Cellular Roles of PKN1

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PROTEIN KINASE NOVEL 1 (PKN1), which in part resembles yeast PKCs, has been shown to be under the control of Rho GTPases and 3-Phosphoinositide Dependent Kinase 1. Initial studies tested the hypothesis that Rho-PKN1 inputs control PKB phosphorylation. Despite a demonstrable intervention in Rho function, no evidence was obtained that indicated a role for Rho-PKN1 in PKB control.

In seeking a cellular role for PKN1, it was found that GFP tagged PKN1 has the ability to translocate in a reversible manner to a vesicular compartment following hyperosmotic stress. PKN1 kinase activity is not necessary for this translocation and in fact the PKN inhibitor HA1077 is also shown to induce PKN1 vesicle accumulation. PKN1 translocation to these vesicles is dependent on Rac1 (and not Rho) activation although the GTPase binding HR1abc domain is not sufficient for this recruitment. The PKN1 kinase domain however localises constitutively to this compartment and it is demonstrated that this behavior is selective for PKNs. Associated with vesicle recruitment, PKN1 is shown to undergo activation loop phosphorylation and activation. It is established that this activation pathway involves PDK1, which is shown to be recruited to this PKN1 positive compartment upon hyperosmotic stress.

Further studies employing PKN1-KO MEF cells place PKN1 upstream of the MKK4-JNK pathway in response to hyperosmotic stress. This PKN1 requirement is shown to be a selective hyperosmotic-induced response and to be specific for JNK, not affecting the closely related ERK1/2 and P38 MAPK pathways. Taken together these findings present a pathway for the selective, hyperosmotic-induced, Rac1
dependent PKN1 translocation, that leads to its endocytosis and PDK1-dependent activation. The role of PKN1 appears to be to facilitate MKK4 activation and its own catalytic activity is indicated to play critical role in this context. This provides a distinctive insight into PKN1 and its specificity of action.
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Abbreviations

ATP  Adenosine 5'-triphosphate
BSA  Bovine serum albumin
DAG  Diacylglycerol
DNA  Deoxyribonucleic acid
DTT  Dithiothreitol
ECL  Enhanced chemiluminescence
EDTA  Ethylene diamine tetra-acetic acid
ES  Embryonic stem (cells)
FCS  Foetal calf serum
GDP  Guanosine 5'-diphosphate
GST  Glutathione S-transferase
GTP  Guanosine 5'-triphosphate
GTPase  GTP hydrolase
h  hours
HEPES  N-(2-hydroxyethyl)-piperazine-N'-(2-ethanesulphonic acid)
IPTG  Isopropyl-β-D-thiogalactopyranoside
KDa  Kilo dalton
LY294002  2-(4-morpholinyl)-8-phenyl-4H-1 Benzopyran-4-one
M  Molar
m  mili
μ  micro
MAPK  Mitogen activated protein kinase
MEFs  Mouse embryonic fibroblasts
MBP  Myelin Basic protein
Mins  Minutes
n  nano
OD  Optical density
PAGE  Polyacrylamide gel electrophoresis
PBS  Phosphate buffered saline
PCR  Polymerase chain reaction
PDK1  PtdIns (3,4,5)P_3-dependent kinase -1
PH  Pleckstrin homology (domain)
PtdIns (3,4,5)P_3  Phosphatidylinositol (3,4,5) triphosphate
PtdIns (4,5)P_2  Phosphatidylinositol (4,5) bisphosphate
PI3K  Phosphatidylinositol 3-kinase
PKB  Protein kinase B
PKC  Protein kinase C
PKN  Protein kinase N
PLC  Phospholipase C
PLD  Phospholipase D
rpm  revolutions per minute
PRK  Protein kinase C related kinase
SDS  Sodium dodecyl sulphate
Sec  Seconds
SH2/3  Src homology 2/3 domain
v/v  volume/volume
w/v  weight/volume
Chapter 1

Introduction

1.1 Signal transduction

Signal transduction pathways provide the means by which cells respond to their surroundings and elicit distinct functions. A key theme relating to how cell signalling mechanisms achieve such responsiveness is specificity. This is achieved through discrete protein-protein and protein-lipid interactions. The resulting modifications of these components form pathways or cascades that are responsible for highly specialised cellular functions. Defective signal transducers have been widely demonstrated to result in a multitude of diseases. The targeting of signalling components using small molecular weight inhibitors allows the intervention in signalling cascades and can therefore be of therapeutic benefit. Such drugs, for example Gleevec, are in fact already in clinical use (see recent review (Fabbro and Garcia-Echeverria, 2002)). The elucidation of signalling pathways along with their associated functions is clearly critical to understanding the full potential and consequences of such therapy in refining these approaches. The work in this thesis concerns one such signalling module. By way of introduction, a general commentary on relevant protein modifications and signalling pathways follows.

1.1.1 Phosphorylation; kinases and phosphatases

Phosphorylation is one of the most widely studied post-translational modifications and was first described as the reversible modification of phosphorylase kinase. This was shown to not only involve a kinase, but also a phosphatase, the function of which is to act antagonistically and catalyse dephosphorylation (Riley et al., 1968).
In mammalian cells, protein kinases covalently attach γ-phosphate groups to the hydroxyl side chain of either serine, threonine or tyrosine residues of specific protein substrates. The completion of the human genome project has enabled the elucidation of the complete human “kinome” which has recently been shown to comprise over 500 genes (Manning et al., 2002).

The dynamic nature of protein phosphorylation as a reversible modification to allow the rapid transduction of signals requires an “off” switch to counteract the “on” signals provided by protein kinases; this role is fulfilled by phosphatases. Phosphatases operate as distinct holoenzyme complexes comprised of various regulatory and catalytic subunits that confer distinct cellular localisation and substrate specificity. Protein phosphatases are reviewed (Cohen, 1989; Depaoli-Roach et al., 1994).

Phosphorylation has widely been shown to confer activity on substrate proteins, however it is also known to render certain substrates inactive as is the case with Src (Kmiecik and Shalloway, 1987). This ability to act in a stimulatory and/or inhibitory manner on proteins is often conferred by the phosphate group in manipulating protein-protein interactions.

1.1.2 Protein-Protein interactions

Interacting domains of proteins provide recognition and target proteins to specific cellular locations. These modules thereby confer discrete modes of action on specific proteins such as the substrate specificity of kinases.

Src homology 2 (SH2) domains, first identified in the tyrosine kinase Src, along with phospho-tyrosine binding (PTB) domains, bind to regions that contain short stretches of amino acids which contain phospho-tyrosine residues. These are
present within a number of signalling molecules such as PLC-\(\gamma\), SHP-2, the p85 subunit of PI3-kinase and the adaptor proteins Nck and Grb2 (Pawson and Nash, 2003). In terms of their responsiveness to phosphorylated tyrosine residues, SH2 and PTB domains highlight the capacity of phosphorylation as a mechanism for exerting control over signalling cascades.

Src homology 3 (SH3) domains, also identified in Src, bind to proline rich regions, examples include those found in the signalling intermediates SOS and POSH. Of particular interest to this study, PKN2 (see section 1.6) contains such a proline rich region and has been shown to bind the SH3 containing adaptor protein Nck (Quilliam et al., 1996).

Other protein-protein interaction motifs include: PDZ domains, WW domains, WD-40 domains and 14-3-3 domains (see recent review (Pawson and Nash, 2003)). These are variously used in recognition of protein motifs including for example phospho-serine residues. These recognition modules serve to permit assembly of higher order signalling complexes that drive specificity and efficiency in relaying signals. Examples of these are described in the following section.
1.2 Mitogen activated protein kinase (MAPK) cascades

Perhaps the best characterised signalling pathways are the mitogen activated protein kinase (MAPK) cascades. The first MAPK to be discovered was initially described as a 42KDa protein that upon tyrosine and threonine phosphorylation becomes a serine/threonine kinase (Ray and Sturgill, 1988). This protein is now more commonly known as extracellular signal-regulated kinase (ERK), while the term MAP kinase refers to the wider superfamily.

In multi-cellular organisms three parallel MAP kinase pathways exist, all sharing common elements; ERK, c-Jun NH$_2$-terminal kinase (JNK) and p38. Related components of these pathways operate as multi-level phospho-relay systems and comprise three sequentially activated serine/threonine kinases. MAP kinases are substrates for MAPK kinases (MAPKK). MAPKKs are dual specificity kinases that recognise ThrXTyr motifs within the activation loop of MAPKs, the “X” residue is glutamate for ERKs, proline for JNKs and glycine for the p38s. MAPKKs are in turn substrates for MAPK kinase kinases (MAPKKK). The ERK, JNK and p38 signalling cascades are summarised in Figure 1.1.

The activation of MAPKs by receptor tyrosine kinase (RTK) mediated signalling is well established, this cascade also highlights the role of protein-protein interactions in triggering these pathways. The RTK stimulated activation of the ERK1/2 cascade is illustrated in Figure 1.2. Upon ligand binding to a receptor, such as the platelet derived growth factor receptor (PDGF), dimerisation and transphosphorylation of receptors is followed by the binding of GRB2 via its SH2 domain to phosphorylated tyrosine residues on the receptor. GRB2 binds directly to the Ras nucleotide exchange factor SOS via an SH3 domain. The recruitment of SOS to the membrane results in Ras activation and the subsequent stimulation of the ERK cascade (see review (Pawson and Nash, 2000)).
Fig 1.1 MAP-kinase phospho-relay systems.

MAP kinase signalling components are organised according to their levels in the phospho-relay cascade; Activator, MAPKKK, MAPKK, MAPK and substrate. Members of the ERK, JNK, and p38 MAPK pathways are given and for many of the pathway constituents shown, multiple family members exist.
Fig 1.2 Receptor tyrosine kinase signalling and Protein-Protein interactions.

A schematic representation of receptor tyrosine kinase signalling. Receptor dimerisation and autophosphorylation is followed by the recruitment of SH2 and/or SH3 containing adaptor molecules and subsequent signalling events.
As well as growth factor stimulated activation, the MAPKs are well known to be activated by a range of environmental stresses. These responses are critical for the ability of cells to sense and adjust to their surroundings. Such stresses include changes in the surrounding osmolarity, temperature and production of reactive oxygen species (by, for example, aerobic metabolism). The highly conserved MAP-kinases are known to be key elements in responding to the harmful effects of these stress conditions. By transducing signals from the cell surface these pathways lead to the stimulation of protective responses (for review see (Kyriakis and Avruch, 2001)). This aspect of MAPK signalling, in particular osmotic stress, is relevant to the work described in this thesis.

1.2.1 Extracellular signal-regulated Kinase (ERK)

The ERK family comprises five proteins (ERK1-5) although ERK5 does have marked differences in structure. ERK1 and 2 are involved in the regulation of cell division and also post mitotic functions, they are activated by a range of stimuli including: growth factors, cytokines and heterotrimeric G protein coupled receptor activation. Mutations in Ras that activate ERK1 and 2 are commonly found in tumours, this activation is thought to increase proliferation. Indeed recent evidence has highlighted BRAF (part of the ERK cascade) mutations to be critical in the development of a large proportion of melanomas (Davies et al., 2002). Inhibitors of the ERK1 and 2 pathway therefore represent potential anti-cancer agents (see review (Johnson and Lapadat, 2002)).

1.2.2 c-Jun NH₂-terminal kinase (JNK)

The JNKs along with p38 MAP kinases (described below), form the stress activated protein kinase group (SAPK). JNKs comprise three genes (JNK1-3) and alternative splicing has been shown to produce 10 isoforms, all of between 46 and
55 kDa, although the significance of this splicing is not fully understood (Gupta et al., 1996). JNKs are activated by inflammatory cytokines and a variety of stress conditions and activation is achieved by phosphorylation of both Thr and Tyr residues by MKK4 and/or MKK7. While MKK7 is thought to be primarily activated by cytokines and MKK4 by stress conditions, the preferential phosphorylation of a Tyr residue for MKK4 and a Thr residue for MKK7 would suggest that these MKKs may act cooperatively (Fleming et al., 2000). MKK4 and MKK7 are activated by a variety of MAPK Kinase Kinases. To date, 13 MKKKs have been described that regulate JNK activity and this diversity of JNK activators is thought to enable multiple levels of stimulus specificity. A good example of such specificity is demonstrated for the MAPK Kinase Kinase, TAK1 which has been shown to be essential for JNK activation in response to LPS. In contrast, multiple MAPK Kinase Kinases (TAK1, MLK2, ASK1 and MEKK1) were found to be required for MKK4 activation under hyperosmotic stress conditions (Chen et al., 2002).

Recent studies have highlighted the role of scaffolding proteins in the activation of the JNK pathway. JNK interacting protein (JIP) is known to scaffold MKK7 and JNK, indeed JIP1 knockout mice demonstrate an in vivo and in vitro requirement for JIP1 under specific stress conditions (Whitmarsh et al., 2001). JIP1 however does not interact with the other JNK upstream kinase, MKK4. The retention of MKK4 signalling through JNK might explain the lack of penetrance upon the JNK pathway found in JIP1 knockout mice (Yasuda et al., 1999). The protein, “plenty of SH3 domains” (POSH) has been shown to scaffold MKK4 and JNK in promoting apoptosis (Xu et al., 2003), and JNK stress-activated protein kinase-associated protein 1 (JSAP1) is also thought to have a role in JNK scaffolding (Ito et al., 1999). As discussed in section 1.4.2.3, the scaffolding protein POSH also binds the small GTPase Rac and regulation of JNK signalling by Rac is well established.

The most well described target of JNK is c-Jun which is part of the activator protein 1 (AP-1) complex and is important for transcriptional responses. JNK was shown to
bind c-Jun and phosphorylate the Ser-63 and Ser-73 residues, thereby promoting transcriptional activity (Derijard et al., 1994). Other JNK substrates include ATF2, ELK-1, p53 and c-Myc. Signals mediated by JNK are known to regulate a plethora of cellular responses including; cell proliferation, tumorigenesis, embryonic development and apoptosis (Dunn et al., 2002).

2.1.3 p38 MAP kinase

The p38 MAP kinase family comprises four isoforms (α, β, γ and δ). The p38 MAP kinases are activated by a variety of stress conditions such as hyperosmotic stress, heat shock and UV. They are also activated by inflammatory cytokines, hormones and heterotrimeric G protein coupled receptor activation. The importance of this family was highlighted by the identification of p38 as the target of a class of anti-inflammatory drugs which block the expression of interleukin (IL-1) and tumour necrosis factor α (TNFα) upon stimulation with bacterial lipopolysaccharide (LPS) (Lee et al., 1994). The p38 MAP kinases are known to regulate the expression of many cytokines and are therefore also thought to be involved in asthma and autoimmunity (see recent reviews (Clark et al., 2003; Johnson and Lapadat, 2002)).

2.1.4 Yeast MAP-kinases

The high degree of conservation within MAP-kinase pathways from yeast to man has made yeast a valuable model system, particularly for the study of stress activated signalling. This conservation was highlighted by the fact that human stress activated JNK and p38 MAP-kinases can partially substitute for the related S.cerevisiae high osmolarity glycerol (HOG1) pathway in mediating the yeast response to osmotic stress (Galcheva-Gargova et al., 1994; Han et al., 1994).
The *S.cerevisiae* MAP-kinase system, while more simplified when compared with the mammalian MAP-kinase pathway, retains the basic elements of the cascade. In the case of the HOG1 pathway two MAPKKKs (Ssk2 and Ssk22) phosphorylate the MAPKK Pbs2, which in turn phosphorylates the MAPK HOG1. It has been shown however that unlike mammalian stress activated MAP-kinases, the HOG1 pathway is insensitive to stress conditions other than high osmolarity (Brewster *et al.*, 1993; Schuller *et al.*, 1994).
1.3 Lipid Signalling

The triggering of signalling cascades is often influenced by the availability and cellular location of specific phosphoinositides. This section will discuss these lipids, the kinases and phosphatases responsible for their metabolism and the protein domains which mediate specific lipid interactions.

1.3.1 Phosphoinositides

Phosphoinositides are composed of a glycerol backbone with fatty acids at positions 1 and 2, and an inositol 1-phosphate on position 3. Where the inositol ring contains no additional phosphates it is referred to as phosphatidylinositol (PtdIns) (Figure 1.3 A). The inositol ring can however be specifically phosphorylated on positions 3,4 and 5, and the kinases responsible for these modifications are referred to as phosphoinositide PI3, PI4 and PI5 kinases respectively. The production of PtdIns (3,4)P₂ and PtdIns (3,4,5)P₃ is known to be induced rapidly upon agonist stimulation (Stephens et al., 1993) and is associated with a variety of signalling events.

1.3.2 Phosphoinositide 3-kinase (PI3-kinase)

The importance of PI3-kinases is highlighted by their implication in cell growth, proliferation, survival, differentiation and cytoskeletal changes (see review (Vanhaesebroeck and Alessi, 2000)). There are three classes of PI3 kinases, all of which generate specific inositol lipids by phosphorylating phosphatidylinositol lipids at the D-3 position of the inositol ring (Figure 1.3 A). In conjunction with other lipid kinases, PI3-kinases can produce 3 lipid products; PtdIns3P, PtdIns (3,4)P₂ and PtdIns (3,4,5)P₃; such lipid metabolism is illustrated in Figure 1.3 B. Class II PI3-
Fig 1.3 Phosphoinositide metabolism.

A. The structure of phosphatidylinositol, relative positions on the inositol ring are numbered.
B. A schematic representation of phosphoinositide metabolism, highlighting the role of PI3-kinase, PI4-kinase, PI5-kinase and Phospholipase C in lipid metabolism.
kinases are also able to produce PtdIns3P and in addition PtdIns (3,4)P₂ from PtdIns (4)P while class III PI3-kinases phosphorylate PtdIns to form PtdIns3P receptors (for review see (Vanhaesebroeck and Alessi, 2000)). The production of PtdIns (3,4,5)P₃, performed by class 1 PI3-kinases, is of particular relevance to the studies described here. This section will therefore focus on Class 1 PI3-kinases.

Class 1 PI3-kinases can be further divided into class 1ₐ and class 1ₛ PI3-kinases. While class 1ₐ are linked to receptor tyrosine kinases, class 1ₛ are linked to heterotrimeric G-protein coupled receptors (for review see (Vanhaesebroeck and Alessi, 2000)). Class 1 PI3-kinases are heterodimers of; for class 1ₐ, 110 kDa catalytic and 85 kDa regulatory proteins and for class 1ₛ, p110γ catalytic and 101kDa regulatory proteins. For class 1ₐ PI3-kinase, the regulatory subunit mediates activation of the catalytic subunit. This is achieved by direct interaction with phosphotyrosine residues on growth factor receptors via the SH2 domain of the regulatory subunit, in a similar manner to that described for the RTK stimulated MAPK activation in section 1.2. Active PI3-kinase is known to catalyse the production of PtdIns(3,4,5)P₃ from PtdIns(4,5)P₂ which is thought to be its preferred substrate (Stephens et al., 1998). This induces the accumulation of pleckstrin homology (PH) domain containing proteins such as PDK1 and PKB to these sites of PtdIns(3,4,5)P₃ production on membranes for which certain PH domains have a high affinity (PH domains are discussed in section 1.3.5). This leads to the modified functions of these PH domain containing proteins and often leads to the activation of subsequent downstream effectors, ultimately mediating their associated cellular responses.

The small molecule inhibitors LY294002 and wortmannin are widely used as tools to study PI3-kinase function. Both bind to the catalytic domains of PI3-kinases and exhibit IC₅₀ values of 1μM and 5nM respectively against class 1 PI3-kinase (Domin et al., 1997; Vlahos et al., 1994).
1.3.3 PI4 and PI5 kinases

A PI4 kinase activity against PtdIns3P to form PtdIns(3,4)P$_2$ has been described in platelets and a PI4 kinase activity against PtdIns to form PtdIns4P was shown to be stimulated by EGF treatment (Banfic et al., 1998). The PI4 kinases are divided into two classes; type II and type III, each of which include an $\alpha$ and $\beta$ member (see review (De Matteis et al., 2002)). In yeast, two PI4 kinases have described: Pik1 and Stt4p, which are homologous to type III PI4 kinase $\beta$ and to type III PI4 kinase $\alpha$ respectively (Gehrmann and Heilmeyer, 1998).

To date there are three classes of PI5 kinase activity. Type I and type II PI5 kinases catalyse the formation of PtdIns(4,5)P$_2$. The preferred substrate of type I enzymes is thought to be PtdIns4P (Anderson et al., 1999), however the type II enzymes were actually shown to phosphorylate PtdIns5P at the D-4 position to produce PtdIns(4,5)P$_2$ and may therefore require a change in nomenclature (Rameh et al., 1997). The type III PI5 kinase activity was demonstrated in yeast to be Fab1 and was shown to phosphorylate PtdIns3P to form PtdIns(3,5)P$_2$ (Cooke et al., 1998).

1.3.4 Phosphoinositide Phosphatases

Lipid phosphatases play a major role in controlling the PI3-kinase pathway. The lipid phosphatase, "phosphatase and tensin homolog deleted in from chromosome ten" (PTEN), dephosphorylates the 3 position of the inositol ring of PtdIns(3,4,5)P$_3$ to form PtdIns(4,5)P$_2$, thereby acting antagonistically to PI3-kinase. The loss of heterozygosity of PTEN is widely found in cancer, the resulting uncontrolled PI3-Kinase signalling is therefore thought to contribute to cancer progression (Maehama and Dixon, 1999).
Several phosphatases are known to dephosphorylate the 5 position of the inositol ring of PtdIns(3,4,5)P$_3$ to produce PtdIns(3,4)P$_2$. These include; SH2 containing inositol phosphates (SHIPs), GAP containing inositol 5-phosphatases (GIPs) and Sac domain containing inositol 5-phosphatases (SCIPS). Synaptotagmin1, a SCIP, was shown to be active against both PtdIns(4,5)P$_2$ and PtdIns(3,4,5)P$_3$ in vitro (Woscholski et al., 1997). Thus there are multiple routes of manipulation of PtdIns(3,4,5)P$_3$ available to modulate signalling events.

1.3.5 Phosphoinositide-binding modules

Of critical importance to phosphoinositide signalling is the link to signalling proteins. A number of conserved phosphoinositide binding domains have been discovered and these are known to preferentially bind different phosphoinositide species. These domains are therefore able to confer localisation and activation on specialised pathways.

