The Cloning and Characterisation of a Novel Mammalian Phosphatidylinositol Transfer Protein, MrdgBβ

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Phosphatidylinositol transfer proteins (PtdIns-TPs) have historically been described as proteins that constitutively catalyse the exchange of phospholipid monomers between membrane bilayers, thereby facilitating lipid distribution within cells. Recent data have identified an acute requirement for PtdIns-TPs during several essential cellular processes.

The various PtdIns-TPs, including PITPα, PITPβ, retinal degeneration B (rdgB) and PYK2 amino-terminal domain interacting (Nir) proteins, can be divided into two structural families. The small, soluble PITP isoforms contain only a PtdIns transfer domain and have been implicated in phosphoinositide signalling and vesicle trafficking. In contrast, the rdgB proteins, which include the Nir proteins, contain an amino-terminal PITP-like domain, an acidic, Ca\(^{2+}\)-binding domain, six putative transmembrane domains, and a conserved carboxy-terminal domain. Although the rdgB protein was originally identified as an invertebrate phototransduction protein, the biological function of rdgB proteins in vertebrates is unclear.

This thesis describes the molecular cloning and characterisation of a novel rdgB protein, rdgBβ. The 38kDa, mammalian rdgBβ (MrdgBβ) protein contains an amino-terminal PITP-like domain and a short carboxy-terminal domain. Cytogenetic analysis reveals that the human rdgBβ gene is localised on chromosome 17q23. In contrast to other rdgB-like proteins, MrdgBβ contains neither transmembrane motifs nor the conserved carboxy-terminal domain. Northern analysis has demonstrated that MrdgBβ mRNA is ubiquitously expressed. Furthermore, immunofluorescence analysis of ectopic MrdgBβ showed cytoplasmic localisation.

The phospholipid-binding specificity and transfer activities of MrdgBβ, relative to other PtdIns-TPs, have been addressed using radiolabelled- and pyrene-labelled phospholipid transfer assays. The functional significance of the results of these assays is discussed.

While early reports found functional degeneracy in vitro, the work presented in this thesis supports more recent results, which suggest that different members of the PtdIns-TP family have distinct functions in vivo.
This thesis is dedicated to my family.
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1.0 Introduction

1.1 Phospholipid transport

Phospholipids are essential constituents of membrane bilayers in cells. These membranes serve essential roles as selective barriers between the extracellular environment and the interior of the cell, and also act to create discrete biochemical compartments that segregate specific cellular functions. Membranes also provide important chemical interfaces that support the activity of membrane-associated enzymes. Given these important roles of membrane bilayers, cells have evolved elaborate mechanisms dedicated to the establishment and maintenance of membrane integrity and identity. This latter critical feature is most strikingly apparent in eukaryotic cells, which harbour diverse, intracellular, membrane-enclosed organelles. An understanding of how organelle membranes acquire and maintain their unique lipid composition requires knowledge of the mechanisms of lipid transport and sorting.

The mechanism and regulation of the intracellular trafficking of phospholipids are still poorly defined. The major sites of lipid synthesis in eukaryotic cells are the endoplasmic reticulum (ER), mitochondria and peroxisomes (Moreau and Cassagne, 1994). Since no single organelle (with the possible exception of the ER) houses the complex machinery required for the synthesis of its entire complement of resident lipids, lipid trafficking and sorting reactions are likely to be essential processes. Moreover, the fact that organelle membranes maintain their unique compositional identities in the face of lipid flux highlights the efficacy of lipid and protein sorting and trafficking events.

Lipid transfer between different biological membranes was first recorded in the experiments of Hahn and Hevesy, and Hagerman and Gould (Hahn and Hevesy, 1939; Hamilton et al., 1997). Since then several different types of lipid transfer have been defined: spontaneous transfer of lipids by monomer diffusion through a membrane, the spontaneous transfer of lipids through transient contacts formed by intermembrane collision events, lipid transfer mediated by intermembrane trafficking of vesicles, and lipid transfer mediated by soluble protein carriers. For the purpose of this thesis, attention will be restricted to the soluble phospholipid protein carriers the phospholipid transfer proteins (PLTPs) and their potential roles in mediating lipid transport, and the emerging ideas of their mechanisms of function in living cells. Current views of how lipids may traffic between organelles have been
comprehensively reviewed elsewhere (Moreau and Cassagne, 1994), and will not be discussed in detail here.

1.1.1 Phospholipid transfer proteins

The functional parameters used for classifying modes of transport are numerous, but the primary ones include the pharmacology, kinetics, temperature and ATP dependence of transport (Moreau and Cassagne, 1994). Vesicular and collisional modes of transport are generally characterised as exhibiting temperature and ATP dependence. In contrast, the monomeric transfer of phospholipids by soluble protein carriers exhibits characteristics that are temperature and ATP independent. Such carrier proteins act by desorbing lipids from one bilayer and subsequently depositing the bound lipid into a second bilayer.

Early evidence for the existence of the soluble protein carrier mechanism of lipid transport came from the observation that the transfer of newly synthesised radiolabelled phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEth), from the ER to the plasma membrane in intact cells, is an extremely rapid process ($t_{1/2} = 1$ min; Kaplan and Simoni, 1985). Moreover, this rapid transport occurred in an energy and temperature independent manner. Since spontaneous trans-cytoplasmic diffusion of lipids between membranes occurs with a $t_{1/2}$ of 2-24 hours (Roseman and Thompson, 1980), it became clear that such rapid energy and temperature independent lipid transport modes must be carrier protein mediated. Subsequent biochemical analysis has led to the biochemical characterisation of a large family of proteins that fulfil the expected functional criteria for lipid carrier proteins (Wirtz, 1991b). Many studies have since demonstrated that all eukaryotic cells contain a diverse array of cytoplasmic phospholipid transfer proteins (Wirtz, 1991b).

The PLTPs have been classified into three categories: the mono-, oligo, and non-specific PLTPs, on the basis of their in vitro substrate specificities (Rogers and Bankaitis, 2000). The non-specific lipid transfer proteins (nsLTPs) catalyse the in vitro transfer of a wide variety of phospholipids, sterols and glycolipids (Wirtz, 1991b). nsLTPs have been isolated from a variety of plant and animal sources. Another group of nsLTPs, the glycolipid transfer proteins (GLTPs), bind to and transfer glycosphingolipids and glycoglycerolipids between membranes (Sasaki, 1990). The oligo-specific PLTPs transfer a more restricted set of phospholipids. This group includes the phosphatidylinositol (PtdIns)/PtdCho transfer proteins (PtdIns-TPs) isolated from mammalian, plant and yeast sources (Rogers and
Bankaitis, 2000), and the PtdCho/sphingomyelin (SM) transfer protein (Dyatlovitskaya et al., 1982). The mono-specific PLTPs bind and transfer only one phospholipid species and include the PtdCho transfer protein (Teerlink et al., 1982), the phosphatidylglycerol (PtdGly) transfer protein (van Golde et al., 1980) and the SM transfer protein (Koumanov et al., 1982). In this thesis, discussion is limited to the oligo-specific PtdIns-TP family. The characterisation and properties of other PLTPs have been recently reviewed (Rogers and Bankaitis, 2000; Wirtz, 1997).

1.1.2 Phosphatidylinositol transfer proteins (PtdIns-TPs)

PtdIns-TPs appear to be widely expressed throughout evolution as revealed by their characterisation from mammalian, fungal, insect and plant sources. Members of the PtdIns-TP family include the mammalian PITPs, the yeast Sec14ps and the multi-domain rdgB protein in Drosophila. While PtdIns-TPs can bind and transfer several classes of phospholipid, this family of proteins has classically been characterised as small, soluble proteins that can bind and transfer PtdIns or to a lesser extent, PtdCho, in vitro (Rogers and Bankaitis, 2000). This definition derives from the demonstration that a particularly well characterised mammalian PtdIns-TP (PITPa) and yeast PtdIns-TP (Sec14p) contain a single phospholipid-binding site that can accommodate the mutually exclusive binding of PtdIns or PtdCho (Li et al., 2000a; Wirtz, 1991a). However, during the past few years new members of the PtdIns-TP family have been identified that do not fit the narrow initial criteria used to define a PtdIns-TP. As described in the following sections of this thesis, recent data have demonstrated that PtdIns-TPs are more biochemically and structurally diverse than had been appreciated.

PtdIns-TP sequence can be divided into two distinct unrelated branches: the fungal and metazoan. These two branches exhibit a high level of primary sequence conservation across a wide evolutionary range, a feature that suggests an important in vivo function for these proteins. The first characterised fungal and metazoan PtdIns-TPs are Sec14p and PITPa, respectively, both of which are small soluble proteins with a mass of approximately 35kDa (Bankaitis et al., 1990; Helmkamp et al., 1974). Despite the complete lack of primary sequence homology between fungal and metazoan PtdIns-TPs, these proteins appear biochemically similar in their in vitro phospholipid transfer activity. Additionally, Sec14p shares significant primary sequence homology with other proteins that are not PtdIns-TPs, namely, the
mammalian retinaldehyde and α-tocopherol-binding proteins and several other uncharacterised mammalian proteins (Salama et al., 1990; Sato et al., 1993).

The characterisation of the physiological roles of the PtdIns-TPs remains in its infancy. Recently a convergence of evidence from reconstitution studies has identified essential roles for PtdIns-TPs in the co-ordination and coupling of phospholipid metabolism with vesicle trafficking and in the regulation of important signalling cascades (reviewed by Cockcroft, 1997; Kearns et al., 1997). Nevertheless, many basic questions are unresolved, including the structural basis for the recognition of specific phospholipids by the various PtdIns-TP isoforms and the mechanism by which PtdIns-TPs exchange bound phospholipid ligand for one that is confined to the bilayer. From a biological perspective, fundamental questions include defining the true biological functions of these proteins, and defining the individual contributions of the PtdIns and PtdCho binding to in vivo function. The following sections will consider these four basic issues in relation to the existing knowledge regarding known members of the PtdIns-TP family.

1.2 The Sec14 family of PtdIns-TPs

1.2.1 Sec14p function in yeast secretory pathways

The Sec14 gene was originally identified in a large collection of conditional lethal secretory pathway mutants generated by Schekam and colleagues (Novick et al., 1980). These mutations manifest themselves as stage-specific blocks in the Saccharomyces cerevisiae secretory pathway, as determined by biochemical and morphological criteria, and 23 Sec genes were initially identified in this mutant collection. 21 of these genes, including Sec14 play direct roles in the S. cerevisiae secretory pathway (Novick et al., 1980).

The Sec14 gene product (Sec14p) represents the major PtdIns-TP of this budding yeast and plays an essential role in protein transport from the yeast Golgi complex (Bankaitis et al., 1990; Bankaitis et al., 1989; Li et al., 2000a). Temperature sensitive Sec14 (Sec14°) mutants block transport out of the Golgi, accumulate dysmorphic Golgi membranes and arrest growth at a non-permissive temperature (Bankaitis et al., 1989; Novick et al., 1980).

A dissection of the mechanism by which Sec14p translates its PtdIns and PtdCho exchange activity to biological function has been facilitated by the isolation and characterisation of mutations that alleviate the normally essential requirement of
Sec14p in Golgi secretory function and cell viability. Several bypass Sec14p mutants are discussed below.

Insight into the mechanism by which Sec14p could be bypassed was gained through the demonstration that three of the known "bypass Sec14p" genes define loss-of-function mutations in three genes for enzymes encoding the CDP-choline pathway (choline kinase, choline phosphate cytidyltransferase (CCT) and choline phosphate transferase; Cleves et al., 1991). This pathway consumes diacylglycerol (DAG) in a reaction with CDP-choline to generate PtdCho. Inactivation of a second pathway for PtdCho biosynthesis, the PtdEth-methylation pathway, does not reduce the cellular requirement for Sec14p (Cleves et al., 1991). These data were interpreted to indicate that the CDP-choline pathway is somehow specifically toxic to Golgi secretory function in yeast (Cleves et al., 1991). It subsequently demonstrated that Sec14p effects an in vitro and in vivo inhibition of CCT, the rate-determining enzyme of PtdCho biosynthesis (Skinner et al., 1995). Furthermore, phospholipid-loading experiments have demonstrated that PtdCho-bound Sec14p is a more potent CCT inhibitor than PtdIns-bound Sec14p. Furthermore, mammalian PITPα does not inhibit this enzyme in vitro (Skinner et al., 1995).

Various data collected from biochemical studies have given rise to the proposal that Sec14p maintains a critical pool of DAG in the Golgi that is required for Golgi secretory function (Kearns et al., 1997). According to this hypothesis yeast Golgi DAG pools are at least partially maintained through negative regulation of the CDP-choline pathway by the PtdCho-bound form of Sec14p. In this manner, genetic inactivation of the CDP-choline pathway bypasses Sec14p because it recapitulates a principle Sec14p function. This proposed mechanism implies that the phospholipid exchange activity of Sec14p acts as a molecular switch that modulates the ability of Sec14p to function as a negative regulator of DAG consumption (Figure 1.1).

A second body of evidence that PtdCho-bound Sec14p is a functionally important form of Sec14p comes from the analysis of a Sec14p double mutant that is specifically deficient in PtdIns-binding and transfer activity in vitro (Phillips et al., 1999). This mutant effectively transfers PtdCho in vitro and, when expressed at physiological levels, can rescue the Sec14 null mutation, in vivo. Furthermore, this Sec14p mutant retains the ability to down regulate CDP-choline pathway in vivo (Phillips et al., 1999). Direct proof that PtdCho bound Sec14p plays an important physiological role awaits the identification of a mutant Sec14p specifically defective in PtdCho binding.
Figure 1.1 Sec14p regulation of diacylglycerol (DAG) in yeast Golgi membranes. Sec14p is proposed to maintain the Golgi DAG pool in two ways: (1) by inhibiting DAG consumption by the CDP choline-pathway for phosphatidylcholine biosynthesis (PtdCho), and (2) by promoting Golgi DAG generation through the facilitation of inositol phospholipid metabolism. The PtdCho bound form of Sec14p (Sec14p-PtdCho) inhibits the activity of cholinephosphate cytidylytransferase (CCT). The phosphatidylinositol bound form of Sec14p (Sec14p—PtdIns) might provide PtdIns turnover than resupplies the Golgi DAG.
The studies discussed above suggest that the PtdIns binding and transfer activity of Sec14p is remarkably dispensable *in vivo*. However, it has been proposed that the PtdIns-bound form of Sec14p may somehow increase the rate of PtdIns metabolism such that the Golgi DAG pool is replenished after consumption (Figure 1.1; Kearns *et al.*, 1997; Li *et al.*, 2000a). Several lines of evidence support this concept. For example, high levels expression of mammalian PITPa or PITPB rescues *sec14Δ* growth and secretory defects, but failed to rescue the lethality associated with *sec14* null mutations (Alb *et al.*, 1995). It has been demonstrated that mammalian PITP mutants, which exhibit a specific reduction in PtdIns transfer activity without significantly affecting PtdCho transfer, do not rescue Sec14p defects (Alb *et al.*, 1995). These observations suggest that the PtdIns binding and transfer activity of mammalian PITP is able to rescue the specific Sec14p defects. Additionally, high-level expression of a novel Sec14p homologue from soybean (Ssh2p) exhibits PtdIns but not PtdCho transfer activity, also partially rescues the *sec14Δ* mutant phenotype (Kearns *et al.*, 1998b). Finally Sec14p stimulates the activity of two distinct PtdIns kinases in several reconstitution studies using permeabilised cell reconstitution studies (Cunningham *et al.*, 1996; Xie *et al.*, 1998 and Section 1.4.2). Taken together, these studies demonstrate that regulation of PtdIns metabolism is an important activity of Sec14p, which requires further clarification.

The concept that the PtdIns and PtdCho bound forms of Sec14p exhibit unique and co-operative functions in Golgi DAG maintenance in yeast offers an alternative interpretation of how a PtdIns-TPs might employ its phospholipid exchange activities. In the case of Sec14p, this activity may provide a mechanism to regulate Sec14p effector function. Thus, it has been proposed that Sec14p may not primarily function as a *bona fide* phospholipid transfer protein *in vivo* (Skinner *et al.*, 1995). The observation that mutant mammalian PITPs that exhibit essentially wild type proficiency for both PtdIns and PtdCho transfer are incapable of rescuing Sec14p defects, supports this hypothesis (Alb *et al.*, 1995).

**1.2.1.a crystal structure of Sec14p**

Sha and co-workers have recently resolved the crystal structure of Sec14p at a resolution of 2.5Å (Sha *et al.*, 1998). Sec14p consists of 12 α-helices, 68 strands and 8 3_{10}-helices (tightly wound helices that contain three amino acid residues per helical turn rather than the 3.6 residues per turn of α-helices) that co-operate to form a single large hydrophobic pocket. The dimensions of this pocket are compatible...
with the accommodation of a single phospholipid molecule. This hydrophobic pocket is gated by a hydrophobic surface helix whose conformational flexibility has been proposed to represent an important component of the catalytic mechanism for phospholipid exchange. Sha and co-workers have proposed a "bulldozer" model of phospholipid exchange. According to this model, the hydrophobic helix swings into the membrane to sweep open a cavity and Sec14p subsequently deposits the exposed incoming phospholipid. The helix then swings back to abstract the outgoing phospholipid (Sha et al., 1998).

β-octylglucoside (βOG) was required both for crystal formation and crystal stability. Thus even though a PtdCho-bound form of Sec14p was initially prepared for crystallisation, Sec14p containing two molecules of βOG within the lipid-binding site, was ultimately crystallised. The authors have proposed that this crystal structure of Sec14p represents that of a transition intermediate, in which upon interaction with the detergent micelles, Sec14p had discharged its bound PtdCho ligand as the first step in the exchange reaction (Sha et al., 1998).

The structure of this detergent-bound form has guided the successful identification of at least three Sec14p residues (K66, E207 and K239) that are specifically required for PtdIns-binding and transfer activity. Analysis of a Sec14p double mutant, in which K66 and 239 are mutated to alanine, reveals that this protein is defective in PtdIns but not PtdCho transfer activity (Phillips et al., 1999). Furthermore, residues E207 and K239 represent a salt bridge pair that is conserved in all members of the Sec14p family known to exhibit either PtdIns transfer or binding activity (Sha and Luo, 1999).

1.2.1.b Signalling lipids and vesicle budding from the yeast Golgi complex

While the DAG homeostasis model for Sec14p function appears to account for most of the available data (Section 1.2.1), it remains unclear why DAG might be required for Golgi secretion. DAG may serve as a signalling lipid to promote secretory vesicle formation (Kearns et al., 1997).

Golgi DAG may also represent a precursor reservoir for PtdOH biosynthesis and modulation of PtdOH might be the function of the Sec14p pathway. This concept is generally in accord with the evidence that PtdOH and PLD, whose activity generates PtdOH as a direct product of PtdCho hydrolysis, are down stream effectors of ADP ribosylation factor (ARF) in a pathway requiring the synthesis of a phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P₂) pool for mammalian vesicle
budding reactions (Liscovitch and Cantley, 1995 and Section 1.4.3). Additional weight for this view comes from the observation that a large proportion of Sec14p bypass mutations require functional PtdIns(4,5)\(P_2\)-activated dependant phospholipase D (PLD), for full manifestations of their "bypass Sec14p" phenotypes (Xie et al., 1998). These observations raise the possibility that the bypass Sec14p mutations to exert their bypass effects through PLD. Ethanol-challenge experiments indicate that PLD supports bypass Sec14p phenotypes by generating a PtdOH pool that is somehow utilised in promoting Golgi function. According to this view, the proposed DAG requirement could be rationalised by DAG serving as a precursor for PtdOH (Xie et al., 1998). However, to date, there is no evidence to suggest that yeast express a DAG kinase that would catalyse such a metabolic conversion. Furthermore *E. coli* DAG kinase expression is detrimental to Sec14p-dependent Golgi secretory function (Kearns et al., 1997).

An alternative interpretation of the data demonstrating that PtdIns(4,5)\(P_2\)-activated PLD activity is essential, but not sufficient, for bypass Sec14p function is that the PtdOH generated by PLD may serve as a precursor for DAG production (Li et al., 2000a). According to this view, yeast PtdOH phosphohydrolase would function downstream of PLD by converting PtdOH to DAG. Indeed, yeast express multiple PtdOH phosphohydrolases, however no role for any of these enzymes in the Sec14p pathway is yet known (Toke et al., 1998).

### 1.2.1.c Other Sec14p-like proteins in yeast

The lethal consequences associated with the *sec14* null mutations indicate that Sec14p executes an essential cellular function, and that it does not share substantial functional redundancy with other yeast proteins in executing that function. Recently, a family of five open reading frames that encode proteins with significant homology to Sec14p have been identified. Individual expression of three of these proteins (SFh2p, SFh4p and SFh5p) effected a PLD-dependent rescue of *sec14*-assoicated growth defects, suggesting the possibility of some functional redundancy with Sec14p (Li et al., 2000b). The protein that shares the greatest primary sequence identity (64%) with Sec14p (SFh1p), did not offer any detectable rescue and did not exhibit PtdIns or PtdCho transfer activity *in vitro*. The genes encoding these Sec14p homologues can be deleted *en bloc* without affecting cell viability, thus the physiological functions of these proteins remain to be elucidated (Li et al., 2000b). Nevertheless, the identification of multiple Sec14p-like proteins
raises the topical issue of whether yeast expresses a family of PtdIns-TPs dedicated to divergent sets of cellular functions.

1.2.2 Sec14p function in dimorphic fungi

*Yarrowia lipolytica* express at least one Sec14p form (Sec14p\(^{YL}\)), which shares 65% primary sequence identity with *S. cerevisiae* Sec14p. Expression of Sec14p\(^{YL}\) in *S. cerevisiae* efficiently rescues the essential function of Sec14p. Moreover, Sec14p\(^{YL}\) exhibits both PtdIns and PtdCho transfer activity and localises to the Golgi bodies (Lopez *et al*., 1994). However, Sec14p\(^{YL}\) is neither required for the cellular viability of *Y. lipolytica* nor for efficient secretion. Instead Sec14p\(^{YL}\) is required for execution of a developmental switch that governs differentiation of *Y. lipolytica* from the yeast to the mycelial mode of growth (Lopez *et al*., 1994). The involvement of Sec14p-like PtdIns-TPs in mediating the dimorphic switch extends to fungal pathogens as well. Genetic screens for *Candidia albicans* mutants defective in filamentous growth yields mutants harbouring an insertion mutation in the *Sec14* gene (Riggle *et al*., 1997).

1.2.3 Higher plant Sec1p homologues

Recent analysis of higher plant PtdIns-TP-like proteins has uncovered soybean proteins (Ssh1p and Ssh2p) that define novel and non-classical members of the Sec14p homology family (Kearns *et al*., 1998b). The expression of Ssh1p and Ssh2p was shown to phenotypically rescue sec14\(^{\prime}\) growth and secretory defects. However, it is of note that neither protein afforded complete rescue of the mutant phenotype. These cDNAs encode two distinct soybean polypeptides (Ssh1p and Ssh2p) that share roughly 25% primary sequence identity and 50% similarity with Sec14p and with each other (Kearns *et al*., 1998b). While Ssh2p possesses PtdIns transfer activity, it fails to transfer PtdCho in vitro. Ssh1p is also atypical in that it does not transfer PtdIns or PtdCho in vitro. Competitive photolabelling experiments suggested that, in contrast to Sec14p, both Ssh1p and Ssh2p are high affinity PtdIns(4,5)\(P_2\) and phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)\(P_2\)) binding proteins (Kearns *et al*., 1998b).

The synthesis of these proteins appears to be developmentally regulated in the soybean plant. Ssh2p is highly expressed in developing seeds and poorly expressed in roots and leaves. In contrast, Ssh1p is highly expressed in the roots and leaves and poorly expressed in developing seeds. The biological functions of Ssh1p
and Ssh2p remain to be elucidated, however the differential expression patterns of Ssh1p and Ssh2p indicates that these two proteins have distinct physiological functions within the plant. The observation that Sshp1 is rapidly phosphorylated when Ssh1p-producing yeast strains, young soybean roots, or transgenic tobacco leaf discs are subjected to hyperosmotic stress has lead to the proposal that Ssh1p may be involved in the plant response to environmental stresses associated with environmental conditions of high osmolarity (Kearns et al., 1998b).

1.2.4 Mammalian Sec14p like genes

As indicated above, Sec14p is a member of a family of Sec14p-like molecules being discovered in diverse organisms. As mentioned previously, Sec14p does not exhibit any significant primary sequence identity with mammalian PITPa or PITPβ. However, several mammalian proteins do exhibit significant sequence similarity to Sec14p, including the Sec14p-like protein (Sec14Lp), retinaldehyde-binding protein and α-tocopherol binding proteins (Salama et al., 1990; Sato et al., 1993). Retinaldehyde binding protein acts as a substrate carrier protein for 11-cis-retinol and 11-cis-retinal and plays a role in the regeneration of 11-cis-retinal following light activation during phototransduction (Crabb et al., 1991). While, the function of Sec14Lp remains unknown, it is clear that some Sec14p-like proteins are novel or classical PtdIns-TPs, while other members of the Sec14p-like family are clearly not PtdIns-TPs. The comparative functions of yeast Sec14p and mammalian PITPa and PITPβ are discussed later (Section 1.5).

1.3 Mammalian PITPa and PITPβ

To date, mammalian cells contain two small soluble PtdIns-TPs, PITPa and PITPβ. PITPa was purified from bovine brain cytosol more than 20 years ago (Helmkamp et al., 1974). The PITPa polypeptide consists of 269 amino acids. Mouse and human PITPa share 99% sequence identity and even the flanking untranslated regions (UTR) of human and rat PITPa are remarkably conserved (Wirtz, 1991b). PIPTβ was cloned more recently, on the basis of its ability to complement Sec14α mutants (Tanaka and Hosaka, 1994; Tanaka et al., 1995). In an independent study, PITPβ was subsequently partially purified from bovine brain cytosol and purified to homogeneity from chicken liver (Westerman et al., 1995). PITPβ consists of 271 amino acids and exhibits 77% sequence identity with PITPa. The high degree of sequence conservation between mammalian PITP isoforms
suggests a strong dependence of the functions of these proteins on their complete primary structure. Furthermore, the high sequence similarity and cross-immunological reactivity between rat, bovine and human PITPα or β isoforms suggests that eukaryotic PITPs are highly conserved between species (Rogers and Bankaitis, 2000).

The activities of PITPα and PITPβ have been estimated by measuring the time dependent transfer of labelled phospholipids from a "donor" membrane to an "acceptor" membrane. In the most commonly used transfer assays, the phospholipids are either radiolabelled or carry a fluorescent acyl chain (typically, parinaric acid or pyrene-labelled acyl chains). An incomplete range of lipids is available to establish the transfer activity of the PtdIns-TP of interest. Radiolabelled phospholipid transfer assays are discontinuous, since the estimation of transfer activity requires the separation of the donor and acceptor vesicles at the end of the incubation (Thomas et al., 1994). Fluorescently-labelled phospholipids are employed in a continuous assay, which makes use of the change in spectral properties of the probe (Van Paridon et al., 1988b). These techniques are discussed in detail later (Chapter 6).

In vitro studies have demonstrated that PITPα and PITPβ can bind and transfer PtdIns and to a lesser extent, PtdCho (Van Paridon et al., 1987b). However, PITPβ is unique in its ability to effect a robust transfer of SM (Westerman et al., 1995). Thus, PITPβ may possess more a relaxed specificity, as it is able to accommodate either the ceramide backbone of SM or the glycerol backbones of PtdIns and PtdCho. Early in vitro studies demonstrated that PITPα also exhibits a very low level of PtdGly transfer activity (Somerharju et al., 1983). However, no PtdIns-TP activity has been observed for PtdEth, PtdSer or PtdOH. To date, all studies indicate that PITPα and β mediate a strict molar exchange reaction in the sense that the net flux of phospholipids from donor to acceptor membrane equals that in the reverse direction (Rogers and Bankaitis, 2000; Wirtz, 1991a).

Steady-state and time-resolved fluorescence spectroscopy have demonstrated that PITP_ contains a single hydrophobic site for phospholipid binding (Van Paridon et al., 1987a). This is reflected in the existence of two species of PITPα with isoelectric points of 5.5 and 5.7, representing the PtdIns and PtdCho-bound forms, respectively (Van Paridon et al., 1987a). To date, neither comparable charge isomers nor the existence of one or multiple lipid-binding sites in PITPβ have been reported.
However, the high level of sequence homology between PITPα and PITPβ suggest that this isoform also contains a single phospholipid-binding site.

Each molecule of PITPα, purified from either mammalian tissue or from recombinant bacterial systems, always contains one molecule of non-covalently bound phospholipid. Comparisons of holo and apo-species of native PITPα indicate that the binding of phospholipid ligand is required to obtain the correct, more compact structure of holo-PITP (Voziyan et al., 1996). Using apo-PITPα has been found to possess a significant relaxation of tertiary structure (Voziyan et al., 1996).

