstly, to compare the influence of the presence or absence of PTEN in one cell-type, and secondly, to perform a study without being concerned about the effects of some cells expressing larger quantities of PTEN than others, or some cells not being co-transfected with both nPKC isoform and a PTEN construct. Two PTEN-stabily expressing UM-UC-3 cell clones were provided, PTEN1 and PTEN2. They expressed approximately equivalent levels of PTEN protein, as determined by Western blotting (Figure 10 reproduced with kind permission from Dr R Katso).

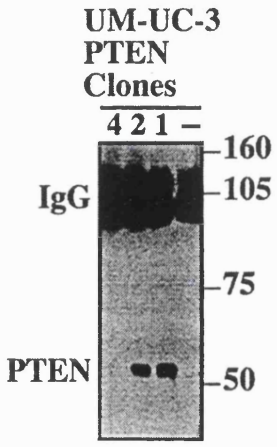


Figure 10. PTEN expression in stably transfected UM-UC-3 cells.
UM-UC-3 cells were transfected with a PTEN vector and selected by treatment with neomycin (800μg/ml) for two weeks. PTEN in these clones was immunopurified using an anti-PTEN antisera (Santa Cruz A2B1) to identify PTEN expression. The immune complexes were incubated in SDS-PAGE sample buffer at room temperature to promote the formation of IgG heavy-chain dimers (100KDa) so the IgG heavy-chain monomers (50KDa) would not mask the PTEN protein. Three clones are shown. Clones 1 and 2 with approximately equivalent levels of PTEN expression, was identified by Western blotting with A2B1. Non-transfected UM-UC-3 cells (-) are also shown for comparison.

These clones were chosen because the PTEN levels were not too high so as to suppress an insulin-induced PKB/Akt activation (Figure 11 reproduced with kind permission from Dr R Katso). It was interesting to note that the null clones ($PTEN^{-/-}$) had high basal PKB kinase activity which did not appear to increase significantly with IGF-1 stimulation. This was also been observed with the PTEN null mouse embryo fibroblasts (Dr R Katso communication). Furthermore, it seemed that the IGF-1 induced phosphorylation at the hydrophobic site (S473) in PKB was not higher in the null cell lines. The reason for this could be that the hydrophobic site in PKB was maximally phosphorylated, and IGF-1 treatment did not have any significant effect on the phosphorylation of this site.

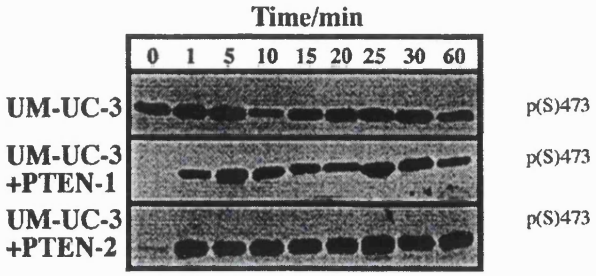


Figure 11. Effect of IGF-1 treatment on the phosphorylation of the hydrophobic site in PKB/Akt.
Serum deprived UM-UC-3 cells, and UM-UC-3 clones 1 and 2, stably expressing PTEN, were deprived of serum for 24 hours and then treated with 20ng/ml IGF-1. Cell lysates were analysed for phosphorylation at the hydrophobic site (S473) using a phospho-specific polyclonal antibody.

Most experiments were performed by transfecting only the UM-UC-3+PTEN1 clone, while some experiments were repeated in UM-UC-3+PTEN2 clone for comparison. Both UM-UC-3 and UM-UC-3+PTEN1 cells were transfected with PKCδ and then deprived of serum for 24 hours while adherent, or in suspension. The T loop and hydrophobic sites in PKCδ were unphosphorylated only in the UM-UC-3+PTEN1 cells, which had been maintained in a suspension culture without serum for a period of 24 hours (Figure 12).

This behaviour is in contrast to what was observed at these sites when PKCδ was expressed transiently in serum-starved UM-UC-3+PTEN1 adherent cells. Under adherent conditions, both the T loop and hydrophobic site were phosphorylated. PTEN appeared to have had little effect on the phosphorylation at either the T loop or hydrophobic site when UM-UC-3+PTEN1 cells were cultured in adherent conditions without serum for 24 hours. The high basal level of phosphorylation at the T loop and hydrophobic site in serum-starved adherent UM-UC-3+PTEN1 cells, resembles the situation that is observed for these sites when PKCδ or PKCε is transiently expressed in HEK/293 cells and then deprived of serum for 24 hours

while adherent. Therefore, it seems that PTEN only has a negative effect on the phosphorylation at the T loop and hydrophobic sites in nPKC isoforms when the cells are deprived of serum for 24 hours while in suspension. Only after the prolonged absence of both growth factor and β1-integrin signalling of HEK/293 or UM-UC-3+PTEN cells, will nPKC isoforms accumulate as unphosphorylated proteins at the T loop and hydrophobic sites.

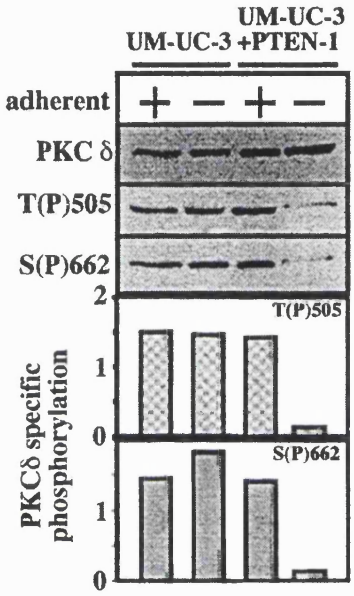


Figure 12. Effect of PTEN and adherence on the phosphorylation of PKCδ in UM-UC-3 cells.
UM-UC-3 cells and clone 1 of a PTEN-expressing UM-UC-3 cells (UM-UC-3+PTEN-1) were transiently transfected with PKCδ and then deprived of serum for 24 hours while either in an adherent or in suspension (adherent -) culture. Whole cell lysates were analysed by Western blotting for PKCδ protein expression and phosphorylation at the sites indicated on the left. The phospho-specific bands were quantified by densitometry, and expressed as a function of the quantified PKCδ protein expression, shown in the lower panels. This is one of three similar experiments.

To determine whether the behaviour of the T loop and hydrophobic site in PKCδ or PKCε are cell-type specific, a pharmacological characterisation of these sites was

carried out in UM-UC-3+PTEN cells. When UM-UC-3+PTEN cells were transfected with PKCδ, deprived of serum for 24 hours in suspension, and then treated with serum for 30 minutes, there was a serum-induced phosphorylation detected at the T loop and the hydrophobic site. The T loop site was only sensitive to acute pre-treatment with LY294002, while the hydrophobic site was sensitive to pre-treatment with either rapamycin or LY294002 (Figure 13).

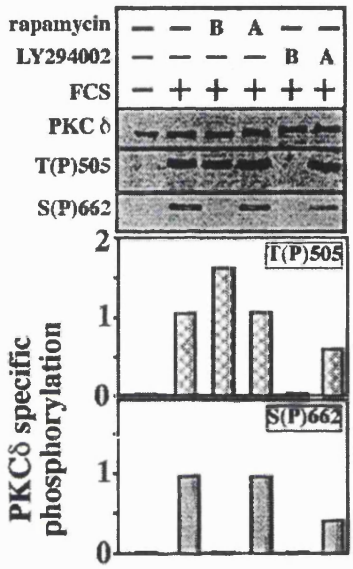


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Figure 13. Effect of PTEN on the sensitivity of the T loop and hydrophobic sites in PKCδ to inhibitor treatment in UM-UM-3 cells.
Clone 1 of PTEN-expressing UM-UC-3 cells (UM-UC-3+PTEN-1) were transiently transfected with PKCδ and then deprived of serum for 24 hours while in suspension culture. The cells were treated with either 20nM rapamycin or 10μM LY294002, for 30 minutes, before (B) or after (A) serum treatment (10%) for 30 minutes. Whole cell lysates were analysed by Western blotting for PKCδ protein expression and phosphorylation at the sites indicated on the left. The phospho-specific bands were quantified by densitometry, and expressed as a function of the quantified PKCδ protein expression, shown in the lower panels. This is one of two similar experiments.

However, if the serum-starved UM-UC-3+PTEN cells in suspension were stimulated with serum for 30 minutes and then treated with either LY294002 or rapamycin for 30 minutes, the T loop or hydrophobic site were no longer sensitive to their

inhibitory effects. This mimicked the behaviour of the T loop and hydrophobic site in nPKC isoforms examined in HEK/293 cells under the same conditions (Figure 1a and Chapter 3 and 4). Hence, the phosphorylation sites in the nPKC isozyme examined behaved similarly in both cell types.

The next step was to determine whether the catalytic activity of PKCδ, immunopurified from UM-UC-3+PTEN cells, reflected the changes in the phosphorylation at the T loop and hydrophobic site following serum deprivation in suspension (Figure 14).

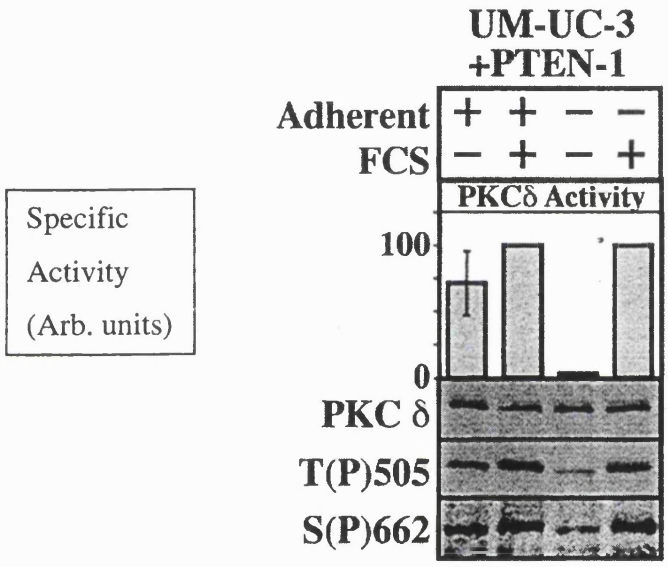


Figure 14. The influence of PTEN and adherence on the catalytic activity of PKCδ.
Clone 1 of PTEN-expressing UM-UC-3 cells (UM-UC-3+PTEN-1) were transiently transfected with myc-tagged PKCδ and then deprived of serum for 24 hours while either in an adherent or in suspension (adherent -) culture. The cells were treated with serum (10%) for 30 minutes, and then lysed. Myc-tagged PKCδ was immunopurified using an anti-myc antiserum, and then assayed for catalytic activity using myelin basic protein (MBP) as a substrate. The activity was quantified by Cerenkov counting of SDS-PAGE-purified MBP. The relative PKCδ concentrations were determined by densitometry of SDS-PAGE-purified PKCδ bands. Specific activities were calculated as kinase activity/PKCδ concentration. These represent the means of duplicate determinations from three independent experiments. The immunopurified PKCδ protein used in the in vitro kinase assay was also assayed for

phosphorylation at the T loop and hydrophobic site by Western blotting (lower panel).