Many functionally diverse proteins contain pleckstrin homology (PH) domains, these are globular domains of around 100 amino acids. Nearly all PH domains require membrane association for their function (Bottomley et al., 1998). Examples of PH domain containing proteins include PDK1 and PKB and are discussed in sections 1.5.1 and 1.5.4.

The epsin amino-terminal homology domain (ENTH) is a phosphoinositide binding domain recently shown to confer specific PtdIns(4,5)P$_2$ binding to a number of proteins involved in endocytosis. This finding highlights the possibility that PtdIns(4,5)P$_2$ could be important for endocytic processes (Kay et al., 1999).

The FYVE domain is known to preferentially bind PtdIns3P and this interaction has been shown to target FYVE domain containing proteins to endosomal compartments. Proteins which contain FYVE domains are typically involved in
membrane trafficking events such as: early endosomal antigen 1 (EEA1), Vac1, and Fab1 (see review (Cullen et al., 2001)).

The phox homology (PX) domain is found in a wide range of proteins from signalling molecules like phospholipase D (PLD) to vesicle trafficking proteins such as the human sorting nexin, SNX3. For SNX3, the PX domain has been shown to specifically bind PtdIns3P and to target this protein to endosomal compartments (Xu et al., 2001). The issue of whether PX domains can function to target phosphoinositides other than PtdIns3P remains to be addressed (see review (Cullen et al., 2001)).
1.4 GTPases

GTPases are responsible for guanine nucleotide hydrolysis and are divided into two main groups; heterotrimeric and small. While these proteins share a common enzymatic mechanism, it is well established that different GTPases operate within highly specialized signalling pathways. GTPases operate as molecular switches by cycling between “active” GTP bound forms and “inactive” GDP bound forms (Figure 1.4).

1.4.1 Heterotrimeric G proteins

Heterotrimeric G proteins comprise three subunits (α, β and γ), which associate with the cytoplasmic domains of receptors with seven membrane spanning regions (serpentine receptors). These proteins transduce signals arising from stimulation of serpentine receptors and are implicated in a range of diverse processes (see review (Neves et al., 2002)).

There are 20 known Ga subunits, these contain a GTPase domain which is similar to that of the small GTPases. There are 6 Gβ and 11 known Gγ subunits, Gβγ subunits are tightly complexed and operate as obligate dimers. It is thought that Gβγ subunits interact with Ga subunits via an interface that covers the switch domain of the GTPase domain. Upon ligand binding to serpentine receptors, rearrangements in the receptor promote particular Ga domain(s) to exchange GDP for GTP (Bourne, 1997). This results in the loss of Gβγ subunit binding and both functional subunits (Ga-GTP, free Gβγ) activate downstream effectors (Hamm, 1998).
Fig 1.4 The GTPase cycle

Schematic representation of the GTPase cycle. Conversion between "active" GTP bound form and the "inactive" GDP bound form is performed by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) as indicated. Some Rho proteins bind guanine nucleotide exchange inhibitors (GDIs) which hold them in an inactive GDP bound state.
The Gα subunits are known to transduce signals to a variety of effectors including; adenyl cyclase, photoreceptor cGMP phosphodiesterase, phospholipase C-β and Bruton’s tyrosine kinase (Btk). The Gβγ subunits are known to signal to; phospholipase C-β2, several ion channels and the kinases Raf1, Tsk and Btk (Hamm, 1998). Additionally Gβγ subunits are known to bind the novel regulatory PI3-kinase P101 subunit, this is thought to explain the increase in PtdIns(3,4,5)P3 upon stimulation of heterotrimeric G protein-linked receptors (Stephens et al., 1997).

1.4.2 Small GTPases

The Ras superfamily of small GTPases comprises over 70 mammalian members and includes; Ras, Rho, Rab, Arf and Ran families. Most of these proteins contain a C-terminal “CAAX” motif where the “X” confers a specific posttranslational modification on the cysteine residue, such as; prenylation, proteolysis or methylation. For Rho this confers a geranylgeranylation modification since “X” is either leucine or phenyalanine whereas for Ras, farnesylation is specified.

This thesis is focussed on the PKN family of kinases which are known Rho family effectors. This section will therefore primarily discuss the Rho family of small GTPases.

The most widely studied Rho family GTPases include Rho (A, B, C isoforms), Rac (1, 2, 3 isoforms) and Cdc42 (Cdc42Hs and G25K), (see review (Bishop and Hall, 2000)). These proteins are widely thought to function by the disruption of auto-inhibitory intramolecular interactions of effector proteins, allowing exposure of functional domains. This is the case for kinase effectors such as the PKNs where Rho binding was shown to promote PDK1 interaction and catalytic activity and also
non-kinase effectors such as the scaffolding Cdc42 effector, WASP (Flynn et al., 2000; Kim et al., 2000).

Rho GTPases are regulated by guanosine nucleotide exchange factors (GEFs) which facilitate the exchange of GDP for GTP, and GTPase activating proteins (GAPs) which increase the rate of GTP hydrolysis of Rho GTPases (see Figure 1.4) (Lamarche and Hall, 1994; Van Aelst and D'Souza-Schorey, 1997). Rho GEFs contain a Dbl-homology (DH) domain which functions as the catalytic domain and also a PH domain which is thought to mediate membrane localisation. The PH domain has also been suggested to affect the activity of the Dbl homology domain (Soisson et al., 1998). Furthermore Rho and Rac are also thought to be modulated by guanine nucleotide dissociation inhibitors (GDIs) which bind Rho and Rac sequester GDP bound Rho/Rac, thereby inhibiting GDP-GTP exchange as illustrated in Figure 1.4 (Olofsson, 1999).

Rho GTPases have been associated with a variety of cellular functions although the major function is thought to be the regulation of the actin cytoskeleton (Hall, 1998). Additionally, however, Rho small GTPases have been shown to regulate several known biochemical pathways such as the serum response factor (SRF), nuclear factor κB (NF-κB) and the c-jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase pathways (Coso et al., 1995; Hill et al., 1995; Minden et al., 1995b; Perona et al., 1997). These pathways are associated with a plethora of cellular functions and highlight the importance of this class of proteins.

1.4.2.1 Small GTPase mutants and inhibitory toxins.

In dissecting the role of individual Rho-GTPases, activated and dominant negative mutants along with bacterial toxins have proved useful tools. For Rac, the amino acid substitution of Gly with Val at residue 12 (V12) suppresses GTP hydrolysis and is therefore considered to confer constitutive activation (Ihara et al., 1998).
Conversely the substitution of Asn for Thr on position 17 (N17) acts as a dominant negative by titrating endogenous GEFs (Feig, 1999).

Several bacterial toxins have also been employed for their ability to modify and inactivate Rho GTPases. The exoenzyme C3 transferase, isolated from Clostridium botulinum, has been shown to ADP-ribosylate Rho proteins on the Asn41 residue thereby inactivating the Rho sub-family of Rho GTPases, in particular specificity has been demonstrated over Rac (Aktories and Just, 1995; Wilde et al., 2000). Toxin B, from Clostridium difficile, has also been shown to inhibit Rho GTPases. Toxin B has a broader specificity and is thought to inhibit most Rho family GTPases (Aktories et al., 2000).

1.4.2.2 Rho

Rho is typically associated with actin stress fibers and Rho effector domains are thought to be different from those of Rac and Cdc42. The Rho effector domain, Homology region 1 (HR1), contains a leucine zipper like motif and is found in the Rho effectors PKN, Rhotekin and Rhophilin (see also section 1.6). However, the PKNs have also been shown to bind Rac, implying that such selectivity is not totally exclusive (Flynn et al., 1998). Non-HR1 domain containing proteins have also been shown to bind Rho, for example ROCK and Kinectin (see review (Bishop and Hall, 2000)).

The well characterised Rho effectors ROCK and Dia are thought to be required for Rho induced actin stress fibre formation. Substrates of ROCK include MLC phosphatase which has been shown to stimulate actin-activated ATPase activity of myosin II, thereby promoting actomyosin filaments (Amano et al., 1996a; Bresnick, 1999; Kawano et al., 1999). Lim kinase (LIMK), another ROCK substrate, also promotes actin stress fibres by phosphorylating and inhibiting cofilin, this in turn stabilizes filamentous actin (Maekawa et al., 1999). The Rho effector Dia has been
shown to induce actin stress fibres in combination with ROCK, promoting the idea that both ROCK and Dia are necessary for stress fibre formation. Dia contains two formin homology domains (FH) which have multiple proline-rich motifs and allow interaction with the G-actin-binding protein profilin which promotes actin polymerisation (Wasserman, 1998; Watanabe et al., 1999).

1.4.2.3 Cdc42 and Rac

The conserved GTPase binding motif known as the CRIB (Cdc42/Rac interactive binding) domain is contained within several Rac and Cdc42 binding proteins including ACK, WASP and PAK (see review (Bishop and Hall, 2000)). Differences within the CRIB domain have revealed that these effectors have a different selectivity for Rac and Cdc42 binding. While ACK and WASP are specific Cdc42 targets, it remains unresolved whether the PAKs are targets for Cdc42, Rac or both. It should be noted that not all Cdc42 and Rac effectors mediate interaction via the CRIB domain, non-CRIB domain containing effectors include WAVE, POSH and CIP-4 (see review (Bishop and Hall, 2000)).

Rac is associated with lamellipodia formation and several Rac binding partners establish the role of Rac in cytoskeletal organisation. The Rac binding protein Partner of Rac (POR-1), was shown to be involved in Rac-induced Lamellipodia formation (Van Aelst et al., 1996). Another target of Rac is the lipid kinase PI-4-P5 Kinase, this GTP-independent interaction is thought to mediate actin filament uncapping, a requirement for actin assembly which was shown to be dependent on an increase in PIP$_2$ levels (Hartwig et al., 1995). WASP-like verprolin-homologous protein, known as WAVE, has also been shown to bind Rac and to localise to membrane ruffles. Mutants of WAVE have been shown to prevent Rac induced membrane ruffles (Miki et al., 1998b).
Cdc42 is most typically associated with the formation of filopodia (see review (Bishop and Hall, 2000)). Cdc42 binds N-WASP and contains several domains involved in protein-protein interactions. These include; an N-terminal PH domain, an SH2 domain, an actin monomer-binding WASP homology domain-2 (WH2) and an acidic C terminus which binds ARP2/3. N-WASP and Cdc42 overexpression have been shown to cause exaggerated Cdc42 effects such as large microspikes suggesting that they are both involved in the formation of filopodia (Miki et al., 1998a). Furthermore, since ARP2/3 binds actin monomers and is thought to act as a nucleation site for actin polymerisation, a mechanism for Cdc42 controlling the cytoskeleton is detailed (Welch, 1999).

Of particular relevance to this thesis, the overexpression of Rac and Cdc42 was shown to stimulate the activation of the stress activated JNK and p38 MAP kinase pathways (Coso et al., 1995; Minden et al., 1995a). Rac and Cdc42 have been shown to bind MLK1,2,3, MEKK1 and MEKK4, direct activators of the JNK pathway (Fanger et al., 1997; Teramoto et al., 1996), as well as the JNK scaffolding protein POSH (Xu et al., 2003). The Cdc42 and Rac effectors mediating p38 and JNK activation however remain unclear. A potential common target for Rac and Cdc42 is p21-activated kinase 1 (PAK1), since it was shown to induce both filopodia and membrane ruffles and to phosphorylate both LIMK and MLC kinase in vitro, two enzymes involved in these subcellular processes (Edwards et al., 1999; Sanders et al., 1999). The role of PAK1 in relation to p38 and JNK activation has therefore been investigated. PAK1 has been shown to enhance p38 activation in conjunction with both Cdc42 and Rac (Zhang et al., 1995). It was also reported that PAK1 could activate JNK (Brown et al., 1996). However it has also been suggested that Rac activation of p38 and JNK is independent from PAK1 (Westwick et al., 1997).
Small GTPases are known to be involved in different stages of membrane trafficking (see review (Zerial and McBride, 2001)). While the Rab subfamily of small GTPases are perhaps most widely associated with membrane trafficking, the Rho subfamily have also been shown to be important for various membrane trafficking events. Rho associated trafficking events include; pinocytosis, phagocytosis, clathrin mediated endocytosis and exocytic pathways. These effects on vesicular traffic are thought to occur in part as a result of small GTPase effects on the actin cytoskeleton and the microtubule network. Cdc42 is thought to drive Arp2/3 mediated actin polymerisation at the vesicle surface and to act on different stages of endocytic pathways (Kroschewski et al., 1999). Rac is known to stimulate pinocytosis and phagocytosis and Rho is thought to act on vesicular traffic post endocytosis (Dharmawardhane et al., 2000; Ellis and Mellor, 2000; Leverrier and Ridley, 2001). There is also evidence that small GTPases influence microtubule mediated trafficking events since Rho was shown to bind kinectin and Rac was shown to bind tubulin (Best et al., 1996; Cook et al., 1998).

Important in small GTPase control of traffic is the integration with signalling molecules, for example activated Rac/Cdc42 in co-operation with p21-activated kinase (PAK) has been shown to stimulate pinocytosis (Dharmawardhane et al., 2000). Of particular relevance to the work of this thesis, PKN1 has also been shown to co-operate with RhoB to control EGF receptor traffic (Mellor et al., 1998). The role of controlling vesicle traffic places small GTPases central in transducing signals from the plasma membrane and resulting in transcriptional responses (see review (Ridley, 2001)).
1.5 AGC kinases

The "AGC" kinases comprise an extended superfamily of proteins including; PKA, PKG, PKC, PKB, p70^sk and p90^sk (for definitions and review see http://pkr.sdsc.edu/html/index.shtml). These enzymes have a high degree of homology with respect to their kinase domain and are all modulated by phosphorylation. The need for activation loop, or 'T' loop, phosphorylation to achieve optimal activity is a common feature of this group. Furthermore, there are two additional classes of phosphorylation that are associated with activation loop phosphorylation; autophosphorylation and hydrophobic site phosphorylation. The relative conservation of these sites among AGC kinases is illustrated in Figure 1.5.

1.5.1 Activation loop phosphorylation

The activation loop motif T(F/L)CGT is shared among AGC kinases and was first identified in PKCa where it was shown to be essential for catalytic activity (Cazaubon et al., 1994). Bacterial expression studies have shown that PKA autophosphorylation is sufficient for PKA activation loop phosphorylation, however for other AGC kinases such as PKBs, PKCs and PKNs this is not the case. Here the PI3-kinase pathway has been shown to trigger activation loop phosphorylation through 3-phosphoinositide dependent kinase 1 (PDK1) (Alessi et al., 1997b; Flynn et al., 2000; Le Good et al., 1998; Stephens et al., 1998).

PDK1 is a 63KDa serine/threonine kinase which was isolated as an activity responsible for PKBα activation loop phosphorylation. This activation was shown to be dependent on inclusion of PtdIns(3,4)P_2 or PtdIns (3,4,5)P_3 in the in vitro reaction (Alessi et al., 1997b) (Stephens et al., 1998). PDK1 has more recently
Fig 1.5 Sequence alignment of key phosphorylation sites of the AGC kinases.

Activation loop, autophosphorylation and hydrophobic sites among several AGC kinases are indicated. The stars highlight conservation of residues. The two stretches of amino acids are separated by approximately 125 residues.
been demonstrated to phosphorylate equivalent residues on many other AGC kinases including; p70<sup>60K</sup>, PKA, and PKCs. The PH domain of PDK1 has a higher \textit{in vitro} affinity for PtdIns (3,4,5)P<sub>3</sub> and PtdIns(3,4)P<sub>2</sub> than PtdIns(4,5)P<sub>2</sub>, implying that PDK1 can be responsive to PI3-kinase activity (see review (Vanhaesebroeck and Alessi, 2000)).

The activation loop site of several AGC kinases has been shown to undergo phosphorylation upon stimulation by regulatory effectors. For example DAG stimulates 'T'-loop phosphorylation of classical and novel PKCs (Le Good \textit{et al.}, 1998). The activation loop phosphorylation of PKN1/2 is also subject to regulation by small GTPases (Flynn \textit{et al.}, 2000). These regulatory controls are thought to induce a conformational change in PKN which is consistent with the need for such a change to allow PDK1 docking and phosphorylation (Biondi \textit{et al.}, 2000).

\subsection{1.5.2 Autophosphoryiation site phosphorylation}

Initially described as an autophosphoryiation site on PKC \( \beta I \) (Thr 642) and on PKC \( \alpha \) (Thr 638), this conserved AGC kinase motif is also known as the TP site or turn motif (Cazaubon and Parker, 1993). The occupation of this site has variable effects on function. For PKC\( \beta \), mutation of this site led to the phosphorylation of proximal sites resulting in a fully active kinase (Newton, 1997). However, for PKC\( \alpha \) this site is specifically thought to play a role in affecting the conformation of the kinase, thereby affecting the rate of dephosphorylation and inactivation (Bornancin and Parker, 1996).

\subsection{1.5.3 Hydrophobic site phosphorylation}

The highly conserved FSY motif is set within a hydrophobic sequence of the V<sub>5</sub> region of the AGC kinase domain (see figure 1.6) and has been shown to contribute to the activation and stabilisation of the kinase. Controversy surrounds
the regulation of this phosphorylation site with several reports suggesting that this is an autophosphorylation site (Keranen et al., 1995; Toker and Newton, 2000a), whilst other studies on PKB suggested this is in fact a target for transphosphorylation (Alessi et al., 1997b). The mechanism of phosphorylation is unclear, although interestingly, this region can act as a binding site for PDK1 (Biondi et al., 2000).

The putative kinase that mediates hydrophobic site phosphorylation is referred to as 3-phosphoinositide dependent kinase 2 (PDK2). The hydrophobic kinase for AGC kinase family members remains elusive although several proteins have been suggested to play a role in phosphorylating this site. It has been suggested that integrin-linked kinase-1 (ILK1) may have a role in phosphorylation of the hydrophobic serine residue at amino acid 473 of PKB (S473). PI3-kinase activated ILK1 was proposed to enhance PKB S473 phosphorylation in NIH3T3 cells and also in vitro, although this is thought to be an indirect mechanism since a kinase inactive form of ILK1 also enhanced PKB S473 phosphorylation (Lynch et al., 1999).

An alternate hypothesis for the origin of PDK2 activity involves protein kinase N 2 (PKN2), which was identified as a potential PDK1 interacting partner by a yeast two-hybrid screen. A C-terminal PKN2 fragment termed PDK1 interacting fragment (PIF), was isolated and shown to convert PDK1 to a form that will phosphorylate PKB on S473 in vitro, raising the possibility that PDK2 is in fact a modified PDK1 (Balendran et al., 1999). However more recent work involving full length PKNs has cast doubt on this hypothesis and would seem to indicate that they play negative rather than positive roles on PKB phosphorylation (section 1.6). Candidate PDK2 activities are reviewed (Chan and Tsichlis, 2001).
1.5.4 Protein Kinase B (PKB)

PKB was originally identified by its homology to PKC and PKA and was also known as RAC1 (related to A and C kinases) (Bellacosa et al., 1991; Coffer and Woodgett, 1991; Jones et al., 1991). PKB is a major target of PI3-kinase lipid products and is recruited to cell membranes by PtdIns(3,4,5)P_3 via its Pleckstrin Homology (PH) domain where it is subsequently phosphorylated by PDK1. PKB has been shown to require phosphorylation at its activation loop site, (Thr 308) which is mediated by PDK1, and also at the hydrophobic site (Ser473) site for full activation, for which the upstream kinase or mechanism of phosphorylation remains elusive (see sections 1.5.1 and 1.5.3). Targets of PKB include Glycogen synthase kinase 3 (GSK3), the phosphorylation of which results in its inactivation and hence subsequent stimulation of glycogen synthesis (Cross et al., 1995).

PKB is of great interest since it is overexpressed in a range of cancer cell types and is known to have anti-apoptotic properties. Indeed one mechanism for this has been proposed since PKB was shown to phosphorylate the proapoptotic protein BAD. This phosphorylation event is thought to promote binding of BAD with the 14-3-3 protein, rendering BAD non-functional (Downward, 1998; Franke et al., 1997; Sabbatini and McCormick, 1999). Other targets of PKB are also involved in promoting cell survival including: human caspase-9, forkhead transcription factors and IκB kinases (see review (Vanhaesebroeck and Alessi, 2000)).

1.5.5 Protein kinase C (PKC)

PKC was one of the first protein kinase families to be identified, originally reported as a histone kinase activity that is activated by proteolysis (Inoue et al., 1977). PKC was shown to be activated by phosphatidylserine (PS) and diacylglycerol (DAG) in a Ca^{2+} dependent manner. The tumour promoting phorbol esters (such as PMA) were also shown to activate PKC (Castagna et al., 1982; Nishizuka, 1984). To date
the PKC superfamily comprises twelve distinct genes and these have varying capacities for Ca\(^{2+}\) and DAG responsiveness (see figure 1.6). However, it is likely that the number of gene products is higher than this as PKC\(\beta\) was shown to be alternatively spliced to give two proteins which differ at the C-terminus (Coussens et al., 1987).

PKC isoforms have been classified according to their enzymatic properties. The classical PKCs (cPKC) include \(\alpha,\beta\) and \(\gamma\) isoforms and are activated by phosphatidylserine in a calcium dependent manner, they are also activated by DAG. The novel PKCs (nPKC) comprise \(\delta,\theta,\varepsilon\) and \(\eta\) isoforms and while independent from calcium they are activated by DAG. The atypical PKCs (aPKCs) comprise \(\iota\) and \(\zeta\) isoforms and are both calcium and DAG independent. The most recent PKC superfamily members to be identified are the Protein kinase Ns (PKN) (also known as protein kinase C-related kinase PRK). Human PKNs comprise at least three members (1, 2, and 3) of which only 1 and 2 are fully cloned and characterised. These are activated independently from calcium and DAG although they share significant homology with the catalytic domain of other PKCs. They have been shown to be regulated by the Rho family of small GTPases and are discussed in more detail in section 1.6. The comparative domains structures of PKC superfamily members are summarised (Figure 1.6).

A generalised model of PKC activation has emerged in recent years and is illustrated in Figure 1.7. Classical and novel PKCs are recruited to the plasma membrane in response to increased levels of DAG through lipid hydrolysis by either phospholipase C (PLC) or indirectly via phospholipase D (PLD); this is mediated via the C1 domain. Classical PKCs additionally require Ca\(^{2+}\) binding which occurs via inositol 1,4,5-trisphosphate (IP\(_3\)) induced calcium release and is mediated by the C2 domain (Berridge, 1993). C1 and C2 domains are discussed below. This co-factor binding to PKCs is thought to precede a conformational change resulting in the displacement of the autoinhibitory pseudosubstrate domain.
Fig 1.6 PKC superfamily domain structure.