When incubated with vesicles of various phospholipid compositions, the endogenous phospholipid can be readily exchanged with other phospholipids during the process of intermembrane transport. For example, analysis of recombinant PITPα revealed that it was loaded with PtdGly and to a lesser extent PtdEth. These bacterial phospholipids were readily exchanged for PtdIns or PtdCho when PITPα was incubated with PtdIns and PtdCho vesicles (Hara et al., 1997). Although, the existence of apo-forms of PITPβ has not been investigated, it is assumed that this protein will also always contain a readily exchangeable, endogenous phospholipid.

1.3.1 Phospholipid specificity: Recognition sites for the polar head group

Competition-binding experiments have estimated that PITPα has a 16-fold higher affinity for PtdIns than for PtdCho (Van Paridon et al., 1987b). Likewise, from kinetic analysis it was estimated that PITPα exchanges its bound PtdIns 20-times faster for PtdIns than for PtdCho and its bound PtdCho 5-times faster for PtdIns than for PtdCho (Van Paridon et al., 1987b). The only reason that PITPα and possibly PITPβ carry PtdCho is that in most membranes the PtdCho pool greatly exceeds the size of the PtdIns pool. Oxidative cleavage of the inositol ring results in a 10-fold reduction in the rate of transfer of the PtdIns derivative (Somerharju et al., 1983). Thus, it appears that an intact inositol ring is a prerequisite for PtdIns to be preferentially transferred. Moreover, PITPα does not transfer phosphatidylinositol 4-phosphate (PtdIns4P) or PtdIns(4,5)P₂ in vivo, indicating that the phosphorylation of the inositol moiety inhibits transfer by PITPα (Schermoly and Helmkamp, 1983). However, PITPα does exhibit a high affinity for PtdIns(4,5)P₂ vesicles (Van Paridon et al., 1988c and Section 1.3.1.b).

Pyrene-labelled phospholipid studies have revealed that PITPβ exhibits a greater affinity and transfer rate for SM compared with PtdCho and PtdIns (Westerman et al., 1995). PtdCho and SM contain the same polar head group,
suggesting that PITPβ may specifically recognise the sphingosine moiety. However, polar head group recognition also influences binding because PITPβ does not bind and transfer analogous ceramide species (Westerman et al., 1995).

Thus, it is apparent that there is competition between transferable phospholipid species and that the specificity of binding of different species is in part due to specific recognition of the polar head groups of the phospholipid.

1.3.1.a Phospholipid specificity: Specific recognition sites for the acyl chains

In addition to headgroup specificity, extensive studies have demonstrated that phospholipid binding is sensitive to the acyl chain structure of PtdCho, PtdIns and SM (Westerman et al., 1995; Wirtz, 1991a). This suggests that phospholipid acyl chains participate to stabilise interactions of the PtdIns-TP with the phospholipid and may contribute to the energy required to extract the phospholipid from a membrane. By measuring the binding of fluorescent parinoyl- and pyrene-labelled PtdIns and PtdCho analogues carrying both pyrene/parinoyl-labelled and unlabelled saturated acyl chains of different lengths at either the sn-1- or sn-2-position, several groups have investigated the 1-acyl and 2-acyl binding sites of PITPa and PITPβ. Such studies have demonstrated that the length of the acyl chains has a marked effect on the binding and transfer of various PtdIns and PtdCho analogues (Westerman et al., 1995; Wirtz, 1991a). Van Paridon and co-workers have demonstrated that the binding of pyrene PtdIns-species, with varied acyl chain lengths, increased in the order of C_{16:1}>C_{16:0}>C_{18:0} (Van Paridon et al., 1988b). Likewise, the binding of pyrene labelled PtdCho species revealed that the optimal length of the unlabelled acyl chain increased in the order of C_{14:0}>C_{16:0}>C_{18:0}>C_{20:0} (Kasurinen et al., 1990; van Paridon et al., 1988b). Irrespective of the chain length, PITPa preferred PtdCho analogues with a prenyl acyl chain in the sn-1 rather than the sn-2 position, suggesting that the sn-2 position is less suited to accommodate a bulky acyl group (Van Paridon et al., 1988b). Comparisons of the binding profiles of PtdIns and PtdCho species demonstrated a strong resemblance with respect to the sn-2 acyl chain specificity for both phospholipid classes (Kasurinen et al., 1990; Van Paridon et al., 1988b). This suggests that PtdIns and PtdCho share the same sn-2 binding site and possibly the sn-1 binding site on PITPa.

More recently, fluorescence spectroscopy studies have demonstrated that the binding of phospholipid to PITPβ is also dependant on acyl chain length, optimum binding of PtdIns, PtdCho and SM was observed with C_{12:0}, C_{14:0}, C_{16:0} and C_{19:0} groups in
the sn-2 position, respectively (Westerman et al., 1995). These studies suggest that PITPa and PITPB will preferentially bind phospholipids with short chains rather than the more physiological species. However, these conclusions did not take into account the influence of unsaturation within the acyl chain.

Several studies have demonstrated the importance of hydrophobic interactions between the acyl chains of the phospholipid and the binding domain of the PtdIns-TP (Wirtz, 1991a). Unsaturation of an acyl chain also has an affect on the affinity for a phospholipid. Indeed, the affinity of PITPa for a range of PtdCho species steadily increased with unsaturation up to a C_{18:0} species carrying 3 double bonds in the sn-2 chain (Kasurinen et al., 1990). However, thereafter it declined strongly with C_{16:0}C_{20:4} PtdCho and C_{16:0}C_{22:6} PtdCho binding more weakly (Kasurinen et al., 1990). This would suggest that steric factors prevent a strong association between C_{16:0}C_{20:4} PtdCho and C_{16:0}C_{22:6} PtdCho and PITPa.

The studies discussed above indicate that PITPa and PITPB have distinct binding sites for the sn-1 and sn-2-chains of PtdIns, PtdCho and SM. Owing to the different properties of these sites, both proteins can discriminate between molecular phospholipid species as well as between positional isomers of these species. This suggests that PITPa and PITPB have a preference for certain molecular species of PtdIns, PtdCho and SM. It is possible that the different affinities of various PtdCho, PtdIns or SM species are connected to their functional and/or metabolic properties. The majority of mammalian PtdCho species contain saturated acyl residues, usually palmitic and stearic acid, with unsaturated chains in the sn-2 position. Polyunsaturated, C_{20:4} and C_{20:6} residues are the most common. Likewise, the most abundant species of PtdIns in the membrane is C_{18:0}C_{20:4}. In vitro studies suggest that PITPa will not preferentially bind these abundant species, but that the protein will display a high affinity for the more minor species, such as C_{16:0}C_{18:2} and C_{18:2}C_{18:2} PtdIns species. It is of note in this regard that both of the later species have been proposed to be highly metabolically active (Nakagawa et al., 1989). Van Paridon and co-workers have demonstrated that the differences between optimally and poorly bound species is larger for PtdCho than for PtdIns, suggesting that the fractional contributions of the acyl chains to the binding affinity are much greater for PtdCho than for PtdIns (Van Paridon et al., 1988b).
1.3.1.b Other factors affecting binding and transfer of a phospholipid by PITPa and PITPβ

Interactions of a PtdIns-TP with the hydrophilic surface of the membrane presumably precede the exchange of endogenous phospholipid for the membrane bound phospholipid. This suggests that membrane properties should influence phospholipid binding. Indeed, steady-state and time-resolved fluorescence spectroscopy and radiolabelled transfer assays have established that phospholipid binding and transfer is affected by such variables as phospholipid composition, pH, vesicle size and curvature (Wirtz, 1991a).

Kinetic analyses of the binding and transfer of various pyrene-labelled phospholipid species by PITPa demonstrated that the incorporation of negatively-charged species such as PtdOH, PtdIns4P and PtdIns(4,5)P₂ into PtdCho-containing vesicles increased the affinity of PITPa for these vesicles, and subsequently decreased pyrene labelled phospholipid transfer (Van Paridon et al., 1988c). While some affinity of a PtdIns-TP for both donor and acceptor vesicles is essential, if the affinity is too great, transfer activity will be reduced or eliminated. Thus it is clear that there is competition between transferable and non-transferable phospholipid species at the vesicle interface.

Using a pyrene-labelled phospholipid, Van Paridon and co-workers have demonstrated that an increase in vesicular pH above the isoelectric point of PITPa appears to decrease the affinity of the protein for negatively-charged vesicles (Van Paridon et al., 1988c). This is primarily due to enhanced vesicle repulsion the large increase in negative charge on PITPa. In contrast, below the isoelectric point of PITPa the transfer activity of PtdCho was diminished as a result of the strong binding of an overall positively charged protein to the negatively charged vesicle surface (Van Paridon et al., 1988c). Divalent cations, amphiphilic amines, such as local anaesthetics, and polyamines also have an effect on the binding and transfer activity of PITPa, presumably as a result of the accumulation of these cationic agents at the interface of the membrane and the protein (Wirtz, 1991a). The effects of membrane charge and pH have been accounted for by changes in the membrane and protein surface charge density, rather than specific protein-phospholipid interactions (Wirtz, 1991a). These observations indicate that the membrane interaction site of PITP is positively charged.

In addition to electrostatic effects, PtdIns-TPs appear to be very sensitive towards membrane curvature and the fluidity of the membrane. Several studies have
demonstrated that PtdIns transfer activity displays a marked preference for a more disordered fluid environment of a liquid crystalline phase than a gel phase (Helmkamp, 1980a; Helmkamp, 1980b; Somerharju et al., 1983). Likewise chemicals that disorder or fluidise membranes, for example ethanol, enhance PITPα activity. The analysis of the influence of planar and curved vesicles on PITPα mediated transfer of radiolabelled PtdIns, has demonstrated that activity is enhanced in the presence of more curved vesicles in comparison with more planar vesicles (Komatsu et al., 2000). The efficiency of van der Waals interactions between lipids will be reduced in highly curved vesicle systems. This will reduce the energy barrier for transfer of the PtdIns in and out of the vesicle membrane. In this regard, the activity of PITPα has been shown to depend inversely on the size of the vesicles employed in the assay system (Wirtz, 1991a). This can be explained by the fact that smaller vesicles exhibit greater local convex curvature, greater head group areas and smaller cohesive forces in the outer leaflet. These observations are a further indication that the physical organisation of lipids in participating membranes is an important aspect of protein catalysed phospholipid transfer.

Kinetic analysis indicates that membrane properties influence transfer by influencing the insertion and desorption rates without significantly affecting phospholipid binding (Wirtz, 1991a). This suggests that the interaction of PITPα, and most probably PITPβ, with a membrane surface and the exchange of an endogenous lipid molecule from the interface, are two separate processes. This two-stage mechanism may involve distinct protein domains, thereby increasing the ultimate specificity and efficiency of the process. It has been suggested that PITPα and PITPβ may initially scan a lipid bilayer for general phospholipid properties such as charge and acyl chain structure, and subsequently effect a sample of neighbouring head groups for high specificity binding. It would be predicted that the initial low-affinity step might trigger the release of the endogenous phospholipid, thereby permitting the headgroup sampling reaction (Rogers and Bankaitis, 2000). That PITPα and PITPβ transfer activity is very sensitive to membrane properties and acyl chain structure, together with the observation that challenge of the transfer protein with an excess of non-substrate lipids effects a specific reduction in the lipid transfer activity, provide support for this model.

Although the conclusions that a number of factors affect the transfer activity of PITPα and PITPβ are drawn from model in vitro systems, they may also apply to the situation in the intact cell. However, it remains difficult to predict relative
affinities and transfer in vivo mainly due to the lack of (1) information regarding the relative abundance of various PtdIns, PtdCho or SM species on the cytoplasmic leaflets of different organelle membranes, (2) a strict correlation between the affinity and rates of transfer of pyrene labelled phospholipids with more natural species and (3) understanding of acyl chain dependent interactions of the phospholipids with other membrane phospholipids and proteins. Nevertheless, it is clear that that the membrane composition and structure can direct protein catalysed movements of phospholipids in and out of biological membranes (discussed in Section 1.4.4).

1.3.2 Tissue distribution and intracellular localisation of PITPα and PITPB

Until recently, the analysis of the tissue and cellular distribution of PITPα and PITPB has been hampered by the apparent lack of specific antibodies that are able to distinguish between the two isoforms. While the published literature describes several antibodies that recognise both isoforms of PITP, few studies have described isoform-specific antibodies. In some studies PITPα specific-antibodies have been employed together with antibodies that recognise both isoforms to successfully analyse the distribution of PITPα and PITPB (De Vries et al., 1995; De Vries et al., 1996).

Northern and immunoblot analyses of various tissues have revealed that PITPα and PITPB are ubiquitously expressed (Rogers and Bankaitis, 2000). Both isoforms are expressed at high levels within the brain, kidney, liver and lung. However, unlike PITPα, PITPB is weakly expressed within the testis (Tanaka et al., 1995; Wirtz, 1991a). While PITPα and PITPB are both expressed at high levels within the brain, in situ hybridisation studies have revealed that the two isoforms exhibit a distinct cellular distribution in the mature nervous system (Imai et al., 1997). The differential distribution of PITPα and PITPB cDNA most probably reflects different functional roles for each of the gene products.

Analysis of the intracellular localisation of PITPα and PITPB has revealed that they are targeted to distinct intracellular sites. Indirect immunofluorescence of either microinjected protein or cDNA, in Swiss 3T3 fibroblasts and heart endothelial cells, have revealed that PITPα preferentially localises to the nucleus and cytoplasm, whereas PITPB is preferentially associated with the perinuclear Golgi system (De Vries et al., 1996). However this localisation is not absolute since low levels of PITPB are detected within the cytoplasm and the nucleus. Furthermore, neither isoform was found associated with the plasma membrane (De Vries et al., 1996). The
mechanism by which PITPβ is normally localised to the Golgi in mouse fibroblasts, while the more abundant PITPα localises to the cytoplasm and the nucleus has not been identified.

Immunocytochemistry using an anti-PITPα polyclonal antibody has revealed that within the rat lung, PITPα is abundantly expressed within the non-ciliated bronchial epithelial cells (Clara cells; Ibrahim and Funkhouser, 1997). The Clara cell is a non-ciliated secretory cell containing abundant secretory granules in the apical regions of the cell. No PITP immunoreactivity was detected in alveolar type-2 cells, which have a major role in the synthesis and selective sorting of phospholipids into lamellar cell bodies (Ibrahim and Funkhouser, 1997).

In contrast to studies on Swiss 3T3 fibroblasts and heart endothelial cells, further investigation of the Clara cells by electron microscopy revealed that PITPα accumulated at the apical surface plasma membrane and around the limiting membranes surrounding secretory granules (Ibrahim and Funkhouser, 1997). The authors proposed that the accumulation of PITPα at the plasma membrane probably represent a transient interaction (Ibrahim and Funkhouser, 1997). This is because the protein is considered to be a cytosolic protein on the basis of its purification and from soluble fractions in cells (Helmkamp et al., 1974).

By immunofluorescence microscopy, Wirtz and co workers have investigated the intracellular localisation of PITPα in Swiss 3T3 cells under conditions of agonist induced activation (Snoek et al., 1993a). The stimulation of cells by PMA or bombesin, which activate PKC and PLC dependent PtdIns(4,5)P₂ hydrolysis, respectively, resulted in a redistribution of cytoplasmic PITPα to the Golgi membranes. The same group has subsequently demonstrated a similar translocation of PITPα to the Golgi in PMA or EGF-stimulated NIH3T3 cells (van Tiel et al., 2000a).

Immunoprecipitation of PITPα from prelabelled Swiss 3T3 cells revealed that stimulation by PMA significantly increased the phosphorylation of PITPα (Snoek et al., 1993a). However, no significant increase in phosphorylation was observed after the stimulation of these cells by bombesin. While, Wirtz and co-workers demonstrated that PITPα is a substrate for PKC in vitro (Snoek et al., 1993a), it remains to be demonstrated whether PMA stimulation increases phosphorylation through the activation of PKC or whether an alternative enzyme, such as MAP kinase, is involved. Nevertheless, since protein phosphorylation is known to affect the activity and localisation of many proteins, the authors proposed that the PMA-
mediated phosphorylation of PITPα might induce the translocation of this protein to the Golgi membrane (Van Tiel et al., 2000a and Section 1.6). A possible role for this translocation is discussed later (Section 1.6).

1.4 Cellular roles of PITP

Ptdlns-TPs have historically been described as proteins that constitutively catalyse the exchange of phospholipid monomers between membrane bilayers, thereby facilitating lipid distribution within cells. Recent data have identified an acute requirement for Ptdlns-TPs during several essential cellular processes. Work from several groups has provided the first insights into the physiological roles of the metazoan Ptdlns-TPs. The data implicate Ptdlns-TPs as potentially important regulators of Ptdlns metabolism, mammalian vesicle trafficking and receptor mediated signal transduction. To adequately describe what is currently known about PITPα and PITPB it is necessary to digress slightly to provide a brief summary of Ptdlns metabolism, phosphoinositides and Phosphoinositide kinases.

1.4.1 The phosphoinositide cycle

Although Ptdlns represents only a small percentage of the total cellular phospholipid, this phospholipid plays a crucial role in signal transduction as the precursor of several second messengers. The first established phosphoinositide signalling pathway was described in the 1980's and involves the agonist induced sequential phosphorylation of Ptdlns to PtdIns4P and then PtdIns(4,5)P₂ by phosphoinositide kinases at the plasma membrane (Berridge, 1983; Michell et al., 1981; Streb et al., 1983 and Figure 1.2). PtdIns(4,5,)P₂ is subsequently cleaved by phospholipase C (PLC) to generate the second messengers DAG and inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃), which are involved in the activation of PKC and the release of Ca²⁺ from the ER, respectively. DAG can be phosphorylated to PtdOH thereby terminating signalling through PKC, while Ins(1,4,5)P₃ can be metabolised to generate a vast number of inositol polyphosphates. Inositol is produced by the action of various Ins phosphatases, and this is used together with PtdOH to regenerate Ptdlns within the ER. This pathway is considered to form a cycle within which key intermediates are exploited for their biological signalling ability (reviewed by Berridge, 1983; Hsuan and Tan, 1997). There is some evidence that this cycle defines a closed pool or compartment.
Figure 1.2 The phosphoinositide cycle

Ins(1,4,5)P$_3$ and DAG generated by the hydrolysis of PtdIns(4,5)P$_2$ can be used to resynthesise PtdIns via Ins and the activated precursor CDP-DAG, a reaction catalysed by PtdIns synthase. The scheme shown is simplified for the sake of clarity and it should be noted that several of the steps shown above are known to take place in distinct subcellular compartments. Inositol phosphates are also known to undergo a variety of phosphorylation and dephosphorylations which are beyond the scope of this thesis. Figure adapted from Hsuan and Tan, 1997.
of phosphoinositide metabolites, termed the agonist-sensitive pool. This agonist-sensitive pool is somehow distinguished from an agonist-insensitive pool, even during prolonged signalling. However, the physical nature of these compartments is not understood and in other studies their existence was not apparent (reviewed by Monaco and Gershengorn, 1992). Mechanisms of the metabolic compartmentation of the phosphoinositides are considered later (Section 1.4.4).

1.4.1.a Phosphoinositide kinases

Phosphorylation at the D3, D4 and D5 hydroxyls of the inositol head group of PtdIns, by various phospholipid kinases generates a diverse array of phosphoinositides, each with a unique function. A vast body of work has accumulated over the past forty years on the properties of phosphoinositides and the kinases involved in their synthesis, however due to length constraints and the scope of this thesis, it is impossible to give a full review of the literature. Therefore, the present discussion will be a brief summary of the current knowledge regarding the properties and roles of the various phosphoinositides and kinases. Where possible reviews will be cited.

Many phosphoinositide kinases were initially described as enzymatic activities capable of transferring a phosphate to a particular position on the inositol head group of PtdIns or one or more of its phosphorylated derivatives. Studies of these purified enzymes led to the characterisation of the PtdIns and phosphoinositide kinases into three general families: phosphoinositide 3-kinases, PtdIns 4-kinases and PtdIns-phosphate (PtdInsP) kinases (reviewed by Fruman et al., 1998; Martin, 1998).

The cloning of genes encoding several members of these families has accelerated research into this area. Subsets of each family have been identified in yeast and lower eukaryotes; each shares substantial protein sequence homology with its mammalian counterpart. Early purification studies have showed that the PtdIns 4-kinase family consists of multiple isoforms that were biochemically characterised as type II or type III PtdIns 4-kinases. Type II PtdIns 4-kinases were defined as single-subunit, membrane-bound enzymes whose lipid kinase activity was inhibited by adenosine and stimulated by non-ionic detergents (Fruman et al., 1998). Although type II PtdIns 4-kinase activity is the most potent PtdIns 4-kinase in most cell types, the gene encoding this enzyme has yet to be cloned. Type III PtdIns 4-kinases were defined as membrane-bound enzymes whose activity is relatively resistant to adenosine and is maximally activated in non-ionic detergent (Fruman et al., 1998).
Additionally, two other PtdIns 4-kinases have been cloned, whose characteristics do not strictly follow the typing described above and have therefore been called PtdIns 4-kinase α and PtdIns 4-kinase β (Fruman et al., 1998).

The phosphoinositide 3-kinase family consists of three distinct classes, each class being defined on the basis of their primary structure, regulation and in vitro lipid substrate specificity. Depending on the class, the phosphoinositide kinases phosphorylate the D3-position of the inositol moiety of PtdIns, PtdIns4P and PtdIns(4,5)P₂. Phosphoinositide 3-kinase activity has been implicated in multiple cellular events, including cell proliferation and survival, metabolism, cytoskeletal reorganisation and membrane trafficking (reviewed by Toker, 2000).

Two distinct families of PtdInsP enzymes have been purified on the basis of their ability to produce PtdIns(4,5)P₂. Until recently, both families (type I and type II) were believed to be PtdIns4P 5-kinases. However, it is now firmly established that only type I isoform possesses PtdIns4P 5-kinase activity. They have also been found to phosphorylate the D5’ position of phosphatidylinositol 3-phosphate (PtdIns3P) in vitro (Fruman et al., 1998). The type II isoform is actually a PtdInsP 4-kinase and phosphorylates the D4- hydroxyl of PtdIns5P and PtdIns3P. Although the type I isoform is likely to be a bona fide constituent in the classical biosynthetic pathway for the formation of PtdIns(4,5)P₂, the type II isoform may catalyse an alternative mode of PtdIns(4,5)P₂ synthesis from PtdIns5P and/or catalyse PtdIns(3,4)P₂ synthesis from PtdIns 3P (Fruman et al., 1998).

Phosphoinositides are localised to the cytoplasmic face of cellular membranes where they can act as substrates for phosphoinositide kinase, phosphatase, PLC and PLD isozymes. Additionally, in particular PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ are now recognised as signalling molecules in their own right (reviewed by Czech, 2000; Hsuan et al., 1998; Martin, 1998). For example PtdIns(4,5)P₂ can directly modulate the in vitro activity of a number of signalling enzymes, including PKC, PLD, and casein kinase I, and antagonise ATP inhibition of ATP-sensitive potassium channels (Shyng and Nichols, 1998; reviewed by Hsuan et al., 1998). Furthermore, phosphoinositide-binding sites are found on a range of cellular proteins, including cytoskeletal and actin-binding proteins and modules that allow protein-phospholipid interaction, such as SH2 and PH domains (reviewed by Hsuan et al., 1998). Thus, PtdIns(3,4,5)P₃ and PtdIns(4,5)P₂ act as site-specific signals on membranes that recruit and/or activate proteins for the assembly of spatially-localised, functional complexes. It is clearly established that
phosphoinositides are involved in the regulation of diverse cellular responses including proliferation, survival, cytoskeletal organisation, and vesicle trafficking, glucose transport and platelet function (reviewed by Hsuan et al., 1998). As such, it is necessary to identify and characterise the many proteins that are involved in determining the balance of different phosphoinositides within the cell.

1.4.2 PITPs: a requirement in PLC-mediated signal transduction

One long standing speculation regarding PtdIns-TP function has been that these proteins might serve to replenish PtdIns stores in the plasma membrane that are subject to depletion as a result of membrane signalling events (Michell, 1975). A body of work directed at a reconstitution of trans-plasma membrane signalling now provides strong experimental evidence to support a role of PITP in transmembrane signalling.

A large number of extracellular signals stimulate the hydrolysis of PtdIns(4,5)P_2 by inositol lipid-specific phospholipase C. To date, three classes of mammalian PtdIns(4,5)P_2-specific PLCs have been identified and are all differentially regulated (reviewed by Katan, 1998; Rhee and Bae, 1997). The PLCβ family are activated by interaction with the subunits of pertussis toxin insensitive G_q family of heterotrimeric G-proteins. The G protein coupled receptors that are known to utilise this pathway include those for bradykinin, muscarinic and α1 adrenergic receptors. PLCβ isoymes are also activated by the βγ subunits of pertussis-toxin sensitive G proteins from the G/G_α family. The m2 and m4 muscarinic acetylcholine receptors, fMetLeuPhe receptor and interleukin 8 receptor appear to be linked to the Gβγ/PLCβ pathway. PLCγ isoymes are regulated by receptor and non-receptor linked tyrosine kinases. Agonists of the PLCγ pathway include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), nerve growth factor (NGF) and T-cell receptor agonists and ligands. PLCδ is regulated by micromolar concentrations of Ca^{2+} however the physiological role and regulation of this PLC isoform is poorly understood (Katan, 1998).

Thomas et al. (1995) demonstrated that following permeabilisation of HL60 cells, GTP-binding protein (G-protein) mediated PLCβ signalling could not be activated by the addition of exogenous PLCβ, GTPγS, and/or the ligand fMetLeuPhe (Thomas et al., 1993). When rat brain cytosol was also added back to these permeabilised cells PLCβ responsiveness to GTPγS was restored, clearly suggesting that a novel cytosolic factor(s) was required for G-protein-regulated PLC signalling.
Purification of a single 35kDa cytosolic factor from bovine brain cytosol and tryptic peptide sequencing experiments revealed that this factor was PITPα (Thomas et al., 1994). Cell reconstitution studies have also demonstrated that PITPα is a critical cytosolic factor required for EGF-dependent PLCγ activity in A431 cells and IgE-dependent PLCγ1 activity in RBL-283 cells (Cunningham et al., 1996; Kauffmann Zeh et al., 1995). More recent permeabilisation studies have revealed that the Ca²⁺-stimulated activity of PLCδ was also greatly enhanced in the presence of PITPα (Allen et al., 1997).

The requirement for PITPα during agonist induced PLCβ-, PLCγ- and PLCδ-catalysed PtdIns(4,5)P₂ hydrolysis suggests that PITPα functions at a common point in these differentially-regulated signalling pathways. PITPα, PITPβ and yeast Sec14p are equally effective in restoring PLCβ and PLCγ signalling in HL-60 and RBL-2H3 cells, respectively (Cunningham et al., 1996). Since the PtdIns and PtdCho binding and transfer activity is the common feature shared by all three PtdIns-TPs, it has been suggested that this is the relevant activity that determines their abilities to restore PLC signalling. Thus, it has been proposed that one possible function of PITPα in PLC signalling is in the provision of PtdIns for the synthesis of PtdIns(4,5)P₂ (Kauffmann Zeh et al., 1995). Exploring this concept, it is possible that the activities of other plasma membrane enzymes utilising PtdIns(4,5)P₂ as a substrate also depend on PITPα or PITPβ. Indeed, a recent reconstitution study has demonstrated a requirement for PITPα in the phosphorylation of PtdIns(4,5)P₂ to PtdIns(3,4,5)P₃ by phosphoinositide 3-kinase, following stimulation of the G-protein coupled fMetLeuPhe receptor in neutrophils (Kular et al., 1997).

1.4.2.a Mechanisms of action during PLC-mediated signal transduction

The deduction that PITP is involved in the provision of PtdIns for the synthesis of PtdIns(4,5)P₂ and other phosphoinositides has not been questioned. However, different empirical systems have produced different conclusions regarding the specific function of PITPα at the plasma membrane, and the mechanism remains controversial (reviewed by Cockcroft, 1998; Kearns et al., 1998a).

PITPα might simply function in a passive supply capacity, that is, PITPα might effect a sustained influx of PtdIns to a membrane that is consuming a limited supply of phosphoinositide (the replenishment model; Currie et al., 1997). As the major site of synthesis in most cell types, the ER would be expected to provide the
main source of PtdIns for transfer. A distinguishing prediction of the replenishment model is that PITPα supports sustained PLC mediated hydrolysis of PtdIns(4,5)P_2 but does not affect the initial rate of PtdIns(4,5)P_2 hydrolysis. Using a heterologous turkey erythrocyte ghost preparation, Downes and co-workers have provided support for the replenishment model (Currie et al., 1997). It was observed that the initial rate of agonist-induced PtdIns(4,5)P_2 hydrolysis in erythrocyte ghosts was not influenced by ectopic PITPα (Currie et al., 1997). Furthermore, while PITPα did not enhance initial PtdIns 4-kinase activity, it did increase the unstimulated steady-state, levels of both PtdIns4P and PtdIns(4,5)P_2 by a catalytic mechanism. Based on these observations, the authors proposed that in turkey erythrocyte ghosts, PITPα acts by catalytically transferring PtdIns down a chemical gradient so as to replenish PtdIns depleted sites within the cell (Currie et al., 1997). However, it should be noted that turkey erythrocyte ghosts do not represent a typical eukaryotic cell since these cells are known to synthesis PtdIns within the plasma membrane (Vaziri and Downes, 1992).