Consistent with earlier observations in HEK/293 cells under the same conditions, PKCδ was fully active when extracted from an adherent culture of serum-starved UM-UC-3+PTEN cells. Furthermore, only a small increase in the catalytic activity of PKCδ was observed following serum treatment of the cells for 30 minutes. However, a serum-induced increase in catalytic activity of PKCδ was observed following treatment of a suspension culture of serum-starved UM-UC-3+PTEN. The PKCδ protein, immunopurified from UM-UC-3+PTEN cells for determination of catalytic activity, was also assayed for phosphorylation at the T loop and hydrophobic site by Western blotting. The *in vitro* catalytic activity of PKCδ paralleled the phosphorylation at the T loop and hydrophobic sites. This was consistent with earlier findings.

5.2.5 Analysis of long-term inhibition of PI 3-kinase activity on the phosphorylation of the T loop and hydrophobic site in nPKC isoforms.

The above data imply that adherent HEK/293 cells, are still receiving stimulation from the β1-integrin-mediated signalling pathway following 24 hours of serum deprivation. The phosphorylation at the T loop and hydrophobic sites are presumably maintained in serum-starved adherent cells due to the β1-integrin-induced activation of the PI 3-kinase pathway.

Since the hydrophobic site in PKB/Akt (S473) does not accumulate in a phosphorylated form under the same conditions (Figure 2), it suggests that the level of PI 3-kinase activity, and hence concentration of $PI(3,4,5)P_3$/ $PI(3,4,)P_2$, required

for inducing phosphorylation at the sites in question, differs between the nPKC isoforms and PKB/Akt.

Figure 15 a

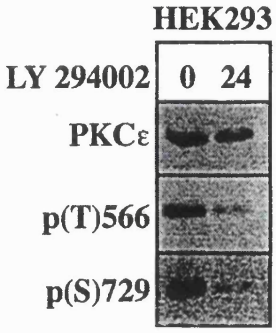


Figure 15 b

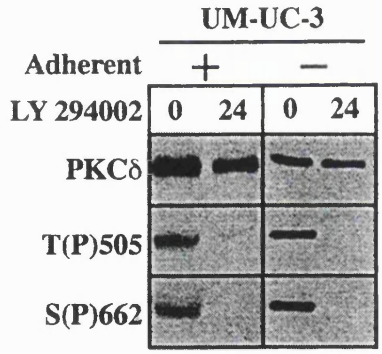


Figure 15 c

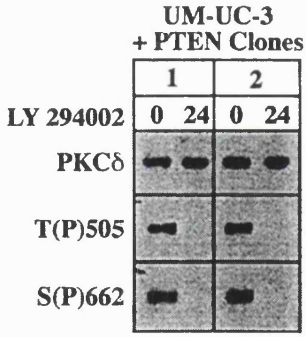


Figure 15. Effect of chronic PI3-kinase inhibition on the phosphorylation of the T loop and hydrophobic site in nPKC isoforms.
HEK/293 cells were transfected with PKCε (a), while UM-UC-3 cells and clone 1 of PTEN-expressing UM-UC-3 cells (UM-UC-3+PTEN-1) were transfected with PKCδ (b and c). The HEK/293 and UM-UC-3+PTEN-1 cells were deprived of serum under adherent culture conditions with or without the presence of 10μM LY294002 treatment. However, the UM-UC-3 cells were deprived of serum in either adherent or suspension (adherent -) culture conditions, with or without the presence of 10μM LY294002 treatment. Whole cell extracts were analysed for PKCδ and PKCε protein expression and phosphorylation at the T loop site (T505 and T566, PKCδ and PKCε, respectively) and at the hydrophobic site (S662 and S729, PKCδ and PKCε, respectively) was monitored by Western blotting. These were one of two similar experiments.

To determine whether the phosphorylation at the T loop and hydrophobic sites in nPKC isoforms is maintained under conditions of persistent PI 3-kinase activation, adherent cells were treated chronically with the PI 3-kinase inhibitor, LY294002, while undergoing serum deprivation. It was predicted that as the adherent cells were deprived of serum in the presence of LY294002, newly synthesised nPKC protein would not be influenced by the activation of the PI 3-kinase pathway. Although other β1-integrin-mediated signalling pathways were expected to function under these conditions (e.g. $p125^{FAK}$-mediated signalling, and integrin LFA-1 mediated modulation of AP-1 activity (Bianchi et al., 2000; Sieg et al., 2000; Sieg et al., 1999)), chronic LY294002 treatment permitted the absence of the PI 3-kinase input to be assessed in the phosphorylation at the T loop and hydrophobic sites in nPKC isoforms.

Chronic LY294002 treatment of adherent HEK/293 cells, adherent or suspension UM-UC-3 cells and the adherent PTEN-expressing UM-UC-3 cell line, all led to reduction in phosphorylation at the T loop and hydrophobic sites in PKCδ after 24 hours (Figure 15 a, b, c). Since the effects of chronic LY294002 treatment on the phosphorylation at the T loop and hydrophobic site was also observed in PKCε, it implied that the behaviour of these sites were not a PKCδ-specific phenomenon, but general to the nPKC sub-group.

These findings appeared to support the argument that both β1-integrin activation and PTEN activity control the phosphorylation at the T loop and hydrophobic site in the nPKC isoforms by controlling the PI 3-kinase pathway.

5.2.6 Examining the dual phosphatase activity of PTEN in controlling the phosphorylation of the T loop and hydrophobic sites in nPKC isoforms.

Both the lipid and tyrosine phosphatase activities of PTEN have been implicated in the control of the β1-integrin-mediated signalling pathway (Li et al., 1997a; Tamura et al., 1999c). The tyrosine phosphorylated $p125^{FAK}$ had been implicated in the SH2-domain mediated recruitment and activation PI 3-kinase, following β1-integrin activation (Tamura et al., 1999a; Tamura et al., 1999b). This suggests that the tyrosine phosphatase activity of PTEN negatively regulates the PI 3-kinase pathway by dephosphorylating the tyrosine residues on $p125^{FAK}$ and blocking PI 3-kinase recruitment (Tamura et al., 1999a). However, more recently the lipid phosphatase activity has been thought to be responsible for the regulation of the PI 3-kinase pathway and also the tumour suppressive activities of PTEN (Leslie et al., 2000; Myers et al., 1998; Stambolic et al., 1998).

To determine which of the dual phosphatase activities of PTEN was most influential in controlling the phosphorylation at the T loop and hydrophobic site in the nPKC isoforms, a G129E substitution mutant was employed. The G129E substitution mutant of PTEN retained the tyrosine phosphatase activity, but was deficient in phosphatidyl inositol 3-phosphatase activity (Myers et al., 1998). UM-UC-3 cells were co-transfected with PKCδ and the G129E substitution mutant of PTEN, and then deprived of serum for 24 hours, while in suspension. For controls, UM-UC-3 cells were also co-transfected with PKCδ and wild-type PTEN or the empty vector, and treated in the same way. The cells were stimulated with serum for 30 minutes and then analysed for phosphorylation at the T loop and hydrophobic site (Figure 16).

Both the T loop and hydrophobic site remained highly phosphorylated in PKCδ when co-expressed with the G129E substitution mutant of PTEN in the UM-UC-3 cell line. Furthermore, there did not appear to be a serum-induced increase in phosphorylation at either the T loop or hydrophobic site. When PKCδ was co-expressed with the vector control, the basal level of phosphorylation at these sites remained high, implying that the observed effects at the T loop and hydrophobic site were not due to the presence of the vector.

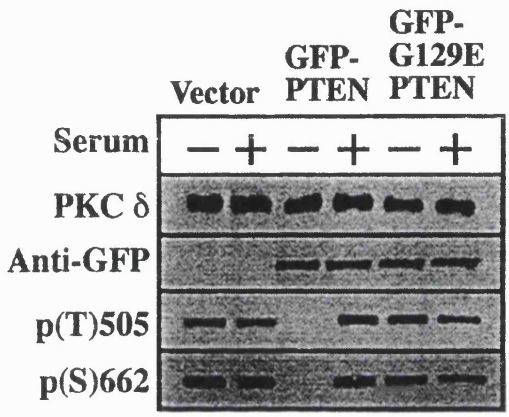


Figure 16. The effect of G129E mutation in PTEN on the phosphorylation at the T loop and hydrophobic site in PKCδ.
UM-UC-3 cells were transiently co-transfected with PKCδ and either a green fluorescent protein (GFP) vector control, a GFP-tagged PTEN construct, or a GFP-tagged G129E substitution mutant of PTEN. The cells were deprived of serum for 24 hours while in suspension, and then treated with serum (10%) for 30 minutes. Whole cell lysates were analysed by Western blotting for PTEN protein expression using an anti-GFP antisera, and PKCδ protein expression and phosphorylation at the sites indicated on the left. This was one of two similar experiments.

The basal level of phosphorylation at these sites in PKCδ, when transiently co-transfected with wild-type PTEN in the UM-UC-3 cell line, mimicked the previously observed response when PKCδ was transiently transfected into the PTEN-stable UM-UC-3 cells (Figure 12). This implies that the transient transfection procedure was not responsible for the observed effects. Therefore, it

seems that it was the phosphatidyl inositol 3-phosphatase activity, and not the tyrosine phosphatase activity, of PTEN that was responsible for controlling the phosphorylation at the T loop and hydrophobic site of the nPKC isoforms when deprived of serum in a suspension culture.

5.3 Discussion

This study aimed to investigate the observations relating the effects of serum deprivation in suspension on the phosphorylation at the T loop and hydrophobic site in nPKC isoforms. In summary, adherent cultures of HEK/293 cells, deprived of serum for 24 hours did not lead to a loss of phosphate at the T loop and hydrophobic site in the nPKC isoforms examined. Furthermore, the level of phosphorylation at these sites did not appear to change following serum treatment of these cells for 30 minutes. The phosphorylations at the T loop and hydrophobic sites seemed to reflect the catalytic activity of PKCδ under these conditions. It appeared that the interactions between the cells and the extracellular matrix (e.g. collagen, a β1-integrin substrate) were sufficient to induce and maintain the phosphorylation at the T loop and hydrophobic sites in the nPKC isoforms. This was supported by observations in which the β1-integrin pathway was activated following engagement and cross-linking of the extracellular domain of the β1-integrin polypeptides on the surface of serum-starved HEK/293 suspension cells.