A schematic representation of PKN superfamily domain structure. C1 domains are indicated in green, C2 and C2-like domains in grey, catalytic domains in red and the V5 regions in purple. The HR1 regions are indicated in blue and the PKN2 proline rich region is in pink.
Fig 1.7 Model for classical and novel PKC activation.

PKCs are activated by agonist dependent production of second messengers DAG and Ca$^{2+}$ (classical) which bind C1 and C2 domains (classical) respectively in the context of membrane lipids. This relieves the autoinhibitory pseudosubstrate domain from the active site. This allows targeted phosphorylation at the activation loop site by PDK1, the hydrophobic site phosphorylation by the "V5 kinase" and subsequent autophosphorylation resulting in a fully active PKC.
from the active site. These events facilitate PDK1 docking and multi-site phosphorylation and result in a fully functional kinase (see figure 1.7 and (Le Good et al., 1998; Parekh et al., 2000)).

Conventional and novel PKCs contain a C1 domain which is composed of two repeated zing-finger motifs (Hurley et al., 1997). This domain is thought to fold into a distinct structure unlike other zinc finger domains (Hommel et al., 1994). It has been shown that diacylglycerol (DAG) binds to the C1 domain and that the phorbol ester $[^{3}H]$phorbol-12,13-dibutyrate (PDBu) competes with DAG for C1 binding, hence DAG and PDBu are thought to have the same C1 domain point of contact (Kaibuchi et al., 1989; Ono et al., 1989) (Sharkey and Blumberg, 1985). The atypical PKCs, while unresponsive to phorbol esters, do however contain a single C1 zinc-finger motif.

Conventional PKCs contain a C2 domain which is located C-terminal to the C1 domain. These are thought to bind phospholipid in a calcium dependent manner, indeed this has been shown for PKCβ (Shao et al., 1996). While the calcium binding C2 domain is missing from the novel and atypical PKCs, these calcium independent PKCs contain a closely related region that is termed C2-like or ‘V₅’ in novel PKCs, a PB1 domain in atypical PKCs and an ‘HR2’ domain in PKNs. Although these regions lack aspartate residues that are required for calcium binding, it has been suggested that they could mediate phospholipid binding or protein-protein interactions (Mellor and Parker, 1998). The crystal structure of the C2-like domain of PKCδ has been solved and was shown to be a C2-fold (Pappa et al., 1998).

PKCs have a wide range of substrates in vitro, however precise in vivo targets remain largely unresolved, particularly in relation to physiological outputs. Well characterised PKC substrates include MARCKS protein (myristoylated alanine-rich C-kinase substrate) and various STICKS (Substrates that interact with C kinases)
such as the adducins, syndecan-4 and PAR-3. As a consequence of the highly conserved catalytic domain among PKCs, isoform specific substrates are not distinguished in vitro. It is likely therefore that isoform specific binding partners serve to target individual classes of PKC to their precise in vivo substrates (Jaken and Parker, 2000).

1.5.5.1 **In vivo PKC studies**

Knockout PKC mice have been generated for most PKC isoforms and phenotypes have been found. For example, PKCε knockout mice present an attenuated response to interferon γ stimulation and macrophage activation (Castrillo et al., 2001). PKCβ knockout mice were shown to have a marked immunodeficiency resulting from impairment of B cell receptor signalling (Leitges et al., 1996). PKCδ and PKC ζ knockout mice were also shown to have B cell defects (Martin et al., 2002; Mecklenbrauker et al., 2002). PKCθ knockout mice however were shown to be defective for T cell receptor signalling (Sun et al., 2000b). Mice deficient in PKCγ were shown to be resistant to neuropathic pain syndrome after partial sciatic nerve section, implying a role for PKCγ in the central nervous system (Malmberg et al., 1997). The possibility remains that PKC isoform redundancy can partially compensate for the normal PKC responses. Future studies involving multiple isoform knockout mice will answer more comprehensively the full extent of PKC involvement on cellular control.

1.5.5.2 **Yeast PKC**

The yeast *S.cerevisiae* contains one PKC homologue, Pkc1 while *S.pombe* contains two PKCs; and Pck1 and Pck2. These homologues show close structural resemblance to mammalian PKC and related PKNs as reviewed (Mellor and Parker, 1998). As indicated in figure 1.6, Pkc1 contains a putative DAG sensitive
C1 domain and a C2-related domain. Also present are two HR1 repeats similar to those found in mammalian PKNs, these are thought to mediate binding of the yeast Rho homologue Rho1 (Nonaka et al., 1995).

The yeast Pkc1 is known to activate the MAP kinase cascade involving BCK (MAPKKK), MKK1 and MKK2 (MAPK), and MpK1. This pathway is also controlled by Rho1, and has been linked to the control cell wall integrity (Arellano et al., 1999; Irie et al., 1993; Levin et al., 1994). Interestingly, this control mechanism has been likened to the osmotic stress response (Alonso-Monge et al., 2001).
1.6 Protein Kinase N (PKN).

PKNs (Protein Kinase Novel, also known as PRKs (Mukai and Ono, 1994) (Palmer et al., 1994)) are a subfamily of serine/threonine kinases identified independently by molecular cloning, protein purification and polymerase chain reaction based screens for PKC related kinases. PKNs currently comprise three isoforms, PKN1, PKN2 and PKN3 (formerly known as PKN or PRK1, PRK2 and PKNβ respectively). Two human isoforms are fully cloned and characterised; PKN1 and PKN2 which migrate on SDS-PAGE at 120 and 140 kDa respectively. PKN1 and PKN2 were found to be expressed ubiquitously (Mukai and Ono, 1994; Quilliam et al., 1996). However human PKN2 was also shown to have a distinct pattern of expression (Palmer et al., 1995b). PKN3, while undetected in adult tissue has been found abundantly in cancer cell lines (Oishi et al., 1999).

The C-terminal kinase domains of these proteins are closely related to those of PKC. At their amino termini they have a conserved repeated domain known as homology region 1 (HR1a,b,c) followed by a C2-related domain; overall these proteins have a domain organisation related to that of the yeast PKC-related proteins excepting the lack of a C1 domain (see (Mellor and Parker, 1998) and figure 1.6). The HR1 domain was identified as a Rho interacting region and incorporates a leucine zipper-like motif (Amano et al., 1996b; Vincent and Settleman, 1997; Watanabe et al., 1996). Other examples of this domain have been identified such as the Rho binding regions of Rhotekin and Rhofilin (Watanabe et al., 1996). The C2-like domain does not confer calcium sensitivity to PKNs since it does not contain the critical aspartate residues needed, although it is thought to act as an autoinhibitory region (Mukai et al., 1995).

PKNs are activated by fatty acids and phospholipids in vitro; arachidonic acid,
linoleic acid, cardiolipin, PtdIns (4,5)P$_2$, PtdIns (3,4,5)P$_3$ and LPA have all been shown to activate PKNs in vitro although the in vivo significance of this remains unclear (Morrice et al., 1994; Mukai and Ono, 1994; Palmer et al., 1995a; Peng et al., 1996). PKN activation has also been shown to be under the influence of small GTPases. The interaction of Rho with PKN1 has been demonstrated to facilitate PKN1 activation loop phosphorylation by 3-Phosphoinositide Dependent Kinase 1 (PDK1). The same study demonstrated an in vivo ternary complex of Rho-PKN1-PDK1 which was shown to be dependent on PI3-kinase activity and to be critical for the catalytic activation of PKN1 (Flynn et al., 2000).

The role of PDK1 in relation to PKN1/2 was studied using PDK1 null embryonic stem (ES) cells (Balendran et al., 2000). These cells were found to have a much reduced level of PKN1 expression when compared with WT ES cells. The expression level of PKN2 was unaffected in PDK1 null cells and some residual PKN2 activation loop phosphorylation was detected when compared with WT ES cells. These findings imply that for PKN1, PDK1 mediated activation loop phosphorylation may be critical for the stability of the protein. However, some residual PKN2 activation loop phosphorylation was found in PDK1 null ES cells.

The role of PKNs in relation to the downstream PDK1 effector PKB has also been the subject of debate. A C-terminal PKN2 fragment was shown to convert PDK1 to a form that will phosphorylate PKB in vitro on the serine residue at amino acid 473 (S473) functioning as the elusive PDK2 activity (Balendran et al., 1999). This work raises the possibility that PKN signals can influence PKB S473 phosphorylation thereby acting as a “PDK2” complex. However, a recent study has suggested that full length PKN1 and PKN2 may negatively regulate PKB phosphorylation (Wick et al., 2000). PKN2 cleavage during apoptosis has also been shown to inhibit PKB phosphorylation (Koh et al., 2000). The role of PKNs in regulating other AGC kinases has also been investigated since a C-terminal PKN2 fragment was found to inhibit PDK1 mediated PKC$_{\zeta}$ phosphorylation (Hodgkinson and Sale, 2002).
Much like PKCs, *in-vitro* PKN substrates have been described, although the *in-vivo* significance of these remains unclear. PKN substrates include, MARCKS protein, vimentin, Tau, and CPI-17 *in vitro* (Hamaguchi *et al.*, 2000; Kawamata *et al.*, 1998; Matsuzawa *et al.*, 1997; Palmer *et al.*, 1996). Pharmacological inhibition of the PKNs is achievable since the ROCK inhibitors HA1077 and Y27636 have been described to inhibit PKN2 with IC$_{50}$ values of 4μM and 600nM respectively (Davies *et al.*, 2000), however these have not been exploited to correlate with potential PKN functions.

Perhaps the most striking structural difference between PKN1 and other PKNs is the proline rich region between the C2 and the catalytic domains of PKN2 and PKN3. PKN2 and PKN3 contain 1 and 2 proline rich sequences respectively which are thought to target SH3 domains. Indeed, PKN2 has been shown to bind the SH3 domain containing molecules Nck and GRB4 (Braverman and Quilliam, 1999; Quilliam *et al.*, 1996), whilst PKN3 was shown to bind the SH3 domain containing GRAF (Shibata *et al.*, 2001). Since these adaptor proteins can bind directly to receptor tyrosine kinases, these interactions could conceivably couple PKN2 and PKN3 to receptor mediated events thereby conferring selective localisation and stimulus specificity.

PKNs have been implicated in several MAP-kinase signalling events; however a definitive role for PKNs in these pathways has not emerged. PKN2 was shown to bind MEK Kinase 2 (MEKK2) and this interaction was shown to be mediated through the proline rich region of PKN2, not present in PKN1. This interaction was shown to promote PKN2 activation although this effect was independent of MEKK2 activity (Sun *et al.*, 2000a). Recent evidence has also placed PKN1 downstream of Rho, at the MAPK Kinase Kinase level of the p38γ cascade leading to c-Jun activation (Marinissen *et al.*, 2001). Further implicating PKN1 in the p38 pathway, PKN1 was shown to phosphorylate the novel p38 MAPK Kinase Kinase, MLTK.
The loss of PKN function in *Drosophila* highlighted a defect in dorsal closure. This phenotype resembles the loss of function of the GTPase Rho1 and the Rac mediated JNK cascade. The study however, indicated that PKN1 was not operating within the JNK pathway but a parallel system that is also important for dorsal closure (Lu and Settleman, 1999).

The small GTPase, RhoB has been shown to target PKN1 to an endosomal compartment where it is implicated in controlling the kinetics of epidermal growth factor (EGF) receptor traffic (Mellor et al., 1998) (Gampel et al., 1999). PKN1 was also found by electron microscopy, to be enriched in a variety of membrane compartments including ER-derived vesicles, late endosomes and multi-vesicular bodies (Kawamata et al., 1998), further implicating PKN1 in vesicle transport. Furthermore, PKN1 was shown also to bind and activate Phospholipase D (PLD) in the presence of PtdIns(4,5)P₂ in a manner independent of PKN1 activity (Oishi et al., 2001). PLD has been demonstrated to be activated by EGF stimulation in endosomes (Hughes et al., 2002). These findings raise the possibility that PKN-PLD interactions may be part of vesicular trafficking events. Furthermore, PKN1 may act upstream of PLD in stimulating a variety of events directly and/or indirectly, such as classical and novel PKC activation of PLD mediated via lipid hydrolysis and the production DAG.

Several reports have indicated that PKNs play a role within cytoskeletal structures. This notion is consistent with PKNs being small GTPase effectors which are also well established as cytoskeletal regulating proteins (see section 1.4). PKN1 was shown to stimulate actin reorganisation (Dong et al., 2000) and the kinase inactive form of PKN2 has been shown to induce the disruption of the actin cytoskeleton (Vincent and Settleman, 1997). However, care does need to be taken when interpreting data concerning the overexpression of PKNs since these proteins may
well titrate Rho proteins and highlight Rho functions which are in fact mediated by Rho effectors other than PKNs. Further establishing the link between PKNs and the actin cytoskeleton, PKN1 was however shown to bind the actin crosslinking protein α-actinin (Mukai et al., 1997). Another potentially interesting PKN interaction concerns PKN2 which was shown to bind the phosphatase PTP-BL which has been implicated in cytoskeletal rearrangement. PKN2 and PTP-BL are found to co-localise in lamellipodia-like structures (Gross et al., 2001).

In highlighting a functional role for PKNs, PKN2 has been placed on a pathway controlling cell adhesion. A Rho mutant that fails to bind PKN2 was unable to recruit E cadherin and promote keratinocyte differentiation. Furthermore PKN2 was shown to enhance keratinocyte cell-cell adhesion and induce phosphorylation of the tyrosine kinase Fyn and also β and γ catenin. This work implicates PKN2 as a direct mediator between Rho and Fyn signalling (Calautti et al., 2002).

PKNs have also been implicated in signalling to the nucleus; PKN activation, known substrates and transcriptional responses are summarised in Figure 1.8. PKN1 was described to translocate to the nucleus in response to heat shock (Mukai et al., 1996). Furthermore, PKN1 was shown to drive c-fos expression from the serum responsive element (SRE) and also reporter expression from the ANF promoter which contains an SRE like sequence. This would be consistent with PKNs driving transcriptional responses downstream of Rho activation (Morissette et al., 2000). Additionally PKN1 was shown to drive stimulation of ATF2 and MEF2A which acts on the c-jun promoter by signalling through ERK6 and P38γ (Marinissen et al., 2001). PKN1 has also been shown to bind the neuron specific Helix-Loop-Helix (bHLH) transcription factor, and to enhance the transactivation of NDRF/NeuroD2 (Shibata et al., 1999). Recently PKN1 was shown to induce transcriptional activation of the androgen receptor (Metzger et al., 2003). Taken together, these studies suggest that PKNs may have multiple roles in gene transcription events.
Fig 1.8 PKN Signalling.

A summary of PKN signalling including a model for PKN activation, potential downstream substrates and transcriptional responses.
Current literature presents a model for the activation of PKNs. Other studies have identified several PKN binding partners and associated transcriptional responses, however, a definitive cellular role for the PKNs remains elusive. This work seeks to increase our understanding of PKN function by monitoring PKB as a candidate for Rho-PKN signals, and also by screening for potential PKN agonists.
Chapter 2
Materials and Methods.

2.1 Materials

All reagents were obtained from Sigma-Aldrich unless otherwise indicated. Cy3 anti mouse antibody were from Jackson Immunoresearch laboratories. AcrylaGel and Bis-AcrylaGel was from National Diagnostics. Ethanol, Methanol, TritonX-100 and Tween-20 were from BDH. Glutathione-Separose, ultra pure dNTPs, radioisotopes, protein standard markers, hyperfilm and ECL western blotting detection reagent were from Amersham-Pharmacia. PVDF membrane was from Millipore. Lipofectamine 2000 reagent, Optimum media, and agarose were from Gibco-BRL. Protein A, Protein G and restriction enzymes were from New England Biolabs. Phosphatidyl serine lipids were from Lipid Products. The Rac1 activation assay was purchased as a kit from Totam Biologicals. Actigel resin was from Sterogene.

2.1.1 Cell Types

NIH3T3 cells were from Cancer Research UK cell services, WT and PKN1-KO primary mouse embryonic fibroblast (MEF) cells were generated by Dr Adele Cassamassima.

2.1.2 Buffers

Luria-Bertani (LB), PBS, Trypsin-versine, EDTA, Dulbecco’s MEM (DMEM) were from CR-UK research services.
<table>
<thead>
<tr>
<th>Buffer Type</th>
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### 2.1.5 Primary antibodies

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<td>W, IF</td>
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<td>Transduction Laboratories</td>
<td>Mouse monoclonal</td>
<td>W</td>
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<tr>
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</table>

(W= Western, IF= Immunoflorescence, IP = Immunoprecipitation, P- = Phospho-specific antibody)

2.1.4 Secondary antibodies

<table>
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<td>Sheep-HRP</td>
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<tr>
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2.1.5 Pharmacological Agents

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2.1.6 DNA constructs

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<td>pGEX (Amersham)</td>
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<td>Dr Harry Mellor (Flynn et al., 1998)</td>
<td>pCDNA3 (Invitrogen)</td>
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<td>Myc-PKN1myc</td>
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<td>pCDNA3</td>
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<td>Myc-PKN2</td>
<td>Dr Harry Mellor (Flynn et al., 1998)</td>
<td>pCDNA3</td>
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<td>GST-PKB</td>
<td>Dr Brian Hemmings</td>
<td>pBC (EMBL X78316)</td>
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<td>Myc-Rac1 wt</td>
<td>Dr Alan Hall</td>
<td>pCDNA3</td>
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<tr>
<td>HA-HR1abc</td>
<td>Dr Harry Mellor (Mellor et al., 1998)</td>
<td>pCDNA3</td>
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<tr>
<td>GFP-PKN1</td>
<td>See section 2.2.1.6</td>
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</tr>
<tr>
<td>GFP-PKN1-KR</td>
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<td>pEGFP-C1</td>
</tr>
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</table>
2.2 Methods

2.2.1 Molecular biology

Standard molecular biology procedures such as agarose gel electrophoresis were as described (Maniatis et al., 1989). Restriction digests were carried out according to the manufacturer’s instructions.

2.2.1.1 PCR Reactions

Polymerase chain reactions incorporated: 1 unit of Pfu Turbo in combination with the recommended buffer, 200µM of dNTPs (from a 10mM stock of ultrapure dNTPs with respect to each deoxynucleotide triphosphate), 1µM of each sense and antisense primer, and 50ng of double stranded DNA template to a final volume of 50µl. The program used for PKN1 cloning into pEGFP and pDS-Red vectors (as described below) is as follows; 94°C for 5min followed by 30 cycles of 94°C denaturation step for 45 seconds, a 65°C annealing step for 45 seconds, and an extension step of 68°C for 6.5min. PCR products were separated by agarose gel electrophoresis, the desired bands were excised and purified using the Qiagen QIAquick gel extraction kit.

2.2.1.2 Ligation reactions

Inserts and vectors were digested and separated using agarose gel electrophoresis. Bands were excised and purified using the QIAquick gel extraction kit from Qiagen. Ligation reactions typically incorporated 0.5µl of T4 DNA ligase with the appropriate buffer and a ratio of 3:1 insert to vector to a volume of 10µl.
2.2.1.3 Transformation of *E. Coli*

Aliquots of *E. coli* DH5α competent cells (50μl) were incubated with 500ng of plasmid DNA (4μl of ligation reaction) for 20min on ice. Cells were then heat shocked at 42°C for 45 seconds and then replaced on ice for 2min. LB media (1ml) was added and cells incubated at 37°C for 1hour. The bacterial inoculate (50μl) was spread onto an LB plate containing the appropriate selection antibiotic; 100μgml⁻¹ ampicilin, and 50μgml⁻¹ kanamycin and incubated overnight at 37°C.

2.2.1.4 Plasmid DNA preparation.

A colony from transformed DH5α cells was picked and grown in a 5ml culture of LB containing the relevant selection antibiotic. From this, small scale DNA purifications utilised the Qiagen Mini-prep kit. Large scale DNA purifications added the 5ml overnight culture as described above to 200ml of selecting LB and after a further overnight culture prepared DNA using the Qiagen Plasmid Maxi-prep kit. A ratio OD_{260}/OD_{280} of 1.8 from maxi prep prepared DNA was considered an acceptable DNA quality.

2.2.1.5 DNA sequencing

DNA sequencing was carried out using the ABI prism Dye Terminator kit.

2.2.1.6 Construction of GFP-PKN1, GFP-PKN2, DS-Red-PKN1 and DS-Red-PKN2.

GFP-PKN1 and GFP-PKN2 constructs were generated by polymerase chain reaction using as templates PKN1 and PKN2 constructs respectively. PKN1 was amplified using the primer (1) 5'-
GCAGAAGCTTGCATGGCCAGCGACGCCGTGCAGAGTGAGCC-3' and (2) 5'-GCAGCGGTACGTTAGCAGCCGGCGGCCACGAAGTCGAAGTCCAGGAAGGC-3' and for PKN2 (3) 5' -GCGCAAGCTTGCATGGCCAGCGACGCCGTGCAGAGTGAGCC-3' and (4) 5' -
GCAGCGGTACGTTAGCAGCCGGCGGCCACGAAGTCGAAGTCCAGGAAGGC-3'. Primers 1 and 2 incorporated a HindIII restriction site, primers 3 and 4 contained a Kpnl site. The resulting products contained the full length PKN sequences and were cloned into PCR blunt (Invitrogen). This was subsequently digested with HindIII and Kpnl and cloned into pEGFP-C1 (Clontech) enabling the fusion of an N-terminal GFP tag in frame with the full length PKN1 and PKN2. The DS-Red PKN1 was constructed in the same manner as GFP-PKN1, using the pDS-Red-C1 vector from Clontech.

### 2.2.2 Cell Culture and transfection

NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium and supplemented with 10% foetal calf serum (10%E4). Transient transfections were performed using Lipofectamine 2000 (Invitrogen). The lipid DNA mix was incubated for 6 h according to the manufacturer's protocol and all subsequent manipulations (see text and figure legends) were performed 48 h post transfection.