Alternatively PITPα might define the pool of PtdIns that will become the substrate for phosphoinositide-specific PLC activities. Specifically, PITP may act as a cofactor during signalling to specifically present PtdIns to a plasma membrane PtdIns 4-kinase for which the PtdIns bound to PITPα is the preferred substrate (the cofactor model; Cockcroft, 1998; Hsuan, 1993; Hsuan and Tan, 1997). Based on this hypothesis and the inability of PtdIns-TP to transport polyphosphoinositides, although they are strongly bound (Section 1.3.1), a model has been proposed in which phosphoinositides remain bound to PITPα after their formation. PITPα sequentially then presents substrate to downstream phosphoinositide kinases and to PLC (Cockcroft, 1998; Hsuan, 1993; Hsuan and Tan, 1997). In addition to allowing efficient and rapid signalling, this model provides a partial biochemical explanation for the apparent compartmentation of phosphoinositides into agonist sensitive and agonist insensitive pools (Section 1.4.4).

There are several experimental observations that point to the cofactor model of PITP activity. Firstly, cytosol-depleted HL60 cells which have lost their endogenous PtdIns-TP are capable of producing a weak but sustained Ins(1,4,5)P_3 increase in response to GTPγS (Cunningham et al., 1995). The addition of exogenous PLCβ to the same cells increases the rate of PtdIns(4,5)P_2 hydrolysis suggesting that the rate of hydrolysis is determined by the amount of PLCβ. Furthermore, addition of exogenous PITPα greatly enhances the initial rate of PtdIns(4,5)P_2 hydrolysis.
(Cunningham et al., 1995). Taken together these observations may suggest that the main effect of PITPα is to increase the rate hydrolysis at all concentrations of PLCβ isozymes and are inconsistent with the replenishment model. Further evidence that lipid transfer activity is insufficient to account for PLC reconstitution comes from analysis of carboxy-terminal truncation mutants. Systemic deletion of the carboxy-terminal of PITPα revealed that removal of just five amino acids is sufficient for the complete loss of function in PLC signalling (Hara et al., 1997). However, this deletion did not significantly alter phospholipid-binding properties. Moreover, bulk PtdIns transfer is reduced by only 30% and full transfer of PtdCho was maintained, although higher concentrations of protein were required (Hara et al., 1997). The dominant negative nature of this mutant was interpreted to result from the interaction of the mutant with a putative receptor for PITP (Hara et al., 1997). However, the effects of the truncation mutant may also be due to strong non-specific ionic interactions between the mutated protein and the membrane (Tremblay et al., 1998).

The co-factor model gained additional support from native immunoprecipitation experiments using cell lysates prepared from intact A431 cells. These studies demonstrated that PITPα associates with EGF receptor, type II PtdIns 4-kinase and PLCγ in an agonist dependent manner (Kauffmann Zeh et al., 1995). This has lead to the proposal that PITPα, PtdIns 4-kinase and PLC are components of multi-enzyme signalling complexes formed during G-protein and receptor tyrosine kinase signalling (Kauffmann Zeh et al., 1995). Subsequent immunoprecipitation experiments demonstrated that PITPα also associates with a PtdIns 3-kinase, Vps34, in Jurkat cells (Volinia et al., 1995).

However the observation that PITPα, PITPβ and Sec14p were found to be essentially interchangeable in a reconstituted signalling assay limits a convincing argument for the cofactor role (Cunningham et al., 1996). These would be unexpected results if PITPα were to enter into a specific physical interaction with multi-component signalling complexes. Nonetheless, taken together with the lack of any apparent binding domain, it is possible that putatively specific interactions with the PtdIns 4-kinase at the plasma membrane or with Vps34 at the post Golgi membrane is primarily due to the binding of cytosolic substrate.

More recently, Downes and co-workers have investigated the compartmentation of phosphoinositides in intact and permeabilised 1321N1 astrocytoma cells (Batty et al., 1998). These studies have provided evidence for integrated PtdIns pools and have led to the proposal of a targeted replenishment
model for PITP activity. In this model, compensatory PtdIns synthesis occurs across an intracellular substrate gradient, with resynthesis being most prominent near sites of PtdIns depletion, as suggested previously by the same group in the replenishment model (Currie et al., 1997). As in the replenishment model, PITP has an essential role in the ensuing rapid equilibration of PtdIns between intracellular membranes and the plasma membrane. However, in contrast to the replenishment model, PITP is postulated to function in the cycle by efficiently targeting PtdIns somehow to sites of depletion at the plasma membrane and elsewhere, possibly by direct interaction with receptor or lipid kinase complexes (Batty et al., 1998). This would ensure the supply of substrate to PtdIns dependent pathways in the plasma membrane and perhaps elsewhere. This possibility is in agreement with the observations that PITPa might associate with activated signalling complexes and with the truncation studies described above.

Models invoking phospholipid presentation suggest that PITP functions only at its site of membrane association and thus the cytosolic PITP pool represents a disengaged reservoir of protein that is not a functional reaction intermediate. These models have led to the proposal of yet another paradigm for phospholipid-presentation. Bankaitis and colleagues suggested a liftase model for PITP function (Kearns et al., 1998c). According to this model PITP stimulates phosphoinositide synthesis by lifting the PtdIns monomer, thereby supposedly rendering the headgroup of the PtdIns molecule a good substrate for modification by a PtdIns kinase (Kearns et al., 1998c). While there is no direct evidence to suggest that PITP does indeed function as a liftase, precedent for such a mechanism is provided by function studies of the ganglioside GM$_2$ activator protein (Meier et al., 1991). This protein is an obligate cofactor for $\beta$-hexosaminidase-A-mediated degradation of sphingolipid in mammalian lysosomes (Meier et al., 1991). The studies of Meier and co-workers suggest that the role of the activator protein appears to be to lift a GM$_2$ monomer away from the plane of the bilayer (in a manner that does not involve desorption from the bilayer) such that GM$_2$ is rendered susceptible to attack by $\beta$ hexosaminidase A (Meier et al., 1991).

Thus, there are currently several unresolved views of how PITPa and possibly PITPB stimulate phosphoinositide reactions in vitro. Although the studies considered above clearly illustrate an essential requirement for PITPa and PITPB during PLC signalling, the cellular preparations or assay conditions that they employ are likely to compromise endogenous PtdIns metabolism. Consequently the exact
role and mode of action of PITPa and PITPβ during agonist-induced PLC hydrolysis of PtdIns(4,5)P₂ remain to be determined. Likewise, the exact mechanism by which PITP is targeted to metabolically active sites within the membrane requires clarification. Several studies suggest the existence of a PITP "receptor" in the form of a lipid kinase or a signalling complex. However, the marked sensitivity of PITP to membrane structure (Section 1.3.1.b), provides an alternative mechanism by which PITP could direct the transfer of phospholipids between sites of de novo synthesis and metabolism and during increased turnover of PtdIns in a variety of stimulated cells.

Finally, it is of note that the distinct localisation of PITPa and PITPβ appears to be at odds with the observations that both forms are equally effective in PLC reconstitution studies. Furthermore, while Ibriam et al. (1997) do demonstrate that PITPα does localise to the plasma membrane in clara cells, further studies are required to identify a plasma membrane localisation. It is possible that a combination of phospholipid binding specificity and intracellular localisation would allow specificity of the system.

1.4.3 PITP and vesicle trafficking

Inositol phospholipids have been implicated in membrane trafficking events that occur constitutively in all eukaryotic cells, as well as those associated with the regulated secretion mediated by secretory granules and synaptic vesicles. Specifically the machinery operating these membrane traffic steps has been shown to include phosphoinositide kinases, phosphatases and PITPs. Due to the complexity of this field, only vesicular processes that have been demonstrated to require PITPα and PITPβ will be discussed. (For a comprehensive review of membrane trafficking and the roles of the phosphoinositides in membrane trafficking see Martin, 1998).

1.4.3.a A role for PITPα and β in exocytosis

Ca²⁺-activated secretion can be considered in two sequential steps: (1) an ATP-dependent priming of secretory granules for fusion to the plasma membrane and (2) a Ca²⁺-dependent, ATP-independent fusion event, which have been biochemically resolved (Martin and Walent, 1989). Martin and co-workers have successfully reconstituted the Ca²⁺-activated secretion of norepinephrine from dense core granules, in permeabilised PC12 cell (Hay and Martin, 1993; Martin and Walent, 1989). Efficient release of norepinephrine required Ca²⁺ and cytosol, and ATP to a lesser extent. Three cytosolic factors were identified and designated
priming in exocytosis proteins (PEP1-3). Purification of the PEP3 priming factor from rat liver cytosol lead to the identification of this protein as PITPa (Hay and Martin, 1993). The PEP1 factor was found to co-purify with PtdIns4P 5-kinase activity and was subsequently identified as the type I PtdIns4P 5-kinase (Hay et al., 1995). The characterisation of PEP1 and PEP3 provided strong evidence that the generation of PtdIns(4,5)P₂ is required for the priming event. The authors proposed that PITPa specifically co-operates with type I PtdIns4P 5-kinase to prime regulated exocytosis by catalysing the synthesis of PtdIns(4,5)P₂ (Hay et al., 1995). In agreement, recent immunocytochemical studies with PtdIns(4,5)P₂-specific antibodies conducted with permeabilised PC12 cells indicated that a high concentration of PtdIns(4,5)P₂ is synthesised on secretory granule membranes during ATP-dependent priming (Martin, 1998). The collective data suggest that the priming event represents the synthesis of a PtdIns(4,5)P₂ pool and that intact PtdIns(4,5)P₂ acts as a cofactor during the priming step, rather than a derived metabolite (Martin, 1998).

Additionally, there is evidence to suggest that PtdIns(4,5)P₂ functions in the regulated exocytosis of granules in hematopoietic cell (Fensome et al., 1996). Hexosaminidase secretion in neutrophil-related HL60 cells becomes refractory to GTPγS after cytosol depletion. Exocytosis was restored upon the addition of recombinant PITPa or PITPB and the small G-protein, ARF (Fensome et al., 1996). One possible explanation for the ARF requirement is that this protein stimulates PtdIns(4,5)P₂ via phospholipase D (PLD) production of PtdOH and stimulation of PtdIns4P 5-kinases. The authors proposed that ARF and PITPa or PITPB both restore exocytosis by promoting PtdIns(4,5)P₂ synthesis (Fensome et al., 1996).

Based on the observations that PLD and type I PtdIns(4)P 5-kinase are regulated by PtdOH and ARF1, respectively, a model has been proposed whereby the biosynthesis of PtdIns(4,5)P₂ and the activation of PLD by ARF1 may be coordinated in the regulated exocytosis of coated vesicle (Liscovitch and Cantley, 1995). The concerted action of PITP and resident phosphoinositide kinases on the membrane is proposed to generate PtdIns(4,5)P₂. This PtdIns(4,5)P₂ effects the stimulation of a dedicated guanine-nucleotide exchange factor that drives ARF to its GTP form. According to this model, coated vesicles containing GTP-bound ARF and a PtdIns 4-kinase interact with acceptor membranes containing PLD and PtdIns4P 5-kinase. A positive feedback loop is initiated in which PtdOH stimulates PtdIns4P 5-kinases activity to provide a transient pool of PtdIns(4,5)P₂ for further
stimulation of PLD. This positive feedback loop will rapidly lead to the generation of negatively charged phospholipids at the intermembrane interface, leading to membrane fusion. The activity of the system has been proposed to be regulated by the activation of a PtdIns(4,5)P_2-dependent ARF-GTPase–activating protein or by a phosphoinositide phosphatase (Liscovitch and Cantley, 1995). Inactivation of ARF results in the inactivation of PLD and the uncoating of the docked transport vesicle, thereby allowing for fusion with acceptor membranes. According to this model, phosphoinositide biosynthesis influences vesicle trafficking by affecting the proteins that regulate vesicle formation and by affecting the docking of accessory proteins. Although this model encompasses some of the known in vitro properties of participating proteins and provides a basis for generating a spatially-restricted membrane budding site (reviewed by Martin, 1998), more evidence is required to support the existence of this model in vivo.

1.4.3.b A role for PITPα and β in vesicle biogenesis

The demonstration that PITPα is a priming factor for Ca^{2+}-regulated exocytosis is consistent with the function of Sec14p in that it identifies an execution point for mammalian PITPs at a late stage of the secretory pathway. However, the nature of these two execution points differ in that, while Sec14p is involved in the biogenesis of Golgi-derived secretory vesicles, PITPα is involved in preparing secretory granules for fusion with the plasma membrane. Studies by Ohashi, Wieland and co-workers extended the role of PITP in exocytosis by identifying a critical requirement for PITP in the biogenesis of secretory vesicles and immature secretory granules on the surface of trans-Golgi-network (TGN) membranes in neuroendocrine cells (Ohashi et al., 1995). Analysis of fractionated cytosol from adrenal medulla isolated two distinct fractions, CAST1 (Cytosolic Activity Stimulating TGN vesicle formation) and CAST2, that could fully restore biogenesis of both constitutive secretory vesicles and immature secretory granules in a permeabilised PC12 cells. CAST1 was identified as both PITPα and PITPβ (Ohashi et al., 1995). However, the identity of CAST2 remains unresolved although it has been suggested to contain a cytosolic PtdIns4P 5-kinase (Ohashi et al., 1995).

Further studies have demonstrated that the formation of both regulated and constitutive TGN-derived vesicles in PC12 cells, is stimulated by PLD and PITP in an additive fashion that is inhibited by geneticin, which binds to PtdIns(4,5)P_2 (Tuscher et al., 1997). Although PITPα clearly play a role in PtdIns(4,5)P_2
biosynthesis during regulated exocytosis, it is not clear whether it plays such a role in the formation of TGN-derived secretory granules and vesicles. However, the localisation of various PtdIns 4-kinases at the Golgi membrane and the apparent dependence of TGN vesicle formation on ATP, support a role for PITP in supplying substrate for phosphorylation during the formation of TGN derived vesicles (Martin, 1998).

Using a hepatocyte cell-free system, Howell and colleagues have identified another cytosolic factor, a novel PtdIns-specific PtdIns 3-kinase that acts synergistically with PITPa or PITPB to stimulate the production of poly IgA receptor containing exocytic vesicles from TGN membranes (Jones et al., 1998). The authors proposed that PITP and PtdIns-3 kinase generate a specific pool of PtdIns3P that somehow promotes vesicle biogenesis (Jones et al., 1998).

The yeast Vps34 gene encodes a PtdIns 3-kinase that is essential for protein transport from the late Golgi apparatus to the vacuole. Inhibitors of PtdIns 3-kinases in mammalian cells cause specific defects in TGN to prelysosomal compartment trafficking, resulting in the defective exocytosis of lysosomal proteins (Stack et al., 1995). A mammalian homologue of Vps34 has been cloned and characterised (Volinia et al., 1995). The combined use of PtdIns 3-kinase inhibitors and biochemical approaches has demonstrated that human Vps34 plays a direct role in recruiting the early endosomal autoantigen proteins (EEA1) to membranes (Patki et al., 1997). PITPB and to a lesser extent PITPa have been shown to associate with human Vps34p (Volinia et al., 1995). However, while PITPa co-immunoprecipitate with Vps34 in Jurkat cells (Volinia et al., 1995), it remains to be shown whether the function of human Vps34p is dependent on PITPa or PITPB activity.

It has been proposed that the biosynthesis of PtdIns(4,5)P2 and the activation of PLD by ARF1 may interact in a co-ordinated mechanism (Section 1.4.3.a), during vesicle budding from the TGN. However, an impressive body of evidence has been obtained that indicates that ARF stimulates Golgi PtdIns(4,5)P2 synthesis by recruiting PtdIns 4-kinase to the Golgi membrane where it acts to generate a PtdIns(4,5)P2 in a PLD independent manner (Godi et al., 1998).

Taken together the biochemical and genetic data suggest that PITPa and PITPB play a dual role in vesicular transport, affecting both the formation and consumption of vesicles. These conclusions are consistent with the observation that PITPB preferentially localises to the Golgi. Although the exact function and execution point of PITPa and PITPB in these processes remains unclear, it is possible that PITP may
act by promoting the remodelling of the phospholipid content of the plasma membrane. By increasing the synthesis of PtdIns(4,5)P$_2$ or other phosphoinositides or by modulating the abundance of a specific phospholipid pool, containing for example, DAG or PtdCho, PITP could alter the physical properties of the membrane or generate binding sites for the recruitment of heterologous proteins.

1.4.3.c The roles of phosphoinositides in vesicle trafficking

Collectively, the studies presented above suggest that phosphoinositide kinases and the phosphoinositides that they produce participate in regulating vesicle trafficking. The immediate downstream effectors of the phosphoinositides remain unclear. However, the regulated production of phosphoinositides may be important for determining the local phospholipid composition of specific subcellular membranes. Although there have been few direct studies of the biophysical effects of PtdIns(4,5)P$_2$ in membrane bilayers, it is likely that the highly-charged hydrophilic headgroup alters the physical properties of a membranes that are otherwise antagonistic to fusion. The strong positive curvature imparted to the membrane might destabilise a stalk intermediate envisioned for bilayer fusion (Chernomordik, 1996). Alternatively, PtdIns(4,5)P$_2$ and other phosphoinositides may be required for the recruitment of proteins to the site of budding, priming or fusion. In this regard, a number of proteins that function in the constitutive and regulated secretory pathway bind to PtdIns(4,5)P$_2$ and PtdIns(3,4,5)P$_3$ in vitro (reviewed by Hsuan et al., 1998). Among these proteins are ARF-1, several guanidine nucleotide exchange factors including ARNO and Sec7p, α-COP1 vesicle coat protein, dynamin, synaptojanin and CAPS145 (Hsuan et al., 1998). As an example, during Ca$^{2+}$-regulated exocytosis in PC12 cells, it has been proposed that PtdIns(4,5)P$_2$ serves to recruit CAPS145 to membranes for the Ca$^{2+}$-dependent fusion event (Loyet et al., 1998). This recruitment may allow PtdIns(4,5)P$_2$-mediated regulation of the fusagenic properties of CAPS145 (Loyet et al., 1998). If so it follows that the sites at which any of these given proteins interact would be influenced by the activity of lipid kinases that increase the concentration of the respective phosphoinositide ligand. In addition to acting in translocation, phosphoinositides may influence the activity of various proteins, for example PtdIns(4,5)P$_2$ stimulates the nucleotide exchange by ARNO on ARF in vitro (Paris et al., 1997).

The cytoskeleton is crucial for the efficient and polarised transport of vesicles in intracellular membrane-sorting pathways (reviewed by Kamal and Goldstein,
2000). In addition to associating with other elements of the vesicle trafficking machinery, phosphoinositides, in particular PtdIns(4,5)P$_2$ may associate with the actin cytoskeleton. Indeed recent evidence suggests that ARF enhances the association of a specific form of β-spectrin (βIΣ*) to the Golgi membrane by increasing PtdIns(4,5)P$_2$ levels (Godi et al., 1999).

### 1.4.4 The metabolic compartmentation of the phosphoinositides

It is likely that phosphoinositides and the proteins that they recruit are localised at focal sites in specific membranes. Phosphoinositides and the phosphoinositide kinases are not uniformly distributed in cellular membranes and there is evidence that phosphoinositides are metabolically compartmentalised (Monaco and Gershengorn, 1992). Several mechanisms have been proposed to allow the compartmentation of agonist-sensitive and insensitive phosphoinositide pools including compartmentation by substrate recognition and the segregation of substrates and signalling enzymes into so-called membrane rafts (reviewed by Hsuan et al., 1998). The proposed role of PtdIns-TPs in many phosphoinositide-dependent cellular activities indicates that these proteins may also play a critical role during the metabolic compartmentation of the phosphoinositides.

*In vitro* biochemical characterisation of multiple PtdIns 4-kinase, PtdIns 5-kinase, PLC and PKC activities has revealed distinct preferences in the mode of substrate presentation (reviewed by Fruman et al., 1998; Katan, 1998; Mellor and Parker, 1998). Furthermore, different isoforms of each of these enzymes possess distinct subcellular localisations, and modes of regulation. These observations may provide clues to the *in vivo* specificities of these enzymes and has led to the suggestion that a specific mode of substrate presentation *in vivo* may delimit an agonist–sensitive pool of substrate. The observed differential expression, lipid preference and subcellular localisation of the different PtdIns-TP isoforms suggest that these proteins may also facilitate the compartmentation.

The existence of glycerosphingolipid- and cholesterol-rich microdomains or raft within the plasma membrane has recently been described (Waugh et al., 1999; Waugh et al., 1998; reviewed by Kurzchalia and Parton, 1999). In the past few years, these cholesterol-rich plasma membrane domains have been implicated as organising centres for signalling molecules. The list of signalling molecules apparently localised to these various domains is ever increasing and includes Src family kinases, nitric oxide synthase, EGF and PDGF receptors, PLC$_\gamma$, PKC$_\alpha$, PKC$_\beta$, ...
ras and heterotrimeric G-protein Ga subunits (Kurzchalia and Parton, 1999). Furthermore, low buoyant density rafts derived from the plasma membrane have been found to contain high local concentrations of PtdIns, PtdIns4P, PtdIns(4,5)P$_2$ and type II PtdIns 4-kinase activity (Liu et al., 1998; Pike and Casey, 1996; Pike and Miller, 1998; Waugh et al., 1998). A smaller pool of PtdIns is localised to cholesterol-rich, 50-100nm invaginations of the plasma membrane termed caveolae, which have a buoyancy and detergent insolubility that is characteristic of membrane rafts termed DIGS (Pike and Casey, 1996). Based on these observations, it has been proposed that PtdIns4P, PtdIns(4,5)P$_2$ and agonist-stimulated hydrolysis may be highly compartmentalised into cholesterol-rich plasma membrane domains (Pike and Casey, 1996). A functional link between membrane rafting and signal transduction is supported by the observation that disruption of membrane rafts by the removal of cholesterol led to the loss of receptor-dependent PtdIns(4,5)P$_2$ hydrolysis (Pike and Miller, 1998).

While, uncertainty exists with regard to the nature of membrane microdomains in which agonist-induced PtdIns(4,5)P$_2$ biosynthesis and hydrolysis takes place, the inference from a number of studies is that rafts, rather than the bulk ER and plasma membrane contain the vast majority of PtdIns (reviewed by Kurzchalia and Parton, 1999). As such the apparent compartmentalisation of agonist-induced PtdIns(4,5)P$_2$ hydrolysis suggests an acute requirement for PtdIns-TPs, in the flux of PtdIns between membrane rafts during signalling. Indeed, Waugh et al. (1998) have demonstrated that upon the addition of PITP$\alpha$ to caveolin-rich light membranes (CLM) from A431 cells enhances type II PtdIns 4-kinase activity. Waugh and co-workers have thus proposed that CLMs may represent a site for PITP$\alpha$ dependent supply of PtdIns to the plasma membrane-associated sites of PtdIns4P synthesis. This notion is supported by the observation that in the presence of decreased PITP$\alpha$ activity, a depletion of phosphoinositides was only observed in caveolin rich membrane fractions isolated from normal rat kidney cells (Section 1.6 and Speed and Mitchell, 2000).

1.4.5 PITP and the nucleus

A nuclear response is an important consequence of many signalling pathways; the response may be elicited as events such as gene transcription, DNA cleavage, DNA repair, DNA replication and cell cycling events, potentially resulting in cell differentiation, proliferation and apoptosis. Direct and indirect involvement
of the phosphoinositides, their precursors and the products of their metabolism, have been indicated and implied in a number of nuclear responses. For example, during chromatin remodelling during gene-transcription events (reviewed by D'Santos et al., 1998). Evidence for phosphoinositide signalling in the nucleus is reviewed elsewhere (D'Santos et al., 1998) and therefore will be briefly discussed here.

Diverse methods, ranging from activity measurements in various subfractions to electron microscopy have been used to demonstrate and establish that many of the key lipids and enzymes responsible for the metabolism of phosphoinositides are resident in nuclei. PtdIns4P, PtdIns(4,5)P2 and PtdOH are all present in the nucleus, as well as the corresponding enzyme activities required to synthesis and metabolise these compounds (D'Santos et al., 1998). These observations suggest that nuclei possess an autonomous phosphoinositide signalling system that involves lipid kinases and PLC. In addition other non-inositol-containing phospholipids such as PtdCho constitute a significant percentage of the total nuclear phospholipid content (D'Santos et al., 1998).

Membrane free nuclei are able to phosphorylate PtdIns to PtdIns4P and PtdIns(4,5)P2 in a manner dependent on the differentiation state and the growth conditions of the parent cell (Cocco et al., 1987; Smith and Wells, 1983a; Smith and Wells, 1983b). However, few experiments addressed the nuclear availability of PtdIns in the nuclei, which may depend on transport systems able to redistribute PtdIns from its site of synthesis. In this context, it has been suggested that PITPa maybe involved in nuclear phosphoinositide distribution (Capitani et al., 1990). Initially, Capitani et al. (1990) showed that isolated rat liver nuclei could take up [3H] PtdIns from vesicles or microsome membranes in the presence of purified rat brain PITPa (Capitani et al., 1990). The same group subsequently demonstrated that both intact murine Friend erythroleukemia cell nuclei and nuclei deprived of an outer membrane, contained PITPa and a small proportion of PITPB (Rubbini et al., 1997b). Friend erythroleukemia cells have a nuclear phosphoinositide cycle, which is related to both mitogen-stimulated cell growth. DMSO treatment causes friend cells to differentiate along the erythroid pathway (Cocco et al., 1987; Martelli et al., 1995). Compared to proliferating control cells, nuclei isolated from DMSO-treated cells, have altered phosphoinositide metabolism, including an increase in 32P-labelled PtdIns(4,5)P2 and a down regulation of PLCB1 (Divecha et al., 1995; Martelli et al., 1994). The later observation possibly results in the apparent reduction in nuclear DAG and a consequent commitment of the cell to differentiation. Rubini
et al. (1997) have demonstrated a reduction in nuclear PITPα, in a manner analogous to PLCβ, in DMSO-treated Friend cells. In contrast, the low level of nuclear PITPβ was unaffected (Rubbini et al., 1997a).

It is not clear what role PITPα may play in the nucleus. PITPα could be required for the transfer of PtdIns to the nucleus possibly into discreet regions of the nuclear matrix or envelope, in a manner analogous to the proposed role of PITP during PLC signalling at the plasma membrane. The presence of PtdCho within the nuclear matrix and membranes suggests that the exchange of these two phospholipids by PITPα would provide a mechanism for importing PtdIns into the nucleus. An analysis of the species of DAG present in the nucleus suggested that DAG is predominantly derived from PtdCho, the levels of which decreased upon the differentiation (D’Santos et al., 1998). Thus, it remains possible that PITPα may regulate the level of nuclear DAG by a mechanism similar to that by which Sec14p regulates Golgi DAG levels in yeast. Whether PLCβ1, PLD or an alternative PtdCho-PLC isoform generates DAG in the nucleus, and which substrate is used remain contentious (reviewed by D’Santos et al., 1998).

1.4.6 PITPα and PLA activation

The studies described above have employed permeabilised cells and cell-free systems. More recently, Snoek and co-workers have investigated PtdIns metabolism in a cell line in which the level of expression of PITPα was altered (Snoek et al., 1999). Snoek et al. (1999) generated stable NIH3T3 fibroblast cell lines that expressed a 2 to 3-fold increase in the level of PITPα compared with wild type cells. Analysis of these cells revealed that overexpression of PITPα led to an increase in the growth rate in these cells. Specifically, analysis of exponentially growing cultures by fluorescent activated cell sorting demonstrated that the increased growth rate was due to a decreased G1 phase during cell cycling (Snoek et al., 1999). These observations are consistent with the earlier report that a reduction in PITPα results in a decrease in the growth rate of NIH3T3 cells (Monaco et al., 1998).

Equilibrium-labelling studies indicated that the intracellular levels of lysoPtdIns and the metabolites glycerophosphoinositol, Ins1P and Ins2P were constitutively increased in NIH3T3 cells overexpressing PITPα (Snoek et al., 1999). The authors subsequently proposed that a PtdIns-specific PLA is activated in cells overexpressing PITPα. Furthermore, no evidence was obtained that in NIH3T3 cells, the overexpression of PITPα had any effect on the phosphorylation of PtdIns by PtdIns kinases (Snoek et al., 1999). Glycerophosphoinositol and its metabolic
derivatives have been shown to act as mitogens in various cell lines (Falasca et al., 1995) and may contribute to the enhanced growth rate observed in the PITPα-overexpressing cells.

In contrast to the wild type cells, stimulation of the PITPα over expressing cells with bombesin and PDGF did not result in any apparent activation of the PLC dependent degradation of PtdIns(4,5)P2, indicating a desensitisation of this pathway (Snoek et al., 1999). Whether this observation is an artefact of the experimental procedure or overexpression, or whether elevated levels of PITPα do in fact activate a PLA and simultaneously desensitises the PLC pathway remains to be determined.