The observation by several groups on the involvement of PTEN in suppressing the β1-integrin pathway was verified. PTEN appeared to uncouple the β1-integrin-mediated input to the phosphorylation at the T loop and hydrophobic sites in both the nPKC isoforms examined. It was determined that the phosphatidyl inositol 3-phosphatase activity of PTEN was most likely responsible for this observation. However, it is unclear whether the conformation of the phosphatidyl inositol 3-phosphatase domain in the G129E substitution mutant of PTEN differs from the wild-type PTEN, or the capability of PTEN to interact with other proteins, which

may also influence the phosphorylation of these sites, examined in the two nPKC isoforms. The effect of chronic treatment of adherent cells, or cells lacking PTEN, with LY294002 not only supported the involvement of PI 3-kinase in this pathway, but also suggested that long-term PI 3-kinase inhibition is required to reduced the $PI(3,4,5)P_3$ and $PI(3,4)P_2$ levels sufficiently before nPKC, unphosphorylated at the T loop and hydrophobic sites, accumulates in the cell. These observations also implied a difference in the threshold levels of $PI(3,4,5)P_3$ and $PI(3,4)P_2$ required to induce the phosphorylation at the hydrophobic site, perhaps by a PDK-1-dependant mechanism, between the nPKC isoforms and PKB/Akt. The same could be true for the T loop site between the nPKC isoforms and PKB/Akt, but the anti-p(S)308 antiserum, for detecting the phosphorylated T loop site in PKB/Akt, did not work effectively.

It seems clear that the reduced β1-integrin outside-in signalling pathway in cells deprived of serum only while in suspension seems to lead to an accumulation of nPKC protein unphosphorylated at the T loop or hydrophobic site. PTEN only seems to have an influence on the phosphorylation of the T loop and hydrophobic sites when the cells are cultured in this way. Although the TP site is still phosphorylated under these conditions, the nPKC protein displays very low *in vitro* kinase activity toward an MBP substrate. This suggests that the nPKC protein, in serum-starved suspension cells, has sufficient catalytic activity to maintain the autophosphorylation of the TP site, but is still low (~10% of stimulated) in comparison to the nPKC protein in serum stimulated cells.

Several groups had reported that β1-integrin signalling to the PI 3-kinase is mediated through the $p125^{FAK}$ activation directly or indirectly (King et al., 1997; Sieg et al., 1999; Tamura et al., 1999a) (Cary and Guan, 1999; King et al., 1997; Reiske et al., 1999; Sieg et al., 1999; Tamura et al., 1999a). Phosphorylation of $p125^{FAK}$ leads to the recruitment of class I PI 3-kinases to the plasma membrane, and a subsequent rise in the level of 3-D-OH-phosphorylated phosphatidyl inositols,

e.g. $PI(3,4,5)P_3$ and $PI(3,4)P_2$. The activation of the β1-integrin signalling pathway, by a number of different mechanisms, was sufficient to promote the phosphorylation of the T loop and hydrophobic site in the nPKC isoforms.

The influence of the β1-integrin pathway on the phosphorylation of these sites in nPKC isoforms, was uncoupled when the cells were chronically treated with the PI 3-kinase inhibitor, LY294002. It is proposed that under conditions of chronic PI 3-kinase inhibition, the levels of $PI(3,4,5)P_3$ and $PI(3,4)P_2$ in the plasma membrane fall below a threshold level needed to recruit PDK-1 and perhaps another kinase, that would otherwise phosphorylate both the T loop and hydrophobic site in the nPKC isoforms. As described in chapter 4, the loss of phosphate at the T loop site probably also leads to the phosphate at the hydrophobic site becoming more unstable, or more susceptible to dephosphorylation by an okadaic acid-sensitive protein phosphatase(s) (i.e. PP1 and PP2A). It is postulated that the V5 region of the nPKC polypeptide becomes more flexible as a consequence of the dephosphorylation at the T loop. As a result the phosphate at the hydrophobic site becomes more accessible for protein phosphatases and is dephosphorylated. This inter-dependence between the T loop and the hydrophobic site has also been observed between the equivalent sites (T308 and S473 respectively) in PKB/Akt (Andjelkovic et al., 1999). However, it is unclear why the 'turn over' of these sites differs so greatly between the two protein kinases.

In UM-UC-3 cells the T loop and hydrophobic site in the nPKC isoforms do not seem to change significantly whether the cells have been deprived of serum or not, or whether deprived of serum in suspension or in adherent conditions. This may reflect the levels of $PI(3,4,5)P_3$ and $PI(3,4)P_2$ in the plasma membrane. The changes in the phosphorylation at the T loop and hydrophobic site appears to be directly reflected in the in vitro catalytic activity of PKCδ immunopurified from this cell-type under these conditions. However, it remains unclear whether these phosphorylations in the nPKC isoforms in serum starved adherent cells, expressing

PTEN, are regulated less effectively due to persistent β1-integrin-mediated activation of the PI 3-kinase pathway or termination of PTEN function. However, it does seem that it is the action of the lipid 3-phosphatase activity of PTEN that mediates the regulation of the phosphorylation at T loop and hydrophobic site of these nPKC isoforms in suspension cultures.

PKB/Akt does appear to be dephosphorylated at the hydrophobic site in adherent HEK/293 cells following serum-deprivation for 24 hours. The contrasting behaviour of the equivalent site in PKCδ and PKCε demonstrate one of a number of important differences in the regulation of phosphorylation between these two groups of protein kinases. Furthermore, since the regulation of the hydrophobic site also appears to differ between the nPKC isoforms examined and PKC βII(1) (Behn-Krappa and Newton, 1999), it is possible that similar differences exist in the regulation of the hydrophobic site in all the ACG kinase superfamily, such as PKB/Akt and p70 S6kinase.

These findings suggested an additional level of control exist in the regulation of the phosphorylation at the T loop and hydrophobic sites in nPKC isoforms. The involvement of the tumor suppresser protein PTEN in this mechanism supported findings by other groups for a role of nPKC isoforms in cellular transformation (Basu et al., 1996; Li et al., 1996b; Wang et al., 1998; Zang et al., 1997). This forms the basis of the next chapter, where a PKCε-knockout mouse embryo fibroblast ($PKC\varepsilon^{-/-}$ MEF) cell line is examined in parallel with stably expressing clones of wild-type and phosphorylation mutants of PKCε.

6.0 Generating phosphorylation site-specific mutations in PKCε, and examining their properties in stably transfected PKCε$^{-/-}$ MEFs.

6.1 Introduction

The opportunity arose to study the effects of the absence of PKCε in a PKCε$^{-/-}$ cell line. A PKCε-knockout mouse was generated by Dr D Pennington in the laboratory headed by Dr M Owen at the Imperial Cancer research fund. This was part of a collaborative study between the laboratories headed by Dr P Parker and Dr M Owen. The effects of PKCε absence in this PKCε-knockout mouse are still on going. However, Messing R, O and co-workers investigated the function of PKCε by employing a similar approach. They characterised several different phenotypes in their PKCε-knockout mice. For example Aley and colleagues showed that an inflammatory nociceptor sensitisation was mediated by a PKCε-dependent process, and in the absence of PKCε, there was a marked reduction in nociceptor function (Aley et al., 2000; Khasar et al., 1999). It was also noticed that PKCε$^{-/-}$ mice were hypersensitive to allosteric γ-amino butyrate typeA (GABA (A)) receptor modulators. Furthermore, it seemed that PKCε$^{-/-}$ mice displayed a reduced tendency to consume alcohol (Hodge et al., 1999). Exactly what mechanism is affected and how is not clear. It was also unclear whether the expression levels of the other members of the PKC family varied in the PKCε$^{-/-}$ mice, adapting to the absence of PKCε.

Investigations by England and co-workers revealed that the phosphorylation status of PKCε changed as Swiss 3T3 and 3T6 fibroblasts were grown to confluency and quiescence (England and Rumsby, 2000). The study showed that phosphorylation at

the hydrophobic site in PKCε affected the cellular location and function. Furthermore, Balciunaite and co-workers reported two phases of activation of PKCε following PDGF stimulation of HepG2 cells (Balciunaite et al., 2000). It seemed that the latter prolonged phase of activation was responsible for inducing the cells to proceed from G0 to S phase of the cell cycle. In support of this, Perletti and co-workers demonstrated that if PKCε is over expressed in colonic epithelial cells, these cells can develop an oncogenic phenotype by prolonged induction of the ras signalling pathway (Perletti et al., 1998; Perletti et al., 1996). This seems quite the opposite for the behaviour described for PKCδ, which is thought to suppress the growth of colonic epithelial cells, and tumours derived from this cell-type, if over-expressed (Perletti et al., 1999). Therefore, it seems that PKCε may be responsible for, or contributes to a number of different cellular responses.

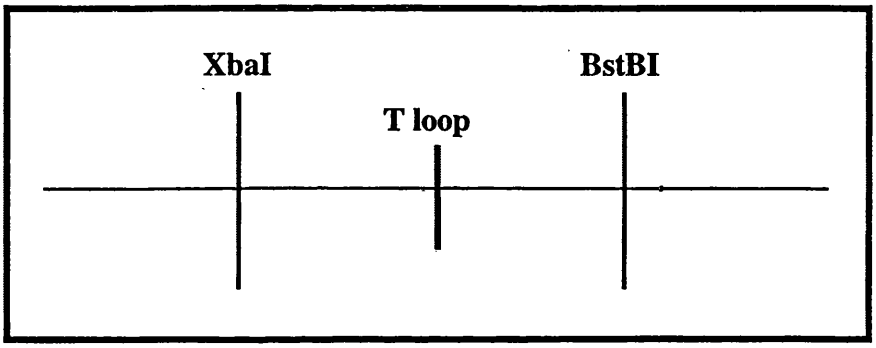
The aim of these investigations was to determine whether phosphorylation mutants of PKCε affected growth/survival responses in mouse embryo fibroblasts (MEFs). Mr R Whelan, in the laboratory, isolated fibroblasts from an embryo of a PKCε$^{-/-}$ mouse, and cultured them until they were immortalised. I generated a PKCε construct, which also encoded a hygromycin-B resistance gene to act as a selection marker. This was employed by Mr R Whelan to create a stable wt-PKCε–expressing cells line, using the MEF cells isolated from the PKCε$^{-/-}$ mice. The characterisation of these cells under a number of different conditions is ongoing.