#### 2.2.2.1 Hyperosmotic Stress

Cells were subjected to hyperosmotic conditions for 30 minutes by the addition of Dulbecco's modified Eagle's medium containing 0.4M sucrose and 25mM Hepes pH 7.2. Where indicated, sorbitol was substituted for sucrose at the same molarity. For recovery experiments, cells were shocked for 30 minutes and then medium was replaced with normosmotic medium. The average number of vesicles after 30min of hyperosmotic stress and subsequent 15 and 30 minutes of recovery were determined. From each condition, 10 GFP-PKN1 vesicle containing cells were
sampled. Images were taken as confocal sections through the centre of the cell and the number of vesicles from each image counted.

### 2.2.3 Microscopy

Cells were seeded on acid washed glass coverslips, transfection and stimulation was as indicated in figure legends. Cells were then washed and fixed in 4% paraformaldehyde for 15min. When processing for immunofluorescence, cells were permeablized with 0.2% (v/v) Triton X-100 for 5 min, washed and incubated in 1% (w/v) bovine serum albumin (BSA) for 20min. Coverslips were incubated with primary antibody containing 1% (w/v) BSA for 1 h. Cells were then washed and incubated with fluorescent dye-conjugated secondary antibody for 1 h, washed three times, the final wash in water. All washes and incubations were performed in Phosphate Buffered Saline (PBS) unless otherwise stated. Cells were mounted on glass slides under MOWIOL [100mM TrisHCl pH 8.8, 10% (w/v) MOWIOL 4-88 (Calbiochem) and 25% (v/v) glycerol] containing antiphotobleaching agent [2.5% (w/v) 1,4-diazabicyclo[2.2.2]octane (Sigma)]. Slides were examined using a confocal laser scanning microscope (Axioplan2 with LSM 510; Carl Zeiss Inc.) equipped with 63x/1.4 Plan-APOCHROMAT oil immersion objectives. GFP and Cy3 were excited with 488 and 543 nm lines of Kr-Ar lasers respectively and individual channels scanned in series to prevent bleed through. Each confocal image is representative of a given field of transfected cells. Images are representative of at least three separate experiments unless otherwise indicated. Each image represents a single 1.0μm 'Z' optical section.

### 2.2.4 Polyacrylamide gel electrophoresis (PAGE)

Sodium dodecyl sulphate (SDS) PAGE was performed according to (Laemmli, 1970) using Hoeffer gel apparatus. Routinely, 10% acrylamide running gels and 3% acrylamide stacking gels were used with the exception of Myelin Basic Protein
resolution where a 15% acrylamide running gel was used. Protein samples were typically prepared in a 4X Laemmli sample buffer (see buffers) unless otherwise stated and heated to 95° for 10min. Molecular weight standards were run in parallel for all samples to determine apparent molecular weight. Proteins were visualised by either coomassie blue staining, western blotting followed by subsequent immunoblotting or where indicated gels were dried and autoradiographed.

2.2.5 Western blotting

Proteins were transferred electrophoretically onto polyvinylidene difluoride membrane (PVDF). Membranes were pre-incubated with TBS containing 3%BSA for one hour followed by overnight incubation with TBS containing 3%BSA and primary antibody typically 1/2000 dilution unless otherwise indicated. Membranes were then washed 3X15 min with TBS followed by incubation with the relevant secondary antibody (see secondary antibodies) in TBS containing 1%BSA to a dilution of 1/7000. Membranes were then washed 3X15min with TBS and visualised with enhanced chemiluminescence ECL according to the manufacturer’s guidelines, using hyperfilm.

2.2.6 C3-GST Protein Purification and cell loading

2.2.6.1 Preparation of FY810-P.lys electro-competent cells.

A 10ml culture of LB, inoculated with FY810 p.lys cells containing 34μg/ml of chloramphenicol was incubated overnight at 37°C. Cells were pelleted and the supernatant discarded. Cells were then added to a 500ml LB culture with 34μg/ml of chloramphenicol and incubated at 37°C until an OD₆₀₀ of 0.4 was reached. The culture was then incubated at room temperature until an OD₆₆₀ of 0.6 was achieved. The culture was cooled on ice for 30min and subsequently pelleted (20min 4200 rpm in a JA20 rotor at 2°C). Cells were then washed in 500ml ice cold
H₂O and pelleted (20min 4200 rpm in a JA20 rotor at 2°C) twice. Cells were resuspended in 80ml 10% glycerol (in ice cold H₂O) and pelleted. Cells were then resuspended in 1.5ml 10% glycerol, 50µl aliquots dispensed on dry ice and stored at -70°C.

2.2.6.2 Transformation of FY810-P.lys electro-competent cells.

Cells were added to electroporation cuvettes on ice for 10min, 1-2µg of DNA was added and the mixture was subjected to electroporation (2.5KV, 25µF with pulse of 200 ohms). Cells were incubated at 37°C in LB, pelleted and spread onto an LB plate containing the appropriate selection antibiotic; 100µg/ml ampicilin, and 50µg/ml kanamycin with 34µg/ml of chloramphenicol and incubated overnight at 37°C.

2.2.6.3 Purification of C3-GST

FY810 plys cells were transformed with the C3-GST plasmid, and used to establish a 5ml overnight culture containing ampicilllin (100µg/ml) and chloroamphenicol (34µg/ml). This was used to inoculate 500ml of LB with the same antibiotic concentration and grown to an OD₆₀₀ of 0.6 while shaking at 37°C for approximately 3h. Protein synthesis was induced by the addition of 500µl 1M IPTG and followed by further shaking for 2.5h. Cells were harvested by centrifugation at 6000 rpm in a Beckman JA-20 rotor for 20min. The cell pellet was frozen in liquid nitrogen and then resuspended in 40ml of ice-cold extraction buffer (50mM Tris; pH7.5, 105mM NaCl, 0.1mM PMSF, 1mM EDTA, 1mM DTT). The cells were then incubated on ice for 30min followed by the addition of 20% Triton X-100 to a final concentration of 1% and then sonicated for 20 seconds at 18 microns (using the MSE probe sonicator 150). The samples then underwent centrifugation at 12000 rpm for 30min in a Beckman JA-20 rotor. 250µl of Glutathione-Sepharose beads
equilibrated in extraction buffer were added to the supernatant and then incubated with agitation in the cold room for 30 min. The beads were pelleted by centrifugation, and after 2X washes in wash buffer (50mM Tris; pH7.5, 105mM NaCl, 1mM EDTA, 1mM DTT), C3-GST was eluted from the beads by a final wash in buffer containing 10mM glutathione pH8.0. C3-GST protein samples were then aliquoted and stored at -70°C.

2.2.6.4 Cell loading with C3-GST

NIH3T3 cells were incubated with C3-GST at a concentration of 5μg/ml for 6 hours. The effectiveness of cell loading was measured by phalloidin staining and \textit{in vitro} ribosylation assay (see below).

2.2.6.5 Ribosylation assay

\textit{In vitro} ADP-Ribosylation of Rho by C3-GST was essentially as described in (Aktories and Just, 1995). NIH3T3 cells were seeded (500,000 cells per 6cm dish), grown in 10% FCS E4 and subsequently starved for 16 hours. Cells were then treated with 5μg/ml of C3-GST or GST protein as indicated for 6h, harvested in 400μl lysis buffer (20mM Tris HCl pH7.5, 0.25M Sucrose, 5mM MgCl₂, 1mM EDTA, 1mM DTT, 0.5mM PMSF, 5mM Leupeptin, 1mM Benzamidine) and sonicated for 20 seconds at 18 microns (MSE probe sonicator 150). A volume of 150μl of lysate was added to 50μl of reaction buffer (400mM TrisHCl pH8, 40mM Thymidine, 40mM Nicotinamide, 4mM DTT, 20mM MgCl₂, 200μM NAD, 10μM \[^{32}\text{P}]\text{NAD}\) and incubated at 30°C for 30 min. Reactions were terminated by the addition of 100μl SDS sample buffer. Samples were then processed and loaded onto a 12.5% PAGE gel. Gels were dried and exposed to the Storm (Molecular Dynamics) phospho-imager.
2.2.7 Rac1 activation

NIH 3T3 cells (500,000 cells per 6cm dish) were seeded and grown in 10%FCS E4 and subsequently starved for 16 hours with 0.05%FCS E4. Cells were then subjected to treatments of hyperosmotic stress as indicated in figure legends. The Rac activation assay was performed on cell extracts using reagents contained in the kit from Totam biologicals. This assay employs the p21 binding domain (PBD or CRIB domain) of p21 activated kinase 1 (PAK) as a GST fusion protein which selectively binds GTP-Rac1 in a “pull down” assay enabling the detection of activated Rac1. All samples were prepared and processed according to the manufacturer’s guidelines. Quantitation was obtained by image capture and densitometry using NIH™ image software.

2.2.8 Phosphorylation of GFP-PKN1

Cells were transiently transfected on 6cm² plates with GFP-PKN1 and subjected to hyperosmotic stress as indicated. Cells were harvested in 400μl sample buffer and fractionated on a 10% SDS polyacrylamide gel. Activation loopThr-774 phosphorylation of PKN1 was assessed by western blotting using a phospho-specific polyclonal antibody with excess of dephospho-peptide as described (Flynn et al., 2000). Relative PKN1 phosphorylation was determined as a function of PKN1 protein levels defined using the PKN1 monoclonal antibody. GFP-PKN1 phosphorylation is taken from three duplicate experiments, where represented graphically, error bars denote the standard error of the mean.

2.2.9 GFP-PKN1 kinase assay

For each condition a 15cm² plate of cells was transfected with GFP-PKN1 and stimulated with hyperosmotic medium as indicated in the figure legend. Cells were harvested in a lysis buffer comprising: 20mM TrisHCl pH7.5, 0.5% (w/v) CHAPS, 1
complete tablet (protease inhibitor from Roche) per 50ml lysis buffer, 2mM EDTA, 150mM NaCl. Preclearance of cell lysates with pre-equilibrated protein G was followed by incubation with 9μg of GFP polyclonal antibody for 20 min at 4°C. Immunocomplexes were captured by incubation for 1h with protein G. Immunoprecipitates were washed three times with lysis buffer, once with 0.5M NaCl and a final wash in reaction buffer, 20mM Hepes pH 7.5, 10mM MgCl₂. For each reaction, 10μl of immunopurified GFP-PKN1 was incubated in a 40μl reaction mix of: 20mM TrisHCl pH 7.5, 10mM MgCl₂, 25μg bath sonicated phosphatidyl-L-serine, 10μg myelin basic protein, 5μM ATP and 1μCi [γ³²P] ATP for 20 minutes at 30°C with agitation. Reactions were terminated by the addition of 40μl of sample buffer prior to fractionation on a 15% SDS-polyacrylamide gel, subsequently western blotting analysis was performed. To assess MBP phosphorylation, membranes (Immobilon-P from Millipore) were exposed to phospho-imager (STORM, Molecular Dynamics) prior to immunoblotting with anti PKN1. Relative specific activity was determined as a function of immunoreactive GFP-PKN1. The data presented is representative of three duplicate experiments and where represented graphically, error bars denote the standard error of the mean. Additional duplicate reactions incorporated 20μM HA1077 as a control for specific PKN activity (Amano et al., 1999) (Davies et al., 2000).

2.2.10 Cell Fractionation

Samples were subjected to velocity and equilibrium sucrose gradient centrifugation essentially as previously described (Tooze and Huttner, 1992). NIH3T3 cells were seeded onto 15cm² plates and stimulated as indicated. Cells were washed with cold PBS and harvested in 1ml of homogenisation buffer (0.25M Sucrose, 10mM Hepes-KOH pH7.2, 1mM EDTA pH7.5, 1mM MgAc and 1 complete tablet per 50ml). Samples were homogenised using a cell cracker (EMBL) and spun at 3000 rpm for 7min using a bench-top centrifuge. The supernatant or (post-nuclear supernatant, PNS) was loaded onto a velocity gradient ranging from 0.3M to 1.2M
sucrose. This was spun using a Beckman JA20 rotor at 25000rpm for 18min. In all conditions, the top 3, 1ml fractions were then loaded onto an equilibrium gradient composed of; 1ml 1.2M, 2ml 1M, 2ml 0.8M and 1ml 0.4M sucrose. This was spun overnight at 250000rpm, 1ml fractions were subsequently collected, 100μl of which was run on 10% SDS-PAGE gel and subjected to western analysis and immuno blotting as described earlier.

2.2.11 PKN3 Antibody generation

The PKN3 specific peptide “QQAAFRDFDFVSERFLEP” was used to generate rabbit polyclonal antibodies.

2.2.11.1 Peptide coupling

Keyhole limpet haemocyanin (KLH) (0.6 ml of 21mg/ml) was dialysed overnight against PBS. KLH (10mg) was added to 10mg of peptide dissolved in 440μl of PBS. Glutaraldehyde (10μl) was added and incubated for 15min incubation at room temperature, a further 5μl of glutaraldehyde was added followed by another 15min incubation. Glycine (200μl of 1M pH6.0) was added to quench glutaraldehyde and this was diluted to a volume of 19.2ml with PBS from which 6 X 3.2ml aliquots were sent for rabbit immunisation.

2.2.11.2 Affinity purification

Actigel resin (5ml) was washed X3 with water by centrifugation at 3000rpm for 5min. Coupling buffer (5ml) containing 0.5ml coupling solution (actigel), 0.5ml of 1M PBS pH 6 (a pH away from the isoelectric point, Pi, of the peptide) and 5mg of peptide. This mixture was agitated for 4h followed by a 0.5M NaCl and buffer wash.
Serum (5ml) was mixed with PBS/0.02% (v/v) Tween 20 (5ml) and pre-cleared by centrifugation at 3000rpm for 5min. Actigel resin (2ml) (see above) was added to each column used, pre-cleared serum was passed through the column X3 times. The column was washed with 20ml PBS/0.02% (v/v) Tween 20, 40ml 0.5M NaCl in PBS/0.02% (v/v) Tween 20 and finally a further PBS/0.02% (v/v) Tween 20. Antibodies were eluted with 0.1M citrate pH2.5/ 0.02% Tween. Fractions of 600μl were collected, neutralised and tested by western for immuno-reactivity.
Chapter 3
PKN and the PKB pathway.

3.1 Introduction

As discussed in the introduction, regulation of PKN1 and PKN2 is distinct from other PKC superfamily members; they are both calcium and diacylglycerol independent. The PKN regulatory region contains a novel N-terminal domain termed homology region 1 (or HR1) which is comprised of three homologous sequences (HR1a, HR1b and HR1c) and has been shown to interact with the small GTPases Rho and Rac1 (Flynn et al., 1998; Vincent and Settleman, 1997). Other G-proteins may play a role in PKN regulation, indeed a novel GTPase, AWP1 has recently been shown to interact with PKN1 (Duan et al., 2000). The interaction of RhoA with PKN1 operates through binding to the HR1a and HR1b sequences of the HR1 domain. Binding to the HR1a region is GTP dependent while the interaction with HR1b was shown to bind both GDP and GTP forms of RhoA (Flynn et al., 1998). This interaction has also been shown to facilitate the activation loop phosphorylation of PKN1 by 3-Phosphoinositide Dependent Kinase 1 (PDK1) (Flynn et al., 2000).

Another substrate for PDK1 is Protein kinase B (PKB). PKB has two specific phosphorylation sites that are required for full activation, these sites are on residues threonine 308 and serine 473 (Alessi et al., 1996). The T308 site has been shown to be directly phosphorylated by PDK1 (Alessi et al., 1997a; Alessi et al., 1997b; Stokoe et al., 1997). The S473 site is however more controversial in its regulation, it has been suggested that an as yet unidentified kinase, termed “PDK2”, is responsible for PKB S473 phosphorylation since PDK1 was found not to phosphorylate this site directly (Alessi et al., 1997b). Phosphorylation of the serine...
473 has also been suggested to be an autophosphorylation site resulting from threonine 308 phosphorylation by PDK1 (Toker and Newton, 2000b).

Further work on the relationship between PKN2 and PDK1 has implicated PKB as a downstream target for PKN2. A C-terminal PKN2 fragment was shown to convert PDK1 to a form that will phosphorylate PKB on the serine residue at amino acid 473 (S473) \textit{in vitro} functioning as the elusive PDK2 activity (Balendran \textit{et al.}, 1999). This chapter seeks to test the hypothesis that Rho-PKN signals can influence PKB S473 phosphorylation thereby acting as a complex with PDK2 activity.
3.2 Lack of a Rho/PKN input to PKB serine 473 phosphorylation

To investigate the putative interaction of PKN2 and PKB, the Rho sensitivity of PKB was tested by employing the Rho inhibitor C3 transferase which is known to specifically inhibit Rho over other GTPases such as Rac1 (Wilde et al., 2000). GST-C3 and GST (control) protein were purified from GST beads (Figure 3.1) and NIH3T3 cells were loaded with GST-C3 protein. The modification of Rho activity was measured in vitro using a post extraction ribosylation assay (Aktories and Just, 1995). Following 6 hours of pretreatment with GST-C3, a reduction in in vitro ribosylated Rho of approximately 80% could be detected, the result of two duplicate experiments (Figure 3.2 A). Since Rho controls actin dynamics, the effectiveness of cell loading GST-C3 in vivo was also measured by phalloidin staining. Following 6 hours of treatment with GST-C3, a loss of stress fibre formation was observed, consistent with the ribosylation and loss of function of Rho (Figure 3.2 B).

Having established that Rho can be inactivated by cell loading with GST-C3, the pattern of PKB 473 phosphorylation was observed in NIH-3T3 cells after either 6 hours of treatment with GST or GST-C3. This was examined under conditions of serum starvation followed by stimulation with various agonists including; total serum, FGF, LPA, PDGF and EGF (Figure 3.3). No difference in the pattern of PKB 473 stimulation with respect to these agonists was detected between GST and GST-C3 treatments indicating no Rho-PKN inputs into the regulation of the PKB 473 site.
Fig 3.1 Purification of C3-GST and GST protein.

Fy810-Plys bacteria were transformed with C3-GST and GST plasmids. C3-GST and GST proteins were purified from Glutathione Sepharose beads as described in chapter 2. Total lysate, beads and eluted protein were run on SDS-PAGE and proteins visualised by coomassie staining.
Fig 3.2 6hours of pretreatment with C3-GST in NIH3T3 cells inhibits Rho.

A. NIH3T3 cells were seeded onto 6 cm² plates and subjected to *in vitro* ribosylation assay; (1) excluded C3-GST *in vitro*, (2-5) incorporated C3-GST in the reaction. Cells in (3) were harvested after 6h of 25μg GST pretreatment, (4) had 6h of 2μg C3-GST pretreatment and (5) had 6h of 25μg C3-GST pretreatment prior to harvesting and ribosylation assay.

B. NIH3T3 cells were treated for 6h with GST or for 6h with C3-GST prior to fixation (as described in chapter 2). Visualisation of the actin cytoskeleton was achieved by staining with Alexa-488 phalloidin. Images are representative of single 1.0μm 'Z' optical sections and the scale bar is equivalent to 10μm.
Fig 3.3 Serum and growth factor stimulation of PKB S473 phosphorylation is unaffected by Rho inhibition.

NIH3T3 cells were seeded onto 6 cm² plates, subjected to 16 hours of serum starvation and 6 hours incubation with either 5μg/ml C3-GST or 5μg/ml GST. Cells were stimulated with: 10% FCS, 10ng/ml FGF, 500ng/ml LPA, 20ng/ml PDGF or 100ng/ml EGF as indicated prior to harvesting in Laemmli sample buffer. Samples were run on SDS-PAGE and subjected to western analysis.
3.3 The HR1abc domain of PKN1 can inhibit PKB S473 phosphorylation; rescue by Rac1 but not Rho

Although the above experiments appear to exclude Rho dependent effects of PKN on PKB 473 phosphorylation, the possibility remains that other inputs (i.e. C3 insensitive) to the HR1abc domain may influence a PKN driven PKB response. To test this issue, we used the overexpression of this domain enabling the titration of HR1abc binding partners and monitored PKB phosphorylation. The co-expression of GST-PKB and HA tagged HR1abc shows an inhibition of PKB S473 response to serum stimulation (Figure 3.4). The loss of PKB S473 phosphorylation is also detected by co-expression of full length PKN1 with GST-PKB (Figure 3.5). HR1abc induced inhibition of PKB 473 phosphorylation can be recovered upon co-expression with the constitutively active RacV12, but not active RhoB QL (Figure 3.5). This observation is consistent with GST-C3 experiments showing a lack of Rho involvement in PKB 473 phosphorylation. The upregulation of HR1abc depleted GST-PKB phosphorylation by the co-expression of RacV12 raises the question of whether Rac1-PKN inputs can influence PKB 473 phosphorylation.
Fig 3.4 Overexpression of PKN1 HR1 domain inhibits GST-PKB S473 phosphorylation.

NIH3T3 cells were seeded onto 6 cm² plates, transfected with either GST-PKB or GST-PKB and HA-HR1 as indicated and subjected to 16 hours of serum starvation. Cells were stimulated with 10% FCS for the time-points indicated prior to harvesting in Laemmli sample buffer. Samples were run on SDS-PAGE and subjected to western analysis.
Fig 3.5 Rac1 can recover HR1 induced inhibition of GST-PKB S473 phosphorylation.

NIH3T3 cells were seeded onto 6 cm² plates, transfected with either GST-PKB, HA-HR1, RhoBQL, Rac1V12 and PKN1 as indicated and subjected to 16 hours of serum starvation. Cells were stimulated for 5 min with 10% FCS as indicated prior to harvesting in Laemmli sample buffer. Samples were run on SDS-PAGE and subjected to western analysis. Band intensities were analysed using NIH image™.
3.4 Co-expression of Rac1V12 and PKN2 induces expression from the pBC plasmid

To investigate the possible PKN involvement in Rac1 mediated PKB S473 phosphorylation, co-expression studies were performed involving GST-PKB, RacV12, HA-HR1abc, PKN1 and PKN2. Increasing plasmid concentrations of transfected HA-HR1abc or PKN1 combined with Racv12 and GST-PKB, resulted in a decrease in GST-PKB 473 phosphorylation and protein level. This could perhaps be an effect of increasing plasmid concentration. However, with increasing amounts of PKN2 plasmid in combination with RacV12 and GST-PKB, an increase in phosphorylation paralleled by an increase in overexpressed GST-PKB protein level is detected (Figure 3.6 A). Further investigation shows that co-expression of RacV12 and PKN2 induces increased expression of GST protein from the pBC plasmid as used for GST-PKB (Figure 3.6 B); this expression vector is driven by an SV40 promoter.
Fig 3.6 Co-expression of PKN2 and Rac1V12 stabilises expression from the GST expression plasmid.