1.4.7 PITPα and PtdCho metabolism: a role for PtdCho-bound PITPα?

The data discussed so far concentrate on the PtdIns-bound form of PITPα and PITPβ and do not invoke a direct role for the PtdCho-bound form of these PtdIns-TPs, other than passive binding and transfer. The demonstration that PITPα is capable of catalysing PtdCho transfer in the absence of any PtdIns transfer activity suggests that there is no obligatory link between the two transfer activities. While, it is clearly established that PtdCho-bound Sec14p maintains Golgi DAG concentrations by inhibiting the activity of CCT (Section 1.2.1), few studies describe a function for PtdCho-bound PITPα and PITPβ. A recent study by Wirtz and co-workers using the expression of antisense RNA suggests that PITPα regulates PtdCho metabolism (Monaco et al., 1998). Investigation of PtdIns and PtdCho metabolism in WRK1 cells having a 25% reduction in PITPα, revealed a 50% reduction in PtdCho metabolism compared with wild type cells (Monaco et al., 1998). In contrast, PtdIns metabolism was similar in both experimental and control clones. Furthermore, the fractional change distribution of phosphoinositides upon stimulation of the antisense clones with vasopressin or bradykinin was not significantly different from that observed with the control cells (Monaco et al., 1998).

Equilibrium labelling of under expressing cells with [3H]choline or [14C]choline and subsequent analysis of the distribution of the radioactivity in pathway intermediates indicated that the rate-limiting enzyme, CCT, was the affected step (Monaco et al., 1998). These observations are reminiscent of those observed with yeast Sec14p mutants in yeast. Thus, by analogy to the function of Sec14p, it is possible that the PtdCho-bound form of PITPα regulates Golgi PtdCho synthesis. However, in contrast to the under expression of PITPα in WRK1 cells,
overexpression of Sec14p activity resulted in a decreased rate of PtdCho biosynthesis (Cleves et al., 1991). The precise mechanism by which PITPα may be involved in the regulation of PtdCho levels and other functions of PtdCho-bound PtdIns-TPs remain to be determined.

1.4.8 PITPβ and SM metabolism

Analogous to the reported roles of PITP in phosphoinositide signalling together with the high affinity of PITPβ for SM, it has been proposed that PITPβ might be involved in the metabolism of SM (Westerman et al., 1995). SM is formed by SM synthase, which transfers the choline phosphate group from PtdCho to ceramide, thereby generating SM and DAG (Bernert and Ullman, 1981; Luberto and Hannun, 1999; Ullman and Radin, 1974). Recent studies have demonstrated that SM synthase activity is localised in the early Golgi, the plasma membrane and possibly recycling endosomes (Levade and Jaffrezou, 1999). Ceramide is a lipid mediator that is involved in regulating cell growth, cell differentiation and cell death (Perry and Hannun, 1998). Upon cell stimulation by a range of extracellular stimuli, including tumour necrosis factor-α and interferon-γ, SM is hydrolysed by sphingomyelinase to ceramide (Luberto and Hannun, 1999). The signalling roles of the ceramide/SM cycle and sphingomyelinases (SMases) are reviewed elsewhere (Levade and Jaffrezou, 1999).

Van Teil et al., (2000) have investigated SM metabolism in NIH3T3 fibroblasts that over express PITPβ. Equilibrium-labelling studies revealed that overexpression of PITPβ resulted in the ability to rapidly resynthesise SM from ceramide produced in the plasma membrane by the action of exogenous bacterial sphingomyelinase (bSMase; Van Tiel et al., 2000b). For example, in the presence of a low bSMase concentration (50 munits/ml), 50% of the [3H]SM in WT cells was degraded, whereas the levels in PITPβ overexpressing cells appeared unaffected presumably as a result of the rapid resynthesis. However, the rates of de novo SM and PtdCho synthesis were similar in PITPβ overexpressing and control cells, indicating that the resynthesis of SM observed upon treatment with bSMase may involve a SM synthase activity that is distinct from the activity involved in de novo SM synthesis (Van Tiel et al., 2000b). Previous studies using baby hamster kidney cells have also demonstrated that the SM synthases involved in de novo synthesis and resynthesis of SM are present at different sites (Kallen et al., 1993). It has been postulated that the SM synthase acting on plasma membrane ceramide plays a role
in the SM/ceramide cycle (Andrieu-Abadie et al., 1998; Luberto and Hannun, 1998). Furthermore, there is convincing evidence for the existence of an agonist-sensitive pool of SM, which may be resynthesised by the same SM synthase that employs plasma membrane ceramide (Andrieu-Abadie et al., 1998; Luberto and Hannun, 1998). It remains to be established whether PITP6 plays a role in this in the regeneration of this SM pool and how PITP6 maintains steady state SM levels.

1.5 A common mechanism for yeast and mammalian PtdIns-TPs?

Despite the absence of any apparent primary sequence homology and cross immunoreactivity between Sec14p and PITPs, Sec14p can substitute for PITPα and PITPβ in the priming and the TGN-derived vesicle biogenesis assays (Hay et al., 1995; Ohashi et al., 1995). However, stimulation with Sec14p was not sustained to the same maximum as was achieved by PITPα or PITPβ (Ohashi et al., 1995). Likewise, Sec14p, PITPα and PITPβ are essentially interchangeable in the reconstitution of transmembrane signalling assay (Cunningham et al., 1996). This interchangability in vitro may underlie the ability of PITPα and PITPβ expression to rescue growth and secretion defects in Sec14Δ strains (Skinner et al., 1995; Tanaka and Hosaka, 1994). These observations suggest that there is some conservation of biological function between mammalian and yeast PITPs. However, PITPα could not rescue Sec14 null mutants (Skinner et al., 1995). The limited complementation was suggested to arise from transfer activity (Skinner et al., 1995). Since PITPα did not associate with the Golgi it was suggested that a stable interaction of Sec14p with the Golgi might be essential for rescuing the null mutants. Thus, it has been proposed that PITPβ is the mammalian counterpart of Sec14p (Tanaka and Hosaka, 1994; Tanaka et al., 1995). Alternatively, the inability of PITPα to inhibit CCT may also account for the lack of total rescue of the mutant phenotype (Skinner et al., 1995). The ability of PITPβ to regulate CCT activity awaits further studies.

The demonstration of limited Sec14p/PITP interchangability both in vivo and in vitro raises the question of whether a unified biological function exists among yeast and mammalian PITPs. The sensor model for Sec14p is not obviously congruent with a role of PITPα and PITPβ in PtdIns(4,5)P₂ biosynthesis (Section 1.2.1). Although PtdIns(4,5)P₂ is synthesised and hydrolysed by PLC in yeast, a role for a PtdIns-TP in this pathway has yet to be addressed. Furthermore, neither the formation of DAG from PtdIns via PtdIns(4,5)P₂ hydrolysis or from inositol phosphoceramide biosynthesis has been demonstrated to occur at the Golgi
membrane (Carman and Henry, 1999; Daum et al., 1998). Thus although, it remains possible that Sec14p and mammalian PITP functions may be supplied by a common lipid precursor pool, the identification of multiple Sec14p-like proteins in mammalian species suggests that Sec14p and PITPs have distinct biological functions. This notion is supported by the observation that the eukaryotic microbe, Dictyostelium discoideum also expresses PITPα, PITPβ and Sec14p homologues (Swigart et al., 2000).

1.6 PITP mutants with specific biochemical defects: clues to mechanisms of PtdIns–TP regulation

Reconstituted empirical systems implicate PITPα, and possibly PITPβ, as cofactors in diverse cellular reactions, suggesting a critical functional role for the PtdIns-transfer activity of PITPs. These studies suggest that PITPs may effect a vectorial transfer of PtdIns to target membranes. While a vectorial PtdIns transfer can conceivably sustained by counter transfer of PtdCho, there may be a mechanism to regulate the PtdIns and PtdCho-transfer activities of PITP at the appropriate membrane. Two ways for such a differential regulation of these transfer activities have been proposed (Rogers and Bankaitis, 2000). First vectorial PtdIns transfer may be accomplished through local modification of the phospholipid cargo such that it is rendered non-transferable. A PtdIns kinase could effect such a modification, and the cumulative findings that PITP has the ability to bind PtdIns kinases in vitro are consistent with such a concept (Kauffmann Zeh et al., 1995; Volinia et al., 1995). A second possibility is that post-translational modification of PITP itself imposes extrinsic and selective regulation of transfer substrate specificity. It follows from the later concept that PITP has specific modification sites allowing regulation of PtdIns and PtdCho transfer can be imposed. If PITP activity does determine the magnitude of PLC signalling, as suggested by reconstitution studies (Section 1.4.2), the nature of extrinsic mechanisms for regulating PITP activity becomes a critical issue in the understanding of how cells control signal transduction cascades. A combination of genetic and biochemical studies now provide strong evidence for extrinsic regulation of PITP function (reviewed by Rogers and Bankaitis, 2000).

Utilising random mutagenesis and activity screening in a sec14 mutant system, Alb et al. (1995) obtained mutant mammalian PITPα proteins that exhibit specific defects in PtdIns transfer activity with out effecting PtdCho transfer activity (Alb et al., 1995). These studies provided the first demonstration that PtdIns and
PtdCho transfer activities could be uncoupled. These mutants contained single amino acid change in the PITPa sequence, including: S25F, T59I, P78L and E248K (Alb et al., 1995). Alignment of the sequences of all mammalian PtdIns-TPs revealed that three of these four residues are invariant, and the fourth, S25 is highly conserved. The specification of the four mutants suggests that these residues cooperate to form the PtdIns head group recognition/binding site (Alb et al., 1995).

Two of these mutations, T59I and E248K, lie within a consensus PKC phosphorylation motifs, suggesting an intrinsic mechanism for regulating PITPa activity (Alb et al., 1995). T59 is a potential PKC phosphorylation site that lies within one of the most highly conserved regions in all of the presently known insect and mammalian PtdIns-TPs. Replacement of T59 with other amino acid residues (including S, D and E) selectively inactivated the PtdIns transfer activity of PITPa. The sensitivity of PtdIns transfer activity to T59 mutation, suggests that phosphorylation of this residue will also inhibit the PtdIns transfer activity of PITPa (Alb et al., 1995). PKC is an attractive candidate for such a reaction since PITPa has been shown to be phosphorylated, in response to challenge of Swiss 3T3 cells with PKC agonists and is an in vitro substrate for PKC (Snoek et al., 1993a). PKC is recruited and activated by membranes engaged in PtdIns-dependent signalling events, thus, this enzyme is well placed to inhibit PITPa mediated PtdIns, allowing to feedback regulation of PtdIns signalling. Modification of PITP by PKC would be predicted to be reversed by protein phosphatase activity. Such a regulatory phosphorylation/dephosphorylation mechanism has been suggested to promote vectorial PtdIns transfer in the cell (Alb et al., 1995; Figure 1.3).

Using a model normal rat kidney cell system in which the expression of an inositol phosphate 5-phophatase was reduced by the transfection of antisense cDNA, Speed and Mitchell have provided additional evidence that the activity of PITPa may be regulated by PKC (Speed and Mitchell, 2000). Since the 5-phosphatase specifically hydrolyses Ins(1,4,5)P3 but not PtdIns(4,5)P2, basal Ins(1,4,5)P3 levels are elevated and are further increased following agonist stimulation, compared to wild type cells. Correlated with this increase in Ins(1,4,5)P3 is a reduction in the total cellular mass of PtdIns(4,5)P2, PtdIns4P and PtdIns (Speed and Mitchell, 2000). In agreement with other studies, the depletion of cellular PtdIns(4,5)P2 is confined to a Triton-insoluble compartment enriched in caveolin (Pike and Casey, 1996; Waugh et al., 1998 and Section 1.4.4). Analysis of the PtdIns transfer activity of total cell
Figure 1.3 Regulation of PtdIns-TP by a PKC/protein phosphatase (PPase) cycle. Upon the discharge of PtdIns (I) or PtdCho (C) into the acceptor membrane (upper part of the cycle), a resident PKC phosphorylates PtdIns-TP on T59 and renders it unable to reload with PtdIns. PtdIns then disengages with the acceptor membrane as a phospholipid-free protein or as a PtdCho bound species (lower part of the cycle). Upon reengagement with the donor membrane, a resident PPase dephosphorylates PtdIns-TP allowing reloading with PtdIns for the next round of transfer (Alb et al., 1995). Regulation of PtdIns-TP in this manner is proposed to allow vectorial transfer of PtdIns from a donor to an acceptor membrane.
lysates revealed that while PITPα expression was unaffected, PtdIns transfer activity was decreased by 70% in transfected cells (Speed and Mitchell, 2000). In contrast, no inhibition of PtdIns 4-kinase or PtdIns4P 5-kinase or activation of PLC isozymes was observed (Speed and Mitchell, 2000). These observations indicated that depletion of phosphoinositides in the agonist-sensitive pool was due to the inhibition of PtdIns supply by PITP. While the authors proposed that feedback inhibition of PITP activity occurs by increased cellular Ins(1,4,5)P₃ levels, the potential role of PKC in modifying the activity of PITP offers an alternative mechanism of regulation. Cells that under express the 5-phosphatase exhibit increased and sustained Ca²⁺ oscillations presumably because of the elevated levels of Ins(1,4,5)P₃ (Speed and Mitchell, 2000). Thus, it is possible that cellular DAG levels would also be elevated, leading to a subsequent prolonged activation of PKC.

**In vitro** kinetic analysis of the PKC-dependent phosphorylation of PITPα indicates that while the affinity for rat brain PKC is the same for both species, PtdCho-bound PITPα was phosphorylated at a greater rate than the protein containing a PtdIns molecule (Van Tiel et al., 2000a). In contrast to the predictions discussed above, phosphoamino acid analysis of the **in vitro** phosphorylated protein revealed that PITPα is mainly phosphorylated on serine residues (Van Tiel et al., 2000a). Peptide analysis and site directed mutagenesis suggested that the phosphorylation of PITP by rat PKC is restricted to S166 (Van Tiel et al., 2000a). While the transfer activity of the phosphorylated protein was not addressed, replacement of S166 with A or N completely abolished both PtdIns and PtdCho activity, indicating that the identity of this residue is critical for the transfer activity of PITP (Van Tiel et al., 2000a).

As discussed previously, activation of PKC in Swiss 3T3 and NIH3T3 cells induced a translocation of Myc-tagged PITPα from the cytoplasm to the Golgi compartment (Section 1.3.2). Thus it has been proposed that phosphorylation of PITPα is required for interaction with Golgi membranes (Snoek et al., 1993a). Based on these observations, together with the observation that PITPα affects the intracellular levels of lyso PtdIns, possibly through the regulation of a PLA₂ (Section 1.4.6), Van Teil et al. (1999) proposed that the PKC-dependent translocation of PtdCho-bound PITPα to the Golgi allows more PtdIns to become available from the Golgi for degradation by PLA₂ (Van Tiel et al., 2000a). An alternative to the model proposed by Alb et al. (1995) was presented whereby receptor-dependent activation of PKC results in a more rapid phosphorylation of PtdCho-bound PITPα (Van Tiel et
al., 2000a). At the Golgi, this PtdCho is rapidly exchanged for PtdIns. A subsequent dephosphorylation step will release the PtdIns bound PITPα from the Golgi, allowing for the delivery of the PtdIns to PLA₂. Although the two models differ, they both provide a mechanism whereby the relative amounts of PtdIns and PtdCho-containing PITPα can be regulated. The roles of T59 and S166 and other residues in the regulation of PITPα and PITPβ require further investigation, as does the functional significance PKC-mediated phosphorylation of PITPα. Nevertheless, the isolation and characterisation of mutant PtdIns-TPs with selective functional defects represent important steps forward in the analysis of PtdIns-TP function. Such mutants will prove very useful in a detailed dissection of what PtdIns-TP functions are relevant in vivo.

1.7 A Role for PtdIns-TPs in neuronal viability in mammals

The studies described above, suggest that PITPα and PITPβ have a number of functional roles at several distinct intracellular localisations. However, the physiological function(s) of PITPα and PITPβ and how transfer activity pertains to this function remains unclear, as does the physiological relevance of two isoforms. Studies have established a role for metazoan PtdIns-TPs in the protection of neurons from degenerative processes (Hamilton et al., 1997; Vihtelic et al., 1993). While the initial observations of this effect were made in Drosophila (Section 1.8), PtdIns-TP deficiency is now recognised as the molecular basis for neurodegeneration disease in the vibrator (vb) mouse (Hamilton et al., 1997). The vb allele is a fully recessive autosomal mutation that, in homozygous newborn mice, evokes a whole-body action tremor whose onset coincides with the acquisition of co-ordinated muscle control. This tremor develops into a progressive ascending motor paralysis that is then fatal in juvenile mice (Hamilton et al., 1997). Hamilton et al., (1997) cloned the vibrator mutation using an in vivo positional approach and complete sequencing of the resultant 76kb critical region from the vibrator and wild type chromosome. The mutation is a retrotransposon insertion into intron 4 of the PITPα gene, which results in the vb animal exhibiting an 80% reduction in PITPα expression (Hamilton et al., 1997). Levels of PITPβ appear to be independent of the vb genotype, since no change in the level of PITPβ mRNA was observed in the vb mouse (Hamilton et al., 1997). Thus, the identification of the vb mutant provided evidence that the PITPα and PITPβ are not redundant, suggesting that the encoded proteins have different functions in vivo.
The vb phenotype, while clearly devastating, shows a highly restricted neurodegeneration in specific neurons of the spinal chord, brain stem, and dorsal root ganglia of mice analysed at near death-stage (Hamilton et al., 1997). Thus, specific neuronal cells are sensitive to PITPα deficiency. Histological analysis of the brains and spinal cord from affected animals revealed three distinct pathologies that were not seen in the controls: vacuolated or spongiform neurons, swollen neurons that appeared to be undergoing central chromolysis, and less frequently, fading neurons in which the cytoplasm and nucleus were poorly stained (Hamilton et al., 1997). It is unclear whether these cytological entities represent different temporal stages of the same degenerative process or distinct processes. The vacuolated pathology suggests that defective intracellular transport may result in the degenerative phenotype and is in agreement with the proposed roles of PITPα in vesicle flow (Section 1.4.3). However, the mechanisms by which PITPα prevents neurodegeneration remain to be determined.

1.8 Drosophila retinal degeneration B (DrdgB): A non-conventional member of the PtdIns-TP family

As demonstrated by Sec14p function in yeast, genetic approaches have significantly contributed to the elucidation of PtdIns-TP function in vivo. The identification of a critical role for a novel member of the PtdIns-TP family in the Drosophila phototransduction cascade has provided a model system with which to study higher eukaryotic PtdIns-TP function (Vihtelic et al., 1993). This multi-domain PtdIns-TP, termed Drosophila retinal degeneration B (DrdgB), was initially discovered in three independent screens for mutants that exhibited either abnormal photoreceptor physiology or retinal degeneration (Hotta and Benzer, 1970; Pak et al., 1970; Vihtelic et al., 1991). The assignment of DrdgB as a PtdIns-TP protein is based on the observation that the amino-terminal 281 amino acids of the 1,054 amino acid DrdgB primary sequence share more than 40% sequence identity with PITPα (Vihtelic et al., 1993). As described in the preceding sections, PtdIns-TPs are soluble proteins operationally defined by their ability to bind and transfer PtdIns and PtdCho. While transfer has yet to be demonstrated for the full-length protein, in vitro studies have demonstrated that the PtdIns-TP-homologous domain of DrdgB, when expressed as a soluble polypeptide, exhibits PtdIns transfer activity (Vihtelic et al., 1993).
DrdgB is unique amongst PtdIns-TPs in that it is a large multidomain protein with a molecular weight of around 160kDa. In addition to the amino-terminal PtdIns-TP-like domain, the protein consists of a small acidic domain, which possesses Ca\(^{2+}\)-binding activity \textit{in vitro}, six putative membrane-spanning domains and a small carboxy-terminal domain. The presence of six putative membrane-spanning domains indicates that DrdgB is a transmembrane protein (Vihtelic \textit{et al.}, 1993). Indeed, alkaline Na\(_2\)CO\(_3\) treatment was unable to extract DrdgB from membranes (Vihtelic \textit{et al.}, 1993). However, recent data suggest that, while DrdgB is membrane bound, it is not an integral membrane protein (Section 1.9.1).

The DrdgB mutant phenotype is characterised by retinal degeneration whose onset, while discernible in dark-reared flies, is greatly accelerated by raising the flies in the light. Typically, \textit{DrdgB} mutant flies begin to exhibit the hallmarks of photoreceptor degeneration several days after eclosion (Harris and Stark, 1977; Milligan \textit{et al.}, 1997b). In addition, these mutant flies exhibit an abnormal termination of the light response, as revealed by the rapid deterioration of the electroretinogram (ERG), shortly after the flies initial exposure to light (Harris and Stark, 1977; Milligan \textit{et al.}, 1997b). The ERG is an extracellular recording that measures the total electrical potential across the eye (reviewed by (Pak, 1979). The primary feature of the ERG, the light-coincident receptor potential (LCRP), reflects activity in the photoreceptor and consists of transient spikes induced by the activation and cessation of the light stimulus generated from activity post-synaptic to the photoreceptor cell (Pak, 1979).

The DrdgB gene encodes at least five different mRNA species that are expressed within photoreceptor cells, chemosensory neurons within the antenna and sensory processing centres of the central brain (Vihtelic \textit{et al.}, 1993). Thus, unlike many other proteins previously identified by their role in visual physiology, DrdgB protein expression is not restricted to the visual system. The expression of DrdgB within the antennae is consistent with the olfaction defects associated with some DrdgB alleles (Woodard \textit{et al.}, 1992). Genetic and electrophysiological approaches have demonstrated that DrdgB function is necessary for olfactory responses of both adult flies and larvae suggesting that visual and olfactory transduction may share at least one common molecular step in \textit{Drosophila} (Woodard \textit{et al.}, 1992). The role of DrdgB in olfaction remains unknown but the restricted tissue distribution of rdgB suggests that this PtdIns-TP may perform functions specific to rapid neurosensory transmission in invertebrates.
Within the fly photoreceptor, DrdgB localises to both the axon and the subrhabdomeric cisternae (SRC; Suzuki and Hirosawa, 1994). The SRC is an extension of the ER that lies adjacent to the rhabdomere that runs the length of the photoreceptor cell. The SRC functions as an intracellular Ca\(^{2+}\) store (Baumann et al., 1991) and has been implicated in the maintenance of the rhabdomeres (Matsumoto Suzuki et al., 1989). Indeed, the degeneration of the rhabdomere seen in light-exposed rdgB mutant flies correlates with the collapse of the SRC network in retinal cells (Suzuki and Hirosawa, 1994). Such a destruction of the SRC may trigger an inappropriate turnover of the rhabdomeric membranes and thus retinal degeneration. DrdgB was the first identified protein required for phototransduction that is not localised to the photoreceptor rhabdomere.

The DrdgB mutant ERG light response is defective within hours after eclosion and completely lost within the first day after exposure to light (Harris and Stark, 1977; Milligan et al., 1997b). This ERG defect is manifested before any physical signs of retinal degeneration (Harris and Stark, 1977), suggesting that the degeneration is a likely consequence of the photoreceptors' abnormal light response. However, Hyde and colleagues have isolated several DrdgB suppressers that dramatically slowed rdgB dependent retinal degeneration without significantly improving the ERG response (Paetkau et al., 1999). The identification and characterisation of these suppressors reveal that DrdgB-dependent degeneration may not be caused by a defective light response.

Genetic and biochemical studies have provided valuable information regarding the role of DrdgB during Drosophila phototransduction. To adequately describe what is currently known regarding DrdgB function in vivo, it is necessary to digress slightly and provide a summary of the basic forms of mammalian and Drosophila phototransduction pathways. Comprehensive reviews of both the vertebrate and fly visual signalling cascade are available (Jindrova, 1998; Montell, 1999).

### 1.8.1 Invertebrate and vertebrate phototransduction

During the past few years, considerable progress has been made towards describing the molecular mechanisms underlying Drosophila phototransduction. Much of this success can be attributed to the identification and characterisation of many phototransduction mutants. The strategies through which mutations affecting phototransduction have been identified have employed behavioural and
morphological screens and the analysis of ERGs. Vertebrate phototransduction takes place within the rod and cone outer segments of the photoreceptor cell (Jindrova, 1998). Instead of the rod and cone structures, which are elaborated cilia, invertebrate photoreceptors usually employ arrays of microvilli termed rhabdomeres (Montell, 1999). In contrast to the situation with vertebrate rods, where most of the rhodopsin is contained in internal disc membranes, Drosophila phototransduction occurs on the rhabdomeric plasma membrane. Drosophila and vertebrate phototransduction exhibit notable similarities and differences. Phototransduction in both vertebrates and invertebrates is initiated by light activation of the photopigment rhodopsin and its subsequent interaction and activation of a heterotrimeric G protein. In vertebrates the G-protein is transducin (G_t) and its effector is 3',5'-cyclic guanosine monophosphate (cGMP) phosphodiesterase (PDE), which hydrolyses cGMP to 5'-guanosine monophosphate. During phototransduction, PDE induced decreases in cGMP levels elicit transient closure of cGMP-gated cation channels and hyperpolarisation within the photoreceptor by the reduction or termination of Na^+ and Ca^{2+} influx (Jindrova, 1998).

In contrast to vertebrate phototransduction, the Drosophila visual cascade utilises the inositol phospholipid signalling system. A G-protein of the G_a family activates an eye-specific PLCβ, encoded by the norpA gene (Montell, 1999). Activation of eye-specific PLCβ leads to the opening of the cation influx channels TRP and TRPL (Hardie and Minke, 1992; Phillips et al., 1992), the subsequent influx of Na^+ and Ca^{2+}, and depolarisation of the photoreceptor (Montell, 1999). However, the precise mechanism by which the stimulation of eye PLCβ activates light dependent channels remains controversial. Until recently, it was generally assumed that excitation was mediated by Ins(1,4,5)P_3, as is believed to be the case for example, in the Limulus ventral photoreceptor (Minke and Selinger, 1992). However, in Drosophila the only evidence to support a role for Ins(1,4,5)P_3, is limited and includes a recently reported correlation between light-induced Ca^{2+} and sensitivity to light in a trp mutant (Cook and Minke, 1999). By contrast, a mutation in the Ins(1,4,5)P_3 receptor (Ins(1,4,5)P_3R) was reported to have no detectable effect on phototransduction (Acharya et al., 1997), and Ins(1,4,5)P_3 is incapable of mimicking excitation (Hardie and Raghu, 1998). It has recently been demonstrated that arachadonic acid and other polyunsaturated fatty acids (PUFAs) can activate both TRP and TRPL channels in Drosophila photoreceptors, as well as recombinant TRPL channels expressed in cell lines (Chyb et al., 1999). This suggested that
excitation may be mediated via the DAG branch of the PtdIns pathway since PUFAs can be released from DAG via DAG lipase. Eukaryotic genes for DAG lipase have yet to be cloned, however, this enzyme is believed to play important signalling roles in a number of vertebrate cells by releasing arachadonic acid (reviewed by Topham and Prescott, 1999), which in some cases can also activate cation channels in the plasma membrane. The observation that mutations in the *Drosophila rdgA* gene, which encodes DAG kinase, result in constitutive activation of light-sensitive channels and defective termination of the light response (Raghu *et al.*, 2000), support this recent proposal that DAG and its down stream metabolites may be the messengers mediating *Drosophila* photoreceptor excitation.

Following the activation of G-protein coupled cascades the system must be reset to achieve the appropriate response to extracellular signals. The process of deactivation of the response is essential to prevent an excessive response to a single stimulus and to permit responses of normal amplitude during repeated stimulation.

Downregulation of the vertebrate phototransduction cascade occurs via several mechanism including increased hydrolysis of GTP bound G<sub>α</sub> subunit of G<sub>α</sub> by the GTPase activating protein (GAP) RSG9, thereby inactivating transducin. Two other molecules that function to down regulate phototransduction are arrestin and rhodopsin kinase (Jindrova, 1998).

Ca<sup>2+</sup> and calmodulin mediate the deactivation of the *Drosophila* phototransduction cascade at multiple steps (Montell, 1999). While the exact mechanism remains unclear, a large body of evidence suggests that Ca<sup>2+</sup> may be a global mediator of termination because elevated concentrations appear to attenuate the activities of rhodopsin, TRP and TRPL channels, eye PLC8 and the eye PKC encoded by the *inaC* locus (Montell, 1999). Eye PKC has been proposed to act as one of the primary mediators of Ca<sup>2+</sup>-dependent feedback (Adamski *et al.*, 1998; Liu *et al.*, 2000). While termination of the light response appears to involve several steps, the inactivation of G<sub>qα</sub> as a result of GTP hydrolysis seems to be the rate-limiting event (Sagoo and Lagnado, 1997). A recent study by Cook *et al.* (2000) demonstrated that as well as being an activator of the cascade, eye PLC8 also functions as a GAP. PLC induces GAP activity and thereby inactivates GTP-bound G<sub>qα</sub>; furthermore, this GAP activity is required for proper termination of the light response (Cook *et al.*, 2000). On the basis of the data provided by Cook and co-workers and other biochemical studies of mammalian PLCs, it seems likely that eye PLC8 may act as a GAP protein during deactivation (Cook *et al.*, 2000).
The Ca\textsuperscript{2+}-binding protein calmodulin (CAM) also plays an important role in the Ca\textsuperscript{2+}-dependent downregulation of the phototransduction response. CAM is required for rhodopsin deactivation, and modulates the function of TRPL light-activated channels \textit{in vivo} (Montell, 1999; Scott \textit{et al.}, 1997). However, the major CAM binding proteins in the \textit{Drosophila} retina are the unconventional myosin serine/threonine kinases, encoded by the \textit{Nina} gene (Porter \textit{et al.}, 1993). \textit{NinaC} mutants show CAM mislocalisation and light responses with deactivation defects (Porter \textit{et al.}, 1993).