Substitution mutants at the T loop and hydrophobic site in PKCε were generated to examine their physiological relevance when stably transfected into PKCε$^{-/-}$ MEF cells. These clones were characterised on the basis of cell cycle progression, entry into the apoptotic pathway (by identifying the presence of Sub G1-content DNA), and the capacity to form colonies on semi-solid media, as a marker of transformation.

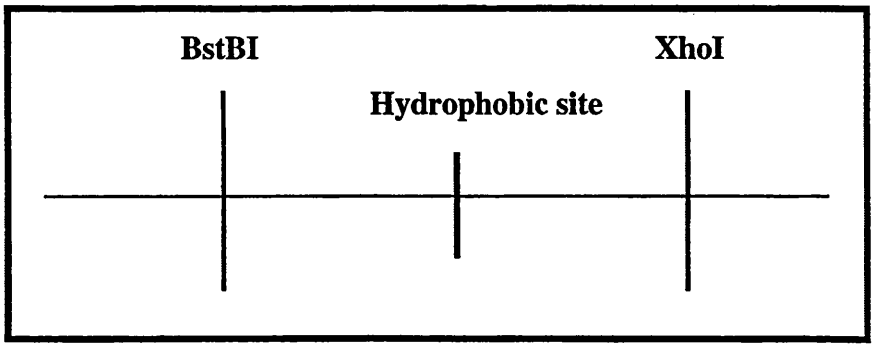
6.2 Results

6.2.1 Designing substitution mutants of the T loop and hydrophobic phosphorylation sites in PKCε.

The PKCε^{wt} cDNA, in the pcDNA3.1 hygromycin (+)^{TM} vector, was used as a template from which the PCR reactions were performed (see Chapter 2). To generate a substitution mutation at the T loop site, the following unique sites were employed:



To generate a similar mutation at the hydrophobic site, a different pairing of unique restriction endonuclease sites were chosen:



The PCR products were purified and ligated into EcoRV-restricted pBlueScript^{TM}, and recombinant colonies identified by blue white colour selection. Finally, the

mutations were verified by sequencing. The T loop or hydrophobic site mutations were excised from the pBlueScript™ vector by treating them with the restriction endonucleases to cut at the restriction sites that flank the mutations.

To substitute these T loop or hydrophobic site mutated DNA fragments back into the PKCε cDNA, changes were required to both the pcDNA3.1 hygromycin (+)™ vector and the PKCε cDNA. The XbaI site in the multiple cloning site (MCS) in the pcDNA3.1 hygromycin (+)™ vector had to be destroyed because insertion of the mutated T loop DNA fragment required the use of a unique XbaI site at the 5' end of it. The pcDNA3.1 hygromycin (+)™ vector was cut with XbaI and then ligated with the following oligonucleotide:

5'-**CTAGGCGCGC**-3'

XbaI site

T CTAGA

AGATC T

XbaI + **CTAGGCGCGC** + Ligation

No XbaI site. New BssHII site

TCTAGGCGCGCCTAGA

AGATCCGCGCGGATCT

This oligonucleotide replaced the XbaI site with a new BssHII site to generate the pcDNA3.1 hygromycin (+)™ vector$^{(-XbaI)}$. If the XhoI site was to be used for introducing the hydrophobic site mutation, the PKCε-cDNA had to be inserted into the pcDNA3.1 hygromycin (+)™ vector$^{(-XbaI)}$ in a different manner than as an XhoI-XhoI cDNA fragment. To do this, the PKCε cDNA in the pMT_2 vector was modifed by removing an AscI restriction site at the 5'-end of the start site, and replacing it

with a unique KpnI site. Treating the PKCε cDNA in the pMT_2 vector firstly with the AscI restriction endonuclease, and then ligating the following oligonucleotide achieved this:

5'-**CGCGGGTACC**-3'

AscI Site

GG▮CGCGCC

CCGCGC▮GG

AscI + **CGCGGGTACC** + ligation

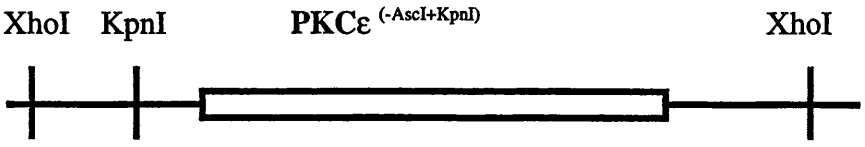
No AscI site. New KpnI site

GG**CGCGGGTACC**CGCG

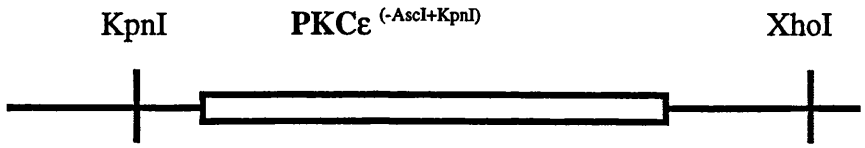
CCGCGC**CCATGGG**CGC

This new PKCε cDNA in the pMT_2 vector (PKCε cDNA $^{(-AscI+KpnI)}$) was treated with KpnI and XhoI, and then ligated into pcDNA3.1 hygromycin (+)^{TM} vector $^{(-XbaI)}$ also cut with KpnI and XhoI:

PKCε cDNA $^{(-AscI+KpnI)}$ in the pMT_2 vector



PKCε cDNA $^{(-AscI+KpnI)}$ in pcDNA3.1 hygromycin (+)^{TM} vector $^{(-XbaI)}$



Finally, the T loop and hydrophobic site mutated DNA fragments were ligated into the PKCε cDNA $^{(-AscI+KpnI)}$ in pcDNA3.1 hygromycin (+)^{TM} vector $^{(-XbaI)}$.

6.2.2 Generating stably-expressing clones of PKCε mutants in the PKCε$^{-/-}$ MEFs.

The phosphorylation site substitution mutants of PKCε were stably transfected into the PKCε$^{-/-}$ MEFs. Selection for transfected cells was performed by incubating the cells in the presence of 200μg/ml of hygromycin B, as described in Chapter 2. Hygromycin B-resistant colonies appeared within 10 days. These clones were picked up with a sterile pipette tip and cultured alone in the presence of hygromycin B, as described in Chapter 2. Finally, Western blotting analysis determined expression of the mutant forms of PKCε.

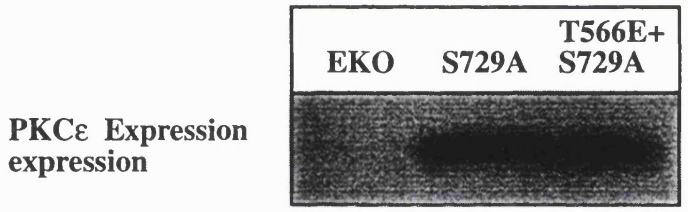


Figure 1. Expression levels of two substitution mutants of PKCε stably transfected into MEFs.
A confluent 90mm dish of two different PKCε phosphorylation mutant clones, derived from PKCε$^{-/-}$ MEF cells (EKO), were analysed for PKCε protein expression. The S729A mutant or T566E and S729A double mutant clones were harvested in 4X sample buffer. The expression level of the PKCε mutants was monitored by Western blotting.

Since it was clear that the mutants expressed, it provided an opportunity to determine whether the phospho-specific antisera detected a change in phosphorylation at either the T loop site (T566) or hydrophobic site (S729) in the

mutants and hence whether the phospho-specific antisera are also detecting other sites (Figure 2).

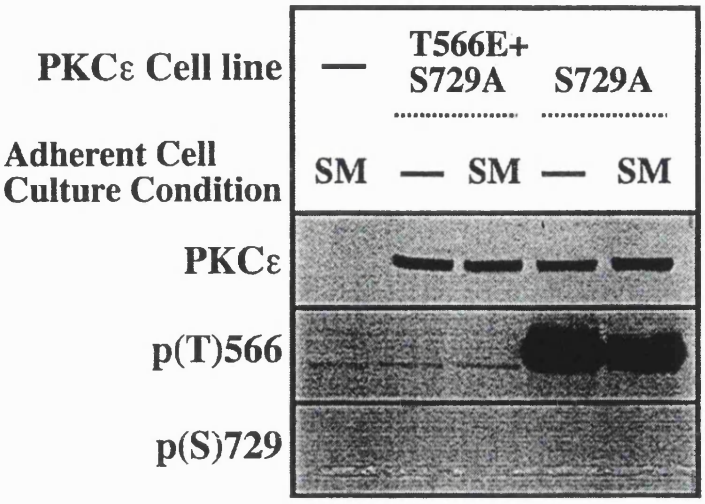


Figure 2. Determining specificity for the phospho-specific antisera for the T loop and hydrophobic sites in two mutants of PKCε under two different culture conditions.

The PKCε$^{-/-}$ MEF clone (-) or the PKCε$^{-/-}$ MEF-derived clones expressing either the T566E+S729A double mutant or S729A single mutant were cultured under adherent conditions. The cells were either maintained in serum for 24 hours (SM), or deprived of 10% serum over the same period (-). Whole cell lysates were monitored for protein expression and phosphorylation at both the T loop site (p(T)566) and the hydrophobic site (p(S)729) by Western blotting.

When the PKCε$^{-/-}$ MEF clones, stably expressing the alanine substitution mutant of the hydrophobic site (S729A), were cultured under adherent conditions in the absence of 10% serum for 24 hours, there was a high basal level of phosphorylation at the T loop site (Figure 2). In the absence of serum, there did not seem to be a loss of phosphate at the T566. These observations were consistent with earlier findings (see chapter 5), where adherence of HEK/293 cells maintained phosphorylation at the T loop and hydrophobic sites.

There appeared to be very low immunoreactivity of the phospho-specific T loop antisera for the glutamate residue from the PKCε$^{-/-}$ MEFs stably expressing the T566E+S729A substitution mutant, but phosphorylation was detected at the T loop site in the PKCε$^{-/-}$ MEFs stably expressing the S729A mutant. This further supported the findings in chapter 3, relating to the specificity of this phospho-specific antisera for the phosphorylated site. The same was also true for the phospho-specific hydrophobic site antisera, as there was no detectable immunoreactivity of this antisera for the alanine residue substituted at this site i.e. the S729A mutant.