A. NIH3T3 cells were seeded onto 6 cm² plates and transfected with either GST-PKB, Rac1-V12, and varying amounts of HA-HR1, PKN1 and PKN2 plasmids as indicated.

B. NIH3T3 cells were seeded onto 6 cm² plates and transfected with GST-PKB, PCDNA3, Rac1-V12 and PKN2 plasmids as indicated.

Samples were harvested in Laemmli sample buffer, run on SDS-PAGE and subjected to western analysis.
3.5 Discussion

This chapter has adopted a candidate approach to investigate PKN targets and has focused on the contribution of Rho-PKN signals to PKB phosphorylation. It is demonstrated that pre-incubation of C3-GST for 6 hours is effective in the intervention of Rho function, however no difference in serum-induced PKB phosphorylation between GST and C3-GST treated cells was observed. The possibility remains that Rho-PKN dependent inputs into PKB could be driven under specific circumstances that otherwise may be obscured by a multitude of inputs controlling PKB 473 phosphorylation under serum stimulation. It was therefore pertinent to investigate the C3-GST sensitivity of specific growth factor stimulation time courses on PKB 473 phosphorylation. Given that such a wide range of stimulation conditions trigger the same pattern of PKB S473 phosphorylation regardless of Rho inhibition, we conclude that there are unlikely to be requirements for Rho-PKN inputs into this phosphorylation site.

It is interesting to note that the overexpression of HR1abc has the ability to deplete PKB 473 phosphorylation. Since Rho dependency on this phosphorylation site has been excluded, attention is drawn to the other HR1 binding GTPase, Rac1. Indeed, Rac1 has been reported to stimulate PKB 473 phosphorylation by PI3 kinase activation in T cells (Genot et al., 2000). It is not possible to attribute the inhibition of PKB 473 phosphorylation by HR1 directly to the influence of PKN as the titration of HR1 binding partners, including Rac1 could compromise Rac1 mediated pathways that are unrelated to PKN. Resolving the question of PKN2 involvement in Rac1 mediated PKB phosphorylation has proved problematic given the effects on expression with the co-transfection of Rac1V12 and PKN2. These effects could be a result of increased transcriptional activity from the SV40 promoter within the pBC vector, driven by the co-operation of Rac1 and PKN2. If this is the case, the
effect of Rac1 and PKN2 on transcriptional activity may prove to be a useful readout for PKN2 activity and future experiments could seek to address this question. These studies could employ a luciferase reporter assay to investigate further the induction of gene expression.

Overall, data presented involving the monitoring of PKB 473 phosphorylation as a potential downstream target for PKN2 indicate no Rho-PKN inputs to this phosphorylation site. Overexpression of HR1abc is shown to inhibit PKB 473 phosphorylation and this was overcome by co-expression with Rac1 but not RhoA or RhoB. The precise role of PKNs in relation to PKB phosphorylation remains unclear, indeed recent co-expression studies along with data presented in chapter 6 suggest that PKNs may even play negative roles in PDK1 mediated PKB phosphorylation (Wick et al., 2000). While Rac1 may influence PKB 473 phosphorylation, and mediation by PKN is a possibility, a direct role for PKNs in PKB 473 phosphorylation has not been established rather it is all but excluded. The evidence that PKN1 does not act on the pathway to PKB indicated that other pathway(s) were likely to lie downstream. The next chapter seeks to adopt an alternative approach in establishing PKN functional roles by screening for potential PKN1 agonists.
Chapter 4

PKN1 is a stress responsive Rac1 effector.

4.1 Introduction

Evidence is accumulating that in order to elicit regulated responses to specific stimuli, signal transduction mechanisms are governed not only by their ability to perform specific functions. The context in terms of complex assembly and cellular location is of increasing interest.

To date, limited information is available regarding the localised control and functions of PKN1. It has been shown that PKN1 can be targeted by RhoB to endosomes and is thought to regulate epidermal growth factor receptor traffic (Gampel et al., 1999). Further studies have also provided evidence for the formation of a ternary complex (Rho-PKN1-PDK1) which is shown by the recruitment of PDK1 in the presence of PKN1 to RhoB containing endosomes (Flynn et al., 2000). PKN1 has also been linked to stress responses, the translocation of PKN1 to the nucleus from the cytoplasm upon heat stress has been shown implying a role for PKN1 in the transduction of signals resulting in transcriptional responses (Mukai et al., 1996).

The role of PKN1 with regard to the cytoskeleton is an interesting question given its control by Rho GTPases. It has been proposed that PKN1 is associated with the actin cytoskeleton, through binding to alpha-actinin (Mukai et al., 1997). A role for PKN1 in the assembly of microtubules through specific phosphorylation of tau, leading to the disruption of tubulin assembly has also been suggested (Taniguchi et al., 2001).
This chapter addresses further the PKN1 localisation question by screening for potential agonists and using GFP-tagged PKN1 to monitor any gross changes in cellular distribution. This approach is taken to understand the context in which PKN1 is functionally active.
4.2 PKN1 localisation is responsive to hyperosmotic stress.

To observe the sub-cellular localisation of PKN1 and PKN2, GFP tagged versions of these proteins were constructed and sequenced. Full length PKN1 and PKN2 constructs were sub-cloned into the pEGFP-C1 vector to give an N-terminal GFP fusion protein as described in the materials and methods section. The expression of full length GFP-PKN1 and GFP-PKN2 is shown (Figure 4.1A). For GFP-PKN1, based on a transfection efficiency of 30%, it is estimated that there is a 10 fold increase in PKN1 protein above endogenous levels.

GFP-PKN1 expressed in NIH3T3 cells displayed a punctate cytoplasmic localisation with an absence of nuclear localisation. The effect of a range of potential PKN1 agonists on the sub-cellular distribution of GFP-PKN1 was investigated including epidermal growth factor, insulin and LPA. No gross alterations in the distribution of PKN1 in response to these growth factor stimulations were detected. Images are shown 30min after growth factor stimulation and are representative from time-courses over a 2 hour period (Figure 4.1B). The effect on GFP-PKN1 localisation of platelet derived growth factor (PDGF) and bombesin was also investigated with no gross changes observed (data not shown). The effect on PKN1 localisation under stress conditions was also investigated (Figure 4.2). Oxidative stress, temperature stress and hypo-osmotic stress conditions did not change the profile of GFP-PKN1 localisation. Notably however, cells treated with hyperosmotic media for 30 min displayed a dramatic change in localisation, with GFP-PKN1 accumulating in large cytoplasmic vesicular structures (Fig 4.2). Since the DS-Red construct is known to be an oligomeric chromophore, DS-Red-PKN1 was co-expressed with GFP-PKN1 to confirm that it shows the same localisation behaviour before and after hyperosmotic stress, indeed this was the case (Figure 4.3). While DS-Red- and GFP-PKN1 are both N-
terminal fusion proteins, a C-terminal myc-PKN1 was localised and upon hyperosmotic stress is shown to
Fig 4.1 PKN1 has a punctate cytoplasmic distribution and is unchanged by agonist stimulation.

A. NIH3T3 cells were transiently transfected with GFP-PKN1 and GFP-PKN2 as indicated, samples were harvested in Laemmli sample buffer, run on SDS-PAGE and analysed by western blotting.

B. NIH3T3 cells were transiently transfected with GFP-PKN1. Cells were either unstimulated (control) or subjected to growth factor stimulation. Images shown are representative of GFP-PKN1 transfected cells after 30 min of; 100 ng/ml EGF, 5 μg/ml LPA and Insulin. The scale bar is equivalent to 10 μm.
Fig 4.2 PKN1 translocates to large vesicles in response to hyperosmotic stress. This translocation response is specific over other stress conditions.

NIH3T3 cells were transiently transfected with GFP-PKN1. Cells were either unstimulated (control), or subjected to various stress conditions including; oxidative stress (H$_2$O$_2$), Oligomycin, hyperosmotic (0.4M sucrose) and temperature stress (12°C or 45°C). Images shown are representative GFP-PKN1 transfected cells after 30min of stress treatment. All images are a representative single 1.0μm ‘Z’ optical section and the scale bar is equivalent to 10μm.
undergo the same translocation response. The translocation response of GFP-PKN1 was shown under conditions of hyperosmotic sorbitol as well as hyperosmotic sucrose (Figure 4.3).

GFP-PKCε has been shown to accumulate in vesicular structures following chronic PKC inhibition in MEF cells (Ivaska et al., 2002). To investigate the selectivity of the PKN1 translocation in response to hyperosmotic stress, DS-Red PKN1 and GFP-PKCε were co-expressed. The ability of DS-Red PKN1 to translocate into vesicles upon hyperosmotic stress was unaffected by the presence of GFP-PKCε, furthermore PKCε did not locate to the DS-Red PKN1 containing structures (Figure 4.4 A), indicating that the observed behaviour of PKN1 in response to hyperosmotic stress is selective. The ability of a second PKN sub-family member to respond to hyperosmotic stress was also followed. GFP-PKN2 when unstimulated was largely cytoplasmic with some limited nuclear accumulation although it was noted to be excluded from the nucleoli (Figure 4.4 B). After hyperosmotic stress, GFP-PKN2 showed the same translocation pattern as GFP-PKN1 (Fig 4.4 B), indicating that the described behaviour is a conserved PKN response.

The dynamic nature of the hyperosmotic-induced structures was investigated by placing cells back into osmotically balanced media following 30min of hyperosmotic stress. Cells containing GFP-PKN1 vesicles were randomly selected and sectional images taken by confocal microscopy. These vesicles were clearly dissipated on removal from hyperosmotic conditions; quantitation showed that the average number of vesicles per cell decreased in a time-dependent fashion after re-addition of osmotically balanced medium. It is evident therefore that the accumulation of vesicular PKN1 is a reversible process (Figure 4.5).
Fig 4.3 GFP-PKN1, DS-Red-PKN1, myc-PKN1 and sorbitol hyperosmotic media display the same hyperosmotic-induced PKN1 translocation response.

NIH3T3 cells were transiently transfected with GFP-PKN1, DS-Red-PKN1 and myc-PKN1 as indicated. Cells were either unstimulated (control) or subjected to a 30min hyperosmotic stress (hyper) prior to fixation. Where indicated, sorbitol hyperosmotic media was used in place of sucrose. All images are a representative single 1.0µm 'Z' optical section and the scale bar is equivalent to 10µm.
Fig 4.4 Hyperosmotic-induced PKN1 translocation is selective over PKC\(_\varepsilon\) but common with PKN2

A. NIH3T3 cells were transiently transfected with DS-Red-PKN1 and GFP-PKC\(_\varepsilon\).

B. NIH3T3 cells were transiently transfected with PKN2.

Cells were either unstimulated (control) or subjected to a 30min hyperosmotic stress (hyper) as indicated prior to fixation. All images are representative of single 1.0\(\mu\)m 'Z' optical sections and the scale bar is equivalent to 10\(\mu\)m.
Fig 4.5 Reversible translocation of GFP-PKN1 in response to hyperosmotic stress.

NIH3T3 cells were transiently transfected with GFP-PKN1. The reversibility of the PKN1 stress response was recorded by monitoring cells after 30 min hyperosmotic stress and also 15 and 30 min of recovery in normal osmotic media post stress treatment. GFP-PKN1 vesicle containing cells were selected randomly for each time point and the average number of vesicles per cell section is indicated graphically. Error bars indicate the standard deviation given by 10 cells from each time-point. Representative cell sections from each time point are shown as an inset. All images are representative of single 1.0μm 'Z' optical sections and the scale bar is equivalent to 10μm.
4.3 Hyperosmotic-induced PKN1 vesicles are not early endosomes or acidified compartments but are associated with actin.

In characterizing the PKN1 response to hyperosmotic stress, the nature of this compartment was investigated. Since PKN1 has been implicated in endosomal traffic control (Mellor et al., 1998), hyperosmotic-induced PKN1 vesicles were co-immunostained with early endosomal antigen 1 (EEA1), a marker for early endosomes. No co-localisation of PKN1 vesicles and EEA1 positive vesicles was observed (Figure 4.6 A). Another possibility is that these compartments could be degradative late endocytic compartments or lysosomes. In order to test this, GFP-PKN1 transfected cells were treated with a marker for acidic compartments, lysotracker™. Hyperosmotic-induced PKN1 vesicles did not co-localise with acidified compartments (Figure 4.6 B).

Small GTPases play an important role in cytoskeletal rearrangements and vesicle trafficking, their association with PKNs is discussed in chapter 1. The relationship between hyperosmotic-induced PKN1 vesicles and the cytoskeleton was therefore investigated. In the first instance the relationship of PKN1 with actin was monitored by immunostaining with FITC-phalloidin. Under control conditions, an association of PKN1 with actin stress fibres was detected (Figure 4.7). It is also shown that hyperosmotic-induced PKN1 vesicles show some association with actin stress fibres as indicated by arrows (Figure 4.7). Other examples of hyperosmotic-induced PKN1 vesicles do not however contain actin stress fibres (Figure 4.7). It is possible that the PKN1 vesicle formation is dependent on actin stress fibres, this was investigated by the disruption of actin polymerisation with cytochalasin D. It is observed that the disruption of actin traps some GFP-PKN1 within actin patches.
Fig 4.6 Hyperosmotic-induced PKN1 vesicles do not co-localise with early endosomes or acidic compartments.

A. NIH3T3 cells were transiently transfected with GFP-PKN1 and stained with anti EEA1.
B. NIH3T3 cells were transiently transfected with GFP-PKN1 and treated with Lysotracker for 30min prior to stimulation. Cells were either unstimulated (control) or subjected to 30min hyperosmotic stress (hyper). The scale bar is equivalent to 10μm.
Fig 4.7 PKN1 is associated with the actin cytoskeleton, hyperosmotic stress induces the down-regulation of stress fibres.

NIH3T3 cells were transiently transfected with GFP-PKN1. Cells were either unstimulated (control) or were subjected to 30min hyperosmotic stress (hyper) as indicated. Two hyperosmotic examples are given so as to highlight the heterogeneity observed. To visualise the actin cytoskeleton alongside GFP-PKN1, cells were stained with FITC-Phalloidin. The scale bar is equivalent to 10μm.
However upon hyperosmotic stress, PKN1 vesicle recruitment is not prevented and PKN1 vesicles appear distinct from actin patches.

The involvement of microtubules in the PKN1 hyperosmotic response was investigated by immunostaining with an anti-tubulin antibody. It is clear that under control conditions PKN1 does not co-localize with microtubules and that under hyperosmotic stress, GFP-PKN1 vesicles do not associate with the disorganised microtubule network (Figure 4.9). These observations are further supported by the disruption of microtubules with nocodazole. Treatment with nocodazole clearly disrupts the microtubule network and this is shown not to affect GFP-PKN1 localisation under control conditions of the ability of GFP-PKN1 to translocate to vesicles under hyperosmolarity (Figure 4.9).
Fig 4.8 Disruption of actin polymerisation traps PKN1 in actin patches. Hyperosmotic-induced PKN1 vesicle accumulation is independent from actin.

NIH3T3 cells were transiently transfected with GFP-PKN1. Cells were treated with 1 hour of 20μm Cytochalasin D or 30min of 20μm Cytochalasin D followed by 30min hyperosmotic stress in the presence of Cytochalasin D, as indicated. To visualise the actin cytoskeleton alongside GFP-PKN1, cells were stained with FITC-Phalloidin. The scale bar is equivalent to 10μm.
Fig. 4.9 Hyperosmotic-induced PKN1 vesicle accumulation is independent from microtubules.

NIH3T3 cells were transiently transfected with GFP-PKN1. Cells were either unstimulated (control) or stimulated with hyperosmotic stress for 30 min (hyper), treated for 1 hour with 20 μm Nocodazole or for 30 min with 20 μm Nocodazole followed by 30 min of hyperosmotic stress in the presence of Nocodazole. After fixation, cells were stained with α-tubulin antibody. The scale bar is equivalent to 10 μm.
4.4 Hyperosmotic-induced PKN1 vesicle recruitment is dependent on Rac1 and not Rho.

PKN has been shown to bind to and become activated by members of the Rho family of small GTPases via the regulatory HR1 domain and indeed has been shown previously to be recruited to an endosomal compartment by RhoB (Mellor et al., 1998). The involvement of Rho proteins in the PKN response to hyperosmotic stress was tested by employing the C3 toxin from *Clostridium botulinum*. C3 toxin has been shown to specifically inhibit the Rho subfamily over other Rho family GTPases such as Rac1 (Wilde et al., 2000). NIH3T3 cells were transfected with GFP-PKN1, then pre-loaded with C3-GST for 6 hours at 5μg/ml followed by subjection to hyperosmotic stress. The effectiveness of C3-GST cell loading was measured by an *in vitro* ribosylation assay and also by monitoring the extent of stress fiber disruption assessed by phalloidin staining (see chapter 3). The accumulation of PKN containing vesicles was independent of C3-GST treatment, implying that Rho function is not critical for this response (Figure 4.10).

The small GTPase Rac1 has previously been implicated in the control of PKN2 (Vincent and Settleman, 1997) and very recently in response to hyperosmotic shock (Lewis et al., 2002). Hence in the absence of a Rho-input, the potential involvement of Rac1 in the translocation of PKN1 was investigated. Myc tagged Rac1 was co-expressed with GFP-PKN1. Under control conditions GFP-PKN1 was cytoplasmic and Rac1 located to lamellipodia, the plasma membrane and to some vesicular structures (Figure 4.11). After 30 min of hyperosmotic stress Myc-Rac1 and GFP-PKN co-localised in vesicles (Figure 4.11). Co-expression of the dominant negative Myc-Rac1-N17 with GFP-PKN1 resulted in no vesicular translocation of GFP-PKN1 after hyperosmotic stress (Figure 4.11). These observations imply that GTP loading of Rac1 is necessary for the hyperosmotic stress induced movement of GFP-PKN1. Rac1 has recently been shown to
Fig 4.10 Hyperosmotic-induced PKN1 vesicle accumulation is independent from Rho.

NIH3T3 cells were transiently transfected with GFP-PKN1. Cells were either unstimulated (control), subjected to 30min hyperosmotic stress (hyper) or treated for 6hr with 5μg/ml of C3-GST with and without hyperosmotic stress prior to fixation. To visualise the actin cytoskeleton alongside GFP-PKN1, cells were then stained with FITC-Phalloidin. The scale bar is equivalent to 10μm.
Fig 4.11 Rac1 dependent hyperosmotic-induced PKN1 translocation.

NIH3T3 cells were transiently co-transfected with GFP-PKN1 and myc-tagged Rac1 or Rac1 N17. Cells were either unstimulated (control) or were subjected to 30min hyperosmotic stress (hyper) as indicated. The scale bar is equivalent to 10μm.
become GTP loaded upon hyperosmotic stress in neutrophils (Lewis et al., 2002). This was confirmed here by employing a Rac1 pull-down assay, using the CRIB domain of PAK which selectively binds GTP bound Rac1. After 30min of hyperosmotic stress, a 1.5-fold increase in the GTP loading of endogenous Rac1 was detected (Figure 4.12).
Fig 4.12 Rac1 activation in response to hyperosmotic stress.

NIH3T3 cells were seeded on 6cm² plates and the Rac1 activation assay was performed. Treatments were: unstimulated cells (1), unstimulated cells with exogenous GTPγS (2), 10min hyperosmotic stress (3) and 30 min hyperosmotic stress (4). One of two experiments performed in duplicate is shown; error bars indicate the range of duplicate observations.
4.5 The regulatory HR1 domain is insufficient for vesicle recruitment whereas the catalytic domain of PKN1 is constitutively vesicular.

Rac1, like Rho, binds to the HR1 domain of PKN proteins (Flynn et al., 1998; Shibata et al., 1996). Thus the impact of the regulatory HR1abc region on hyperosmotic stress induced vesicle recruitment was investigated through localisation studies of the ectopically expressed HA-tagged HR1abc domain of PKN1. This domain constitutively localised around the cell periphery under control conditions and after hyperosmotic stress did not change its distribution. HA-HR1abc was co-expressed with full length GFP-PKN1. The distribution of the HR1abc domain remained unchanged when co-expressed with GFP-PKN1 before and after hyperosmotic stress and the presence of HR1abc did not abolish GFP-PKN1 vesicle recruitment (Figure 4.13).

To assess the potential contribution of other PKN domains in vesicle recruitment, the myc-PKN1 kinase domain was transfected into NIH3T3 cells. Under control conditions, the myc-PKN1 kinase domain already displayed a partially vesicular distribution and upon hyperosmotic stress, this vesicular localisation became more pronounced (Figure 4.14). To test whether the kinase domain locates to the same structures as the full-length protein, the myc-kinase domain was co-expressed with GFP-PKN1; after hyperosmotic stress they were found to co-localise in large vesicular structures (Figure 4.14).

The observation that the PKN1 kinase domain can by itself localise to vesicles, raises the possibility that the kinase domains of the highly related PKC isoforms could behave in a similar manner. To assess further the specificity of the observed PKN behaviour, the myc-PKN1 kinase domain was co-expressed with the GFP-
**Fig 4.13** Rac1 binding is not sufficient for GFP-PKN1 translocation.

NIH3T3 cells were transiently co-transfected with GFP-PKN1 and HA tagged HR1abc. Cells were either unstimulated (control) or were subjected to 30min hyperosmotic stress (hyper) as indicated. The scale bar is equivalent to 10μm.
Fig 4.14 The kinase domain of PKN1 is associated with hyperosmotic-induced PKN1 vesicles.

NIH3T3 cells were transiently transfected with the myc-kinase domain of PKN1. Myc tagged kinase domain of PKN1 was also transiently co-transfected with GFP-PKN1. Cells were either unstimulated (Control) or subjected to 30min hyperosmotic stress (hyper) as indicated. The scale bar is equivalent to 10μm.
PKCa kinase domain. After hyperosmotic stress the kinase domain of PKN1 could be detected in vesicles and in these structures the kinase domain of PKCa was absent (Figure 4.15). These data indicate that vesicle targeting of PKN1 through the kinase domain follows distinct mechanisms when compared with a closely related kinase domain.
Fig 4.15 The kinase domain of PKN1 localises to vesicles selectively over the kinase domain of PKCα.