\textit{Drosophila} phototransduction utilises one of the fastest known G-protein-mediated cascades. Cation influx is activated within 20ms of exposure and termination of the light response occurs within less than 10ms (Montell, 1999). The recent discovery that most of the proteins that function in the \textit{Drosophila} visual transduction cascade associate in a supramolecular complex, known as the transducisome or signalplex (Montell, 1999; Scott and Zuker, 1998; Tsunoda \textit{et al.}, 1997), has provided insight into the mechanisms that facilitate the rapid kinetics of activation and feedback regulation.

The pivotal protein in the transducisome is INAD. The \textit{inaD} gene was isolated using classical and reverse-genetic approaches and was originally characterised as a mutant displaying abnormal electrical responses in the retina (Pak, 1979). Subsequent molecular analysis showed that the \textit{inaD} gene product consists of five, protein binding PDZ domains and acts as a scaffold for assembling signalling complexes in the rhabdomeric membranes of the photoreceptor cell (Tsunoda \textit{et al.}, 1997). PDZ domains were first identified as protein components of synaptic junctions, but now appear to be more widely distributed and are thought to play a role in localising signalling and adhesion molecules (Ranganathan and Ross, 1997).

Protein binding assays indicate that the PDZ domains of INAD interact with many of the components of the signalling cascade, including rhodopsin, TRP and TRPL channels, eye PLC\textsubscript{B}, PKC and NinaC (Montell, 1999). Mutations in INAD disrupt the rhabdomeric specific localisation of TRP, PLC and PKC, however, the presence or absence of INAD does not influence the spatial distribution of the remaining INAD binding-proteins (Montell, 1999). As NinaC is an actin-binding protein, it has been proposed that the actin cytoskeleton may be directly coupled to the transducisome (Montell, 1999).

The ability of INAD to co-ordinate membrane signalling has been demonstrated in considerable detail through a combination of genetics,
electrophysiology and biochemistry. Mutations in the \textit{inaD} gene disrupt both the activation and the feedback regulation of the invertebrate phototransduction (Tsunoda \textit{et al.}, 1998). The organisation of signalling components into a single macromolecular complex or an interconnected network is significant for several reasons. First it has the potential to increase the speed and efficiency of transmission relative to molecular diffusion. Secondly, in view of the findings that INAD forms homo-oligomers \textit{in vitro}, it is possible that homomultimerisation of INAD \textit{in vivo} may facilitate amplification of the cascade (Xu \textit{et al.}, 1998). Finally, the transducisome can increase the specificity of signalling to the down stream effectors, particularly during inactivation of the photoresponse by forcing feedback regulation by the rapid, large amplitude changes in Ca\textsuperscript{2+} concentration that are expected near the intracellular pore of the TRP channel. Consistent with this hypothesis, negative feedback regulation of \textit{Drosophila} photoreceptors has been shown to require Ca\textsuperscript{2+} transients in the tens to hundreds of micromolar range (Montell, 1999). Additionally, recent studies indicate a function of the transducisome in maintaining the proper stoichiometry among proteins implicated in phototransduction (Cook \textit{et al.}, 2000).

1.8.2 Genetic interactions of \textit{rdgB} mutations

Insights regarding DrdgB function have been forthcoming from the analysis of mutations that suppress retinal degeneration in DrdgB null mutant flies. Genetic epistasis analysis suggest that DrdgB functions both down stream of both rhodopsin and PLC\textbeta in the visual transduction cascade since both \textit{ninaE} (encoding the opsin expressed in photoreceptor cells (O'Tousa \textit{et al.}, 1985)) and \textit{norpA} mutations (Bloomquist \textit{et al.}, 1988) suppress the light-enhanced retinal degeneration of \textit{rdgB} null mutant flies. Consistent with this view, constitutive activation of the \textit{Drosophila} G-protein, G\textsubscript{q}, either by the action of nonhydrolyzable GTP analogues or by expression of a constitutively active G\textsubscript{q} subunit, effects rapid degeneration of DrdgB mutant retinas in the absence of light (Lee \textit{et al.}, 1994; Rubinstein \textit{et al.}, 1989). The physical basis that underlies the functional interaction between DrdgB and NorpA /NinaE remains unknown. To date, no physical association has been demonstrated between the proteins encoded by these genes and DrdgB. Furthermore, DrdgB and NinaE and NorpA do not co-localise in photoreceptor cells.

Mutations in the \textit{inaC} gene also weakly suppress DrdgB dependent retinal degeneration (Smith \textit{et al.}, 1991). Consistent with this result, application of phorbol
esters also induces rapid rdgB-dependent retinal degeneration, presumably by stimulating PKC activity (Minke et al., 1990). Furthermore, mutant DrdgB is hyperphosphorylated upon light activation in vivo (Sahly et al., 1992). These observations suggest that DrdgB functions downstream of PKC in the fly visual transduction cascade. However, recent studies suggest otherwise. Paetkau and co-workers have demonstrated that inaC mutations did not suppress degeneration in two distinct DrdgB mutant flies upon a 12-hour light/dark exposure cycle (Paetkau et al., 1999). Furthermore, mutations of T59, a putative PKC phosphorylation site (Section 1.6) dramatically reduced DrdgB activity in vivo (Milligan et al., 1997b and below). In the light of these data, Pakatou et al. suggested that PKC is not directly upstream of DrdgB and is not required for rdgB-dependent retinal degeneration. Rather, they proposed that the inaC-encoded PKC might regulate some aspect of DrdgB function (Paetkau et al., 1999). The observation that mutant DrdgB is hyperphosphorylated is consistent with this hypothesis and further suggests that the function of DrdgB may be regulated by a phosphorylation/dephosphorylation cycle (Sahly et al., 1992).

1.8.3 Mechanisms of DrdgB-mediated retinal degeneration

To date, the physiological role of DrdgB within the Drosophila photoreceptor remains unknown. However, several mechanism and models have been formulated that highlight a PtdIns-supply function on the basis of the presence of an amino-terminal PtdIns-TP-like domain. Models have also been proposed that highlight a role for DrdgB in the regulation of intracellular Ca^{2+}-levels by virtue of its Ca^{2+}-binding activity and localisation to the SRC, have been also proposed.

The presence of an amino terminal PtdIns-TP domain on DrdgB provides an opportunity for DrdgB function to be interpreted in light of PtdIns-TP function in mammals and yeast. For example, by analogy to Sec14p function and the proposed roles of PITPα and PITPβ in vesicle trafficking, DrdgB may be required for the transport of membrane proteins and lipids involved in phototransduction from the SRC to the rhabdomeres. It would be predicted that degeneration of the rhabdomeres would result from the absence of such membrane transport.

Alternatively, DrdgB may function as a PtdIns-presenting component of the visual cascade that facilitates the phosphorylation of PtdIns by dedicated kinases. Consequently, DrdgB function may be considered to be involved in the maintenance of a light-sensitive PtdIns(4,5)P_2 pool in the rhabdomeres. Exploring this concept, the identification that defects in an eye-specific CDP-DAG kinase (eye-CDS), the
rate limiting enzyme in PtdIns synthesis, resulted in light enhanced retinal degeneration and that over production of eye CDS increases the amplitude of the light response, clearly demonstrated that the PtdIns(4,5)P$_2$ supply plays a critical role during phototransduction (Wu et al., 1995). A role for DrdgB in establishing an initial PtdIns(4,5)P$_2$ pool seems unlikely since inspection of the ERG light responses of $rdgB$ mutant flies demonstrates that these flies exhibit a wild type amplitude in the light response (Milligan et al., 1997b). However, ERG response is defective in photoreceptor depolarisation after termination of the light response indicating that a requirement of DrdgB for regeneration of this pool remains a possibility.

How then, could DrdgB efficiently mediate PtdIns transfer in the light of the presumed immobility conferred by the putative membrane spanning domains? The gap between the SRC and the rhabdomere is about 10nm, which may accommodate the direct transfer of PtdIns between these juxtaposed membranes by the PtdIns-TP domain of DrdgB without the need for diffusible PITP or vesicular transport. This would facilitate a more rapid rate of PtdIns turnover that may be required for phototransduction. However, whether the tertiary structure of the PtdIns-TP domain is sufficient to span this gap is unknown.

In vivo studies with DrdgB mutants have demonstrated that the expression of the soluble PtdIns-TP domain of DrdgB, was sufficient for complete rescue of retinal degeneration and the electrophysiological defects associated with the mutant phenotype (Milligan et al., 1997b). This suggests that the PtdIns-TP domain of DrdgB represents the sole domain of this protein required for wild type phototransduction and is consistent with a PtdIns supply function for DrdgB (Milligan et al., 1997b). However, neither the expression of PITP$_\alpha$ protein alone, nor PITP$_\alpha$ in combination with the carboxy-terminal of DrdgB provided any detectable rescue, suggesting that the PtdIns-TP domain of DrdgB and mammalian PITP$_\alpha$ are not functionally equivalent (Milligan et al., 1997b). Furthermore mutations in the PtdIns-TP domain of DrdgB that do not effect PtdIns and PtdCho transfer activity in vitro, still cause retinal degeneration (Milligan et al., 1997b).

While the PtdIns-TP domain of DrdgB is sufficient and essential for in vivo function, the recent identification and characterisation of a novel DrdgB suppresser suggests that other regions of the protein can affect DrdgB activity in vivo (Paetkau et al., 1999). An intragenic suppresser ($rdgB^{100}$) of the retinal degeneration phenotype was identified that contains a H542E missense mutation in the first putative lumenal loop of DrdgB (Paetkau et al., 1999). The identification of this
suppressor and the conservation in multidomain structure between DrdgB and mammalian DrdgB homologues (Section 1.9) suggests that the carboxy-terminal domain of DrdgB does possess an important function.

Alternatively or additionally, DrdgB may play a role in regulating intracellular Ca\(^{2+}\) levels. TRP mutants that exhibit a decreased light-dependent influx of Ca\(^{2+}\) into the retinal cell (Peretz et al., 1994) and the Ca\(^{2+}\)-channel blockers diltiazem and verapamil hydrochloride suppress DrdgB-dependent retinal degeneration (Sahly et al., 1992). These observations are consistent with the demonstration that the large quantity of dense globular bodies, found in DrdgB null mutant photoreceptors possess a high Ca\(^{2+}\) concentration (Sahly et al., 1994). Consequently, effector driven Ca\(^{2+}\) toxicity is a plausible mechanism for DrdgB dependent photoreceptor degeneration. Accordingly, DrdgB may downregulate the phototransduction cascade by lowering cytoplasmic Ca\(^{2+}\), possibly by facilitating the sequestration of this cation to an intracellular compartment. Certainly the Ca\(^{2+}\)-binding capability of DrdgB and the six putative transmembrane domain structures are readily consistent with this view. Moreover, this hypothesis is generally consistent with the observation that stimulation of the phototransduction cascade triggers retinal degeneration in rdgB mutant flies.

This hypothesis suggests that the activity of the DrdgB protein may be closely regulated during phototransduction through the elevation of intracellular Ca\(^{2+}\), a key component of photoactivation. The regulation of DrdgB in this manner may account for several aspects of Drosophila PLC signalling, identified by studies of Drosophila mutations including (1) the availability of PtdIns(4,5)P\(_2\) is the rate-limiting step of the cascade, (2) the PtdIns(4,5)P\(_2\) required for signalling is distinct from the general pool within the photoreceptor cell and (3) the PtdIns(4,5)P\(_2\) required for signalling is generated in response to light activation (Zuker, 1996).

An interesting corollary to this Ca\(^{2+}\)-sequestration model is that the PtdIns-TP domain of DrdgB may represent a regulatory subunit whose effect on a Ca\(^{2+}\)-mobilising activity may be regulated by bound phospholipid, in a manner analogous to the sensor model of Sec14p.

1.8.4 Dominant negative mutant forms of rdgB: existence of associating proteins

Milligan and co-workers have provided information on previously unappreciated aspects of DrdgB function as a result of the identification of several dominant-negative mutants of DrdgB. Milligan et al., (1997) observed that the
expression of Drdgb containing a T59E mutation (DrdgB-T59E) or a PITPα-DrdgB chimera in mutant flies expressing one wild type rdgB allele (DrdgB+) exerted powerful effects that were manifested in dominant retinal degeneration phenotypes. Specifically, expression of rdgB-T59E in Drdgb flies resulted in defective rhodopsin biogenesis leading to reduced rhodopsin levels within the rhabdomere (Milligan et al., 1997b). Electrophysiological and histological analysis revealed that the expression of a PITPα-DrdgB chimera in Drdgb flies appeared to antagonise Drdgb activity within the photoreceptor (Milligan et al., 1997b). Both these dominant phenotypes may suggest that Drdgb associates with at least one other component of the phototransduction cascade. The observation that mammalian Drdgb isoforms bind the protein tyrosine kinase Pyk2 via their conserved carboxy-terminal domain (Sections 1.9 and 1.9.5), together with the high level of sequence identity between the carboxy-terminal domains of Drosophila and mammalian rdgB isoforms raises the possibility that Drdgb may also associate with Pyk2. Whether or not Pyk2 or any other protein-binding partners in Drosophila.

In summary, the data presented in Section 1.8 indicate the complexity of the mechanism of rdgB function and its role in the photoreceptor that is not easily reconciled with a simple role of potentiating signal transduction via a PtdIns-driven signalling pathway.

1.9 Mammalian rdgB homologues

The first mammalian homologue of Drdgb was recently cloned by several independent groups and termed mammalian rdgBα (MrdgBα, m-rdgB1 or PITPnm) (Aikawa et al., 1997; Chang et al., 1997; Guo and Yu, 1997; Rubboli et al., 1997). More recently, using a yeast two-hybrid approach to screen for proteins interacting with the protein tyrosine kinase Pyk2, Lev and co-workers identified MrdgBα and two novel human rdgB proteins (Lev et al., 1999). Since all three proteins bound the amino-terminal domain of Pyk2, they have been designated Pyk2 N-terminal domain interacting receptor (Nir) proteins (Lev et al., 1999). According to this nomenclature, which is employed in this thesis, Nir2 corresponds to MrdgBα. Subsequently, Hyde and co-workers have also cloned Nir1 and Nir3 from mouse and zebrafish cDNA libraries, respectively (Elagin et al., 2000; Lu et al., 1999).

Like Drdgb, the Nir proteins have a multiple domain structure, containing a small acidic domain that exhibits Ca²⁺-binding in vitro, six putative transmembrane domains and a carboxy-terminal domain. Both Nir2 and Nir3 contain an amino-
terminal PtdIns-TP-like domain, however, this domain is absent in Nir1 (Elagin et al., 2000; Lev et al., 1999).

Cloning of Nir1-3 has revealed that these proteins have a predicted molecular weight of around 106, 140 and 150kDa, respectively (Lev et al., 1999). The PtdIns-TP-like domains of Nir2 and Nir3 exhibit 72% identity with each other and 65% and 45-47% identity with DrdgB and PITPa, respectively. The acidic domain and putative membrane-spanning domains of Nir1-3 exhibit 66-68% identity with each other and 41-75% identity with their Drosophila counterpart. The carboxy-terminal domains of Nir1-3 exhibit approximately 40-60% identity with each other and approximately 27-60% identity with DrdgB. Alignment of the full-length primary sequence of Nir1-3 with DrdgB reveals that Nir2 exhibits the greatest identity with DrdgB.

Although Nir1-3 mRNAs possess different tissue expression patterns, all three are abundantly expressed in the brain and retina. Nir1 is expressed exclusively within the retina, brain, spleen and ovary (Lev et al., 1999). While some groups have demonstrated that Nir2 and Nir3 exhibit a ubiquitous or broad tissue expression pattern (Aikawa et al., 1997; Lev et al., 1999; Rubboli et al., 1997), other groups have demonstrated a more restricted distribution (Chang et al., 1997; Guo and Yu, 1997; Lu et al., 1999). Nevertheless, although it has been proposed that rdgB proteins are specialised for a role in retinal, olfactory and neuronal cells, the wide tissue distributions of human Nir1-3 suggest much broader roles for these proteins in vivo.

While Nir1-3 are abundantly expressed within the retina, microscopic inspection of the relative expression of these proteins within the retina reveals distinct patterns of expression. The structure of the retina is shown in Figure 4.1.

Fluorescent immunolocalisation studies have demonstrated that Nir1 and Nir2 are expressed within the inner retina, with Nir1 exhibiting a high level of expression within the inner nuclear and outer plexiform layer and Nir3 exhibiting a high level of expression in both the inner and outer plexiform layers (Lev et al., 1999; Lu et al., 1999). In contrast, Nir2 expression was detected through the retina, most notably in the inner segment of the photoreceptor layer and the inner nuclear layer (Chang et al., 1997; Lev et al., 1999). These studies demonstrate that while Nir1-3 do exhibit overlapping patterns of expression, the different isoforms are differentially expressed in different cell types of the retina. Likewise, the Nir proteins exhibit similar expression patterns in some but not all, neuronal cell populations.
within the brain (Lev et al., 1999; Lu et al., 1999). The differential distribution of Nir proteins within the retina and brain is most likely to reflect specificity in the cellular functions of these proteins.

The observation that Nir proteins are expressed within the inner segment and/or the plexiform layers is consistent with a role for these proteins in intracellular transport, as proposed for other members of the PtdIns-TP family (Section 1.4.3). Within vertebrate retina, phospholipids and proteins required for rod outer segment disc assembly are synthesised within the inner segment. Thus, Nir2 may function in the process of outer segment membrane renewal. Nir2 and Nir3 are also concentrated in neuronal processes and termini comprising the plexiform layer and may therefore participate in synaptic vesicle trafficking, facilitating the high rates of intracellular membrane traffic observed these neuronal cells. Nir1 does not have a PtdIns-TP transfer domain and may therefore have another regulatory function.

Although the intracellular localisation of Nir1 and Nir3 remains to be addressed, Aikawa and co-workers have recently demonstrated that both endogenous and Myc-tagged Nir2 localise to the membranes of the Golgi and the ER in COS cells (Aikawa et al., 1999). Immuno-electron microscopy demonstrated that Nir2 is restricted to the cytoplasmic side of the Golgi membranes and that the amino-terminal PtdIns-TP domain is exposed to the cytoplasmic surface. Furthermore, Nir2 truncation mutations demonstrated that the Golgi localisation was dependent on the presence of the six putative membrane-spanning domains and, to a lesser extent, the carboxy-terminal domain (Aikawa et al., 1999).

1.9.1 Are rdgB proteins integral membrane proteins?

The original model for DrdgB existing as an integral membrane protein was based on alkaline extraction and the presence of six hydrophobic domains. Indeed, using an alkaline extraction method, Aikawa and co-workers provided evidence that Nir2 is an integral membrane protein (Aikawa et al., 1999). However, recent biochemical studies using brain and retinal lysates, have demonstrated that Nir1 and Nir3 can be solubilised from the particulate fraction after treatment with guanidinium chloride, a strong denaturing agent, but not with detergents (Elagin et al., 2000; Lu et al., 1999). Based on these data, Hyde and co-workers have proposed that the rdgB proteins do not assume an integral membrane topology, rather they are likely to form stable protein-protein interactions with either a membrane protein or the cytoskeleton (Li et al., 2000b).
1.9.2 *Nir1-3* are functional homologues of *DrdgB*

Insight into the functional of *Nir1-3* and *DrdgB* function has been gained from the genetic studies of Hyde and co-workers. Specifically, they demonstrated that the expression of the cDNA of Nir2, Nir3 or a zebrafish Nir1 homologue in *rdgB* null mutant flies rescued the mutant phenotype; however, the extent of the rescue differed with each isoform (Chang *et al.*, 1997; Elagin *et al.*, 2000; Lu *et al.*, 1999). These data suggest some conservation of biochemical activity between *DrdgB* and *Nir1-3* with respect to function in the phototransduction pathway of *Drosophila*. The expression of Nir2 cDNA in *rdgB* mutant flies was sufficient for complete rescue of the retinal degeneration and electrophysiological defects associated with the mutant phenotype (Chang *et al.*, 1997). In contrast, Nir1 and Nir3 cDNA expression only delayed *DrdgB*^2^-dependent retinal degeneration. While Nir3 was able to partially rescue the defective ERG response (Lu *et al.*, 1999), Nir1 offered no detectable rescue of the electrophysiological defects associated with the mutant phenotype (Elagin *et al.*, 2000). The differences between *Nir1-3* in suppressing the *DrdgB*^2^-mutant phenotype further suggested that there are functional differences between these mammalian homologues and that Nir2 is functionally more similar to *DrdgB* than Nir1 and Nir3.

The observation that Nir1, which lacks a PtdIns-TP domain, is able to partially rescue the mutant phenotype is consistent with the hypothesis that the carboxy-terminal residues of *DrdgB* are functionally relevant. Furthermore, these data suggest that Nir3 possess an activity that promotes photoreceptor viability but is unable to participate in the PLC-mediated signalling cascade (Elagin *et al.*, 2000). In contrast to Nir1, the expression in *rdgB*^2^ flies of a DrdgB cDNA, in which the PtdIns-TP domain was deleted, offered no detectable rescue of the mutant phenotype (Elagin *et al.*, 2000). Since both proteins appeared to possess similar structural motifs and were stability expressed at high levels on several independent transgenic lines, Hyde and co-workers proposed that an intrinsic functional difference exists between Nir1 and the non-PtdIns-TP domains of *DrdgB* (Elagin *et al.*, 2000).

The observation that all three Nir proteins exhibit some activity during invertebrate phototransduction, together with the observation that Nir1-3 map to three distinct chromosome regions known to contain multiple retinopathy loci (Section 3.1), raises the possibility that these proteins may play a role in vertebrate phototransduction. Evolution has adapted seemingly divergent paradigms for invertebrate and vertebrate visual processing (Section 1.8.1), suggesting that Nir1-3
do not play a direct role in the major vertebrate phototransduction pathway. While a
direct role for PLC isoforms during vertebrate phototransduction has yet to be
established, PLC8, PLCγ and PLCδ isoforms have been detected through out the
retina (reviewed by Giusto et al., 2000). Furthermore, Ghalayini and co workers
have demonstrated that PLC activity under light conditions is significantly higher
than the activity found under dark conditions (Ghalayini et al., 1998). However, this
study did not reveal which PLC isozyme was responsible for the increase inactivity.
A vertebrate retina-specific homologue of the Drosophila PLC encoded by the
NorpA gene, is expressed throughout the retina (Alvarez et al., 1995; Ferreira et al.,
1993; Lee et al., 1993). Specifically, this PLCB4 isoform has been detected within
the photoreceptor cell layer, bipolar cells, horizontal cells and the ganglion cells
(Peng et al., 1997). Although the specific function of mammalian retinal PLC
isoforms remain to be clearly defined, experiments in which murine PLCB4 was
knocked out demonstrated altered visual processing and suggested that PLCB4 plays
a modulatory role in the rod-rod-bipolar signalling cascade, rather than acting as a
primary signalling intermediary as it does during Drosophila phototransduction
(Jiang et al., 1996).

In addition to PLC isoforms, immunolocalisation studies in various vertebrate
retinas, have indicated the presence of eight PKC subspecies with unique cellular
distributions within this tissue (reviewed by Giusto et al., 2000). PKC-catalysed
phosphorylation of rhodopsin, transducin, arrestin and PDE has been reported
(Giusto et al., 2000). While, this phosphorylation causes a decrease in the light
response, the functional significance of phosphorylation by PKC remains to be
examined (Giusto et al., 2000). These data suggest the existence of novel aspects of
the vertebrate phototransduction cascade. The roles of Nir1-3 and PLC- and PKC-
isoforms within vertebrate phototransduction, particularly during light recovery and
adaptation, await further investigation.

Recent data by Womack and colleagues have provided further evidence for a
role of phosphoinositide signalling during vertebrate phototransduction (Womack et
al., 2000). Womack and co-workers investigated the effects of PtdIns(4,5)P2 and
anti-PtdIns(4,5)P2 antibodies on guanidine nucleotide-stimulated PDE activity in a
biochemical assay using membranes from bovine rod outer segments. Using this
assay, it was demonstrated that PtdIns(4,5)P2-enhanced PDE activity, suggesting a
regulatory role for PtdIns(4,5)P2 on PDE activity (Womack et al., 2000). In the same
study it was shown, primarily using electrophysiological recordings, that
PtdIns(4,5)P$_2$ has a strong inhibitory effect on bovine rod cyclic nucleotide gated (CNG) channels when $\alpha$ and $\beta$ subunits were expressed in *Xenopus* oocytes (Womack *et al.*, 2000). In agreement with other studies, similar effects on rod CNG channels were also observed by the application of MgATP to the cytoplasmic face of the inside-out patch. Furthermore, this inhibition was reversed by the application of anti-PtdIns(4,5)P$_2$ antibodies (Womack *et al.*, 2000). Previous studies have indicated that the inhibitory effect of ATP on CNG channels expressed in oocyte membranes is due to tyrosine phosphorylation (Molokanova *et al.*, 1999; Molokanova *et al.*, 1997). Womack and colleagues have thus proposed that the inhibition by PtdIns(4,5)P$_2$ and ATP are interdependent (Womack *et al.*, 2000). This notion is consistent with the observation that the stimulatory effects of cAMP dependent protein kinase phosphorylation and PtdIns(4,5)P$_2$ binding on G protein-sensitive inwardly rectifying potassium (ROMK) channels is synergistic (Liou *et al.*, 1999).

The findings of Womack and colleagues are consistent with previous reports demonstrating that PtdIns(4,5)P$_2$ can modulate the function of several ion channels and transporters, independent of Ins(1,4,5)P$_3$, Ca$^{2+}$, DAG and PKC (Womack *et al.*, 2000). The physiological significance and the mechanism by which PtdIns(4,5)P$_2$ promotes the activation of PDE by transducin, and modulates CNG channel activity is unknown, as is the physiological significance.

### 1.9.3 A role for rdgB proteins during capacitative Ca$^{2+}$ entry in non-excitablc cells

The established role of DrdgB in the PLC-mediated phototransduction pathway in invertebrates suggests that mammalian homologues of this protein may be involved in the PLC-mediated pathway of ligand induced capacitative Ca$^{2+}$ entry in non-excitablc cells (reviewed by Barritt, 1999; Putney and McKay, 1999c). In this pathway, InsP$_3$ generated by PLC acts on the InsP$_3$R on the ER membrane activating the Ca$^{2+}$-permeable channel, thus causing the release of stored Ca$^{2+}$ to the cytoplasm. The release of Ca$^{2+}$ is closely followed by an influx of Ca$^{2+}$ into the cell across the plasma membrane (Putney and McKay, 1999c). Since the signal for activation of the plasma membrane channels follow the depletion of Ca$^{2+}$ within the ER Ca$^{2+}$ stores, this process is called capacitative Ca$^{2+}$ entry or store-operated Ca$^{2+}$ entry. Two general mechanisms for signalling capacitative Ca$^{2+}$ entry have been proposed (reviewed by Barritt, 1999). One involves the generation and release from the ER of a diffusable signalling factor. The alternative suggestion is that the ER InsP$_3$R and
the plasma membrane $\text{Ca}^{2+}$ channels interact directly, giving rise to the conformational coupling model. While there is considerable experimental evidence to support the diffusable messenger model, recently published findings support the idea of a close interaction between ER $\text{Ca}^{2+}$ stores and plasma membrane channels (reviewed by Putney, 1999b). A brief discussion of the simplest model of conformational coupling is considered below (for a more detailed review of capacitative $\text{Ca}^{2+}$ entry see Barritt (1999)).

Recently, Muallem and co-workers provide compelling evidence to support the basic tenet of the conformational coupling model of capacitative $\text{Ca}^{2+}$ entry, namely that store-operated channels are regulated through interaction with the InsP$_3$R (Kiselyov et al., 1999; Kiselyov et al., 1998). These two studies demonstrate that a member of the TRP family of channel proteins, TRP3, functions as a store-operated $\text{Ca}^{2+}$ channel, which is gated by Ins(1,4,5)P$_3$-occupied InsP$_3$Rs.