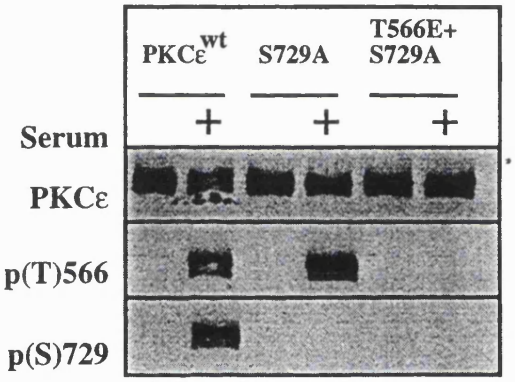


Figure 3. Serum-dependent phosphorylation of the T loop and hydrophobic sites in the wild-type PKCε clone derived from a PKCε$^{-/-}$ MEF cell clone. Clones of wild-type PKCε (PKCε^{wt}), the S729A mutant and the T566E and S729A double mutant of PKCε, derived from a PKCε$^{-/-}$ MEF clone, were deprived of serum while in suspension for 24 hours. The cells were then treated with serum (10% final) for 30 minutes. Whole cell lysates were monitored for protein expression and phosphorylation at both the T loop site (p(T)566) and the hydrophobic site (p(S)729) by Western blotting.

PKCε$^{-/-}$ MEF isolates stably expressing either wild-type PKCε, or the phosphorylation site-mutants, were examined to determine whether a serum-induced phosphorylation is observed at the T loop or hydrophobic sites and whether they behave similarly to PKCε transiently over-expressed in HEK/293 cells. The cells

were deprived of serum for 24 hours while in suspension and then treated with serum for 30 minutes, as before (Figure 3). It seems that both the T loop and hydrophobic sites in the stably transfected PKCε$^{-/-}$ MEFs become phosphorylated in response to serum treatment for 30 minutes. The phosphorylation at the T loop and hydrophobic sites in the stably transfected PKCε^{wt} do behave similar to PKCε^{wt} transiently over-expressed in HEK/293 cells (see chapters 3-5). This implies that the subsequent analysis of the effects of an alanine residue at the hydrophobic site (in the S729A mutant) on the serum-induced phosphorylation of the T loop site, and the effects of all the PKCε forms, stably expressed in PKCε$^{-/-}$ MEFs, is not an artefact of the system.

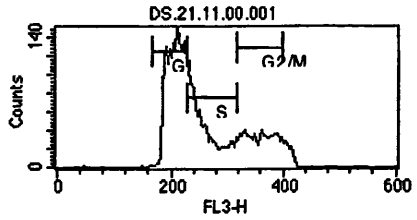
6.2.3 The effects of PKCε expression on cell cycle progression.

Several groups had reported previously that PKCε can affect several other signalling pathways which can promote cell multiplication or induce apopotsis (Chen et al., 1999; Perletti et al., 1998; Perletti et al., 1996; Spitaler et al., 1999; Ueffing et al., 1997; Whelan and Parker, 1998). This seems to be in contrast to the function of PKCδ as some groups have reported that PKCδ has a growth suppressive function, and that v-src-induced phosphorylation of PKCδ leads to its inactivation during cellular transformation (Perletti et al., 1999; Zang et al., 1997). However, Borner, and co-workers stated that PKCε expression was reduced following transformation by oncogenic ras (Borner et al., 1990). Therefore, the aim of these experiments was to determine whether the presence or absence of PKCε^{wt}, or its phosphorylation mutants, in the PKCε$^{-/-}$ MEF-derived clones affect the cell cycle in some way, or the disposition of these cells towards apoptosis.

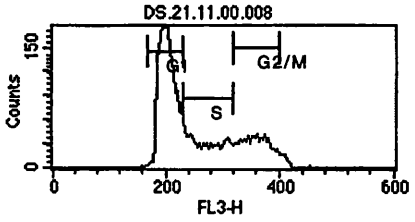
FACS analysis was performed to assess the behaviour of these clones in cell cycle progression and to determine whether they would progress from G1 through to G2/M phase when treated for six hours with serum following 24 hours of serum

deprivation. This approach was also used to detect the presence of Sub-G1 content DNA as a marker of the disposition of these clones to apoptosis. It seems that PKCε has little influence on the behaviour of the MEF cells to induce their progression through the cell cycle in the presence of serum (Figure 4A-4D).

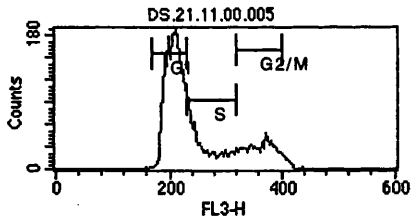
4A. PKCε$^{-/-}$ MEFs



4B. PKCε$^{-/-}$ MEFs, stably expressing PKCε^{wt} (clone 5).



4C. PKCε$^{-/-}$ MEFs, stably expressing the S729A mutant of PKCε.



4D. PKCε$^{-/-}$ MEFs, stably expressing the T566E + S729A double mutant of PKCε

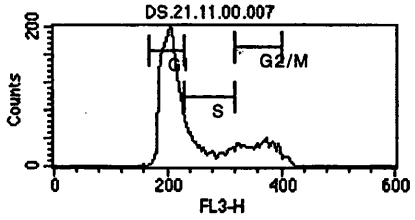


Figure 4A-4D. Cell-cycle progression of PKCε$^{-/-}$ MEF cells and isolates expressing forms of PKCε.

A confluent 90mm dish of PKCε$^{-/-}$ MEF cells, or isolates derived from this clone stably expressing wild-type PKCε or one of two site-specific phosphorylation mutants (S729A or T566E+S729A double mutant), were deprived of serum for 24 hours. The cells were treated with serum (10% final) for 6 hours, and then removed from the dish and fixed in 70% ethanol prior to FACS sorting.

In this system, there does not appear to be a significant difference in the capacity of the different MEF clones to cycle from the G1 phase to the G2/M phase, whether

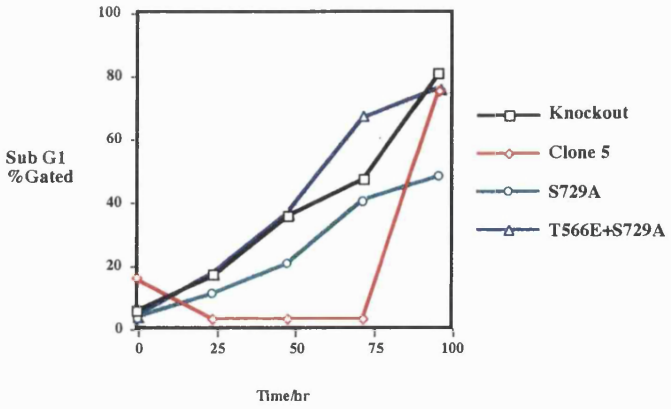
they express PKCε or not. Based on the information in these and other studies, it was thought that the stable over-expression of wild-type PKCε in PKCε$^{-/-}$ MEF-derived clones, might promote some differences in cell cycle progression, compared to untransfected cells, since it was claimed that PKCε can activate the ras-MAPKinase pathway through association with ras or Raf-1 (Cacace et al., 1996; Perletti et al., 1998; Ueffing et al., 1997). However, all these studies are not directly comparable as they were performed in different cell-types, and hence, different experimental conditions.

6.2.4 The influence of PKCε on commitment to apoptosis and growth in soft agar.

The aim of these experiments was to determine if wild-type PKCε, or its phosphorylation mutants, influenced the disposition of these cells to apoptosis if cultured in suspension for 24 hours or more in the absence of serum. There seems to be dependence of PKCε$^{-/-}$ MEF cells upon wild type PKCε expression to avoid early entry into apoptosis (Figure 5a). The PKCε$^{-/-}$ MEF clones expressing either the S729A mutant or the T566E and S729A double mutants of PKCε were not able to mimic the response observed when the PKCε$^{-/-}$ MEF cells were expressing the wild-type PKCε. This was observed in two independent experiments.

There was a possibility that the delay of early commitment to apoptosis, dependent upon wild-type PKCε introduced into the PKCε$^{-/-}$ MEF-derived clone, was due to the high level of PKCε expressed in this clone (clone 5).

5a



5b

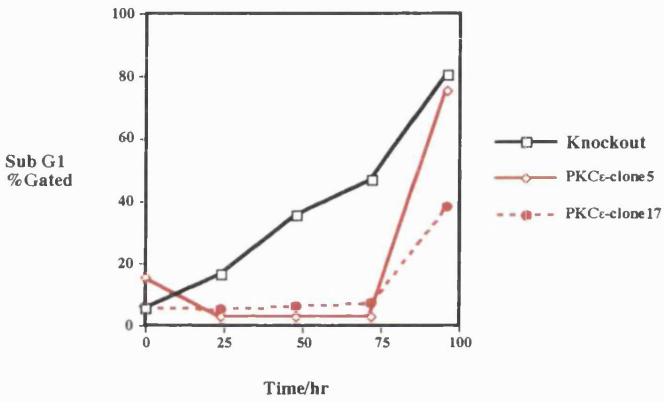


Figure 5. The effect of PKCε expression in PKCε$^{-/-}$ MEF cells on apoptosis by detection of Sub-G1 content DNA

a, The effect of the different mutants of PKCε.

b, A comparison two different wild-type PKCε-expressing clones, clone 5 and clone 17, on apoptosis.

PKCε$^{-/-}$ MEF cells alone, or stably expressing either wild-type PKCε (clone 5 or clone 17) or one of the two phosphorylation mutants (S729A or T566E+S729A) were deprived of serum while in suspension for increasing periods of time. Equal volume samples of cells were removed every 24 hours and fixed in 70% ethanol before FACS sorting for Sub G1 content as an indication of apoptosis. This is one of two independent experiments, which illustrated the same behaviour of these cells.

Therefore, a PKCε$^{-/-}$ MEF clone stably expressing a lower level of PKCε (clone 17) was tested for apoptotic behaviour after increasing periods of serum deprivation

Therefore, a PKCε$^{-/-}$ MEF clone stably expressing a lower level of PKCε (clone 17) was tested for apoptotic behaviour after increasing periods of serum deprivation while in suspension (Figure 5b). It seems clear that the protective effect of PKCε expression, to delay the commitment of the PKCε$^{-/-}$ MEF cells from entering apoptosis, is not simply due the high level of expression of PKCε since clone 17 behaved in the same manner. To confirm the relative expression levels of the different PKCε-expressing cell lines, a 1ml aliquot from a culture of 1X10^6 cells were analysed by Western blotting (Figure 6).