NIH3T3 cells were transiently co-transfected with the GFP-kinase domain of PKCα and the myc tagged kinase domain of PKN1. Cells were either unstimulated (control) or subjected to 30min hyperosmotic stress. The scale bar is equivalent to 10μm.
4.6 Discussion.

The results described here demonstrate that PKN1 is acutely regulated in a reversible manner by hyperosmotic stress. Hyperosmotic-induced PKN1 vesicles are shown to be negative for both early endosomes and acidified compartments, although they do show some relationship with actin. Actin and tubulin disruption is shown not to prevent GFP-PKN1 vesicle formation, indicating that these arise independently from these cytoskeletal components. The assembly of vesicular PKN1 is shown however to be triggered by activation of Rac1 and not Rho. However, Rac1 contacts to the regulatory HR1 domain of PKN1 alone are found to be insufficient for accumulation of vesicular PKN1. A distinct site of interaction between PKN1 and the described compartment appears to be required and this is consistent with the finding that the kinase domain itself is selectively targeted to vesicles.

Upon stimulation with serum and a range of growth factors such as EGF, Insulin, HGF, LPA and PDGF no difference in the pattern of cellular localisation was observed. This observation may place PKN function downstream of receptor mediated events at the plasma membrane. Alternatively, the extent of recruitment to putative receptor complexes could be limited by the concentration of receptors and hence obscured by the bulk of GFP-PKNs. Roles for PKN1 consequent to receptor mediated events are not excluded, they could be simply beyond the detection limits of the expression system used.

It is shown that PKN1 localisation does not change upon stimulation under several stress conditions. Notably PKN1 translocation to the nucleus upon heat shock was not observed although this has been previously reported (Mukai et al., 1996). However, the observed translocation in response to hyperosmotic stress is of
particular interest with regard to PKNs. Yeast PKC homologues share structural similarity to PKNs and have been implicated in controlling cell wall integrity, this response has been compared with hyperosmotic stress (Alonso-Monge et al., 2001). The observed mammalian PKN1 response to hyperosmotic stress could therefore highlight a conserved functional role.

The GFP-PKN1 translocation response to hyperosmotic stress is also demonstrated with DS-Red-PKN1. This control experiment helps to confirm the PKN1 response and the use of a “red” PKN1 widens the scope of localisation studies. The issue of “tagging” is also addressed since the C-terminal myc tagged PKN1 is shown to translocate to vesicles upon hyperosmotic stress in a similar manner to the N-terminal GFP-PKN1. Initial localisation studies sought to address the nature of the PKN1 vesicular compartment. Markers for early endosomes and acidified compartments show that PKN1 vesicles are not positive for either of these compartments. The precise nature of the PKN1 vesicular compartment remains unresolved.

The nature of the PKN1 translocation was also investigated, it is shown that hyperosmotic-induced vesicles dissipate upon the re-addition of norm-osmotic media, demonstrating this response to be a reversible process. These observations add further specificity and provide an insight into the dynamics of this stimulus specific response.

Hyperosmolarity is known to stimulate the remodelling of the actin cytoskeleton (Lewis et al., 2002). PKN1 is shown to partially co-localise with actin stress fibres when unstimulated. Under conditions of hyperosmotic stress PKN1 vesicles are shown to partially co-localise with actin. The idea that initial PKN1 vesicle accumulation is dependent on the actin cytoskeleton was tested by inhibition of actin polymerisation with cytochalasin and PKN1 vesicle formation is shown to occur independently of actin. Further investigation of cytoskeletal inputs to PKN1

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translocation reveals that PKN1 localisation has no relationship with the microtubule network. The disruption of microtubules with nocodazole also shows that PKN1 translocation occurs independently from microtubule disruption.

The regulatory GTPase binding HR1 domain of PKN1 distinguishes PKNs from other PKCs. It was considered that this region of PKN1 could be important for the hyperosmotic induced translocation of PKN1. This idea was tested by inhibiting the HR1 interacting protein Rho, with the ADP-ribosylation factor C3 toxin. C3 has been shown to specifically inhibit Rho over other GTPases (Wilde et al., 2000). These experiments lead to the conclusion that Rho-GTP interaction is not essential for the behaviour of PKN1 in response to hyperosmotic shock.

Since Rac1 has also been reported to bind the HR1abc domain of PKN1 (Flynn et al., 1998), the possible role of this PKN effector was investigated. The ability of the dominant negative Rac1 to suppress PKN accumulation in the vesicular compartment indicates that Rac1 plays a key role in this hyperosmotic-induced entry event. It is shown that Rac1 becomes GTP loaded in response to hyperosmotic shock, and during the course of these studies, this finding was independently reported (Lewis et al., 2002). However this role is permissive for subsequent events rather than sufficient, since the HR1abc domain of PKN1 is not sufficient for vesicle recruitment despite retaining the Rac1 (and Rho) interacting domain (Amano et al., 1996b; Vincent and Settleman, 1997; Watanabe et al., 1996).

The evidence thus indicates that Rac1 recruits PKN1 at the plasma membrane where HR1abc can also interact and that the PKN1-Rac1 complex but not the HR1abc-Rac1 complex is then endocytosed. Since HR1abc was unable to prevent GFP-PKN1 vesicle association, it is likely that either endogenous Rac1 remains in excess under these conditions or that GFP-PKN1 will associate with Rac1 and effectively remove it from (or compete with) the available HR1abc pool. Thus, while
the regulatory input of Rac1 into PKN1 might be essential in priming PKN1 for endocytosis/vesicle recruitment, it cannot be the sole device by which PKN1 vesicle association is mediated.

Selectivity of the PKN1 response to hyperosmolarity is demonstrated over PKCε. PKN2 does show the same translocation pattern in response to hyperosmolarity suggesting that this is however a common PKN response. However, the observation that the PKN1 kinase domain can by itself localise to vesicles raises the possibility that the kinase domains of highly related PKC isoforms could behave in a similar manner. The myc-PKN1 kinase domain was co-expressed with the GFP-PKCa kinase domain. After hyperosmotic stress the kinase domain of PKN1 could be detected in vesicles; in these structures the kinase domain of PKCa was absent. These data indicate that vesicle targeting of PKN1 through the kinase domain is specific for this compartment and must follow distinct mechanisms when compared with a closely related kinase domain.

The finding that the PKN1 kinase domain is in part constitutively vesicular, coupled with the observation that full length PKN1 requires Rac1 activation, leads to the conclusion that Rac1 binding to PKN1 on hyperosmotic stress induces a conformational change exposing the catalytic domain and hence allowing vesicular recruitment. Consistent with this, it has been suggested that the interaction of Rho GTPases at the amino terminal HR1 motif acts to disrupt an autoinhibitory intramolecular interaction thereby allowing activation i.e. an open conformation (Kitagawa et al., 1996).

In summary it is shown that PKN1 is a stress responsive kinase. The induced translocation is shown to be selective over a closely related PKC although common with PKN2. Many stress induced signaling cascades are generalised stress responses, however the described PKN1 response is specific for hyperosmotic stress. PKN1 translocation is shown to be dependent on Rac1 and not Rho
although it is indicated that a distinct contact within the kinase domain is required for this translocation. These findings detail a mechanism and context within which PKN1 is selectively regulated and can potentially elicit specific downstream events. Leading on from these observations, chapter 5 addresses the question of PKN1 activity, specifically is activation loop phosphorylation and catalytic activation regulated under hyperosmolarity and is linked to the translocation response outlined here.
Chapter 5

PKN1 is activated by hyperosmotic stress.

5.1 Introduction

The Rac1 dependent translocation of PKN1 under conditions of hyperosmotic stress is characterized in chapter 4. To further explore the role of PKN1 in relation to hyperosmolarity, the activation state of PKN1 under these conditions was investigated. Previous studies have investigated the mechanism of PKN1 translocation and the interaction of Rho with PKN1 was shown to facilitate PKN1 activation loop phosphorylation by 3-Phosphoinositide Dependent Kinase 1 (PDK1) (Flynn et al., 2000). PDK1 was originally purified as an activity responsible for PKBα activation loop phosphorylation in a manner that is dependent on PI3 kinase activity (Alessi et al., 1997b) (Stephens et al., 1998). PDK1 has more recently been demonstrated to phosphorylate equivalent residues on many other AGC kinases including; p70^S6k^, PKA, and PKCs (reviewed in (Vanhaesebroeck and Alessi, 2000)). The in vivo ternary complex of Rho-PKN1-PDK1 has been shown to be dependent on PI3 kinase activity and is critical for the catalytic activation of PKN1 (Flynn et al., 2000). The idea that GTPase inputs into PKN1 drive catalytic activation raises the possibility that Rac1 dependent PKN1 translocation described in chapter 4 can stimulate catalytic activation of PKN1.

This chapter seeks to address the role of PKN1 activity in response to hyperosmotic stress. Related to this, the role of PKN1 activity in vesicle recruitment is also addressed by employing a kinase inactive mutant PKN1. It was anticipated that these studies will provide an insight into the context within which PKN1 is functionally active.
5.2 PKN1 activity is not required for vesicle recruitment

The influence of catalytic activity on hyperosmotic-induced PKN1 vesicle recruitment was investigated by employing the drug HA1077 which has been shown to inhibit both PKN1 and PKN2 (Amano et al., 1999; Davies et al., 2000). Pre-treatment of GFP-PKN1 transfected cells with HA1077 followed by hyperosmotic stress did not prevent PKN1 vesicle recruitment. Interestingly cells treated with HA1077 alone under norm-osmotic conditions displayed an accumulation of vesicular GFP-PKN1 (Figure 5.1 A). To further investigate the contribution of PKN1 activity, localisation studies were performed using the kinase-dead GFP-PKN-K644R mutant. When unstimulated this inactive PKN1 mutant shows a punctate cytoplasmic distribution and after hyperosmotic stress accumulates in vesicles (Figure 5.1 B) as observed for wt GFP-PKN1. DS-Red-PKN1 and GFP-PKN1-KR were co-expressed to investigate whether either form of PKN1 could behave in a dominant fashion with respect to vesicle recruitment. Both wt and kinase dead PKN1 were cytoplasmic in unstimulated cells and co-localised in vesicles upon hyperosmotic stress (Figure 5.1 C).
Fig 5.1 PKN1 activity is not required for vesicle recruitment.

A. NIH3T3 cells were transiently transfected with GFP-PKN1 and treated with 20μM HA1077 for 1 hour (control) or pre-treated with 20μM HA1077 for 30min followed by 30min hyperosmotic stress maintained with HA1077 (hyperosmotic).

B. NIH3T3 cells were transiently transfected with GFP-PKN1-KR, unstimulated (control) or 30min hyperosmotic stress (hyperosmotic) treatments are shown as indicated.

B. NIH3T3 cell were co-transfected with GFP-PKN1-KR and DS-Red-PKN1, unstimulated (control) or 30min hyperosmotic stress (hyperosmotic) treatments are shown. The scale bar is equivalent to 10μm.
5.3 PKN1 undergoes activation loop phosphorylation and activation in response to hyperosmolarity.

The HA1077 effects on the basal state and osmotic-induced distribution of PKN1 (Figure 5.2), imply that its activity is not required for recruitment to vesicles but could be required for vesicle turnover or exit. However it is not clear whether activation of PKN takes place in response to osmotic stress. Activation loop phosphorylation is required for optimum catalytic activity of PKN1 (Flynn et al., 2000). Phospho-specific polyclonal antibodies were used to assess the effect of hyperosmotic shock on GFP-PKN1 activation loop phosphorylation. A two-fold increase in phosphorylation after 30 min of hyperosmotic stress was detected on GFP-PKN1 (Figure 5.2). An increase in endogenous PKN1 phosphorylation after hyperosmotic shock was also detected. In the example shown, a two fold increase in endogenous PKN1 phosphorylation is detected upon hyperosmotic stress, however in other experiments, our phospho-PKN antibody was unable to detect endogenous PKN1.

Immunoprecipitated GFP-PKN1 from transiently transfected NIH3T3 cells, either prior to or post hyperosmotic shock, was used to determine directly the effect of osmotic stress on activity. After hyperosmotic shock, immunoprecipitated GFP-PKN1 displayed a specific activity approximately two-fold above un-shocked GFP-PKN1 (Figure 5.3). The possibility of associated kinases being responsible for this activity was precluded with parallel control assays incorporating the PKN inhibitor HA1077. This inhibitor has been reported to be specific for PKN1 over kinases which can associate with it (e.g. PDK1, see below) (Amano et al., 1999; Davies et al., 2000). Immunoprecipitated GFP-PKN1 activity after hyperosmotic shock was reduced to background levels in the presence of 20μM HA1077. The fact that
Fig 5.2 PKN1 undergoes activation loop phosphorylation in response to hyperosmotic stress.

NIH3T3 cells were transiently transfected with GFP-PKN1 and subjected to 30min hyperosmotic stress as indicated. Activation loop Thr-774 phosphorylation of PKN1 was assessed by western blotting using a phospho-specific polyclonal antibody. Immunoreactive endogenous and GFP-PKN1 are indicated in the figure. Relative PKN1 phosphorylation was determined as a function of PKN1 protein levels by western blotting and analysed using NIH image™. The relative GFP-PKN1 phosphorylation is taken from three experiments run in duplicate and error bars denote the standard error of the mean.
Fig 5.3 The catalytic activity of PKN1 increases with hyperosmotic stress.

Immunopurified GFP-PKN1 isolated from transfected NIH3T3 cells was incubated with 10mM MgCl₂, 25μg phosphatidyl-L-serine, 10μg myelin basic protein, and 50μM ATP for 20 minutes at 30°C in a total reaction volume of 40μl. A bead control for the immunopurification is shown (1,2). Cells were either unstimulated (1-4) or subject to 30min hyperosmotic stress prior to immunopurification (5,6). All reactions were performed in parallel as duplicates. Additional duplicate reactions incorporated the PKN inhibitor 20μM HA1077. Relative specific activity was determined as a function of immunoreactive GFP-PKN1 determined by western blotting from the same filter used for MBP phosphorylation. Band intensities were analysed using NIH image™ and error bars represent the standard error (n=3).
HA1077 did not reduce un-shocked GFP-PKN1 activity could mean that basal PKN1 activity is very low.

PDK1 has previously been shown to bind to and facilitate the activation loop phosphorylation of PKN1 (Flynn et al., 2000). Given the finding that PKN1 is both phosphorylated and activated after osmotic stress, the potential involvement of PDK1 in the control of vesicular PKN1 was assessed. GFP-PDK1 was co-expressed with DS-Red-PKN1. The localisation of both proteins was cytoplasmic in unstimulated cells, but after hyperosmotic stress, GFP-PDK1 and DS-Red-PKN1 were found to be co-localised in vesicles (Figure 5.4). PI3-kinase influences PDK1 via its PH domain (Alessi et al., 1997a; Stokoe et al., 1997) and the subsequent phosphorylation and activation of PKN1 (Flynn et al., 2000). The effect on osmotic responses following pre-treatment with the PI3-Kinase inhibitor LY294002 was investigated. After hyperosmotic stress in the presence of LY294002, GFP-PDK1 was no longer recruited to DS-Red-PKN1 positive vesicles. Notably, DS-Red-PKN1 was still recruited to vesicles, however these were smaller after the pre-treatment with LY294002 (Figure 5.4).

Chapter 4 shows that PKN1 kinase domain is sufficient for vesicle recruitment, previous studies have described PKN1-PDK1 interactions that occur via the C-terminal PIF (PDK1 interacting fragment) PKN region which has been shown to interact with a hydrophobic pocket of PDK1. This is thought to act as a “docking site” for PDK1 substrates (Biondi et al., 2000). The nature of PDK1-PKN co-recruitment was therefore investigated by co-expressing myc-PKN1 kinase domain with GFP-PDK1. It is shown that PDK1 and PKN1 kinase domain co-localise in vesicles under control conditions and after hyperosmotic stress (Figure 5.5).

The widely studied downstream target of PDK1, PKB is also known to be modulated upon hyperosmolarity (Meier et al., 1998). In the light of PDK1
Fig 5.4 LY294002 sensitive GFP-PDK1 recruitment to DS-Red-PKN1 hyperosmotic induced vesicles.

NIH-3T3 cells were transiently co-transfected with DS-Red-PKN1 and GFP-PDK1. Cells were unstimulated (control), treated with 30min hyperosmotic stress (hyperosmotic) and pre-treated for 20min with $10\mu m$ LY294002 as indicated. The scale bar is equivalent to $10\mu m$. 
Fig 5.5 PDK1 is recruited to PKN1 kinase domain containing vesicles under control conditions and upon hyperosmotic stress.

NIH-3T3 cells were transiently co-transfected with GFP-PDK1 and Myc-PKN1-kinase domain (Kin). Cells were unstimulated (control), or treated with 30min hyperosmotic stress (hyperosmotic). The scale bar is equivalent to 10μm.
recruitment to hyperosmotic-induced PKN1 vesicles, PKB localisation under these circumstances was investigated. It is shown that GFP-PKB and DS-Red-PKN1 when co-expressed do not co-localise in hyperosmotic induced PKN1 vesicles. GFP-PKB was shown to be cytosolic with some nuclear accumulation as others have seen (Watton and Downward, 1999), this localisation did not change upon hyperosmotic stress (Figure 5.6).
Fig 5.6 PKB does not localise to hyperosmotic-induced PKN1 vesicles.

NIH-3T3 cells were transiently co-transfected with DS-Red-PKN1 and GFP-PKB. Cells were unstimulated (control) or treated with 30min hyperosmotic stress (hyperosmotic). The scale bar is equivalent to 10μm.
5.4 Density fractionation reveals the translocation of endogenous PKN1 and PKN2 in response to hyperosmolarity and with kinase inhibition.

To reveal the behaviour of endogenous PKN1 and PKN2 upon hyperosmotic stress, an attempt was made to isolate enriched hyperosmotic induced PKN1 vesicles by density fractionation. NIH 3T3 cells were stimulated with; hyperosmotic media, HA1077 and staurosporine. The PKN inhibitor HA1077 was shown in 5.1 to induce PKN1 vesicular accumulation, staurosporine was used as a second PKN inhibitor albeit a more broad-spectrum kinase inhibitor. Cells were processed according to the fractionation procedure set out in chapter 2 (Figure 5.7). It is shown that endogenous PKN1 is detected in fractions 3-7 under control conditions and fractions 3-8 after hyperosmotic stress. The translocation to higher density fractions is more pronounced with HA1077 treatment, as detected in fractions 3-10 and after staurosporine treatment in fractions 2-9. Endogenous PKN2 is detected principally in fractions 3-8 under control conditions and after hyperosmotic stress is detected in fractions 3-9. After HA1077, PKN2 is present in fractions 3-9 with an enrichment in fractions 7-9 and after staurosporine treatment PKN2 is present in fractions 3-10 with an enrichment in fractions 7-10. The most obvious translocation detected is the effect of staurosporine treatment on PKN2. However a broad distribution of both PKN1 and PKN2 still remains under all conditions tested.
Fig 5.7 Density fractionation of NIH3T3 cells. Endogenous PKN1 and PKN2 move to increasingly dense fractions upon hyperosmotic stress and treatments of HA1077 and Staurosporine.

NIH3T3 cells were either unstimulated (control) or subjected to 30min hyperosmotic stress (Hyper) and treatments of HA1077 (20μM) or Staurosporine (500nM) as indicated. Cells were processed according to the density fractionation procedure in chapter 2, run on SDS PAGE and subjected to western blot analysis.
5.5 Kinase reactions with immuno-purified PKN1 and cell lysate do not reveal hyperosmotic-induced PKN1 substrates.

Since density fractionation did not reveal total translocation of PKN1 upon hyperosmotic stress, the question of whether hyperosmotic-induced PKN1 can phosphorylate specific substrates was addressed. The increase in the catalytic activity of PKN1 after hyperosmotic stress is described in section 5.3. An attempt was made to utilize this increased activity in an in vitro reaction by using NIH-3T3 cell lysate to screen for specific PKN1 substrates. Immunoprecipitated GFP-PKN1 from transiently transfected NIH3T3 cells, either prior to or post hyperosmotic shock, was used to assess whether hyperosmotic induced PKN1 substrates could be visualised. Phosphorylated bands are shown to appear as a result of the incubation of immunoprecipitated PKN1, examples are indicated with arrows (Figure 5.8). These proteins are activated in a manner that is insensitive to pre-heating the cell lysate at 65°C for 10 min. This treatment was included to inactivate endogenous kinases, these reactions therefore represent more stringent conditions for identifying specific PKN1 substrates. However, no hyperosmotic-induced candidate PKN1 substrates were detected.
Fig 5.8 Incubation of immunopurified GFP-PKN1 with NIH3T3 cell lysate reveals several PKN1 dependent heat stable substrates, no hyperosmotic-induced PKN1 substrates are detected.

Immunopurified GFP-PKN1 isolated from transfected NIH3T3 cells was incubated with 10mM MgCl$_2$, 25µg phosphatidyl-L-serine, and 50µM ATP. Kinase reactions were incubated with NIH3T3 cell lysate (A) or NIH3T3 cell lysate pre-heated at 65°C for 10min. Reactions were performed for 20 minutes at 30°C. A bead control for the immunopurification is shown (1). Cells were either unstimulated (2) or subject to 30min hyperosmotic stress prior to immunopurification (3-4). Additional reactions incorporated the PKN inhibitor HA1077 (20µM) (4). PKN1 autophosphorylation is indicated (PKN1 auto) and PKN1 dependent substrates are indicated by arrows.
5.6 Discussion

It is shown that PKN1 activity is not required for hyperosmotic induced vesicle accumulation, although PKN1 is observed to undergo activation loop phosphorylation and catalytic activation under these conditions. This activation is paralleled by PDK1 recruitment to PKN1 vesicles under hyperosmolarity, however PKB, another PDK1 substrate is shown not to be recruited to this compartment. Attempts have also been made to isolate enriched fractions of PKN1 vesicles and to screen for hyperosmotic-PKN1 substrates.

The finding that the kinase inactive GFP-PKN1-K644R mutant localises to hyperosmotic-induced PKN1 vesicles demonstrates that intrinsic PKN1 catalytic activity is not required for vesicle recruitment. The PKN inhibitor, HA1077 does not prevent hyperosmotic induced PKN1 vesicle accumulation but in unstimulated cells does induce PKN1 vesicle accumulation. These findings indicate a role for PKN catalytic activity in either vesicle exit or turnover and maybe part of a constitutive process.