In the simplest form, the conformational coupling model proposes that plasma membrane channels, possibly TRP channels, are loosely associated with underlying InsP$_3$R within the ER membrane (Putney, 1999a). Depletion of intracellular stores then induces strong coupling of these proteins, resulting in the activation of the TRP channel. The observation that this pathway can be activated in the absence of PLC activation indicates that the critical InsP$_3$R involved in communicating with the plasma membrane channels must be at or near saturation with Ins(1,4,5)P$_3$ under basal or unstimulated conditions (Kiselyov et al., 1998). This situation would be expected to occur if these InsP$_3$R sites were located in close proximity to a PLC isoform, such that the concentration of Ins(1,4,5)P$_3$, basal PLC activity is much higher than in the bulk cytoplasm. The co-localisation of InsP$_3$Rs and PLC activity may be mediated through the existence of a PLC-TRP signalling complex involving an as yet, uncharacterised human homologue of the *Drosophila* photoreceptor protein, INAD (Section 1.8.1). The InsP$_3$R involved in regulating TRP channels has been proposed to be distinct from the InsP$_3$R population which is chiefly involved in the release of intracellular $\text{Ca}^{2+}$ when Ins(1,4,5)P$_3$ is released during receptor activation (Kiselyov et al., 1999).

Alternatively, a growing body of evidence suggests that PtdIns(4,5)P$_2$, rather than InsP$_3$, occupies the InsP$_3$R ligand-binding site in unstimulated cells (Kaznacheyeva et al., 2000; Lupu et al., 1998). Furthermore, this association of PtdIns(4,5)P$_2$ has been proposed to inhibit InsP$_3$R activity. Based on *in vitro* functional data and *in vivo* morphological findings, Lipu and colleagues have proposed
that a population of InsP$_3$Rs localised just under the plasma membrane forms a tight inhibitory complex with PtdIns(4,5)P$_2$ in the juxtaposed plasma membrane (Lupu et al., 1998). These same InsP$_3$Rs are directly or indirectly coupled to a Ca$^{2+}$ influx channel in the plasma membrane. In the resting state, these InsP$_3$Rs are constitutively inhibited in the presence of PtdIns(4,5)P$_2$. Agonist stimulation leads to the activation of PLC, cleavage of the InsP$_3$R-tethered PtdIns(4,5)P$_2$, opening of the InsP$_3$R channel and activation of the plasma membrane Ca$^{2+}$-influx channel via direct protein-protein interaction. According to this model, PLC simultaneously removes the InsP$_3$R inhibitor (PtdIns(4,5)P$_2$) and generates the activator (Ins(1,4,5)P$_3$), initiating to Ca$^{2+}$ wave (Lupu et al., 1998). Subsequent feedback by Ca$^{2+}$ on a general population of InsP$_3$R is proposed to sustain Ca$^{2+}$ wave propagation through the cell. As proposed above, the integrity of this preferential coupling will depend on correct spatial arrangements between intracellular Ca$^{2+}$ stores and PLC-linked receptors. Furthermore, a functional link between the InsP$_3$R, PtdIns(4,5)P$_2$ and PM Ca$^{2+}$ channels provides a basis for a local, rapid and efficient coupling between PLC activation and intracellular Ca$^{2+}$ wave initiation in neuronal and non-neuronal cells.

The models discussed above imply that the InsP$_3$R is essential for the activation of capacitative Ca$^{2+}$-entry channels. As discussed previously, current data does not accommodate a role for InsP$_3$R during invertebrate phototransduction (Section 1.8.1) suggesting the existence of at least one additional Ca$^{2+}$ uptake pathway in non-excitable cells. Nevertheless, the conformational-coupling model of the ligand-induced generation of intracellular Ca$^{2+}$ signals in non-excitable cells exhibits some common features with the *Drosophila* phototransduction cascade. As such, mammalian DrdG homologues, in particular Nir2 and Nir3, may also play a critical role in capacitative Ca$^{2+}$ entry in mammalian cells. The observation that Nir2 localises to the ER and exhibits PtdIns(4,5)P$_2$-binding activity, supports this hypothesis (Section 1.9.4).

### 1.9.4 A Role for rdgB during phosphoinositide mediated signal transduction

The presence of a PtdIns-TP domain suggests that Nir2, and Nir3 may function to present PtdIns to cognate kinases. Recently, Aikawa and co-workers investigated a role for Nir2 in phosphoinositide synthesis on the Golgi membranes (Aikawa et al., 1999). Using a combination of an enzyme-linked immunoabsorbant assay and a lipid protein co-sedimentation assay, Aikawa and co-workers investigated the ability of various Nir-2 truncation mutants to bind
phosphoinositides. Such analyses demonstrated that residues 196-257 within the PtdIns-TP domain of Nir2 exhibited PtdIns, PtdIns4P and PtdIns(4,5)P₂ binding activity, in vitro (Aikawa et al., 1999).

In the same study, Aikawa and co-workers showed that full length, Myc-tagged Nir2 and FLAG-tagged type III PtdIns 4-kinase protein could be immunoprecipitated from COS7 cells with anti-FLAG and anti-Myc antibodies, respectively (Aikawa et al., 1999). Taken together with the observation that both these proteins co-localised the Golgi. These results were interpreted to suggest that Nir2 associates with PtdIns 4-kinase on the Golgi membranes in vivo. Subsequent in vitro studies using Myc and GFP-tagged Nir2 truncation mutants, suggested that both the PtdIns-TP and the adjacent acidic domains of Nir2 were required for association with FLAG-tagged PtdIns 4-kinase in vitro. Furthermore, PtdIns 4-kinase activity was detected in Nir2 and the production of PtdIns4P was observed without the addition of exogenous PtdIns, indicating that this protein can interact with endogenous PtdIns 4-kinase in COS cells (Aikawa et al., 1999). Based on these data, Aikawa and co-workers proposed that PtdIns-bound Nir2 forms a complex with type III PtdIns 4-kinase in vivo and that Nir2 specifically supplies PtdIns to the kinase (Aikawa et al., 1999). This hypothesis is consistent with the cofactor model of PtdIns-TP function (Section 1.4.2.a). Furthermore, the observation that Nir2 can bind PtdIns4P and PtdIns(4,5)P₂ in vitro suggested that this protein may also present substrate to PtdIns4P 5-kinase and PLC.

1.9.5 Association between Nir1-3 and Pyk2

Recently, Lev and co-workers have identified an unanticipated role for Nir1-3 in Pyk2 signalling pathways (Lev et al., 1999). As indicated previously, Nir1-3 bind to the amino-terminal domain of the protein tyrosine kinase Pyk2. Using both a yeast two hybrid approach and direct binding experiments, Lev and co-workers found that the conserved carboxy-terminal domain of Nir1-3 associated with the amino-terminal domain of Pyk2.

An association of Nir1-3 with Pyk2 in vivo was confirmed by co-transfection studies in which haemagglutinin (HA)-tagged Nir1-3 cDNA and Pyk2 were co-transfected in to HEK293 cells (Lev et al., 1999). Furthermore Nir1-3 could be detected in anti-Pyk2 immunoprecipitates prepared from the transfected cell lysates. Similar studies, in which Pyk2 was replaced with focal adhesion kinase (FAK), demonstrated the specificity of the association between Nir1, 2 or 3 and Pyk2 (Lev et
Additionally, Pyk2-Nir complexes were detected in lysates prepared from brain tissue.

Analysis of Pyk2 lysates from cells co-expressing Nir2 demonstrated that both Nir2 and Pyk2 were tyrosine phosphorylated and are present in the same immunocomplex. However, while, Nir2 was not tyrosine phosphorylated in cells expressing a kinase-negative Pyk2 mutant protein (PKM), it was still found to associate with PKM (Lev et al., 1999). These data indicate that Nir1-3 are likely substrates of Pyk2 and that tyrosine kinase activity is not required for association of these proteins. Endogenous Nir2 and Pyk2 were also found to co-immunoprecipitate from quiescent HL60 cell lysates following stimulation of the intact cells with agents known to activate Pyk2, such as PMA and the ER Ca\(^{2+}\)-ATPase inhibitor, thapsigargin (Lev et al., 1999). Furthermore, these agonists induced a strong phosphorylation of Nir2 tyrosine residues. While these data strongly suggest that endogenous Nir2 is a substrate for Pyk2, it is possible that tyrosine phosphorylation of Nir proteins is induced by another protein tyrosine kinase that can be activated by Pyk2, such as Src. These observations, together with expression of Nir1-3 with Pyk2 in distinct regions of the rat brain and retina has led to the hypothesis that Pyk2 is an upstream regulator of Nir proteins (Lev et al., 1999).

1.9.5.a Pyk2

Protein tyrosine kinase (PTK) activities transduce many extracellular signals that trigger key cellular events (Avraham et al., 2000 and references therein), such as mitogenesis and cytoskeletal rearrangement, and thereby co-ordinate physiological processes, such as development and oncogenesis. PTKs mediate these responses by activating a variety of intracellular signalling pathways through their intrinsic kinase activity. Protein tyrosine kinases can be divided into receptor (RTKs) and non-receptor kinases, based on the presence of extracellular ligand-binding and transmembrane domains in the former.

Pyk2 (also known as RAFTK, FAK2, CAK-β or CADTK) belongs to a new family of PTKs, which has been named after FAK (reviewed by Avraham et al., 2000; Hanks and Polte, 1997). FAK and Pyk2 have molecular weights of 110-125kDa and exhibit around 48% amino acid identity. Both kinases have a similar domain structure: a unique amino-terminal domain, a central protein tyrosine kinase domain, and two proline rich regions at the carboxy-terminal (Avraham et al., 2000; Hanks and Polte, 1997). Despite their structural similarity, Pyk2 and FAK have
different tissue expressions and different modes of activation. Pyk2 is expressed mainly in the central nervous system and in cells derived from haematopoietic lineages while FAK is expressed in a variety of tissues (Schlaepfer and Hunter, 1998). Immunolocalisation studies have demonstrated that FAK is localised to focal adhesion sites, while Pyk2 is mainly distributed throughout the cytoplasm and is concentrated in the perinuclear region (Hanks and Polte, 1997). Activation of FAK is linked with transmembrane integrin receptors and functions during integrin-mediated signalling pathways (reviewed by Schlaepfer and Hunter, 1998). In contrast, Pyk2 is activated by a variety of extracellular stimuli including growth factors, GPCR agonists, extracellular matrix proteins and stress signals, as well as stimuli, which elevate the intracellular Ca\textsuperscript{2+} concentration. The cell-dependent roles of Pyk2 in various signalling cascades have been recently reviewed (Avraham et al., 2000) and will therefore not be discussed in detail here.

A large body of data indicates that Pyk2 regulates a variety of cellular responses, including neuronal excitability, T- and B-cell receptor signalling, cell growth and survival. Pyk2 is activated in response to stress signals, such as tumour necrosis factor-α, hyperosmotic shock and UV light, thereby inducing Jun N-terminal kinase (JNK) activation (Tokiwa et al., 1996; Yu et al., 1996). In PC12 cells, Pyk2 tyrosine phosphorylation and activation are stimulated by neuronal stimuli and stress signals leading to modulation of the delayed-rectifier-type potassium channel and the JNK signalling pathway, respectively (Lev et al., 1995; Yu et al., 1996). Similar to FAK, Pyk2 is tyrosine phosphorylated and activated by adhesion-mediated signalling in platelets and B-cells (Astier et al., 1997; Tokiwa et al., 1996). Schlessinger and co-workers have demonstrated that Pyk2 plays an important role in MAP kinase signalling cascades mediated by elevation of intracellular Ca\textsuperscript{2+}, by activation of GPCRs, by stress signals or by PKC agonists (Dikic et al., 1996; Tokiwa et al., 1996). Activation of Pyk2 by bradykinin or LPA stimulates ERK activation by a mechanism involving Pyk2 autophosphorylation, association with Src, recruitment of a Grb2-Sos complex and subsequent activation of Ras (Dikic et al., 1996; Lev et al., 1995).

In addition to the Nir proteins, several other intracellular proteins have been shown to interact with Pyk2. Specific structural motifs or tyrosine phosphorylation sites mediate these interactions. Recent studies have described the direct association of Pyk2 with the adapter protein Grb2, the tyrosine kinase Src and the ARF-GAP protein Pap (Pyk2 carboxy-terminal associated protein; Andreev et al., 1999; Dikic
et al., 1996). Pap forms a stable complex with Pyk2 and is tyrosine phosphorylated upon Pyk2 activation. Pap is a multidomain protein that exhibits intrinsic GAP activity towards the small G proteins Arf1 and Arf5 (Andreev et al., 1999). Overexpression of Pap inhibits Golgi vesicle release. Recent studies have also demonstrated that Pyk2 directly interacts with the cytoskeletal proteins paxillin, p130^* and Graf (Rac/Cdc42 GTPase-activating protein; Ohba et al., 1998).

Although the role of Pyk2 in a variety of signalling cascades has been extensively studied, its actual physiological function remains largely unknown. Since Pyk2 is activated by a variety of extracellular stimuli in different cell types, it has been proposed that Pyk2 may facilitate cross talk between different intracellular signalling pathways (Lev et al., 1999). Although phosphoinositides bind many actin-binding proteins and affect cell attachment, the role of the interaction between Pyk2 and Nir1-2 has yet to be addressed. Based on the observations discussed earlier (Section 1.4) and genetic studies in Drosophila, Lev and colleagues have proposed that the rdgB proteins function in concert with Pyk2 and downstream of G-protein coupled receptors as components of an evolutionarily conserved Ca^{2+} and phosphoinositide-dependent signalling (Lev et al., 1999).

1.10 Summary

The PtdIns-TP family consists of the single domain yeast Sec14p and mammalian PITPa and PITPβ and the multidomain rdgB proteins. Numerous studies have indicated a requirement for PtdIns-TPs during phospholipid metabolism, phosphoinositide-mediated signalling and vesicle trafficking. As such, this family of proteins is thought to play a role in a variety of cellular processes. With the possible exception of yeast Sec14p, the actual biological functions of the PtdIns-TPs remain to be characterised. Many basic questions remain essentially unresolved, including the structural basis for the recognition of specific phospholipids by the various PtdIns-TPs and how the phospholipid binding and transfer activity of a PtdIns-TP pertains to its in vivo function. Additionally, the existence of multiple PtdIns-TPs raises the question of functional specificity and degeneracy between different PtdIns-TP domains. At the start of this thesis it was hypothesised that the molecular and biochemical characterisation of the PtdIns-TPs would provide a better understanding of the functional specificity and degeneracy within the PtdIns-TP family. The following studies describe the cloning and characterisation of a novel member of the PtdIns-TP family.
2.0 Experimental procedures

2.1 Materials

All chemical reagents, unless otherwise indicated, were supplied by BDH (at least AnalaR grade) or Sigma.

**AP Biotech:** $[^{32}\text{P}]$-ATP (110 TBq/nmol), $[^{32}\text{P}]$-dCTP (220 TBq/mmol) myo-$[2-{^3}\text{H}]$-inositol (2.96-4.44 TBq/mmol), Enhanced Chemiluminescence (ECL) detection reagent, Rainbow protein markers, Hybond-N membranes, anti-rabbit IgG-Horseradish peroxidase (HRP) and anti-mouse IgG-HRP conjugates, pGEX-4T-2, pGEX-KG, Protein G-Sepharose 4B, Superdex-200 gel filtration column, gel filtration calibration kit (BSA, ribonuclease and chymotrypsin).

**Amicon:** Filter concentrators.

**Biorad:** Affigel 10, Bradford reagent, Coomassie stains and sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) molecular weight markers.

**Calbiochem:** Bovine thrombin, recombinant calmodulin and Mowial.

**Clontech:** λDR2 fetal brain library and multiple tissue Northern blot.

**Eurogentech:** MrdgBB peptide immunogens and anti-MrdgBB antisera.

**Life Technologies:** 1kb DNA molecular weight marker, dNTPs, glycogen, lipofectin transfection reagent, T4 DNA ligase, T4 DNA ligase buffer, penicillin-streptomycin, Superscript II cDNA synthesis kit, random hexamer, yeast tRNA, Trizol reagent, Glutamax and Dulbecco’s modified Eagle medium (DMEM).

**The Human Genome Mapping Resource Centre (HGMPRC):** Human PAC filters, human PAC clones and Genebridge4 hybrid panel.

**ICN:** $[^{45}]\text{CaCl}_2$ (37MBq/mmol).

**Jackson Laboratories:** FITC-labelled anti-mouse antibody.

**Millipore:** Polyvinylidene difluoride (PVDF) membrane.

**Molecular Probes:** 1-heaxadecanoyl(1-pyrenedecanoyl)-sn-glycero-3-phosphocholine, N-(1-pyrenedecanoyl)SM and trinitrophenyldioleyl-PtdEth (TNP-PE).

**MWG:** oligonucleotides

**New England Biolabs:** Restriction enzymes, Klenow DNA polymerase and Vent DNA polymerase.

**Novagen:** pET21a, *E. coli* strains BL21(DE3)pLysS and BL21(DE3) and Ni-NTA agarose resin.

**Perkin-Elmer:** Dye-Deoxy sequencing kit.
Promega: Taq DNA polymerase, Wizard columns and pGEM-T easy cloning system.

Qiagen: Qiaquick gel extraction kit, Tip100, Qiagen spin plasmid DNA purification columns and Qiagen Large construct DNA purification kit.

Roche: (p-amidino)phenylmethylsuphonylfluoride (APMSF).

Stratagene: Oligo-dT cellulose, *E. coli* strain XL-1 Blue.

Santa Cruz: 9E10 anti-Myc antibody.

Whatman: Silica-60 thin-layer chromatography (TLC) plates and filter papers.

Vector Laboratories: Vector shield.

Vysis: CGH nick translation kit.

Miscellaneous: Type Iα and type IIA PtdInsP kinase mammalian constructs were provided by Drs M. Dos Santos and S. Minogue, respectively. PITPα and PITPβ constructs were provided by S.K. Tan. The [PITP]Nir2 construct was provided by Dr J.J. Hsuan. pEFpLink2 and pET21:GST were gifts from Drs C.S. Hill and M. Howel (ICRF).

2.2 Molecular biology

Basic molecular biology techniques were performed essentially as described elsewhere (Sambrook *et al.*, 1989). All restriction enzymes, other modifying enzymes and kits were used according to the manufacturers’ instructions.

2.2.1 Purification of plasmid DNA

Cultures (2-5 ml) of LB, containing the appropriate antibiotic, were inoculated with bacterial colonies and incubated over night. Bacteria were sedimented at 4000 *x*g for 1 min and the supernatants were discarded. DNA was isolated from the bacterial pellet using Qiagen spin columns in accordance with the manufacturer’s instructions.

For the purification of larger amounts of plasmid (100-500 mg), overnight cultures (100 ml) of LB inoculated with single colonies were sedimented in 50 ml centrifuge tubes at 4000 *x*g and the supernatants were aspirated. DNA was isolated from the pellets using a Qiagen Tip100 kit in accordance with the manufacturer’s instructions. Plasmid DNA to be used as template in dideoxy sequencing reactions was prepared using Qiagen spin columns or Qiagen Tip100 columns depending on the size of the culture. Genomic DNA-free PAC DNA was isolated using a Qiagen Large-construct kit, in accordance with the manufacturer’s instructions.
2.2.2 Preparation of mRNA and first strand cDNA

Total RNA was prepared from different murine tissues using Trizol reagent in accordance with the manufacturer’s instructions. Total RNA was resuspended in DEPC-treated, 0.5x SSPE and heated to 68°C for 15 min. Chilled samples were incubated with oligo-dT cellulose, previously equilibrated with 0.5 M SSPE, for 20 min at room temperature. Slurries were loaded onto Wizard columns and washed four times with 0.5 M SSPE. The poly-(A)$^+$ fraction was eluted with DEPC-treated H$_2$O at 70°C and precipitated using ethanol and 2 μg glycogen carrier. 1 μg poly-(A)$^+$ RNA was reverse transcribed using a Superscript reverse transcription (RT) PCR kit in accordance with the manufacturer’s instructions.

2.2.3 Gel purification of DNA

Restriction fragments and PCR products were excised from agarose gels following electrophoresis and purified using the Qiagen Qiaquick gel extraction kit in accordance with the manufacturer’s instructions.

2.2.4 Ligation of DNA

Cohesive ligation of restriction fragments were carried out in 20 μl reactions containing 10 nmol vector DNA, 200 nmol insert DNA, 1x ligase buffer and 2.5 U T4 DNA ligase and incubated at 14-22°C for 3h to overnight before transformation into competent *E. coli*. Ligation of PCR products to pGEM-T easy vector, was performed as directed by the manufacturer.

2.2.5 Preparation and transformation of competent *E. coli*

LB broth was inoculated with freshly grown *E. coli* strains and grown overnight. Each starter culture (0.5 ml) was subsequently used to inoculate 50 ml LB the next morning. When the expanded cultures had reached mid-log phase (OD$_{600}$=0.4-0.6) they were cooled rapidly on ice, pelleted by centrifugation at 400 xg, and resuspended in storage buffer (100 mM KCl, 50 mM CaCl$_2$, 10 mM potassium acetate and 10 % (v/v) glycerol) and incubated on ice for 10 min. Bacteria were repelleted (1000 rpm, 15 min at 4°C) and resuspended in 2 ml storage buffer. 100 μl aliquots were snap-frozen and stored at -70°C.

Competent cells were transformed with 10 μl of each ligation reaction by mixing the cells and DNA on ice for 20 min and then subjecting to heat shock at 42°C for 90 s before returning to ice. After a further 5 min on ice, 250 μl of pre-
warmed LB medium was added and the bacteria incubated at 37°C for 1 h prior to spreading on an agar plate containing the appropriate antibiotic.

2.2.6 Subcloning and plasmid construction

Unless stated, all PCR products that were sequenced were subcloned in to pGEM-T easy vector in accordance with the manufacture’s instructions.

2.2.6.a Preparation of pET21-GST constructs

A PCR fragment from pGEX-KG (Guan and Dixon, 1991) encoding glutathione S-transferase (GST) and containing the NdeI site at the beginning of the open reading frame, was subcloned into the NdeI and XhoI sites in pET21b and designated pET21-GST.

2.2.6.b Construction of PITPa, PITPb and Nir2 HIS6 expression plasmids

PITPa and β isoforms were cloned and expressed as His6-fusion proteins in bacteria as described previously (Cunningham et al., 1996). The PITP-like domain of human Nir2 was obtained using PCR with Vent polymerase from the pDR2 human infant brain cDNA library (forward primer: 5'- AAAACATATGCTCATCAAGGAATACC-3'; reverse primer: 5'- AAAACTCGAGCTCGGTGCTCGGTTTCCC-3'). Products were treated with Taq polymerase to generate A-overhangs and subcloned into pGEM-T easy. Several independent clones were sequenced to check for mutations before insertion into pET21a using NdeI and XhoI restriction sites.

2.2.6.C Construction of carboxy-terminally FLAG-tagged MrdgBβ mammalian expression vector

The FLAG epitope was generated by hybridising complementary oligonucleotides (5'-AATTCCGACTACAAGGACGACGATGACAAGTGA-3' and 5'-GGCTGATGTTCCTGCTGCTACTGTTCACTGATC-3') and subcloned into the mammalian expression vector pEFpLink2 (Marais et al., 1995) at the EcoRI and SpeI sites. A PCR fragment encoding the MrdgBβ open reading frame with terminal EcoRV and EcoRI restriction sites (forward primer: 5'- ATATGATATCAATGCTGCTGAAAGAGTACCGG-3'; reverse primer: 5'- ATATGA ATTCTCAGATTGGGGCCGACATGG-3') was subcloned into the NcoI
and EcoRI sites of the pEFpLink2-FLAG construct. The final open reading frame was verified by sequence analysis.

2.2.7 PCR Methods

2.2.7.a PCR from cDNA libraries, first strand cDNA

PCR using first strand cDNA or the λDR2 fetal brain cDNA library as a template was used to isolate the complete ORF of MrdgBβ and to screen tissues for expression. Typically, 50μl reactions were performed in 1x Vent polymerase buffer containing 100 pmol of each primer, 2 mM MgCl₂, 0.2 mM dNTPs and 2.5U Vent DNA polymerase containing 2 x 10⁵ pfu or 10 ng of first strand cDNA. 40 cycles of amplification (94°C denaturation for 30 s, 50°C annealing for 30 s and 72°C extension for 60s) were performed. The Mg²⁺ concentration, annealing temperature and extension time were optimised for each amplification.

2.2.7.b Rapid amplification of cDNA ends from λDR2 fetal brain cDNA library

5’-rapid amplification of cDNA ends (RACE) PCR was performed to isolate sequences encompassing the initiation codon and absent cDNA sequence of MrdgBβ. Two sequence-specific antisense primers, designated R1 and R2 (R1: 5’-AAAAACTCGAGTGGCTCGTTCAAATTCTCG-3’; R2: 5’-AAAAACTCGAGCCTG TCTATGTCCAATCAGC-3’) and two sense primers flanking the multiple cloning site of the library vector (V1 and V2) were used to screen a λDR2 fetal brain cDNA library. The RACE reaction consisted of two rounds of PCR in which the first utilised the R1 and V1 primers. A small aliquot of this reaction was subsequently reamplified with nested primers R2 and V2.

For the first reaction conditions were essentially as described above, but with 1 x 10⁷ pfu/reaction of library, and a 2 min extension time. An initial denaturation step of 94°C for 3 min was employed to release phage DNA before the addition of Taq DNA polymerase. A 1 μl aliquot of this reaction was used in a second PCR with the nested primers V2 and R2 for a further 25 cycles under the same conditions.

2.2.7.c PCR screening of bacterial colonies

PCR screening of recombinant bacteria was employed when large numbers of clones were to be analysed or the plasmid copy number was too low for adequate plasmid yields using standard plasmid analysis techniques. The size of the insert and the identification of positive clones were determined using primers complementary
to the sequences flanking the multiple cloning site of the vector or using insert-specific primers, respectively.

Single colonies were picked from agar plates and resuspended in 20 μl ddH₂O, heated for 3 min and centrifuged at 14 000 xg for 10 min. 10 μl aliquots of the supernatant were subsequently used as a template in 20 μl reactions containing 1x Taq DNA buffer (Promega), 50 pmol of each primer, 2 mM MgCl₂, 0.2 mM dNTPs and 1.0 U Taq DNA polymerase. 25 cycles of amplification (typically: 94°C for 30 s, 50°C for 25 s and 72°C for 60 s) were performed.

2.2.7.d PCR analysis of the Genebridge4 mapping panel and PAC clones

To determine the chromosomal locus of the MrdgB8 gene, the Genebridge4 mapping panel consisting of 86 hybrid cell lines (obtained from HGMPRC) was screened by PCR using MrdgB8-specific primers. The Mg²⁺ concentration, annealing temperature and extension time were optimised. 10 μl reactions were performed in 1x Taq polymerase buffer containing 1 pmol of each primer, 1.5 mM MgCl₂, 0.2 mM dNTPs, 50 ng template DNA and 1U Taq DNA polymerase. Following an initial denaturation step at 94°C for 4 min, 35 cycles of amplification (94°C denaturation for 30 s, 54°C annealing for 30 s and 72°C extension for 60 s) were performed.

PAC DNA clones were screened using identical cycling parameters to those described above.

2.2.7.e Cycle sequencing

Sequencing reactions were carried out on double stranded plasmid DNA, using the PRISM Dye Deoxy Terminator Cycle Kit (Perkin-Elmer Biosystems) and an automated DNA sequencer (Perkin-Elmer Biosystems PRISM 377). DNA was sequenced in both directions using vector sequence specific primers.

2.2.7.f Sequence analysis

Unless otherwise indicated, sequence analysis, comparisons and alignments were performed using the PEPLOT, BESFIT and the PILEUP programs, respectively (version 7, Genetics Computer Group (GCG)). ClustalW alignments shown in Figures 3.3 and 3.3 were performed using MacVector software (version 6.5, Oxford Molecular).
2.2.8 Fluorescent probe labelling

A CGH nick translation kit (Vysis) was used as directed to incorporate Spectrum Green-labelled dUTP into PAC DNA in order to generate a fluorescently labelled MrdgBβ probe for use in fluorescent in situ hybridisation (FISH) experiments.

2.2.9 Radioactive probe labelling

Radiolabelled probes for Northern analysis and PAC library screening were generated using random priming of a MrdgBβ cDNA template. 25 μl reactions contained 15-30 ng of template DNA, 0.7 mM dATP, 0.7 mM dTTP, 0.7 mM dGTP, and 5-10 μCi [α32P]-dCTP, 10 pmol random hexamer, 2 mM MgCl2, 2.5 mM DTT, 0.1 mg/ml BSA, and 2U Klenow DNA polymerase. All reaction components were heated at 100°C for 4 min and then rapidly cooled on ice, after which the [α32P]-dCTP and Klenow were added and the reactions incubated at 37°C for 45 min. Reactions were stopped by the addition of 75 μl of TEN buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, and 1 mg/ml yeast transfer RNA). Labelled probes were purified from unincorporated radionucleotides by gel filtration through a Sephadex G-50 spin column. Cerenkov counting in a Beckman LS 1801 scintillation counter was used to determine the specific activity.