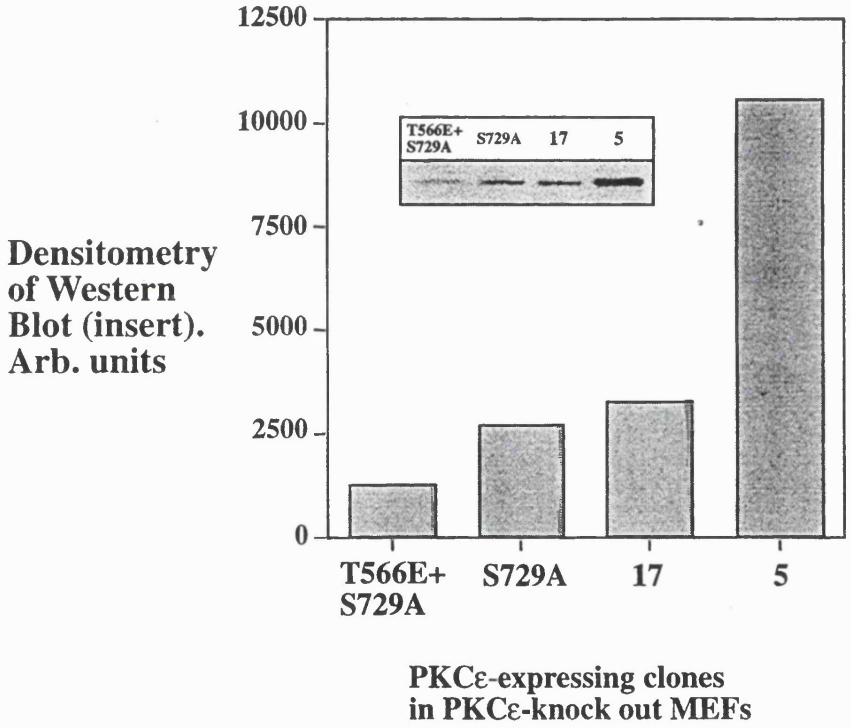


Figure 6. Relative stable expression levels of PKCε in PKCε$^{-/-}$ MEF cells.
A culture of one million PKCε$^{-/-}$ MEF cells/ml, stably expressing one of each of the different types of PKCε, was prepared. The cells from a 1ml aliquot from each culture were harvested and lysed in 100µl of 4X sample buffer. A 10µl aliquot of each of the samples were analysed for PKCε expression by Western blot (insert). The Western blot was scanned and analysed by NIH image to compare to different expression levels.

It seems that there is approximately four times the amount of PKCε stably expressed in clone 5 as there is in the clone 17. It is possible that when PKCε is expressed in lower amounts, as in clone 17, it does not interfere with, or influence other signalling pathways quite so much as when it is expressed so much more, as in clone 5. However, this difference in expression did not seem to influence the delay in commitment to apoptosis induce by wild-type PKCε (Figure 5b).

The capacity of PKCε$^{-/-}$ MEF cells to grow and form colonies in soft agar was examined to further assess the behaviour of these cells. This was part of a collaborative effort between Mr R. Whelan in the laboratory and myself. The clones were set up in triplicate in soft agar containing 10%FBS in DMEM, as described in chapter 2. The purpose of this experiment was to use this technique as a maker of cellular transformation. All the different clones grew with the same efficiency as the vector control.

6.3 Discussion

Wild type and two different phosphorylation mutant clones of PKCε were derived from the PKCε$^{-/-}$ MEF cells. When these cells were deprived of serum in suspension, there was a serum-induced phosphorylation detected at the T loop and hydrophobic sites in PKCε^{wt}, but only at the T loop site in the S729A substitution mutant. The phosphorylation at the T loop and hydrophobic sites in wild-type PKCε clone, behaved similar to when expressed in HEK/293 cells or in UM-UC-3 cells expressing PTEN. Hence, this phenomenon of a requirement of serum deprivation for 24 hours in suspension is not unique to one or two cell types. These findings clearly demonstrate that the T loop site can be phosphorylated in response to serum treatment and that it is not dependent upon the phosphorylation at the hydrophobic site for stability. This confirms earlier observations using rapamycin treatment and

suggests a difference in the stability of a phosphate at the T loop site compared to at the hydrophobic site (see chapter 4). As discussed earlier, this may reflect a difference in the overall conformation of the polypeptide at the V5-domain when phosphorylated at the T loop site (chapter 4).

The expression of wild-type PKCε in the PKCε$^{-/-}$ MEF-derived clone does not seem to influence the ability of these cells to enter into the cell cycle following serum treatment. Although PKCε may have a role to play in proliferation, its function maybe redundant. The absence of this enzyme from the cell has not restricted the entry of serum-deprived PKCε$^{-/-}$ MEF cells from entering the S phase and the G2/M phase after only six hours of serum treatment. These findings are supported by the capacity of all these different PKCε-expressing clones to form colonies in soft agar. Furthermore, the relative differences in expression of PKCε in the cell, do not seem to have affected the timing of the serum-deprived cells to enter G2/M phase of the cell cycle following serum six hours of serum treatment.

As with the cell-cycle data, whether the MEF cells expressed wild-type PKCε or not, or whether they expressed either phosphorylation mutant, the different MEF clones still grew and formed colonies in the absence of an extracellular matrix interaction in soft agar. If there is significant redundancy in the pathways which control cell cycle progression or growth in soft agar, then it is not really surprising to discover that in the absence of PKCε expression the MEF cells are still able to function as 'normal'. Perhaps this is more clearly demonstrated by the finding that the PKCε-knock out mouse does not seem to grow and develop differently from the wild-type mouse.

The ability of an enzyme to promote an aspect of cellular transformation might involve suppressing programmed cell death (apoptosis). This is supported by Bertolotto and co-workers, who reported that both PKCε and PKCθ promoted T cell survival by activating p90rsk and promoting the phosphorylation of the Bad protein (Bertolotto et al., 2000). It seemed that when the PKCε$^{-/-}$ MEF cells were stably

expressing wild-type PKCε (clone 5 or clone 17), there was a delayed entry into apoptosis, as detected by FACS sorting. This was distinct from the PKCε$^{-/-}$ MEF cells stably expressing either the S729A mutant or the T566E+S729 double mutant.

Although the *in vitro* catalytic activity of the various PKCε forms, stably expressed in the PKCε$^{-/-}$ MEF cells, has not been determined, it is attractive to consider that only the stably expressed wild-type PKCε would have had sufficient catalytic activity under these conditions to mediate the protective effects towards apoptosis. The negative charge of the glutamate residue, substituted for the threonine residue at the T loop site (T566E), may not replace the effect of a phosphate at this site. For example, we know that an identical substitution mutation at the T loop site in PKCα (T497E), only yields about 20% of the wild-type activity (Bornancin, 1997). Furthermore, in the absence of a phosphate at the hydrophobic site in PKCε (or PKCδ), the catalytic activity is likely to be very low (see chapter 4). However, we can not rule out the possibility that these mutations have affected the conformation of the protein kinase such that they are no longer able to possess even low catalytic activity.

In summary, it seems that PKCε induces a delay in the mechanism of commitment to apoptosis. This function of PKCε appears to be dependent upon the phosphorylation at the T loop and the hydrophobic site as the substitution mutants investigated did not support this behaviour when expressed at similar levels to the wild-type. Therefore, the pathways examined in the earlier chapters, which play a role in regulating these phosphorylations, are important in this function of PKCε.

7.0 Final Discussion

7.1 Mechanism of activation of nPKCs by phosphorylation at the T loop and hydrophobic sites.

Novel PKCs differ from their cPKC relatives, as they do not respond to intracellular changes in calcium in the cell. However, both groups of PKCs are under the control of lipids, such as DAG and PS. When DAG, in the context of phospholipids, binds at the C1 domain in nPKCs it induces a conformational change that relieves the autoinhibitory mechanism. This process leads to the phosphorylation at the T loop by PDK-1 and the hydrophobic site by a kinase complex that requires catalytically active aPKCζ/ι. Although PDK-1 has been accepted as the protein kinase responsible for phosphorylating the T loop site in most of the members of the AGC superfamily of protein kinases, the kinase responsible for phosphorylating the hydrophobic site in nPKCs is still unresolved. It is possible that different mechanisms operate for phosphorylating the hydrophobic site in the different members of the AGC superfamily.

The hydrophobic site in PKCβ2 (S660) has been reported to be autophosphorylated Behn-Krappa, 1999 #290]. However, questions still remain unanswered with these studies to verify the possible involvement of co-precipitating kinases and protein phosphatases and the stability of the protein kinase when unphosphorylated. This is because it was demonstrated that PKCα was unstable and partially unfolded when unphosphorylated (Bornancin and Parker, 1997). In PKB/Akt, the phosphorylation of the hydrophobic site (S473) has been further complicated by conflicting reports. Initially, it was suggested that a PRK1-directed PIF (PDK-1 interacting fragment) interaction with PDK-1 forms a 'PDK-2' complex. The 'PDK-2' kinase complex then phosphorylates the hydrophobic site in PKB/Akt (Balendran et al., 1999a). However, the same group also stated that the PIF-mediated interaction with PDK-1

would inhibit the phosphorylation of the hydrophobic site in $p70^{S6Kinase}$ (T412) (Balendran et al., 1999b). Therefore, a different mechanism of phosphorylation at the hydrophobic site would appear to operate for these two protein kinases. This could also be true for the hydrophobic site in other members of the AGC super-family of serine/threonine protein kinases, as suggested earlier.

Studies performed by Newton and co-workers demonstrated a similar mechanism of autophosphorylation operated for the hydrophobic site in PKB/Akt, as they had described for the equivalent site in cPKCβ2. They also suggested that the phosphorylation of the T loop site by PDK-1 would drive the autophosphorylation of the hydrophobic site in PKB/Akt (Behn-Krappa and Newton, 1999; Dutil et al., 1998). This was then supported by the Alessi laboratory, which showed that a kinase-dead PKB/Akt is unphosphorylated at the hydrophobic site in response to insulin stimulation of cells. However, this group also demonstrated that in PDK-$1^{-/-}$ embryonic stem (ES) cells, the T loop site is not phosphorylated in response to insulin stimulation, while the hydrophobic site does become phosphorylated (Williams et al., 2000). Several issues remain unclear with many of these studies. For example, it was unclear whether the conformation of a kinase-dead PKB/Akt would be altered whereby the region of the hydrophobic site is no longer recognised by the protein kinase that would phosphorylate it. These studies also imply that the hydrophobic site in PKB/Akt is autophosphorylated by a cis, and not a trans-mechanism, as endogenous PKB/Akt did not phosphorylate the S473 site in the kinase-dead PKB/Akt. Furthermore, the data involving the PDK-$1^{-/-}$ ES cells suggested that there is only one PDK isoform (PDK-1) in the cell that phosphorylates the T loop in PKB/Akt in response insulin stimulation. Firstly, this seems unlikely to be the case if PDK-1 is 'a kinase at the hub of things' as suggested by (Belham et al., 1999) because protein kinases which have such an important role to play in signalling tend to have related isoforms. For example, the PKB/Akt, PKC, PI 3-kinase, and PLC families. However, one of many notable exceptions is cdc 2. Secondly, these investigations do not rule out the possibility of another PDK isoform (e.g. PDK-2) catalysing the phosphorylation at the hydrophobic site in PKB/Akt.