The activation loop phosphorylation of PKN1 has been shown to be a pre-requisite for PKN1 catalytic activity and to be under the influence of Rho GTPases (Flynn et al., 2000). Indeed both activation loop phosphorylation and catalytic activity are shown to increase under hyperosmolarity. These findings provide a context in which PKN1 is activated and can signal to downstream targets.

The recruitment of PDK1 to PKN1 vesicles is described and is shown to occur through the kinase domain of PKN1, this is likely to involve the FXXFDY motif described as a PDK1 docking site (Biondi et al., 2002). This vesicular interaction is not responsible however for the accumulation of PKN1, since this still occurs when
PDK1 recruitment is blocked by the PI3 kinase inhibitor LY294002. Thus the kinase domain docking in the vesicle membrane must be determined by a distinct protein (or perhaps lipid) contact. The inhibitory effect of LY294002 on PDK1 recruitment to PKN1, indicates that PI3,4,5P3 is required to influence the conformation of PKN1 or that of PDK1 to facilitate complex formation. Both of these proteins have been shown to be influenced by PI3,4,5P3 however in the case of PKN1 no specificity was observed relative to the precursor lipid PI4,5P2 (Palmer et al., 1995a). Hence it is likely that the role of PI3,4,5P3 is to enhance membrane occupancy of PDK1 through its PH domain (Alessi et al., 1997a) and so facilitate recruitment to the membrane bound PKN1. The reduction in size of PKN1 positive vesicles on treatment of shocked cells with LY294002 suggests that the larger vesicles observed are a consequence of a PI3 kinase-dependent vesicle fusion event; this is a characteristic of homotypic endosome fusion (Jones and Clague, 1995).

A hyperosmotic stress response has been described previously for the Rac1 effector, p21-activated protein kinase γ-PAK which binds to and is activated by Rac1. γ-PAK has been shown to translocate from a soluble to a particulate fraction and become activated in response to hyperosmolarity (Roig et al., 2000). Interestingly it was demonstrated that the activation but not translocation of γ-PAK was sensitive to wortmannin, suggesting a two-step mechanism for the γ-PAK response to hyperosmotic stress. This parallels the situation described here for PKN1 where inhibition of PI3 kinase does not prevent vesicle accumulation of PKN1, while blocking recruitment of the upstream kinase PDK1. It has been shown previously that inhibition of PI3 kinase will block activation loop phosphorylation of PKN1 (Flynn et al., 2000). Since PKN1 translocation is shown to be dependent on Rac1 and independent from PI3 kinase, it would seem likely that under hyperosmotic stress Rac1 can be GTP loaded independently from PI3 kinase activity. This highlights a perhaps very specialised mechanism of Rac activation given that PI3 kinase has been shown to be an upstream regulator of Rac (Han et al., 1998).
The combined Rac1/PI3,4,5P3 regulatory input that facilitates PDK1 phosphorylation of PKN1 provides further evidence for the view that the specificity of PDK1 actions is driven by the co-association of regulatory inputs to target kinases (Vanhaesebroeck and Alessi, 2000). In this context it is notable that despite the requirement of PI3 kinase for PDK1 recruitment, PKB which is also recruited by 3-phosphoinositides, is not recruited to this hyperosmotic-induced compartment. This suggests that PKB is either actively removed or its affinity for PIP3 is weaker. Indeed the PH domain of PKB was reported to have a lower affinity for PIP3 when compared with PDK1 as reviewed (Vanhaesebroeck and Alessi, 2000).

The hyperosmotic-induced PKN1 compartment was further investigated biochemically by density fractionation. In attempting to isolate fractions that are enriched with PKN1 vesicles, it was anticipated if achieved, that these could be further exploited by 2D electrophoresis and mass spectrometry to identify other constituent proteins which could help explain the functional role of PKN1 in this setting. It is shown that endogenous PKN1 does translocate to more dense fractions (which are indicative of vesicular fractions) with hyperosmolarity and this is also observed for PKN2. The kinase inhibitors HA1077 and staurosporine induce a more robust translocation to higher density fractions. The finding that staurosporine induced the most dramatic change in the profile of PKN2 points to multiple kinase inputs in controlling vesicle turnover or exit. The extent of translocation however is not total and PKN distribution remains dispersed over many fractions. It could be that PKN1 association to this membrane bound compartment is a weak interaction and could be disrupted during lysis or processing. Future experiments to exploit this technique in isolating hyperosmotic induced PKN1 vesicle enriched fractions could involve the use of irreversible crosslinkers to stabilise protein-proteins and protein-lipid interactions such as DTSSP (Jung and Moroi, 1983). Alternatively these could involve “Spiking" by
transfection with vesicle associated proteins such as the overexpressed kinase domain of PKN1.

Since PKN1 catalytic activity is shown to increase under hyperosmolarity, PKN1 substrates were sought using immunoprecipitated PKN1 from cells harvested prior to or post hyperosmotic stress, incubated in a kinase reaction with NIH3T3 cell lysate. This approach is shown to reveal PKN1 specific substrates as is shown by the arrows in Figure 5.8. One of these is approximately 80KDa and previously the 80KDa protein MARCKs was shown to be a substrate of PKN by a similar technique (Palmer et al., 1996). However, no such candidate PKN1 substrates associated with hyperosmotically treated samples were detected. It could be that this is due to the lack of abundance of such proteins and hence beyond the detection limits of this assay. This approach could, however be re-examined as a method for the identification of PKN1 substrates.

In summary PKN1 is shown to be a stress responsive kinase. The selective translocation of PKN1 coupled with its subsequent activation, details a mechanism by which hyperosmotic shock can elicit a particular repertoire of responses through this kinase. The outputs for these events are examined in Chapter 6 which utilises PKN1-KO mouse embryonic fibroblast cells to address this question.
Chapter 6

PKN1 involvement in the hyperosmotic-induced JNK pathway.

6.1 Introduction

The acute translocation to vesicles and associated activation of PKN1 in response to hyperosmotic stress has been detailed in chapters 4 and 5. Hyperosmotic stress is a potent activator of several signalling cascades which include stress activated protein kinase (SAPK or JNK) (Galcheva-Gargova et al., 1994), p38 (Han et al., 1994) and extracellular signal-regulated kinases (ERKs) (Matsuda et al., 1995). In contrast PKB is downregulated by hyperosmotic stress via dephosphorylation of its regulatory Thr308 and Ser473 phosphorylation sites (Meier et al., 1998).

Precedent has also been set for the involvement of PKCs in the hyperosmotic stress response since classical and novel PKC activation is suggested to be a requirement for hyperosmotic-induced ERK activation (Zhuang et al., 2000). The yeast PKCs, which closely resemble PKNs, are known to activate the MAP kinase cascades involving BCK (MAPKKK), MKK1 and M KK2 (MAPK), and MpK1 and have been linked to the control of cell wall integrity (Arellano et al., 1999; Irie et al., 1993; Levin et al., 1994). This control mechanism has been likened to the osmotic stress response (Alonso-Monge et al., 2001). Mammalian PKNs have also been linked to MAP kinase responses since PKN1 was shown to be on a pathway involving p38γ (Marinissen et al., 2001). PKN1 has more recently been shown to phosphorylate the novel MAPK kinase kinase, MLTK, which is also on the p38 MAPK pathway (Takahashi et al., 2003). Furthermore, PKN2 has been shown to
bind MEKK2, although this was independent of PKN1 (Sun et al., 2000a). The related p38, JNK, and ERK MAP kinases therefore represent good candidate pathways in which PKN1 may operate under hyperosmotic stress.

This chapter seeks to address the involvement of PKN1 in stress activated MAP kinase pathways and the hyperosmotic-induced PKB response. Initially these studies involve the transient overexpression of PKN1 and monitoring of these pathways. Subsequent studies also employ primary PKN1 knockout mouse embryonic fibroblast (PKN1-KO-MEF) cells in comparison with wild type (WT) MEFs. The absence of PKN1 should reveal any PKN1 dependency in these stress-activated cascades.
6.2 GFP-PKN1 overexpression and the stress responses

Hyperosmotic stress is known to activate the stress activated protein kinase (JNK) (Galcheva-Gargova et al., 1994), p38 MAP kinase pathway (Han et al., 1994) and is known to down-regulate PKB (Meier et al., 1998). It is shown that transient overexpression of GFP-PKN1 does not appear to influence the basal activity nor hyperosmotic-induction of these pathways (Figure 6.1). Since any differences here could be obscured by low transfection efficiency, an alternative loss of function approach was adopted. This involved primary PKN1-KO versus WT MEF cells.
Fig 6.1 Transient overexpression of GFP-PKN1 has no effect on hyperosmotic-induced PKB degradation, JNK or p38 phosphorylation.

NIH-3T3 cells were transiently transfected with GFP-PKN1. Cells were unstimulated or subject to 30min hyperosmotic stress as indicated. Samples were harvested in Laemmli sample buffer, run on SDS-PAGE followed by western blot analysis. The p38 protein serves as a loading control.
6.3 PKN2 and PKN3 levels are unaffected in PKN1-KO MEFs.

The impact of PKN1 deletion on PKN2 and PKN3 expression levels was investigated by comparing PKN1-KO-MEF cells with WT-MEFs. PKN3 polyclonal antibodies were generated as described in the materials and methods section and used in western analysis (Figure 6.2 A). This antibody is shown to cross-react with PKN1, as confirmed by lack of immuno-reactivity in PKN1 KO cells, see figure. Indeed the peptide sequence used for the generation of the PKN3 antibody shares significant homology with PKN1 (Figure 6.2 B). Both PKN3 and PKN1 signals were competed by immunoblotting PKN3 antibody in the presence of excess PKN3 peptide. It is evident that no differences in PKN3 protein levels were detected between WT and PKN1-KO MEF cells. Commercial PKN2 monoclonal antibodies were used to asses PKN2 levels (Figure 6.2 A), and it is clear that PKN2 levels are not altered in PKN1-KO MEF cells.
Fig 6.2 PKN2 and PKN3 expression is unaffected in PKN1-KO MEF cells.

A. Wt and PKN1-KO primary MEF cell extracts were harvested in Laemmli sample buffer, run on SDS-PAGE and analysed by western blotting. Membranes were incubated with PKN3 antibody, PKN3 antibody with competing peptide and PKN2 antibody as indicated.

B. Alignment of peptide sequence used to generate PKN3 antibody with PKN1 sequence, stars indicate conserved residues.
6.4 PKB, p38 and ERK1/2 responses to hyperosmotic stress are not compromised in PKN1-KO MEFs.

PKB is known to be desphosphorylated and degraded upon hyperosmotic stress (Meier et al., 1998). PKB S473 phosphorylation was monitored in WT and PKN1-KO cells after 16 hours of serum starvation followed by stimulation with hyperosmotic stress (Figure 6.3). It was observed that PKB S473 dephosphorylation is detected in both WT and PKN1-KO MEFs following hyperosmotic stress. It is also clear however, that resting PKB S473 phosphorylation levels in PKN1-KO MEFs are elevated, this experiment has since been repeated and confirmed in conjunction with Dr A. Casamassima.

Both p38 and ERK1/2 are activated in response to hyperosmotic stress and their activation was monitored in PKN1-KO MEF cells (Figure 6.4). It is clear that there is no detectable difference in the pattern of p38 and ERK1/2 activation between WT and PKN1-KO -MEF cells. A sustained activation in response to hyperosmotic stress from ten minutes onwards is shown for both p38 and ERK in PKN1-KO and WT MEF cells.
Fig 6.3 Hyperosmotic-induced dephosphorylation of PKN S473 is detected in both WT and PKN1-KO MEFs.

Wt and PKN1-KO primary MEF cells were subjected to hyperosmotic stress as indicated. Samples were harvested in Laemmli sample buffer, run on SDS-PAGE and analysed by western blotting.
Fig 6.4 Hyperosmotic-induced p38 and ERK1/2 phosphorylation is unaffected in PKN1-KO MEF cells.

Wt and PKN1-KO primary MEF cells were subjected to hyperosmotic stress as indicated. Samples were harvested in Laemmli sample buffer, run on SDS-PAGE and analysed by western blotting.
6.5 PKN1 is required for efficient hyperosmotic-induced JNK and MKK4 activation.

The stress activated protein kinase or JNK pathway is established as being activated by a variety of stress stimuli including hyperosmotic stress (Derijard et al., 1994; Galcheva-Gargova et al., 1994). An increase in phosphorylation of the major JNK isoforms (46Kda and 54Kda) is detected in response to hyperosmolarity and this response is shown to be dramatically reduced in PKN1-KO MEF cells (Figure 6.5). It is also noted that the phospho JNK western shows other bands which are modulated by hyperosmolarity and which are inhibited in PKN1-KO-MEF cells. Since JNK proteins are known to produce many splice variants (Gupta et al., 1996), it is likely that these are indeed other JNK proteins. The JNK pathway was further investigated by monitoring the phosphorylation status of a JNK activator or MAPK kinase namely, MKK4 (Sanchez et al., 1994). MKK4 is also known to be activated following hyperosmotic stress, (Moriguchi et al., 1995) and elevated phosphorylation of MKK4 in response to hyperosmolarity is detected in a similar profile to JNK induction (Figure 6.5). Hyperosmotic-induced MKK4 phosphorylation is also dramatically reduced in PKN1-KO MEF cells in comparison with WT-MEF cells.

It has been suggested that different MAPK kinase Kinases are responsible for JNK activation in a stimulus specific manner (Chen et al., 2002). The selectivity of PKN1 dependent JNK activation was therefore investigated. Following UV stress, a known JNK activator, (Derijard et al., 1994) MKK4 and JNK activation is detected in both WT and PKN1-KO MEF cells with no detectable difference (Figure 6.6). p38 and ERK1/2 responses to UV stress are also unaffected in PKN1-KO MEF cells. TNF stimulation is also know to stimulate JNK activation (Sanchez et al., 1994). It is
**Fig 6.5 Inhibition of hyperosmotic-induced JNK and MKK4 phosphorylation in PKN1-KO MEF cells.**

Wt and PKN1-KO primary MEF cells were subject to hyperosmotic stress as indicated. Samples were harvested in Laemmli sample buffer, run on SDS-PAGE and analysed by western blotting.
Fig 6.6 MKK4, JNK, p38 and ERK1/2 activation is unaffected in PKN1-KO MEFS following UV stress.

Wt and PKN1-KO primary MEF cells were treated with UV stress (dose of 30 J/m²). Samples were harvested post UV treatment at the time point indicated in Laemmli sample buffer, run on SDS-PAGE and analysed by western blotting.
also shown that TNF-induced JNK, ERK1/2, and p38 phosphorylation are unaffected by the absence of PKN1 (Figure 6.7).

Hyperosmotic stress can induce a range of transcription factor responses resulting in gene expression changes and alterations in the activation state of signalling pathways. The possibility that hyperosmotic-induced JNK phosphorylation could arise from changes in gene induction resulting from the activation of parallel pathways such as p38, was investigated by inhibiting protein synthesis using cycloheximide (Figure 6.8). It is shown that pretreatment with cycloheximide does not inhibit hyperosmotic-induced JNK or ERK1/2 activation, although cycloheximide pretreatment did induce some JNK phosphorylation without stress. Cycloheximide pre-treatment in PKN1-KO-MEF cells also elevated resting levels of JNK phosphorylation. Cycloheximide did not however affect the compromised JNK activation with respect to hyperosmolarity in PKN1-KO-MEF cells.
Fig 6.7 MKK4, JNK, p38 and ERK1/2 activation is unaffected in PKN1-KO MEF cells following TNFα stimulation.

Wt and PKN1-KO primary MEF cells were treated with 20ng/ml TNFα as indicated. Samples were harvested in Laemmli sample buffer, run on SDS-PAGE and analysed by western blotting.
Fig 6.8 Cycloheximide pre-treatment does not inhibit the prolonged phosphorylation of ERK1/2 and JNK following hyperosmotic stress.

Wt and PKN1-KO primary MEF cells were treated with cycloheximide (50µg/ml) for 30min where indicated and/or then subjected to hyperosmotic stress for the time-points shown. Samples were harvested in Laemmli sample buffer, run on SDS-PAGE and analysed by western blotting.
6.6 PKN1 activity contributes to hyperosmotic-induced JNK activation.

Previous work has demonstrated PKN1 activity to be dependent on PI3-kinase (Flynn et al., 2000). It is also shown in chapter 5 that PKN1 undergoes catalytic activation following hyperosmotic stress. The role of PKN1 activity under hyperosmolarity with respect to MKK4 and JNK induction was therefore investigated by employing the PI3-kinase inhibitor, LY294002, and the ROCK inhibitor HA1077 which has been shown to inhibit PKN1 directly (Amano et al., 1999).

Pretreatment with LY294002 shows some inhibition of hyperosmotic-induced MKK4 and JNK phosphorylation (Figure 6.9), however this effect is much less than that seen in PKN1-KO-MEF cells. Direct inhibition of PKN1 catalytic function by pretreatment with HA1077 inhibited hyperosmotic-induced MKK4 phosphorylation in WT MEF cells and further inhibited the low level of MKK4 phosphorylation induced in PKN1-KO-MEF cells (Figure 6.10). However, hyperosmotic-induced JNK phosphorylation was largely unaffected in WT MEF cells, a partial inhibition of JNK phosphorylation in PKN1-KO MEF cells is detected. These studies are consistent with recent studies of by Dr A. Casamassima from the laboratory.

The most well characterised target for JNK is c-Jun (Derijard et al., 1994). The role of PKN1 in hyperosmotic-induced c-Jun phosphorylation was monitored by immunostaining for phospho c-Jun in WT and PKN1-KO MEF cells. It was apparent that phospho c-Jun levels increased dramatically upon hyperosmotic stress in WT-MEF cells, consistent with JNK induction. In PKN1-KO MEF cells, basal levels of phospho c-Jun are reduced compared with WT MEF cells. Upon hyperosmotic stress, increased phospho c-Jun levels are detected in PKN1-KO-
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<th>20min LY294002</th>
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<td>Hyperosmotic Stress (min)</td>
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p-MKK4 →

Tubulin →

p-JNK (46 and 54kda) →

Tubulin →

p-ERK1/2 →

Tubulin →

**Fig 6.9 Hyperosmotic-induced MKK4, JNK, and ERK1/2 activation are partially inhibited by LY294002.**

Wt and PKN1-KO primary MEF cells were treated with 10μM LY294002 for 20min and/or then subjected to hyperosmotic stress as indicated. Samples were harvested in Laemmli sample buffer, run on SDS-PAGE and analysed by western blotting.
Fig 6.10 Hyperosmotic-induced M KK4 activation is inhibited by HA1077.

Wt and PKN1-KO primary MEF cells were treated with 20μM HA1077 for 1h and/or then subjected to hyperosmotic stress as indicated. Samples were harvested in Laemmli sample buffer, run on SDS-PAGE and analysed by western blotting.
MEF cells, however these levels are lower compared with WT MEF cells (Figure 6.11).

The influence of PKN1 in driving c-Jun activation was further investigated by transiently transfecting WT and PKN1-KO MEF cells with GFP-PKN1 and the kinase inactive GFP-PKN1-KR mutant. It is shown that in WT MEF cells, under basal conditions, GFP-PKN1 does not increase phospho c-Jun levels, however under hyperosmotic stress, GFP-PKN1 transfectants appear to have higher levels of phospho c-Jun (Figure 6.12). In PKN1-KO MEF cells, GFP-PKN1 has no effect on basal phospho c-Jun levels although upon hyperosmotic stress, GFP-PKN1 transfectants have increased levels of phospho c-Jun (Figure 6.12). Upon transfection of the kinase dead GFP-PKN1-KR mutant, it is shown that GFP-PKN1-KR does not prevent hyperosmotic-induced phospho c-Jun induction in WT MEF cells. However in PKN1-KO MEF cells, GFP-PKN1-KR does not potentiate phospho c-Jun induction (Figure 6.13).
Fig 6.11 Basal and hyperosmotic-induced phospho c-Jun levels are reduced in PKN1-KO MEF cells.

Wt and PKN1-KO primary MEF cells were unstimulated (control) or subjected to 1 hour hyperosmotic stress as indicated. Cells were stained with anti-phospho S63 c-Jun antibody. The scale bar is equivalent to 10μm.
**Fig 6.12** GFP-PKN1 potentiates hyperosmotic-induced phospho c-Jun in Wt and PKN1-KO MEF cells.

Wt and PKN1-KO primary MEF cells were transiently transfected with GFP-PKN1 and were unstimulated (control) or subject to 1 hour hyperosmotic stress as indicated. Cells were stained with anti-phospho S63 c-Jun antibody. The scale bar is equivalent to 10\(\mu\)m.
Fig 6.13 GFP-PKN1-KR does not block nor prevent hyperosmotic-induced c-Jun phosphorylation.

Wt and PKN1-KO primary MEF cells were transiently transfected with GFP-PKN1-KR and were unstimulated (control) or subject to 1 hour hyperosmotic stress as indicated. Cells were stained with anti-phospho S63 c-Jun antibody. The scale bar is equivalent to 10μm.
6.7 Discussion

This chapter set out to establish a role for PKN1 in classical stress responses given the data presented in chapter 5, which outlined the hyperosmotic-induced activation of PKN1. The transient overexpression of GFP-PKN1 was shown not to affect hyperosmotic-induced p38, JNK phosphorylation, or PKB down-regulation. However it is also clear that only limited transfection efficiency is achieved and that another approach is needed to investigate further the role of PKN1 in relation to these known stress activated cascades.

PKN1-KO-MEF cells, generated in the lab by Dr A. Casamassima, were employed as another approach to study the involvement of PKN1 in stress responses. PKN1-KO-MEF cells are shown to express similar levels of other PKNs compared with WT MEF cells. Since no compensation in terms of up-regulated PKNs is detected in these primary MEF cell cultures, these cells are considered valid tools for dissecting the role of PKN1.

Data presented in this chapter show that PKN1 is not required for hyperosmotic-induced PKB dephosphorylation. Since it has been shown that PKB dephosphorylation in response to hyperosmotic stress can be prevented by phosphatase inhibitors (Meier et al., 1998), it is possible that any influence of PKN1 on PKB phosphorylation is masked by phosphatase induction. Interestingly, PKN1 would appear to have some role in PKB control given elevated steady state levels of PKB-S473 phosphorylation in PKN1-KO-MEF cells. The mechanism by which PKN1 exerts a negative influence over S473 phosphorylation was not investigated, however this could conceivably involve competition for access to the upstream kinase shared by both PKN1 and PKB, namely PDK1. The absence of PKN1 could alter levels of free PDK1 and increase the incidence of PKB-PDK1 complexes.
Alternatively, PKN1 may directly have a negative influence on PKB phosphorylation, indeed it has previously been suggested that PKNs can inhibit PDK1 mediated PKB phosphorylation (Wick et al., 2000).