2.2.10 Hybridisation techniques

2.2.10.a Northern blotting

A human multiple-tissue Northern blot (Clontech) was probed under high stringency conditions in accordance with the manufacturer’s instructions with a [α32P]-labelled cDNA fragment of MrdgBβ encoding nucleotides 1 to 600.

2.2.10.b PAC colony screen

In order to isolate MrdgBβ genomic clones for FISH analysis, human PAC gridded library filters (obtained from the HGMPRC) were probed with a 32P-labelled MrdgBβ-specific DNA probe. Human PAC gridded library filters were pre-wetted in 6x SSPE for 2 min and incubated in prehybridisation solution (100 μg/ml denatured, sheared salmon sperm DNA, 50% formamide, 6x SSPE, 5x Denhardt’s solution and 0.5% SDS) for 1-2 h at 40°C. Prehybridisation buffer was removed and the filters were incubated in hybridisation solution (100 μg/ml denatured sheared salmon sperm DNA, 50% formamide, 6x SSPE and 0.5% SDS) containing 1-10 x 10⁵ cpm/ml
denatured probe and allowed to hybridise overnight at 42°C in a rotary oven. Filters were washed twice in 2x SSC, 0.5% SDS at room temperature for 5 min, followed by twice in 2x SSC, 0.1% SDS at room temperature for 15 min, followed by once in 1x SSC, 0.5% SDS for 30 min at 37°C and then at 68°C for 30 min and then monitored to determine the background. Further washes were carried out in 0.1x SSC, 0.1% SDS at room temperature until an acceptable background was obtained. The filters were then exposed to X-ray film.

2.2.10.c FISH analysis of metaphase spreads

Slides containing normal metaphase spreads generated from stimulated lymphocytes were denatured at 73°C for 5 min in denaturation solution (70% formamide, 10x SSC (pH 5.3) adjusted to pH 7.0-7.5). Subsequently slides were dehydrated for 1 min in 70% ethanol solution, followed by 1 min in 85% ethanol solution, and 1 min in 100% ethanol solution and then dried on a 45-50°C slide warmer. 10 μl of denatured probe mix prepared as described in Section 2.2.8 was applied to the slides which were then covered with a coverslip, sealed with diluted rubber seal, incubated in a humidified box and allowed to hybridise for 48 h at 37°C. Post hybridisation, slides were washed to a stringency of 2x SSC containing 0.1% NP-40 at 60°C and allowed to dry in the dark. Chromosomal DNA was counterstained and banded with 10 μl of DAPI counterstain containing an antifading solution (Vectorshield). Stained slides were visualised on an Axioskop microscope fitted with a photometric camera (Zeiss). A filter set specific for DAPI and FITC to view the counterstain and Spectrum Green, respectively. The two separate images were collected and overlaid electronically using Mac Probe software (Version 4.0, Perceptive Scientific Instruments).

2.3.1 Expression of bacterial fusion proteins

In all cases bacterial expression required optimisation of expression and purification parameters for each construct for which specific conditions are not given for reasons of space.

2.3.1.a Purification of GST fusion proteins from bacteria

Starter cultures of E. coli strain BL21(DE3)pLysS containing cDNA constructs in pGEX or pET21:GST vectors were grown overnight and used to inoculate larger flasks at a dilution of 1/10-1/20. Cultures were expanded for 90 min
before induction of recombinant protein expression with 0.1 mM IPTG for 3 h at room temperature, after which bacteria were centrifuged at 4000 xg for 15 min. Bacterial pellets were subsequently sonicated in lysis buffer (50 mM Tris-HCl pH7.4, 150 mM KCl, 0.5 % Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.1 % β-mercaptoethanol, 1 mM PMSF, 1 mM benzamidine, 1 mM leupeptin, 1 mM aprotinin).

For larger scale preparations (100-1000 ml of culture), resuspended bacterial pellets were incubated with 50 μg/ml lysozyme for 15 min on ice prior to sonication. Sonicated lysates were cleared by centrifugation at 20,000 xg for 20 min. Supernatants were incubated for 30 min at 4°C, with glutathione Sepharose, washed extensively with lysis buffer without protease inhibitors, and resuspended in 80 mM Tris-HCl pH7.9 containing 2 mM NaCl. Recombinant proteins required for phospholipid transfer assays were washed in the absence of Triton X-100. Bovine thrombin was added at a concentration of 25 U/mg of fusion protein and incubated for 1 h at room temperature. Cleaved protein was collected following centrifugation at 1000 xg for 3 min in order to pellet the glutathione Sepharose. In order to obtain a similar buffer composition to HIS₆-tagged protein preparations, EDTA was added to a final concentration of 400 mM. Recombinant GST was purified essentially as above, with the exception that GST was eluted from the Sepharose using reduced glutathione.

2.3.2 Purification of HIS₆ fusion proteins from bacteria

Starter cultures of *E. coli* strain BL21(DE3)pLysS or BL21(DE3) containing cDNA constructs in pET vectors were grown overnight and used to inoculate larger flasks at a dilution of 1/10-1/20. The expanded cultures were induced as described above (Section 2.3.1). His₆-tagged proteins were purified using His-Bind resin according to the manufacturer's instructions. Cell lysates were also prepared in the same way as in Section 2.3.1 above except that the lysis buffer contained 20 mM Tris-HCl, pH7.9, 50 mM NaCl, 5 mM imidazole, 1 % Triton X-100, 1 mM PMSF, 1 mM benzamidine, 1 mM leupeptin, 1 mM aprotinin. Recombinant proteins were purified on activated Ni-NTA agarose equilibrated with lysis buffer and washed 4 times in wash buffer (20 mM Tris-HCl, pH7.9, 50 mM NaCl, and 60 mM imidazole). Bound protein was eluted by chelation of Ni²⁺ ions with strip buffer (400 mM EDTA, 2 M NaCl, 80 mM Tris-HCl, pH7.9).
2.3.3 Lipid-mediated transfection of mammalian cells

Lipid mediated transfections using Lipofection (Life Technologies) were performed in 90mm tissues cultures dishes. The amount of plasmid DNA and lipofection was optimised for each transfection. Optimal conditions for transfection of human embryo kidney HEK293 cells were as follows: typically monolayers of 40-60% confluence were washed once in phosphate buffered saline (PBS) and incubated for 6 h with a mixture containing 10 μg plasmid DNA, 20μl lipofectin and serum free medium. After incubation, the medium was aspirated, the cells washed once in PBS, and incubated in complete medium. Cells were harvested 24 h after addition of the DNA mix for analysis of expressed protein.

2.4 Cell culture and transfection

HEK293 cells were cultured in DMEM containing Glutamax (Life Technologies) and 10% fetal calf serum, 50 IU/ml penicillin and 50μg/ml streptomycin.

2.5 Immunological techniques

2.5.1 Western blotting

Proteins separated on SDS-PAGE gels were transferred to pre-wetted PVDF membranes (Immobilon-P, Millipore) in transfer buffer (25 mM Tris-HCl, 192 mM glycine, and 20 % (v/v) methanol) using a transphor electrophoresis unit (Hoefer TE series). Proteins were transferred for 1 h at a charge density of 0.8 mA/cm².

To probe the resulting blot, the membrane was first blocked for 1 h in PBST (1x phosphate buffered saline containing 0.1 % Tween 20) containing 5 % (w/v) skimmed milk powder with agitation on a rotary shaker. After blocking, the membrane was incubated in PBST containing 5 % (w/v) skimmed milk powder and the primary antibody at the appropriate dilution. Standard solutions contained 1:400 M2 anti-FLAG (3.6-4.4 mg/ml) or 1:1000 anti-Myc (200μg/ml) antibodies. The membrane was agitated in the antibody solution for 1 h and then washed 4 times for 10 min each in PBST. The blot was next incubated with the appropriate secondary HRP-conjugated antibody diluted 1:2000 as above for 1 h and washed 4 times for 10 min each in PBST. The specifically bound antibody was detected using the ECL detection reagent (AP Biotech) as directed by the manufacturer.
2.5.2 Immunoprecipitation

Transiently transfected HEK293 cells were lysed on ice in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 % Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.1 % β-mercaptoethanol, 1 mM benzamidine, 10 μM leupeptin, 10 μM aprotinin, 2 mM PMSF, 1 μM pepstatin and 10 % glycerol. Samples were sonicated for 5-10 s at high power and cleared at 20,000 xg for 10 min at 4°C. Supernatants were incubated with M2 anti-FLAG immunoaffinity beads for 2h at 4°C. After extensive washing with lysis buffer, FLAG-fusion protein was competitively eluted using FLAG peptide (0.5μg/μl). Eluates were mixed with an equal volume of 2x sample buffer and separated by SDS-PAGE. Immunoprecipitated proteins required for lipid kinase assays were prepared as above except lipid kinase buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 1mM β-mercaptoethanol) instead of lysis buffer was used to wash the immunoaffinity beads. Eluted proteins were assayed directly for lipid kinase activity or analysed by SDS-PAGE electrophoresis.

2.5.3 Indirect immunofluorescence

Transfected HEK293 cells grown on glass coverslips were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Fixed cells were permeabilised in PBS containing 0.2 % Triton X-100 for 5 min. Cells were incubated for 1 h at room temperature with M2 anti-FLAG monoclonal antibody (Sigma) diluted 1:360 in PBS containing 0.1 % bovine serum albumin. Secondary staining was performed using FITC-labelled anti-mouse anti-serum diluted 1:200 in PBS. Actin filaments were detected by incubation with 0.8 nM TRITC-labelled phalloidin in order to identify untransfected cells. Stained samples were mounted in Mowial and analysed by confocal laser scanning microscopy (Zeiss LSM 510).

2.6 Protein Biochemistry

2.6.1 SDS polyacrylamide gel electrophoresis

SDS-PAGE was performed according to Laemmli (1970). Gels were stained with 0.1 % (w/v) Coomassie brilliant blue R250 in 45 % methanol/ 45 % water/ 10 % (v/v) acetic acid and then destained in the same solution without Coomassie Brilliant Blue R250. SDS polyacrylamide gels containing protein for electroelution were stained with 4M sodium acetate.
2.6.2 Electroelution

Protein bands were excised from SDS polyacrylamide gels following electrophoresis and stained with sodium acetate and equilibrated in elution buffer (0.2 M Tris-acetate, pH7.4, 1.0 % SDS, 100 mM dithiothreitol). Gel slices were transferred to dialysis tubes containing 1.0 ml elution buffer per 0.1 g wet polyacrylamide gel. The dialysis tubing was placed crosswise in a horizontal electrophoresis chamber containing running buffer (50 mM Tris-acetate pH7.4, 0.2 % SDS, 0.5 mM sodium thioglycolate) and eluted for 3 h at 100 mA. Following elution the gel band was removed from the dialysis tubing and stained with Coomassie Blue to ensure that the elution was complete. The tubing containing the protein was dialysed against several changes of 0.2M sodium bicarbonate containing 0.02% SDS. The dialysate was removed and diluted to a protein concentration of 1 mg/ml. The purity of the sample was checked by SDS-PAGE electrophoresis and Coomassie Blue staining.

2.6.3 Gel filtration

Gel filtration chromatography was employed to assay protein purity and to determine molecular weight. 50 µg recombinant protein was fractionated on a precision column PC 3.2/20 prepacked with Superdex 200 using a SMART system (AP Biotech). Absorbance of the eluate was monitored at 280nm. 80 µl fractions were collected at 1 min intervals using a 40 µl/ min flow rate and a running buffer containing 400 mM EDTA, 2 M NaCl, 80 mM Tris-HCl, pH7.9. 40 µl of each fraction was analysed by SDS-PAGE followed by staining with stained with Coomassie Blue.

Characterisation of the solute behaviour was determined by: $K_{av} = (V_e - V_o)/(V_t - V_o)$. $K_{av}$ refers to the coefficient defining the proportions of pores occupied by the solutes, $V_e$ to the elution volume, $V_o$ to the void volume, and $V_t$ to the total packed bed volume. For calibration, three standards were used: BSA (approximate molecular weight 67,000 Da), chymotrypsin (approximate molecular weight 25,000 Da) and ribonuclease (approximate molecular weight 13,500 Da). $K_{av}$ values for the 3 standards were 0.38, 0.64 and 0.76, respectively. $V_o$ was 0.87 ml and $V_t$ was 2.5 ml. Values of the standards were then plotted as a function of their molecular weight using the Graphpad Prism software (Version 2.0. Graphpad Software) A linear relationship was generated using Prism software, from which the approximate molecular weight could be determined.
2.6.4 Pyrene-labelled PtdCho and SM transfer assay

2.6.4.a Preparation of lipid vesicles

Donor vesicles consisting of 1-heaxadecanoyl(1-pyrenedecanoyl)-sn-glycero-3-phosphocholine (Pyr(10)PtdCho) or N-(1-pyrenedecanoyl)SM (Pyr(10)SM), TNP-PtdEth quencher, PtdOH and egg PtdCho (10:10:10:70 mol%, 4 nmol of total phospholipid) and acceptor vesicles consisting of PtdOH and egg PtdCho (5:95 mol%, 52 nmol total phospholipid) were used in all pyrene-labelled phospholipid transfer assays. The appropriate lipid mixtures were sonicated and cooled on ice, dried under inert gas, resuspended in an appropriate volume of PBS and allowed to equilibrate at room temperature.

2.6.4.b Fluorescence measurements

The fluorescence measurements were carried out on an LS Perkin-Elmer spectrofluorimeter equipped with a thermostated cuvette holder and a magnetic stirring device. Excitation was at 346 nm and the pyrene monomer fluorescence was monitored at 378 nm. All experiments were carried out at 28°C. Fluorescence measurements were collected every 0.5 s for 150 s following the addition of PtdIns-TP by an on-line computer using Chart software (version 3.6, ADInstruments Pty Ltd). The change in Pyr(10)PtdCho fluorescence following the addition of 20μg PITPa was recorded for 113 s. After this time fluorescence exceeded the range measurable by the spectrofluorimeter. All data were corrected for background fluorescence and analysed using Prism software. Where applicable differences between values were determined using the unpaired student t-test and linear regression analysis were performed using Graphpad Prism software.

2.6.4.c Pyrene-labelled phospholipid binding assay

The assay used was based on a phospholipid-binding assay described by (van Paridon et al., 1988b). The binding assay was carried out in 2 ml PBS containing donor vesicles (4 nmol of total phospholipid). The assay was initiated by the addition of PtdIns-TPs (20, 10, 5 or 2 μg). Vesicles were disrupted by the addition Triton X-100 at the end of each assay, in order to confirm that fluorescence was not limited by the concentration of pyrene-labelled phospholipid. The increase in pyrene monomer fluorescence was plotted as a function of time. The initial slope during the first 10 s was used as an estimate of the affinity of a PtdIns-TP for the respective pyrene-labelled phospholipid.
2.6.4.4 Pyrene labelled phospholipid transfer assay

The transfer assay is based on the increase of pyrene monomer fluorescence intensity resulting from the transfer of Pyr(10)PtdCho or Pyr(10)SM from quenched donor vesicles to unquenched acceptor vesicles (van Paridon et al., 1988b). The assay was identical to the binding assay except that a 25 to 50-fold excess of acceptor vesicles were mixed with the donor vesicles and PBS. The initial slope during the first 10 s was used as an estimate of the rate of transfer of the respective pyrene labelled phospholipid by the PtdIns-TP.

2.6.5 Radiolabelled phospholipid transfer assay

Phospholipid transfer activity was measured by the transfer of radiolabelled microsomal PtdIns to unlabelled liposomes, according to Thomas et al. (1993). Briefly, \[^3H\]inositol-labelled microsomes were prepared from rat livers. The ratio of liposomes (PtdCho:PtdIns: 98:2) to microsomes was 1 \(\mu\)mol lipid per 1.25 mg microsomal protein. 100 \(\mu\)l microsomes, 100 \(\mu\)l liposomes and 12 \(\mu\)g PtdIns-TP were incubated at 25°C for 30 min. The assay was terminated by the addition of 0.2 M sodium acetate, pH 5 containing 0.25 M sucrose to aggregate the microsomes. Following vigorous mixing, samples were centrifuged at 15,000 xg for 15 min at 4°C to pellet the microsomes. 100 \(\mu\)l of supernatant was removed and counted in 2 ml scintillation fluid using a Beckman LS 1801 scintillation counter. PtdIns transfer activity was calculated from the total counts transferred minus the background counts. Results were expressed as a percentage of the radiolabel transferred to liposomes by the PITP-like domain of Nir2, since this protein exhibited the highest transfer.

2.6.6 \(Ca^{2+}\) overlay assay

Proteins (25\(\mu\)g) were separated by SDS-PAGE and transferred to pre-wetted PVDF membranes (Immobilon-P, Millipore) in 10 mM CAPS, pH 11 containing 10% methanol using an Multiphor II electrophoresis apparatus (AP Biotech). Proteins were transferred for 1 h at a charge density of 0.8 mA/cm². Membranes were then washed 3 times for 20 min each at room temp in wash buffer (10 mM imidazole-HCl, pH 6.8, 60 mM KCl and 5 mM MgCl₂) and incubated in wash buffer containing 2 \(\mu\)Ci/ml \(^{45}\)CaCl₂ for 30 min, with agitation on a rotary shaker. Membranes were then washed for 2 min in H₂O followed by 1 min in 50% ethanol, dried using hot air and exposed to X-ray film.
2.6.7 PtdInsP kinase assay

PtdInsP kinase assays were in a 50-100 μl volume containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 1mM β-mercaptoethanol, 500μM PtdIns4P (Sigma), and 50-100 μM [γ³²P]-ATP (1-10 μCi) and 10 μl of immunoprecipitated protein (Section 2.5.2). After incubation at 37°C for 20 min, reactions were stopped with an equal volume of 1M HCl and extracted with chloroform:methanol reagent. The organic phase was further extracted with methanol:1 M HCl (1:1), dried under vacuum and the lipid products analysed by TLC (Section 2.6).

2.6.8 Separation of phospholipids by TLC

TLC was carried out on Whatman silica-60 plates soaked in 1 % (w/v) potassium oxalate and 1 mM EDTA, and activated by heating to 110°C for 30 min prior to spotting samples at the origin.

PtdIns bisphosphates were separated in an acidic system consisting of propan-1-ol: 2M glacial acetic acid (65:35) containing 1% 5M H₃PO₄.
3.0 Cloning and expression of MrdgBβ

3.1 Introduction

The metazoan PtdIns-TP protein family can be divided into two structural groups: the small single domain PITP proteins, and the multidomain rdgB proteins (Chapter 1). The PITP subfamily consists of the closely related PITPα and PITPβ isoforms (Rogers and Bankaitis, 2000). Although PtdIns-TPs were originally considered to play housekeeping roles within the cell, recent reconstitution studies have demonstrated that PITPα and PITPβ play an essential role in the biosynthesis of PtdIns(4,5)P₂ during PLC and phosphoinositide 3-kinase mediated signal transduction and exocytosis (Rogers and Bankaitis, 2000; Section 1.4). The PtdIns-TP requirement revealed by these experiments is satisfied by either PITP isoform, although the intracellular distributions of endogenous PITPα and PITPβ differ (Cunningham et al., 1996; Currie et al., 1997; Ohashi et al., 1995 and Section 1.3.2). Thus, the physiological relevance of different PITP isoforms remains unclear. Recently, Hamilton and co-workers reported that the murine vibrator (vb) mutation, which causes neuronal degeneration, is due to a hypomorphic mutation in the PITPα gene (Hamilton et al., 1997). Their studies have provided evidence for an essential requirement for PITPα in mammalian cells.

The first rdgB protein was identified in Drosophila. The DrdgB protein is a multidomain PtdIns-TP, which has been exclusively implicated in retinal and olfactory neurosensory signalling (Vihtelic et al., 1991; Woodard et al., 1992). DrdgB is a 160kDa protein containing an acidic, Ca²⁺-binding domain, six putative membrane-spanning regions, and a carboxy-terminal domain. The amino-terminal 281 amino acids of DrdgB share >40% identity with PITPα. Although transfer activity still remains to be shown for the full length protein, in vitro studies demonstrate that the isolated PtdIns-TP domain of DrdgB does exhibit PtdIns and PtdCho transfer activity (Vihtelic et al., 1993).

DrdgB mutations were originally identified by defects in the compound eye: null mutations cause light-induced retinal degeneration and abnormal termination of the light response (Harris and Stark, 1977; Stark and Carlson, 1982 and Section 1.8). A combination of genetic, biochemical and electrophysiological evidence indicates that DrdgB plays a critical role in the PLC-dependent phototransduction cascade in Drosophila, both downstream of PLC and in the recovery phase of the light response (Section 1.8.2). Nevertheless, the exact biochemical role of DrdgB remains to be determined. While the expression of the PtdIns-TP-like domain of DrdgB was
sufficient for complete rescue of DrdgB mutant flies, PtdIns transfer activity alone appears not to be sufficient as PITPα was unable to rescue the same mutants (Milligan et al., 1997a). These observations indicate that the PtdIns-TP domain of DrdgB is not functionally equivalent to PITPα with respect to function in the invertebrate phototransduction cascade.

When this research was initiated one mammalian homologue of DrdgB, Nir2, had already been cloned and partially characterised (Aikawa et al., 1997; Chang et al., 1997; Guo and Yu, 1997). EST databases searches for sequences exhibiting similarity to the PtdIns-TP domain of DrdgB indicated the existence of at least two additional mammalian rdgB isoforms. While this project was in progress two further mammalian rdgB isoforms were cloned and characterised, Nir1 and Nir3 (Lev et al., 1999). Nir3 encodes the complete sequence of one of two mammalian rdgB homologues identified in the EST databases. Like DrdgB, the Nir proteins have a multidomain structure. Both Nir2 and Nir3 contain an amino-terminal PtdIns-TP-like domain, but this domain is absent in Nir1 (Section 1.8.5). Drosophila rdgB rescue studies suggest some conservation of biochemical function between DrdgB and Nir1-3 (Chang et al., 1997; Elagin et al., 2000; Lu et al., 1999). Although Nir1-3 mRNAs possess different tissue expression patterns, all three are abundantly expressed in the brain and retina (Table 3.1 and Section 1.8.5).

Although rdgB was originally identified as an invertebrate phototransduction protein, the existence and wide expression patterns of multiple mammalian rdgB isoforms suggests much broader roles for these proteins in vivo. Furthermore, the existence of multiple mammalian PtdIns-TP-like proteins raises the question of functional degeneracy and specificity of the different PtdIns-TP domains. While reconstitution studies indicate functional degeneracy (Cunningham et al., 1996; Ohashi et al., 1995), the inability of PITPβ to rescue the mouse vb mutant (Hamilton et al., 1997) and specificity of DrdgB rescue in DrdgB mutant flies (Milligan et al., 1997a) suggests that different PtdIns-TP domains possess discrete functions in vivo.

In order to have a more complete picture of the diversity and specificity of the functions of these proteins we need to be aware of the range of isoforms. This chapter describes the isolation of the cDNA sequence and the analysis of the expression pattern of a novel mammalian PtdIns-TP related protein with the aim of defining the PITP family and generating reagents for the study of this protein.
3.2 Results

3.2.1 Cloning and sequence analysis of MrdgBβ

In order to isolate novel human homologues of DrdgB, EST databases were screened for novel human sequences showing similarity to DrdgB. A 301bp human brain EST (GenBank Accession number R24545) was identified. The R24545 clone was obtained, sequenced and found to encode amino acid residues 210 to 333 of the final open reading frame, as well as 1782bp of the 3'-untranslated region (UTR). Since DrdgB, Nir2 and Nir3 proteins consist of 974-1250 aminoacids, the unexpected position of the stop codon required verification using PCR primers designed to anneal either side of the stop site in R24545, a fragment of the expected size was amplified from an infant brain cDNA library. The sequence of this fragment confirmed the position of the stop site in R24545. More recently, several EST sequences identical to the 3'-region of R24545 were identified by performing additional BLAST searches (GenBank accession numbers AA035468, H17821, AA588404 and AA807607). The position of the stop site in these ESTs was identical to that observed in R24545.

In order to extend the sequence, 5'-RACE PCR was used to screen the infant brain cDNA library. Several PCR products, ranging from 300-800bp were amplified by 5'-RACE PCR. A nested PCR reaction using an aliquot of the RACE PCR reaction as a template revealed a specific PCR product of approximately 700bp. The sequence of this nested PCR product extended the 5' region of R24545 by 650bp. Sequence alignments of the RACE PCR product with DrdgB and Nir2 indicated that an additional 12bp were required to extend the sequence to the expected ATG start site. No further extension to the ATG was possible despite numerous attempts using 5'-RACE PCR.

The remaining cDNA sequence encoding residues 1-3 was derived from the EST database by screening for sequences homologous to the cDNA sequence encoding amino acid residues 4 to 8. Three EST sequences were identified from human tissue (GenBank Accession Numbers AA021507, H86340 and AA808293). All three ESTs contained a Kozak consensus sequence containing an initiating methionine and a stop signal upstream of the initiating methionine. As the full-length sequence had
<M  L L K E Y R I C M P L T
V D E Y K I O Q L Y N I S K H S H E Q S
D R E V E V V Q N E P P E D P H H O
A V V P K I F Y V T E K A W N Y Y P Y T
I T E Y T C S P L P K F S I H I E T K Y
E D N K G S N D T I P D S E A K D V E R
E V C F I D I A C D E I P E R Y Y K E S
E D P K H F S K E T G R G Q L R E G W
Figure 3.1. The nucleotide and predicted amino acid sequence of MrdgBB. The initiation methionine is marked by >>> and a red asterisk indicates a stop codon. The positions of the antisense RACE primers R1 and R2, and the primers used to confirm the position of the 3' stop codon, 3'F and 3'R, are underlined.
Figure 3.2 Domain structure of Mammalian PtdIns-TP family (aa amino acid)
been compiled from several independent clones, the complete ORF was verified by PCR using the infant brain cDNA library.

A GenBank search using the compete ORF revealed extensive homology with other PtdIns-TP-related proteins. The closer similarity of the predicted amino acid sequence to rdgB compared with PITP isoforms (see below) suggested that this sequence defined a novel human and murine (Section 3.2.7) rdgB protein, which was therefore termed mammalian rdgBβ (MrdgBβ).

MrdgBβ has an open reading frame of 999bp, which encodes a 333 amino acid polypeptide with a predicted molecular mass of 38.2kDa (Figure 3.1). MrdgBβ contains a PtdIns-TP-like amino-terminal domain and a small carboxy-terminal domain which exhibits no sequence homology to the Ca²⁺-binding nor the conserved carboxy-terminal domains of the Nir1-3 (Figure 3.2). Furthermore, this domain shows no obvious homology to any known proteins present in databases compared using BLAST programs. Hydrophobicity analysis using PEPLOT (GCG) and the lack of any discernible transmembrane segments indicated that MrdgBβ is likely to be soluble.

3.2.2 Analysis of the MrdgBβ amino acid sequence: Homology with PITP-related proteins

Sequence alignments of MrdgBβ, Nir1-3, PITPα, PITPβ and DrdgB are shown in Figure 3.3. The amino-terminal domain of MrdgBβ exhibits 47%, 43% and 41% amino acid sequence identity with DrdgB, Nir2 and Nir3 respectively. These levels of amino acid sequence identity are higher than between MrdgBβ and either PITPα (41%) or PITPβ (39%). These data are represented as a dendrogram in Figure 3.4. While the primary sequences of the PtdIns-TP domains of rdgB proteins and PITPα and PITPβ do differ there are no obvious motifs that are unique to either sub family of PtdIns-TPs.

MrdgBβ exhibits 58% and 40% identity with two Drosophila sequences recently derived from the Drosophila Genome project (GenBank accession numbers AF160934 and AAF61273), both of which are distinct from DrdgB (Figure 3.4). Consequently, from here onwards DrdgB will be referred to as DrdgBα. The predicted amino acid sequence of AF160934 shows a greater level of identity with MrdgBβ than with DrdgBα, Nir2 or Nir3, thereby indicating the existence of a Drosophila rdgBβ (DrdgBβ). Alternatively, as the predicted protein sequence consists of 273 amino acids and contains no carboxy-terminal extension beyond the
Figure 3.3 Comparisons of the amino acid sequences of PtdIns-TP family. Sequence alignment of the PtdIns-TPs domains of the five human and three Drosophila (DrdgB \textalpha{}, DrdgB\textbeta{} and PITP-Dm) PITP-related proteins. Sequence identities are shaded in dark grey and sequence similarities are shaded in light grey. Consensus sequence shows highly conserved residues. Weak conservation is indicated by a dot.
Figure 3.4 The PtdIns-TP family and rdgB sub-family. (A) Dendrogram showing the degree of amino acid sequence similarity of the PITP-like domains of the five human and three Drosophila (DrdgBα, DrdgBβ and PITP-Dm) PITP-related proteins. (B) Dendrogram showing the degree of amino acid sequence similarity of rdgB proteins. rdgB-Ce: rdgB-like protein from C.elegans.
PtdIns-TP domain, this sequence may encode a novel isoform. The second *Drosophila* EST sequence is more similar to mammalian PITPs than to any rdgB protein, and is therefore termed *Drosophila* PITP (PITP-Dm in Fig 3.3 and 3.4). The genome of *Caenorhabditis elegans* appears to contain only one PITP-like (Wormpep Accession number Y71G12A-205.C) and one rdgB-like gene (GenBank Accession number Z77131). The probable functions of the *Drosophila* and *C. elegans* PITPs are unclear from sequence comparisons, as they are less similar to either mammalian PITP isoform than the latter are to each other. The predicted *C. elegans* rdgB protein (CrdgB) has a similar domain organisation to DrdgBα, Nir2 and Nir3, and may therefore bind a Pyk2-like protein.