There also appear to be differences in the regulation of the phosphorylation at the hydrophobic site between PKB/Akt and members of the PKC family. For example, depriving adherent cells for up to an hour can lead to the loss of phosphate at the hydrophobic site in PKB/Akt. However, the equivalent site in PKCδ or PKCε is only detected as unphosphosphorylated when the cells have been deprived of serum for 24 hours while in suspension (Parekh et al., 2000a). A reason for these differences might be a variation in PtdIns (3,4,5)P_3-dependent recruitment of PDK-1 and PKB/Akt to the plasma membrane and a DAG-dependent recruitment of nPKCs to a PDK-1 compartment.

The 'TP' site in PKCδ or PKCε (S643 and T710, respectively) appeared to be dependent upon the catalytic activity of the PKC enzyme. This was unlike the regulation of the T loop or hydrophobic sites. It seemed that PKCδ and PKCε had sufficient catalytic activity to maintain the phosphorylation of the TP site when the cells were deprived of serum for 24 hours in suspension. Treatment of cells with one of two different PKC-specific catalytic site inhibitors led to a loss of phosphate at the TP site. This suggests that protein phosphatase(s) and PKC dynamically regulate the phosphorylation at the TP site. A net loss of phosphate at the TP site is only detected when the catalytic activity is inhibited under conditions where the other sites are unoccupied. However, these findings did not determine whether the TP site is autophosphorylated by a *cis* or a *trans* mechanism.

There seems to be additional levels of control on the phosphorylation at the hydrophobic site in PKCδ and PKCε. The tumour suppresser PTEN/MMAC, mTOR and integrin signalling all seem to influence the phosphorylation at this site. When rapamycin treatment or nutrient deprivation inhibits mTOR, there is no serum-induced phosphorylation at the hydrophobic site in either nPKC isoform investigated. This was not a function of the catalytic activity because the PKC-specific catalytic site inhibitors, BIM-I or Go6983, did not affect the serum-induced

phosphorylation at the hydrophobic site, but they did inhibit the phosphorylation at the TP/autophosphorylation sites in PKCδ and PKCε.

It is not clear at present how the integrin pathway, protein phosphatases (i.e. PP1/PP2A and PTEN), mTOR, and the PKCζ/ι-complexed protein kinase(s) integrate to regulate the phosphorylation at the hydrophobic site. However, it is likely that these players reflect a mechanism of signal cross-talk and signal integration, between the different pathways which relates to the physiological condition of the cell. For example it was demonstrated that the negative influence of the phosphoinositide 3-phosphatase activity of PTEN on the phosphorylation at the T loop and hydrophobic sites, is not experienced if the cells are adherent during serum deprivation, at least at this level of PTEN expression.

7.2 Implications of phosphorylation

It is postulated that when newly made PKCδ and PKCε are phosphorylated at T loop and hydrophobic sites, their catalytic domains are thus 'primed' to induce efficient autophosphorylation at their TP sites (S643 and T710, respectively). These phosphorylations act to fully 'prime' the catalytic domain for activity. The phosphorylations in the catalytic domain and cofactor binding are both needed before PKCs are able to catalyse efficiently. PKCs are effectively phosphorylated *in vivo* when bound to their cofactors at the plasma membrane. When fully phosphorylated, but in the absence of cofactors, PKCs can remain in the cytosol for many hours (>24 hours) after serum withdrawal. Persistent stimulation or phorbol ester (TPA) treatment, generates a cofactor-bound form of PKC that is allosterically activated and targeted for dephosphorylation and degradation (Hansra et al., 1996; Hansra et al., 1999; Lee et al., 1996). The consequence of this is that several factors need to be present before PKCs are fully active, so they can respond quickly to an increase in DAG levels following stimulation without being delayed by requiring phosphorylation. Another issue is that the second more sustained rise of DAG,

derived from PC and other lipids in non-plasma membrane compartments, may serve to direct PKCs to other parts of the cell for more long-term functions, for example, proliferation and differentiation. This is reviewed by Nishizuka and co-workers (Asaoka et al., 1992; Nakamura et al., 1993).

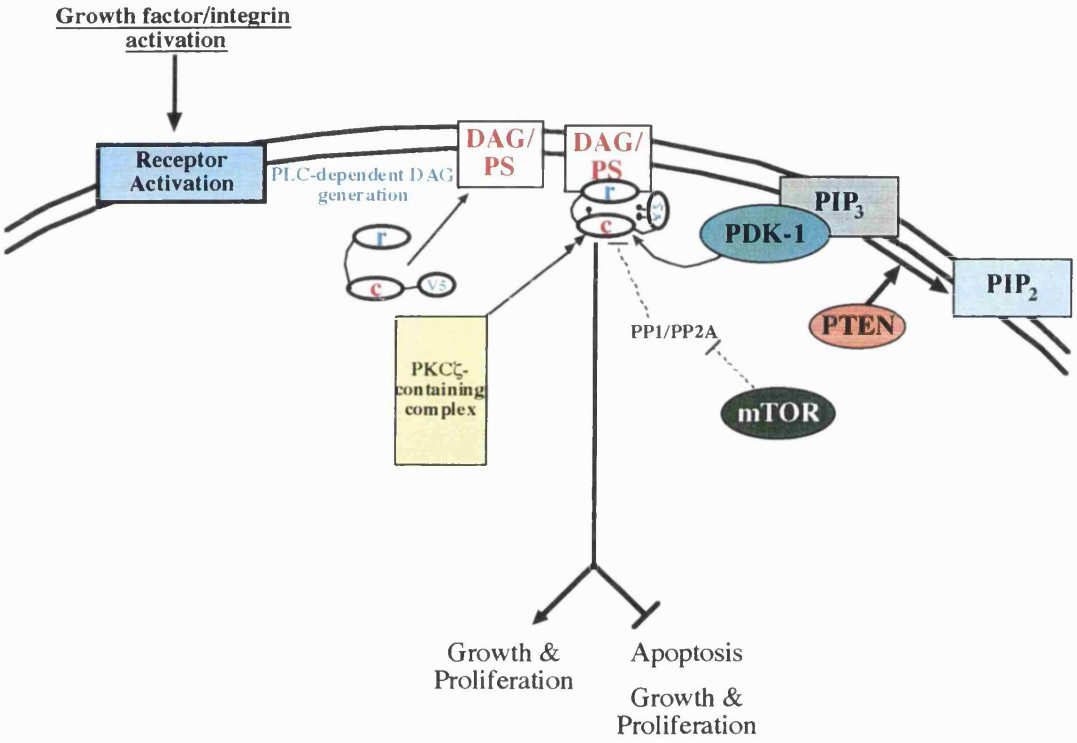


Figure 1. General Scheme.
This scheme has been simplified by only including the elements that have been investigated in this study

7.3 Role of PKCs in apoptosis.

A consequence of these regulatory mechanisms operating on PKC phosphorylation might serve to protect cells from apoptosis (Meinhardt et al., 2000). For example, nutrient withdrawal or removal of growth factors for more than 30 minutes can lead

to an abrupt termination of many synthetic processes, and an inhibition of PKB/Akt activity. However, the cells do not commit to apoptosis immediately. An explanation for these responses may be that the cell has evolved a sensing mechanism so as not to apoptose the moment it experiences periods of nutrient deprivation. The involvement of PKCs in regulating apoptosis may be very important in this process, as suggested by several other groups (Denning et al., 1998; Emoto et al., 1995; Mizuno et al., 1997; Sawai et al., 1997; Shao et al., 1997; Whelan and Parker, 1998). For example, it was demonstrated by several groups that cells were protected from pro-apoptotic stimuli, such as U.V radiation, when over-expressing aPKC isoforms by promoting AP1-dependent transcription (Berra et al., 1997; Diaz-Meco et al., 1996; Emoto et al., 1995; Huang et al., 2000; Huang et al., 1997; Shao et al., 1997). Whelan and co-workers, who demonstrated that the loss of PKCα from cells promotes apoptosis, provide further support of the involvement of PKC in this process (Whelan and Parker, 1998). By contrast, a feature of pro-apoptotic cells is that many PKC isoforms (PKCs α, β_I, δ, ε, θ, and ζ) undergo a caspase 3-mediated proteolysis at the V3 region. This forms constitutively active catalytic domain fragments which have been reported to promote apoptosis (Datta et al., 1997; Denning et al., 1998; Emoto et al., 1995; Ghayur et al., 1996; Mizuno et al., 1997). However, it is not clear whether these PKC-catalytic domain fragments have a function in the mechanism of apoptosis.

All these studies strongly link PKC function with some aspect of apoptotic behaviour; to either promote or suppress apoptosis. However, it is not clear what signalling pathways are activated or why the response varies with the PKC isoform involved, however this may reflect a cell-type-specific effect. Our initial studies using HEK/293 cells, transiently over-expressing PKCδ, showed that when these cells have been deprived of serum for 24 hours while in suspension (low PKC activity), the cells do not display any significant apoptotic behaviour (Parekh et al., 1999; Parekh et al., 2000a). When the PKCε$^{-/-}$ MEF cells were examined, there seemed to be a delay of entry into apoptosis only when these cells were stably expressing wild-type PKCε. This did not seem to be a function of the expression

level of the wild-type PKCε since a different PKCε$^{-/-}$ MEF-derived clone expressing a lower level of wild-type PKCε, had a similar effect. The phosphorylation mutants were unable to mimic the response observed with the wild-type PKCε. This suggests that the absence of phosphorylatable residues at the T loop and hydrophobic sites hinder the anti-apoptotic effect observed with wild-type PKCε in the PKCε$^{-/-}$ MEF cells. This could reflect a difference in the catalytic activity between the wild-type PKCε and the phosphorylation mutants. However, the catalytic activity of the different forms of PKCε under these conditions has yet to be resolved. It will be important in the future to determine the activities of all the forms of PKCε under the various growth conditions employed here.