It is shown that the hyperosmotic-induced JNK is impaired in PKN1-KO-MEF cells. Given that the absence of PKN1 results in defective JNK and MKK4, the possibility is raised that PKN1 could function at the MAP3K level or higher within this cascade. The findings that p38, and ERK1/2 MAP kinase hyperosmotic responses are intact in PKN1-KO-MEF indicate that PKN1 operates selectively through the JNK pathway over parallel MAP kinase signalling events.

MKK4 is known to bifurcate MAP kinase signalling pathways by directly activating both JNK and p38 pathways (Derijard et al., 1995). Results presented here however indicate that PKN1 may engage MKK4-JNK signals independently from p38 since in PKN1-KO-MEFs, p38 induction is unaffected. Such selectivity within these pathways has been described since Rac1 was shown to regulate the JNK response to hypertrophic stimuli independently from p38 activation (Clerk et al., 2001). The data described indicates that PKN1 could be important in conferring specificity to MKK4-JNK induction, whilst other MAPK kinases control p38 activation under these conditions.

The JNK pathway is known to have at least two MAPK kinases, namely MKK4 and MKK7 and several MAPK kinase kinases such as TAK1, MLK1, and ASK1. It has been shown that MAP3 kinases can work on a stimulus specific basis (Chen et al., 2002) and it is thought that these integrate signals from upstream activators such as Rac1. Data presented here indicate that PKN1 controls operating on the JNK pathway lie upstream of MKK4 and are specific for hyperosmotic stress over other JNK activators such as UV stress and TNF stimulation. Direct evidence for the selective MKK4 activation of JNK pathway has come from MKK4-KO-MEF cells that are deficient in JNK signals in response to IL-1, TNFα, hyperosmotic stress
and anisomycin (Ganiatsas et al., 1998). Also in this study, p38 responses were inhibited in MKK4-KO-MEF cells upon stimulation with IL-1, TNFα and anisomycin, however the p38 response was intact upon hyperosmotic stress. These findings support the notion that signalling complexes are assembled under specific circumstances, in this case resulting in conferring specificity to MKK4 and the level of cross talk between MAP kinase signalling. Results described here therefore indicate that PKN1 may play a role in specifying the hyperosmotic-induced MKK4-JNK pathway.

It is concluded that the hyperosmotic-induced JNK phosphorylation arises from direct activation of the JNK cascade rather than the induction of gene expression since the inhibition of protein synthesis is shown not to block hyperosmotic-induced JNK phosphorylation. Cycloheximide treatment is however shown to stimulate JNK phosphorylation in WT-MEF cells and to a lesser extent in PKN1-KO-MEF cells. These results imply that PKN1 may have a direct role in JNK pathway signalling under conditions of hyperosmolarity and also under JNK signalling triggered by cycloheximide.

The described PKN1 requirement for hyperosmotic-induced MKK4-JNK activation, together with the finding that PKN1 is activated under hyperosmolarity, (Torbett et al., 2003) raises the possibility that PKN1 may be a direct MAPK kinase kinase or have an important catalytic role in MKK4 complex assembly. It is also possible that PKN1 dependent MKK4-JNK activation could be the result of a scaffolding role for PKN1 within this cascade. PI3-Kinase inhibition using the drug LY294002 results in the partial inhibition of MKK4 and JNK in wt cells and no difference was detected in PKN1-KO-MEFs (Figure 6.8). Direct PKN1 inhibition with HA1077 resulted in reduced MKK4 activation in WT MEFs and residual MKK4 activation observed in PKN1-KO-MEFs is further diminished with HA1077 treatment. Partial inhibition of JNK induction with HA1077 is also detected in PKN1-KO-MEFs (Figure 6.9). These findings imply that PKN1 activity may contribute to PKN1 dependent hyperosmotic-
induced MKK4-JNK activation. The fact that these inhibitor treatments do not reduce WT-MEF MKK4 or JNK induction to levels seen in PKN1-KO-MEF cells could imply that either PKN1 plays a scaffolding role in this context or that catalytic inhibition is not fully achieved. However the observation that MKK4 and JNK activation is further inhibited by HA1077 in PKN1-KO-MEFs is suggestive of a potential catalytic role for other PKNs such as PKN2 or PKN3. This idea is supported by the finding that PKN2 activity is sensitive to HA1077. Taken together, the data points to a catalytic rather than a scaffolding role for PKN1 in MKK4-JNK hyperosmotic induction.

Consistent with the notion that PKN1 operates within the JNK cascade under conditions of hyperosmolarity, it is shown that PKN1-KO MEF cells have a reduced capacity for c-Jun induction under hyperosmotic stress. Furthermore, the reintroduction of PKN1 is shown to restore the ability of PKN1-KO cells to activate c-Jun after hyperosmotic shock. The observation that the kinase dead GFP-PKN1-KR does not restore c-Jun activation provides further evidence for PKN1 catalytic involvement in the JNK cascade. Since PKN1-KR does not prevent c-Jun induction in WT MEF cells, it is indicated that PKN1-KR does not behave in a dominant fashion over endogenous PKN1.

Overall, the results presented in this chapter establish a requirement for PKN1 in the hyperosmotic-induced MKK4-JNK-c-Jun pathway. The role of PKN1 with respect to MKK4-JNK induction is shown to be selective over parallel MAP kinase pathways and is also demonstrated to be stimulus specific. PKN1 activity is shown to be important for this MKK4-JNK response indicating a direct catalytic role for PKN1 at or upstream from the MAPK kinase kinase level of the cascade. The identification of PKN1 as a critical component of this MAP kinase response provides a new dimension to PKN1 and JNK signalling.
Chapter 7

Discussion

7.1 Overview

A screen for potential PKN agonists has revealed that PKN1 is regulated by hyperosmolarity. The characterised PKN1 response to hyperosmotic stress combines both translocation and activation and a PKN1 requirement for the classically stress activated MKK4/JNK pathway is also established. It is demonstrated that both the PKN1 translocation response and PKN involvement in the JNK cascade behave in a stimulus specific manner. The PKN1 requirement for JNK activation is shown to operate independently from other stress activated cascades and the PKN1 translocation behaviour is demonstrated to be specific over the related PKCe. A model for the PKN1 response to hyperosmolarity is illustrated in figure 7.1.
7.2 PKN1 Regulatory controls

It is intriguing that previous studies have principally focused on Rho as a PKN activator (Amano et al., 1996b; Flynn et al., 1998; Shibata et al., 1996; Watanabe et al., 1996), whereas this study has found no dependence on Rho for the PKN1 response to hyperosmolarity. However several studies have also found Rac-PKN interactions (Amano et al., 1996b; Flynn et al., 1998; Vincent and Settleman, 1997; Watanabe et al., 1996). Taken together the possibility remains that PKN1 is a target of both Rho and Rac small GTPases.

Evidence is presented here for the Rac mediated PKN1 response to hyperosmolarity and is supported by several observations. Rac is shown to co-localise in vesicles and the dominant negative Rac N17 is shown to prevent PKN1 vesicle accumulation. This provides direct evidence for the Rac dependence of PKN1 translocation. Rac is also demonstrated to become GTP-loaded upon hyperosmotic stress as recent studies have found (Lewis et al., 2002). This would provide a mechanism for subsequent PKN induction. Furthermore, PKN1 dependent MKK4-JNK activation under hyperosmolarity is established and this pathway is well characterised to be a Rac dependent cascade (Coso et al., 1995; Minden et al., 1995a).

One alternate hypothesis regarding PKN1 regulation is that there is a dynamic of Rho/PKN and Rac/PKN triggered responses. Perhaps under hyperosmolarity, the activation of Rac predominates and may actually downregulate Rho. Such a mechanism has been shown where Rac mediated production of reactive oxygen species (ROS) results in the downregulation of Rho by inhibiting the p190Rho-GAP (Nimnual et al., 2003). In subverting Rho-PKN signals, hyperosmotic stress would enable Rac-PKN responses to be elicited. This might be tested initially by monitoring the dynamics of Rho and Rac GTP loading under hyperosmolarity.
Many examples of Rac acting downstream of PI3-kinase signalling have been demonstrated and this mechanism is thought to operate through Rac GEFs. A good example is the recently described Rac GEF, P-REX1. Like other Rac GEFs P-REX1 contains a PH domain and is activated by PtdIns(3,4,5)P$_3$, a product of PI3-kinase activation (Welch et al., 2002). The dominant negative Rac N17 is shown here to inhibit hyperosmotic-induced PKN1 translocation. However the inhibition of PI3-kinase is shown not to inhibit hyperosmotic-induced PKN1 vesicle association. These findings would therefore indicate that the Rac-PKN translocation response to hyperosmotic stress is PI-3 kinase independent. It is possible that Rac could actually be triggering PI3-kinase pathways, thereby promoting PDK1 recruitment to the PKN1 positive compartment. Indeed, while the mechanism of Rac induced PI3-kinase activation is unclear, Rac has been shown to bind the p85 regulatory subunit of PI3-kinase (Zheng et al., 1994). The evidence for Rac signalling both downstream and upstream of PI3-kinase has been recently reviewed (Welch et al., 2003).

The mechanism of PKN1 vesicle association has not been fully elucidated, although some indications as to the PKN domain responsible for this association are given. The regulatory HR1abc domain, when localised, is not vesicular while the kinase domain is constitutively vesicular. It is therefore concluded that PKN1 vesicle association is mediated by interactions with the kinase domain. PDK1 is known to interact with PKN1 via the kinase domain of PKN1 and is also present on PKN1 vesicles. However, it is shown that PI3-kinase inhibition can dissociate PDK1 from these PKN1 positive compartments. It is therefore likely that an as yet unidentified PKN1 kinase domain interacting component(s) ("X") mediates this association (a model for this assembly is illustrated in figure 7.1).
7.3 PKN1 activation

The incubation of Rho-GTP with PKN1 has been shown to increase the phosphorylation and catalytic activity of PKN1 (Amano et al., 1996b; Vincent and Settleman, 1997; Watanabe et al., 1996). It is shown that PKN1 undergoes activation loop phosphorylation and catalytic activation upon hyperosmolarity. Consistent with PKN1 activation within the PKN1 vesicle compartment, the PKN1 activator, PDK1, is also recruited. The behaviour of PKN1 described here supports the view that the allosteric input through the amino-terminal HR1 domain is required for complex formation with and subsequent phosphorylation by PDK1.

The role of hyperosmotic-induced PKN1 activation within the vesicular compartment is of interest. Hyperosmolarity is a known apoptotic stimulus (Edwards et al., 1998; Morales et al., 2000) and PKNs have been shown to undergo caspase cleavage in response to apoptotic stimuli and under ischemic conditions (Cryns et al., 1997; Sumioka et al., 2000). However, cleavage of PKN1 under osmotic stress has not been observed. The responses defined here indicate that in fact the behaviour of PKN1 observed under hyperosmotic conditions reflects an underlying constitutive process. The partial vesicular localisation of the kinase domain in the absence of hyperosmotic shock shown in chapter 4 and the observation that HA1077 induces some PKN1 vesicle accumulation described in chapter 5, suggests that this is a constitutive trafficking pathway up-regulated by hyperosmotic shock. The findings indicate that the PKN1 response to hyperosmolarity is not simply targeting PKN1 for degradation but that PKN1 activity is involved in the turnover or exit from this vesicular compartment.

The reversibility of hyperosmotic-induced vesicle formation is described in chapter 3. It would be interesting to monitor whether this reversal correlates with a reversal in PKN1 activation loop phosphorylation and catalytic activation. Perhaps this could
be controlled by the recruitment and/or dissociation of PDK1. The reversal of hyperosmotic-induced PKN1 activation could also be controlled by recruitment of inactivating phosphatases and the effect of phosphatase inhibition on this system would be of interest.

7.3.1 The PKN-PKB question

The PKN1 translocation, activation and PDK1 recruitment in response to hyperosmotic stress is established here. Given that PKB is a well characterised PDK1 substrate, the potential involvement of PKB under conditions of hyperosmolarity was investigated. Consistent with previous reports, it is shown that PKB is dephosphorylated on hyperosmotic stress (Meier et al., 1998). The localisation of PKB translocation under hyperosmolarity was also monitored, it is shown that PKB is not recruited to PKN1 vesicles. The findings gathered from PKN-PKB studies in chapter 3 indicate that Rho-PKN signals do not positively regulate PKB phosphorylation. In fact recent studies have indicated a negative regulatory role for PKNs on PKB phosphorylation (Koh et al., 2000; Wick et al., 2000). These studies, combined with finding that PKB phosphorylation levels are higher in PKN1-KO MEF cells together indicate a negative influence from PKN on PKB phosphorylation.

The observation that PKBS473 resting phosphorylation is higher in PKN1 KO-MEFs raises some interesting questions regarding the influence of PKNs on PKB regulation. This could conceivably involve competition for access to the upstream kinase namely PDK1, shared by both PKN1 and PKB, which would be consistent with our findings that PDK1 is shown to be recruited to the PKN1 vesicular compartment upon hyperosmotic stress. This could affect the stability of PKB and render it more susceptible to degradation. Indeed PDK1 has been shown to affect the stability of AGC kinases given that in PDK1 -/- ES cells, PKN protein levels are compromised (Balendran et al., 2000). The question of how this could relate to
other AGC kinases is an interesting point, indeed a C-terminal fragment of PKN2 was shown to inhibit PDK1 mediated PKCζ phosphorylation (Hodgkinson and Sale, 2002). These findings would also be consistent with a model of competition for access to PDK1 among AGC kinases in general.
7.4 PKN1 and the JNK cascade

The role of PKN1 in classically activated stress pathways was investigated and PKN1 dependence on the hyperosmotic induction of the JNK pathway is described. The question of whether PKN1 is directly activating JNK, perhaps as a MAPKKK or upstream kinase, or is functioning to scaffold components of this pathway is critical to understand PKN1 function. The idea that PKN1 may have a catalytic influence on a MAP-kinase pathway has recently been suggested since PKN1 was shown to phosphorylate \textit{in vitro} the p38 pathway MAPKKK, MLTK. The kinase inactive form was shown to inhibit a hyperosmotic-induced mobility shift of MLTK (Takahashi \textit{et al.}, 2003). The results presented here indicate that PKN1 may have a catalytic contribution to the JNK cascade since the PKN1 inhibitor HA1077 was found to inhibit MKK4 induction, the effect was not total although this discrepancy could be due to the effectiveness of \textit{in vivo} PKN1 catalytic inhibition. Furthermore, the kinase inactive form of PKN1 was found not to potentiate phospho c-Jun staining in MEF cells as was the case with WT-PKN1. While these studies imply a catalytic role for PKN1 in JNK cascade activation, a scaffolding role has not been excluded. Further studies could seek to address these questions by asking whether PKN1 can directly phosphorylate MKK4 or upstream kinases of the JNK cascade. Co-immunoprecipitation experiments to determine whether PKN1 can bind JNK pathway kinases such as MKK4 and/or scaffolding proteins such as POSH, JIP, and JSAP (Ito \textit{et al.}, 1999; Xu \textit{et al.}, 2003; Yasuda \textit{et al.}, 1999) will also be informative with regard to the precise nature of the PKN1 role.

The question of what aspect of PKN1 is necessary and sufficient to engage productive JNK signals could be addressed by transfection studies in PKN1-KO and WT MEF cells. The kinase domain of PKN1 was shown in chapter 4 to be constitutively vesicular, this observation raises the possibility that it may, in lacking an autoinhibitory mechanism, be able to signal to downstream effectors in a
constitutive manner. It would therefore be interesting to ask whether the transfection of PKN1 kinase domain could trigger MKK4/JNK activation under basal conditions. If so, the extent of PKN1 activity involved in JNK signalling could be addressed by employing the kinase dead kinase domain in the same experiment.

It has been shown that PDK1 and Rac are both recruited to the PKN1 induced compartment. Given that this pathway seems to be required for JNK signalling the possibility is raised that these vesicles function as signalling modules. One key question for future studies is to address whether the stimulus specificity of the translocation response correlates with and/or confers stimulus specificity on PKN1 dependent signalling through the JNK pathway. This would need to establish a direct link between hyperosmotic-induced PKN1 vesicle recruitment and JNK pathway constituents. Co-localisation studies could be performed with GFP-PKN1 and constituent members of the JNK cascade such as MKK4 and JNK alongside JNK scaffolding proteins such as JIP, POSH and JSAP (Ito et al., 1999; Xu et al., 2003; Yasuda et al., 1999).

The JNK scaffolding protein JIP1 is known to bind kinesin motors. This may provide a link between vesicle trafficking events such as those observed with hyperosmotic-induced PKN1 translocation and related signalling events such as the triggering of the JNK pathway. The role of trafficking processes in regard to JNK signalling has been reviewed (Goldstein, 2001). The coupling of trafficking and signalling processes may explain how these signals are transmitted from the plasma membrane to the nucleus.

It is possible that other PKNs may operate in a similar manner to that described for PKN1. It is shown that PKN2 displays the same translocation response to that of PKN1. Furthermore the PKN inhibitor HA1077 is shown to inhibit residual MKK4 activation in PKN1-KO-MEF cells. Since what appears to be a highly stimulus
specific and pathway selective role for PKN1 is described, the question of whether
different stimuli and perhaps related MAP-kinase pathways are regulated by other
PKNs and/or related PKCs is raised. This idea is supported from the finding that
hyperosmotic-induced ERK activation was found to be dependent on classical and
novel PKC activation (Zhuang et al., 2000). Further supporting this theory, recently
PKCδ was shown to be required for JNK activation, operating through the
MAPKKK, MKK7 pathway in response to DNA damaging agents (Yoshida et al.,
2002). To investigate this potential cross-over of function, small interfering RNAs
could be employed for PKN2, PKN3 and other PKC superfamily members. As has
been demonstrated, this technique is effective in preventing expression of targeted
proteins (Elbashir et al., 2001). These studies would enable the dissection of the
stimuli and MAP-kinase pathways within which individual isoforms operate.
7.5 Future Directions

The nature of the induced PKN1 vesicular compartment described here is not resolved. Immunostaining indicates that this is not an early endosomal compartment, nor an acidified compartment. However, the effect of PI3kinase inhibition on the induced PKN1-positive compartment, would be consistent with this being part of an endocytic pathway, where inhibition of PI3kinase arrests homotypic fusion (Jones and Clague, 1995). To gain a greater understanding of the precise size and morphology of the vesicles, electron microscopy studies could be performed. This would provide greater resolution and magnification as compared with light microscopy techniques.

Future studies might focus on isolating this compartment and the optimisation of the density fractionation protocol would be a powerful approach to enable the identification of proximal targets for PKN1 and the nature of the PKN1 compartment. This could involve the transfection of constituent components of hyperosmotic-induced PKN1 vesicles, including the kinase domain of PKN1 or perhaps a constitutively activated Rac. These proteins may help achieve greater stability of the PKN1 vesicle association, for example the constitutively activated Rac would prevent GTP hydrolysis upon cell lysis and in doing so may give stability to the complex. As also indicated from the preliminary experiments in chapter 5, kinase inhibitors may prove useful tools in stabilising PKNs on membrane compartments. In purifying PKN1 enriched vesicle fractions, subsequent 2D gel and mass spectrometry analysis may identify novel components such as hyperosmotic-induced binding partners and substrates for PKN1.

The JNK cascade is well known to stimulate a variety of transcription factors resulting in a range of cellular responses such as apoptosis, cell proliferation and tumourigenesis (Dunn et al., 2002). In seeking to understand transcriptional
outcomes of PKN1-JNK responses, gene chip technology could be employed. The comparison of total RNA samples from hyperosmotically stressed WT and PKN1-KO MEF cells may highlight differences in gene induction and could point to precise functional roles for this pathway.

The role of the JNK pathway in relation to cell death has been an active area of research with both pro and anti apoptotic roles for JNK reported, these have been recently reviewed (Lin, 2003). Since hyperosmolarity has been shown to be an apoptotic trigger (Edwards et al., 1998; Morales et al., 2000), it would be of interest to assess a role of PKN1 within the JNK pathway in relation to cell death. This could be achieved by performing apoptotic assays on hyperosmotically shocked PKN1-KO and WT-MEF, such studies have demonstrated involvement for other components of the JNK pathway in cell death (Xu et al., 2003).

A potentially pathogenic property of the JNK pathway is that of invasion, indeed invasiveness has been associated with increased JNK activity. This process has been linked to focal adhesion kinase (FAK) signalling and cells lacking FAK were shown to lack invasive properties. Downstream signalling from FAK in this process was shown to involve the Rac-MKK4-JNK pathway (Hsia et al., 2003). It had been proposed that this pathway involves PAK as an intermediate signalling molecule between Rac and MKK4 (Almeida et al., 2000). Given the studies described in this thesis, it would be interesting to determine any contribution from PKN1 into the invasive process.

A physiological situation where osmotic gradients are important is in the kidney where water re-absorption is a key function. Aquaporin-1 (AQP1) is a water channel that is induced by hyperosmolarity. The activation of ERK, p38 kinase, and JNK pathways were shown to be involved in hyperosmotic-induced AQP1 expression in mIMCD-3 cells (Umenishi and Schrier, 2003). It would be interesting, perhaps to ask whether such induction is impaired in PKN1-KO cells derived from
the kidney. Further studies looking at physiological consequences of loss of PKN could involve water deprivation experiments. In monitoring changes in urine composition, any defect in the ability to reabsorb water would be revealed. Long term studies could involve the generation of PKN2 and PKN3 KO-mice. Assuming viability, multiple crosses of these animals could reveal the total PKN contribution and level of redundancy among these proteins to physiological situations as well as stress activated and other signalling cascades.

In conclusion, a role for PKN1 in an important signalling cascade is demonstrated. Translocation and activation of PKN1 in a vesicular compartment points to a functional role for these vesicles in eliciting downstream signalling. The identification of complex components within these compartments and associated downstream transcriptional responses will help to further understand the function of this hyperosmotic-induced pathway.
References


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