7.4 Downstrean targets of PKCs.

PKCs were originally defined as the cellular receptors for the tumour promoting phorbol esters (see chapter 1). An example of a proximal downstream target of PKCs is the MARCKs protein (Graff et al., 1989; Stumpo et al., 1989). However, PKCs are also thought to influence transcription, in part by promoting the dephosphorylation of c-jun (a primary response gene). The dephosphorylated c-jun interacts with the TPA-response element (TRE) through the regulatory region in c-jun and other TPA-activatable genes (Boyle et al., 1991; Mitchell and Tjian, 1989). This leads to the formation of active homo- and heterdimers of the AP-1 transcription factor (e.g. fos-jun heterodimer, or jun-jun homodimer) which binds at the TRE in TPA-inducible genes (Chiu et al., 1988; Tseng et al., 1994). The AP-1 transcription factor has been shown to be important in tumour formation (Bernstein and Walker, 1999; Young et al., 1999). Although the mechanisms of PKC-inducible activation of TPA-responsive genes are not completely understood, some of these genes lead to the synthesis of proteins that regulate the extracellular matrix. Stromelysin and collagenase are two examples of proteins, which are synthesised in response to TPA treatment, and assist the migration of cells through the extracellular

matrix (Conquer et al., 1992; Gaire et al., 1996; Reifel-Miller et al., 1996; Shoshan and Linder, 1994; Tseng et al., 1994). This is a feature of cellular transformation (Cagliero et al., 1991; Conquer et al., 1992; Gaire et al., 1996; McDonnell et al., 1990; Reifel-Miller et al., 1996; Shoshan and Linder, 1994).

7.5 PKCs in cellular transformation

Various groups have implicated PKC isoforms as essential players in cellular transformation (Cacace et al., 1993; Goodnight et al., 1994; Housey et al., 1988; Mischak et al., 1993a; Mischak et al., 1993b; Wang et al., 1998). For example, Dean and co-workers reported an inhibition of human tumour cell proliferation in nude mice when antisense oligonucleotides to PKCα expression was added to cells (Dean et al., 1996). This observation was supported by findings from Baltuch and co-workers that demonstrated a rise in proliferation of C6 glioma cells when PKCα is over-expressed (Baltuch et al., 1995). Furthermore, it was observed that glioma cells became apoptotic when treated with antisense oligonucleotides to PKCα. This supported earlier discussions of the anti-apoptotic role of PKCs (Dooley et al., 1998).

Many tumour promoters act by constitutively activating PKCs in cells (Bell and Burns, 1991; Huang, 1989; Nishizuka, 1984). Ultimately, these tumour promoters either activate proto-oncogenes, inactivate tumour suppressers, or increase the susceptibility of cells to transformation by other oncogenes such as v-ras or v-src (Borner et al., 1992; Borner et al., 1990; Dotto et al., 1985; Hsiao et al., 1984; Hsiao et al., 1989). However, the use of tumour promoters such as phorbol esters to demonstrate the involvement of PKCs in a mechanism is controversial. This is because PKCs are not the only intracellular receptors for phorbol esters, for example β2-chimaerin, Munc13, RasGRP, and protein disulfide isomerase (Caloca et al., 1999; Lorenzo et al., 2000; Mayumi et al., 2000; Orita et al., 1997). This has been reviewed by Kazanietz and co-workers (Kazanietz, 2000; Kazanietz et al., 2000).

However, the effects of persistent activation of PKCs may be cell-type specific or isoform-specific because in some cases, phorbol ester treatment of cells leads to growth suppression and differentiation (Mischak et al., 1993a; Mischak et al., 1993b; Regazzi et al., 1986; Vandenbark and Niedel, 1984). However, it is not clear from such studies whether this is due to PKC activation, downregulation, or the activation of another phorbol ester-sensitive protein.

To support the action of a PKC isoform-specific effect on cellular transformation, Housey and co-workers reported that Rat 6 embryo fibroblasts were transformed when over-expressing PKC$\beta_{I(2)}$, but not when over-expressing PKCα; this effect was particularly evident when cells were exposed to phorbol esters (Housey et al., 1988). These findings suggested a difference between these two isoforms in respect of inducing cellular transformation. Further examples are provided by Borner and co-workers, who reported a change in expression of certain PKC isoforms in Rat 6 fibroblasts transformed with v-HA-ras or v-src, but not when transformed with the v-myc or v-mos viral oncogenes (Borner et al., 1992; Borner et al., 1990). This demonstrated an oncogene-specific effect on the transformation capabilities, of certain PKC isoforms.

Viral oncogenes such as the polyoma middle T antigen appear to be more efficient at transforming rat FIII fibroblasts in the presence of TPA treatment. However, this appears to be cell-type specific, as the same treatment in NIH 3T3 cells did not have a similar effect (Raptis and Whitfield, 1986). This may reflect the proportion of membrane-bound PKC or the cellular wiring (Raptis et al., 1993). Furthermore, it was reported that PKC activation promoted the oncogenicity of polyoma middle T antigen due to the association of PI 3-kinase (Raptis et al., 1988). It seemed that induction of PKCs at the appropriate time of transformation, by a viral oncogene, was crucial in the synergy between these two factors (Borner et al., 1990; Dotto et al., 1985; Hsiao et al., 1984; Hsiao et al., 1989; Hsiao et al., 1986; Hsieh et al., 1989).

PKCε had been implicated as a protein kinase with oncogenic properties, particularly when it interacts with members of the ras signalling pathway, such as ras and raf-1 (Cacace et al., 1993; Hsiao et al., 1989; Mischak et al., 1993a; Perletti et al., 1998; Ueffing et al., 1997; Wang et al., 1998). This seems specific for PKCε because PKCδ has been reported to inhibit cellular growth and cell-cycle progression (Mischak et al., 1993a; Wang et al., 1998; Watanabe et al., 1992). The availability of a PKCε-knockout MEF cell line ($PKC\varepsilon^{-/-}$MEFs) provided an ideal opportunity to study the effects of this isoform on cell cycle progression, apoptosis and growth in soft agar, as markers of tumourigenicity.

When wild-type PKCε, or phosphorylation mutants at the T loop and hydrophobic sites, were stably transfected back into the $PKC\varepsilon^{-/-}$MEFs, predictions could be tested. These studies revealed that there was little difference in the $PKC\varepsilon^{-/-}$MEF cells from those stably transfected with the different forms of PKCε in terms of cell cycle progression or growth in soft agar. These studies on growth in soft agar and cell cycle progression only examined the effects of the absence of PKCε expression, and did not examine the effects of tumour promoters, such as phorbol esters, or the effects of co-expressing oncogenes, which could have complicated the interpretation of the findings. Another issue is that the clones derived from the $PKC\varepsilon^{-/-}$ MEF clones are already immortalised. It is not clear from these studies whether these cells are also partially transformed, and if so what effect this has on the function of PKCε in the cell.

The effect of an absence of phosphate at the defined regulatory sites in PKCε was determined pharmacologically (chapter 3 and 4); this showed their importance for catalytic activity. If the catalytic activity of these mutants reflect the activity determined for PKCε, when immunoprecipitated from cells treated with pharmacological inhibitors, then it may explain why the phosphorylation mutants, or the knockout cells, were unable to prevent the early entry into apoptosis. However, the catalytic activities of the different forms of PKCε are yet to be determined.

In support of the contention that PKCε can act as an oncogene, PKCε$^{-/-}$MEF cells stably expressing wild-type PKCε did not commit to apoptosis quite as quickly as the PKCε$^{-/-}$MEF cells did or the same cells stably expressing the phosphorylation mutants. It seemed clear from these studies that although PKCε may be important in some aspect of cellular transformation, these fibroblasts were still able to grow and form colonies in soft agar (a transformation phenotype) in the absence of PKCε. This might imply that an element of redundancy exists in this process within these cells. This may be a consequence of the immortalisation of the cells. Therefore, it seems that this feature of PKCε$^{-/-}$MEF cell growth in soft agar is not related to PKCε. However, it is debatable whether this is a fair reflection of transformation. Another way to determine whether an enzyme has an important role in cellular transformation is by using nude mice, which have a defective immune system. Cells expressing the wild-type or phosphorylation site mutants of PKCε are either injected into the circulatory system *via* the tail or under the skin of the mouse, and then the mouse is periodically examined to identify the presence of tumour formation. These investigations are currently the subject of ongoing investigations in the laboratory by Mr Whelan.

7.6 Perspectives

This study has revealed that a number of different pathways are involved in the regulation of phosphorylation of nPKCs. When the kinase is 'primed' through phosphorylation and bound to its cofactors, the PKC is fully active. Although our attention has been focused on the effects of particular phosphorylation sites on the activity of nPKCδ and nPKCε, evidence has accumulated on the effects of PKCs on various aspects of cellular behaviour. It is apparent that some of the effects of PKCs in cells depend on other factors also. This may reflect a cell-type-specific response. In our studies, it has been noticed that the phosphorylation sites in the nPKC isoforms are also under the regulation of many different factors. For example,

integrin and growth factor-induced signalling, the PI 3-kinase pathway via PDK-1 recruitment, PTEN, mTOR and nutrient deprivation, an aPKC-containing kinase complex, and PP1/PP2A-type protein phosphatases. This demonstrates that there is a significant amount of cross-talk between various signalling pathways which feed into the regulation of these phosphorylation sites, and hence the catalytic activity of PKCs.

There did not appear to be any differences in the regulation of the T loop, TP, and hydrophobic phosphorylation sites between PKCδ and PKCε. These phosphorylation sites seem to have an important influence on the catalytic activity of the nPKCs examined. The similarity in the regulation of these phosphorylation sites in PKCδ and PKCε does not seem to reflect the differences between the properties of these two nPKC isoforms in cellular behaviour, for example, transformation and cell-cycle progression, that has been reported by other groups. The differences reported by several groups in the function between the various members of the PKC family in a cell may depend upon many factors. When examining which PKC isotype is causing an effect, it might be as important to consider the cell-type, the activation of other signalling pathways in parallel, the presence (and period of exposure) of tumourigenic or transforming compounds in the study, intra-cellular location, and expression level of the PKC isotype. In the future, to understand the importance of these phosphorylation sites in all the PKC members, on the behaviour of the cell, all these factors ought to be considered.

Future research ought to include the examination of phosphorylation of endogenous nPKC in other cell types, particularly epithelial cells. This is because many cancers in humans are derived from these cells. It would also be interesting to examine the function of PKCs in cells derived from patients suffering from the loss of PTEN/MMAC, such as Cowden syndrome and Bannayan-Zonana syndrome. mTOR has been shown to be involved in the regulation of phosphorylation at the hydrophobic site in PKCδ and PKCε, but it remains to be determined what the mechanism is. Another issue that has remained contentious throughout these studies

is the identity of the V5 kinase. Although PKCζ seems to be an important component, it remains to be determined what the protein kinase is, or what all the components are. Finally, it would be desirable to assimilate all this information as an approach to target PKCs for intervention of cancer.

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