3.2.3 Analysis of the MrdgBβ amino acid sequence: Motifs present in MrdgBβ

Analysis of the deduced MrdgBβ protein sequence using the MOTIFS algorithm (GCG) revealed several potential N-methylation sites and an N-myristylation site. Several potential PKA and PKC phosphorylation sites were also identified, suggesting possible mechanisms of functional regulation. Furthermore, in common with all proteins containing a PtdIns-TP domain, MrdgBβ contains a threonine and a serine residue corresponding to residues 59 and 166, respectively, of PITPa, both of which has been suggested to allow PKC to regulate the PtdIns transfer activity of PITPa (Alb et al., 1995).

3.2.4 Tissue distribution of MrdgBβ

In order to assess the expression of MrdgBβ mRNA and to investigate the existence of possible splice variants or closely related genes, Northern blot analysis was performed. High stringency hybridisation to a 600bp probe representing nucleotides 1-600 indicated that MrdgBβ is ubiquitously expressed in human tissues (Figure 3.5.a). A 2.0kb MrdgBβ transcript was expressed strongly in heart, muscle, kidney, liver and peripheral blood leukocytes and weakly expressed in other tissues. Additionally, two transcripts of about 7.0kb and 1.0kb were present in skeletal muscle and heart and may represent untranslated splice variants of MrdgBβ.

In comparison, DrdgBα shows multiple transcripts ranging from 3.9kb to 9.5kb with expression limited to the brain and retina, Nir2 is ubiquitously expressed as a transcript of 4.5kb, whereas Nir1 and Nir3 exhibit more limited expression patterns with transcripts of around 7.5kb (Table 3.1). The size of the MrdgBβ transcript is consistent with the defined cDNA sequence and the absence of sequence
Figure 3.5 Tissue distribution of MrdgBβ. The expression of MrdgBβ was analysed using (A) a human multiple tissue Northern blot (Clontech) hybridised with a $^{32}$P-labelled DNA fragment of MrdgBβ. Lanes: 1, peripheral blood leukocytes; 2, lung; 3, placenta; 4, small intestine; 5, liver; 6, kidney; 7, spleen; 8, thymus; 9, colon; 10, muscle; 11, heart; 12, brain. (B) RT-PCR with MrdgBβ-specific primers and first-strand cDNA prepared from the indicated murine tissues. The quality of the cDNA was tested by RT-PCR using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) specific primers. Lanes: 1, heart; 2, muscle; 3, eye; 4, brain; 5, liver; 6, spleen; 7, lung.
encoding multiple, putative transmembrane domains, which are present in the DrdGα, CrdG and Nir proteins.

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Size (kb)</th>
<th>Tissue Distribution</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Nir1</td>
<td>7.5</td>
<td>brain, small intestine, spleen, ovary</td>
<td>Lev et al., 1999</td>
</tr>
<tr>
<td>Nir2</td>
<td>4.4, 4.9, 6.4</td>
<td>brain, retina</td>
<td>Chang et al., 1997</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>ubiquitous</td>
<td>Lev et al., 1999</td>
</tr>
<tr>
<td>Nir3</td>
<td>7.5, 9.5</td>
<td>all tissues except kidney</td>
<td>Lev et al., 1999</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>brain, retina, testis</td>
<td>Lu et al., 1999</td>
</tr>
</tbody>
</table>

Table 3.1 Tissue distribution of Nir1-3

In order to confirm the ubiquitous expression of MrdGβ, RT-PCR analysis of cDNA from various mouse tissues was performed. Sequence-specific primers were designed from a murine EST (GenBank accession number A1019450). The predicted amino acid sequence of this EST is identical to residues 1-91 of human MrdGβ. A single PCR product corresponding to the expected size of 270bp was apparent in all tissues analysed (Figure 3.5.b).

3.2.5 Expression of MrdGβ in E. coli

PCR was used to incorporate a 5' EcoRI and a 3' XhoI site into the full ORF of MrdGβ. The ORF was verified by sequence analysis and subcloned into the EcoRI and XhoI site of the pET21:GST construct, allowing for the expression of the GST fusion protein (GST:MrdGβ) under the tight control of the T7 promoter (Section 2.3.1). The recombinant protein was expressed in E. coli BL21(DE3)pLyS in the presence of IPTG. A time course and variations in the concentration of IPTG and incubation temperature were used to determine that the optimum yield of recombinant GST:MrdGβ occurred following induction of protein expression with 0.1mM IPTG, for 3 h at room temperature (data not shown). Affinity-purified proteins were analysed by SDS-PAGE. The fusion protein had an apparent molecular mass of approximately 60kDa, comprising the 38kDa MrdGβ and the 26kDa GST domain (data not shown). Approximately, 50% of the fusion protein was found in the
Figure 3.6 Recombinant expression of MrdgBβ. Full length MrdgBβ was expressed as a GST and an HIS$_6$-fusion protein in bacteria and purified as described in Sections 2.3.1. (A) Thrombin cleaved GST:MrdgBβ and (B) MrdgBβ:HIS$_6$ were analysed by SDS-PAGE using a 15% acrylamide gel (1μg of protein/lane) and visualised by Coomassie Blue staining.
detergent insoluble fraction, resulting in a low yield of soluble protein of approximately 0.3mg/l. Cleavage of GST:MrdoBβ with thrombin typically gave preparations of the 38kDa protein without further degradation as judged by SDS-PAGE (Figure 3.6).

In order to improve the recovery and yield of recombinant protein, MrdoBβ was expressed as a carboxy-terminal HIS₆ tagged protein (MrdoBβ:HIS₆). PCR was used to incorporate a 5’ Nde I and a 3’ Xho I site into the full ORF of MrdoBβ. The ORF was verified by sequence analysis and subcloned in to the 5’ NdeI and a 3’ XhoI sites of pET21a and was expressed as in E. coli BL21(DE3)pLyS in the presence of IPTG. The recombinant protein had an apparent molecular weight of about 40kDa, in agreement with the predicted value (Figure 3.6). In contrast to GST:MrdoBβ, the recovered yields of detergent soluble protein were approximately 2mg/l.

In order to assess the purity of MrdoBβ:HIS₆ and to determine the molecular weight of MrdoBβ, MrdoBβ:HIS₆ was subjected to size exclusion chromatography using a precision column PC 3.2/20 pre-packed with Superdex 200, run on a SMART system (Figure 3.7.A). Five peaks (numbers 1-5) were observed. The $K_a$ values for peak numbers 1-5 are 0.043, 0.342, 0.40, 0.711 and 1.0, respectively, with corresponding $V_e$ values of 0.94 ml, 1.43 ml, 1.62 ml, 2.03 ml and 2.5 ml, respectively. The polypeptides within peak numbers 1-4 have a molecular weight of approximately 115 kDa, 75 kDa, 63 kDa and 18 kDa, respectively. Analysis of fractions with UV absorbing material by SDS PAGE electrophoresis revealed that MrdoBβ:HIS₆ was present in fractions 9-13, corresponding to peak number 2 (Figure 3.7.B). The apparent molecular weight of the polypeptides within this peak suggests that MrdoBβ:HIS₆ exists as a homodimer.

A low level contaminant was eluted with MrdoBβ:HIS₆ in fractions 9 and 10 and no other polypeptides were detected by Commassie Blue staining, suggesting a high level of purity (Figure 3.7).

### 3.2.6 Generation of MrdoBβ-specific antisera.

The analysis of the *in vivo* and *in vitro* function of MrdoBβ would be aided by the generation of a specific antiserum. Alignment of the amino acid sequences of all known mammalian PtdIns-TPs, together with a BLAST searches of the GenBank database confirmed that residues 757-991 were unique to MrdoBβ. Consequently this domain was chosen to minimise cross reactivity of the antiserum with the other
Figure 3.7 Gel filtration analysis of MrdgBB·HIS₆. (A) 50 μg of purified MrdgBB·HIS₆ was fractionated on a precision column PC 3.2/20 prepacked with Superdex 200, using a SMART system. 80 μl fractions were collected at 1 min intervals using a 40 μl/min flow rate and a running buffer containing 400 mM EDTA, 2 M NaCl, 80 mM Tris-HCl, pH7.9. Fractions taken for further analysis are indicated above each peak. (B) Analysis of 40μl of each peak fraction by SDS-PAGE visualised by staining with Coomassie Blue.
PtdIns-TP subtypes and residues 757-991 were expressed as a GST-fusion protein (GST::[3']MrdgBB) in *E. coli*.

Soluble protein was purified with a yield of approximately 2 mg/l. Thrombin cleavage of the fusion protein gave preparations of the expected 8 kDa polypeptide without further degradation. A contaminating protein of around 70 kDa, possibly corresponding to heat shock protein, was also present in the preparation (Figure 3.8). Initially, the two polypeptides were separated by gel filtration. However, insufficient amounts of the 8 kDa polypeptide were recovered, possibly due to the protein aggregating or associating with the column. Therefore, preparative SDS-PAGE and electroelution were employed to isolate the 8 kDa polypeptide (Figure 3.8).

Two rabbit antisera were raised against the purified protein injected subcutaneously with adjuvant. Booster immunisations were carried out at four weekly intervals and test bleeds were taken two weeks after each immunisation. The antisera obtained from such test bleeds were used at 1:50-1:500 dilution in Western blotting studies to test for immunoreactivity against recombinant protein using both the full length protein and the 8 kDa fragment against which the antiserum had been raised. Immunoreactivity against a total cell lysate derived from HEK293 cells transfected with FLAG-tagged MrdgBB (Section 5.2.3) was also assayed by western blotting. Following the final booster immunisation no specific immunoreactivity was observed against either recombinant proteins or the cell lysates (data not shown). Thus, the antisera were not employed in any further studies. It is unclear why no specific immunoreactivity was observed. It is possible that the sequence was not immunogenic or that the polypeptide was rapidly proteolysed or that the small size of the polypeptide to which the antibody had been raised allowed a rapid rate of clearance. In the latter case, conjugation of the protein to itself or to Keyhole Limpet Haemocyanin may have improved the immunoreactivity of the antiserum.

### 3.2.7 Isolation of the partial cDNA sequence encoding a novel MrdgBB protein

As described in Section 3.2.4, MrdgBB specific RT-PCR products appeared to be amplified from all tissues analysed: heart, muscle, eye, brain, liver, spleen and lung (Figure 3.5). On closer inspection, the PCR products from muscle, eye and brain appeared marginally larger than those from the other tissues.

In order to investigate the apparent differences in size, the larger and smaller PCR products from muscle and spleen, respectively, were subjected to sequence analysis. Sequence analysis revealed a 269bp product from muscle that exhibited 100%
Figure 3.8 Recombinant expression of [3′]MrdgBβ. Residues 757-991 were expressed as a GST-fusion protein in *E. coli* and used as an immunogen for the generation of MrdgBβ antisera. Following purification of the recombinant protein, as described in Section 2.3.1, a contaminating protein of around 70 kDa was also present in the preparation. Preparative SDS-PAGE and electroelution were employed to isolate the 8 kDa protein. (A) SDS-PAGE (using a 15% acrylamide gel) showing recombinant GST:[3′]MrdgBβ following cleavage with thrombin (40 μg of protein/lane) visualised with Coomassie Blue. (B) SDS-PAGE showing electroeluted polypeptide (20 μg of protein/lane) visualised with Coomassie Blue.
identity at the amino acid level with MrdgBβ. In contrast the sequence from spleen encoded a 257bp product. The predicted amino acid sequence of this PCR product exhibited 75% identity, to the corresponding sequence in MrdgBβ (Figure 3.9). In addition to 26bp differences, the cDNA sequence derived from spleen lacked 12bp corresponding to the region encoding amino acid residues 55 to 58, GQYT in MrdgBβ (Figure 3.10).

The isolation of a partial cDNA from spleen (designated MrdgBβ’ from here onwards) lacking a region that is contained in all other known PtdIns-TPs suggested the existence of a splice variant of MrdgBβ or yet another rdgB isoform. In order to address this several cloning strategies were employed to obtain the full length sequence. Based on the hypothesis that MrdgBβ’ encoded a splice variant form of MrdgBβ, various combinations of MrdgBβ-specific primers (Table 3.2) were used to isolate the full length sequence from cDNAs prepared from heart.

<table>
<thead>
<tr>
<th>Sense Primer amplified</th>
<th>Antisense Primer</th>
<th>Amino Acid Residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. ATGCTGCTGAAAGAGTAC</td>
<td>CTCAGATTGGGGCGACATG</td>
<td>1-333:full length protein</td>
</tr>
<tr>
<td>2. ATGCTGCTGAAAGAGTAC</td>
<td>CTTGTGGGCTTCCCGTAC</td>
<td>1-247:PtdIns-TP domain</td>
</tr>
<tr>
<td>3. ATGGCAATGAGAAGAAGAG</td>
<td>CTCAGATTGGGGCGACATG</td>
<td>270-333:3’ domain</td>
</tr>
</tbody>
</table>

Table 3.2 Primers used to isolate the full-length sequence of MrdgBβ’.
Underlined sequences are MrdgBβ’-specific sequences. All other primers sequences were derived from the cDNA sequence of MrdgBβ.

liver, kidney, spleen, brain and muscle tissues. PCR products of expected sizes were obtained from each tissue, however, sequence analysis revealed that all the products corresponded to MrdgBβ and not the sequence containing the deletion. This suggested that MrdgBβ’ deviated from that of MrdgBβ within both its PtdIns-TP domain and carboxy-terminal domain.

As the complete cDNA sequence of MrdgBβ could be amplified from all tissues, including spleen (data not shown), it is likely that MrdgBβ is ubiquitously expressed as inferred from the Northern blotting result (Section 3.2.4). While
Figure 3.9 Comparison of the amino acid sequence of MrdgBB and a MrdgBB-related sequence from spleen. Sequence alignment of the predicted amino acid sequence derived from spleen and MrdgBB. Identities are shaded in dark grey and position similarities are shaded in light grey. Consensus sequence shows highly conserved residues. Weak conservation is indicated by a dor.

Figure 3.10 cDNA and predicted amino acid sequence of residues 1-270 of a novel MrdgB isoform from spleen. The antisense RACE primer R3 used to screen an infant brain cDNA library is underlined.
MrdgBβ' appears to be expressed in spleen, heart, liver and lung, expression in brain, muscle and eye can not be excluded (Figure 3.5). In this regard 3' RACE PCR using primers specific to MrdgBβ' produced no specific products from an infant brain cDNA library, suggesting that MrdgBβ' is absent from brain.

Further studies are needed to obtain the full-length clone and to compare the functions of MrdgBβ' with other members of the PtdIns-TP family, in particular MrdgBβ. These studies will require the design and production of antisera directed against unique amino acid sequences in MrdgBβ and MrdgBβ'. To this end, 2 peptides have been designed corresponding to the region containing the absent or conserved GQYT residues. Peptide A comprises a 8 amino acid sequence (GNGQFTEK) derived from residues 20-27 of MrdgBβ. Peptide B comprises an 8 amino acid sequence (HHGNEKKV) derived from residues 18-25 of MrdgBβ'. Specific residue either side of the GQYT sequence were chosen to minimise cross reactivity of the antiserum with other PtdIns-TP proteins. In addition, both peptide sequences contain several hydrophilic and charged residues that are likely to be more immunogenic.

At the time of writing, rabbits were immunised with peptides A or B conjugated to Keyhole Limpet Haemocyanin.

3.3 Discussion

This chapter describes the identification of MrdgBβ, a novel member of the PtdIns-TP family. The cDNA encoding MrdgBβ has been cloned and the encoded protein has been expressed in E. coli. This work was necessary to generate reagents for the experiments described in Chapters 4 and 5. However, several predictions regarding the function of MrdgBβ and the molecular evolution of PtdIns-TPs can be drawn from the data presented in this chapter.

RT-PCR and high stringency Northern analysis revealed that the MrdgBβ transcript is ubiquitously expressed. The differential expression patterns of MrdgBβ and Nir1-3 are most likely to reflect differences in their cellular activity. Furthermore, the existence of multiple isoforms with both distinct and overlapping patterns of expression within the eye (Section 1.8.5) suggests that these proteins are not functionally degenerate.

Sequence alignments of the conserved PtdIns-TP-like domain of MrdgBβ with other members of the PtdIns-TP family revealed that, while it is clearly a member of the rdgB subfamily, it contains no apparent membrane-spanning domains,
nor the domain required for interaction with Pyk2. One possibility is that MrdgBβ could associate with Nir1, which lacks a PtdIns-TP domain, to form a heterodimer containing all of the domains present in other rdgB proteins. However, the restricted tissue distribution of Nir1 suggests that if such an association occurs in vivo, it would not be ubiquitous. Since MrdgBβ defines a novel structural isoform that differs from the generic rdgB and PITP structures this protein may also have a novel function.

The demonstration that the conserved carboxy-terminal domain of each Nir protein forms a complex with Pyk2 and is phosphorylated on tyrosine residues in response to Pyk2 activation, led to the suggestion that Pyk2 is an upstream regulator of Nir proteins (Lev et al., 1999). The absence of the conserved carboxy-terminal domain in MrdgBβ suggests that this isoform of rdgB does not interact directly with Pyk2 and is therefore not a member of the Nir protein family.

Bacterial expression of GST:MrdgBβ resulted in low yields of soluble material. Increased yields of soluble protein were obtained by bacterial expression of MrdgBβ as a HIS₆-tagged protein. Purified recombinant proteins migrated at approximately 40 kDa on SDS-PAGE. Guo and co-workers have shown that immunoblot analysis of a homogenate prepared brain tissue using polyclonal antibodies raised against residues 56-769 of Nir2, revealed a minor cross-reacting polypeptide of approximately 40 kDa in addition to the expected 160 kDa polypeptide (Guo and Yu, 1997). The observed mobility of recombinant MrdgBβ on SDS-PAGE suggests that this cross-reacting endogenous polypeptide could be MrdgBβ.

Fractionation of MrdgBβ:HIS₆ by gel filtration revealed that the recombinant protein had a molecular weight of approximately 75 kDa, approximately twice the predicted size, indicating that, in vitro at least, recombinant MrdgBβ:HIS₆ exists as in a homodimeric form. Previous studies have provided evidence of the heterogeneous behaviour and existence of polymeric forms of PITPα and PITPβ on gel filtration (S.K.Tan, unpublished work). PITPβ was eluted in three peak fractions with corresponding molecular weights of 200 kDa, 60 kDa and 17.5 kDa. PITPα eluted in a major peak fraction consistent with a molecular weight of approximately 17.5 kDa (S.K.Tan, unpublished work). Dimerisation of MrdgBβ requires further investigation, as does the physiological relevance of this observation. Dimerisation may be a general property of PtdIns-TPs and may be important for lipid binding and/or transfer activity.
This chapter also describes the bacterial expression of a recombinant polypeptide consisting of residues 757-991 of MrdgBβ. This polypeptide will provide a useful reagent for future studies in which the function of the unconserved carboxy-terminal domain will be addressed.

During RT-PCR analysis of the tissue distribution of MrdgBβ, a novel cDNA fragment, MrdgBβ', was isolated from spleen tissue. The amino acid sequence of this 270bp fragment exhibited 75% identity with MrdgBβ and lacked 12bp, corresponding to amino acid residues 55 to 58 (GQYT). It is unclear at this stage whether or not this sequence represents a fragment of a novel rdgB protein or a pseudogene. The sequence is of particular interest since residues 55 to 58 are highly conserved among all other members of the PtdIns-TP family. Most notably is the lack of the threonine residue at position 59. This residue has been implicated in the regulation of the PtdIns transfer activity of PITPα via PKC (Alb et al., 1995). The T59 residue is a likely substrate for PKC and mutation of T59 to D, E or S specifically abolishes the PtdIns transfer activity of PITPα (Section 1.6 and (Alb et al., 1995). The sensitivity of PtdIns transfer activity to changes in the T59 residue suggests that MrdgBβ' may have altered PtdIns transfer activity and/or an alternative mechanism of regulation.

The wide distribution of rdgB proteins in metazoan organisms indicates that these proteins have an early origin. The limited tissue distribution of DrdgBα and Nir1 suggests functional specialisation of various isoforms during evolution. The divergence of a single PITP protein in to PITPα and PITPβ appears to be the result of a recent evolution since divergence seemed to have occurred after the invertebrate/vertebrate division since invertebrates have only one PITP isoform, whereas vertebrates have two.

In conclusion the identification of MrdgBβ, a novel, ubiquitously expressed member of the PtdIns-TP family, strengthens the argument for functional specificity of the different PtdIns-TP isoforms. Furthermore, reagents have been prepared to allow the function of MrdgBβ to be investigated. Such studies are described in Chapter 6.
4.0 Chromosomal mapping of the MrdgBβ gene

4.1 Introduction

Human Nir1-3 genes have been mapped to three distinct chromosome regions that contain several retinopathy loci (Table 4.1) identifying rdgB genes as candidate genes for retinal diseases. Although Nir1-3 exist at distinct loci, all three occur at the sites of three different forms of the neurodegenerative disease retinitis pigmentosa. This intriguing observation, together with the DrdgBα phenotype (Section 1.8), suggest that mutation of the mammalian rdgB genes may cause some forms of retinitis pigmentosa. Furthermore, the phenotype of the mouse vibrator mutant (Section 1.7) supports a role for PtdIns-TPs in neurodegeneration (Hamilton et al., 1997).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosomal Localisation</th>
<th>Associated Retinopathy Loci</th>
<th>Reference</th>
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<tr>
<td>Nir1</td>
<td>17p13.1</td>
<td>ADRP:RP13</td>
<td>Lev et al., 1999</td>
</tr>
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<td></td>
<td></td>
<td>Cord5</td>
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</tr>
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<td></td>
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<td>CACD</td>
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<td></td>
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</tr>
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<td>Nir3</td>
<td>7p12-q22</td>
<td>ARDP:RP9</td>
<td>Lu et al., 1999</td>
</tr>
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</table>

Table 4.1 Chromosomal localisation of Nir1-3 and associated retinopathy loci.

Retinopathy diseases that involve retinitis pigmentosa are indicated in bold text. (ADRP: autosomal dominant retinitis pigmentosa; CORD: rod-cone dystrophy; CACD: central aerial choroidal dystrophy; EVRI: familial exudative vitreoretinopathy; VRNI: neovascular inflammatory vitreoretinopathy).

Retinitis pigmentosa denotes a group of hereditary retinal dystrophies and is the most frequent cause of inherited retinal degeneration, affecting about 1 in 4000 people worldwide (Berson, 1996; van Soest et al., 1999). Although retinitis pigmentosa is extremely genetically heterogeneous, many retinitis pigmentosa cases have similar symptoms and retinal histopathology. Retinitis pigmentosa is characterised by progressive retinal degeneration resulting in the early onset of night blindness and
Figure 4.1 The structure of the retina. The outer most layer of cells form the retinal pigment epithelium (RPE) in which the tips of the rods and cones are buried. Light focused from the lense passes through each layer and is absorbed by the rod and cone cells in the outer segment. The axons of each rod and cone cell synapse with many bipolar nuclei the outer plexiform layer. The inner nuclear layer contains the cell bodies of the bipolar cells, horizontal cells and amacrine cells. The horizontal cells connect groups of photoreceptor synapses before the signal is passed to the inner plexiform layer by the bipolar cell. In the inner plexiform layer the bipolar cells commonly synapse with amacrine cells which in turn modify the signal before it is passed to the ganglion cell. (adapted from Lodish, L, Baltimore, D, Berk, A, Zipursky, S.L, Matsundaira, P and Darnell, 1995, J. Molecular biology of the cell, Freeman).
ERG amplitude decline, followed by a progressive loss of the visual field (Berson, 1996). The primary effect of retinitis pigmentosa is the loss of function of rod photoreceptor cells. Subsequently, mostly unknown molecular and cellular mechanisms trigger the apoptotic degeneration of these photoreceptors. Histologic studies of photoreceptors in retinitis pigmentosa retinas have demonstrated outer segment shortening and the progressive loss of the cells within this segment of the retina. Although initial degenerative changes occur in rods, subsequent loss of cone cells is also apparent (Berson, 1996).

Retinitis pigmentosa segregate as autosomal dominant (ADRP), autosomal recessive (ARRP), X-linked (XLRP) or a digenic trait (van Soest et al., 1999). This disorder may also occur in association with certain syndromic disorders such as Bardet-Biedl syndrome (Leppert et al., 1994). For each mode of segregation multiple genes have been identified and so far 23 distinct loci are known (Table 4.2). For 12 loci, the causative genes and the mutations therein have been identified. These genes include rhodopsin, the β-subunit of cGMP PDE and cellular retinaldehyde-binding protein (CRABP) (Farber, 1995; Maw et al., 1997). The remaining 11 loci, including RP9, RP13 and RP17 remain anonymous. So far no clear relationship between genotype and phenotype has been observed and the exact number of genetic loci at which mutations cause retinitis pigmentosa is currently unknown.

In addition to their mode of segregation, the identified retinitis pigmentosa genes and mutations have been classified according to the cellular function disrupted by the mutation (van Soest et al., 1999). Three classes of mutation have been distinguished: (1) mutations affecting disc renewal and shedding and the structure of the rod outer segment (ROS) (2) mutations affecting the visual transduction cascade, and (3) retinal pigment epithelium (RPE)-specific mutations affecting retinol metabolism (Table 4.2). Since a large number of genes still remain to be identified and characterised, is unclear whether any other functional classes of mutation exist. Several examples of various cell functions disrupted in retinitis pigmentosa are discussed below. The structure of the retina is shown in Figure 4.1.

The normal rod photoreceptor sheds about 10% of its outer segment discs at its apex after light onset and renews a corresponding amount of outer segment discs at its base over the course of a day (Bok, 1985). Disruption of the continuous disc renewal, overactive degradation or defects in the structural components of the ROS may lead to shortened and malfunctioning outer segments. Alternatively, deficient degradation
**Table 4.2 Genes and Loci Identified in Retinitis Pigmentosa.** * The cellular functions disrupted by known mutations can be classified into three groups: (1) mutations affecting disc renewal and shedding and the structure of the photoreceptor outer segment (2) mutations affecting the visual transduction cascade, and (3) RPE- specific mutations affecting retinol metabolism (see text for more detail). (ABCR: retinal ATP-binding cassette gene; TULP: Tubby-like gene1; RDS: retinal degeneration slow; RPGR: retinitis pigmentosa GTPase regulator; ROM1: retinal outer segment membrane protein 1)
of the shedded disc material will result in the formation of retinal deposits, obstructing the flow of metabolites through the RPE to the photoreceptor. Defects in the renewal, shedding and the structural proteins of outer segment discs will result in retinal degeneration. One such gene that is a member of this class of retinitis pigmentosa mutations is the rhodopsin gene. Rhodopsin constitutes up to 85% of the total amount of protein present in the ROS (van Soest et al., 1999). Although the major function of rhodopsin lies in the initiation of the phototransduction cascade, the abundance of rhodopsin suggests that the protein may play an important structural role in the ROS. Mutations in the rhodopsin gene represent the most common cause of retinitis pigmentosa, accounting for about 25% of all ADRP cases and 10% of all cases of retinitis pigmentosa (Berson, 1996) (Inglehearn et al., 1998). Furthermore, molecular genetic studies of human retinitis pigmentosa have revealed a wide spectrum of around 100 different mutations in the rhodopsin gene that result in the disease (van Soest et al., 1999). Although it was originally proposed that rhodopsin mutations lead to degeneration via constitutive activation of the visual transduction cascade (Robinson et al., 1992), more recent studies clearly indicate that defective folding and transport of rhodopsin to ROS membranes is the primary defect (Berson, 1996; Li et al., 1996; Sung and Tai, 2000). It is possible that the accumulation of rhodopsin and the subsequent disruption of processes such as the renewal and shedding of ROS trigger cell death.

As discussed in Section 1.8.1, multiple proteins mediate the vertebrate visual transduction cascade. A possible explanation for the finding that mutations in any one of a number of genes can produce the retinitis pigmentosa phenotype lies in the complex nature of the phototransduction cascade. Mutations at many points within this cascade have been linked to retinitis pigmentosa (Table 4.2). As an example, mutations in the genes encoding the α and β subunits of cGMP PDE have been identified in patients with ARRP (Table 4.2). While the mechanisms of disruption by these mutations are unknown, it has been predicted that they disrupt the enzymatic function of cGMP-PDE (Farber, 1995; McLaughlin et al., 1993). A well-characterised model for retinitis pigmentosa is provided by the retinal degeneration mouse (rd mouse) in which the gene encoding the β-subunit of cGMP PDE is disrupted by a large insertion (Bowes et al., 1990; Bowes et al., 1993). In rd mice a phenotype similar to humans is observed in which homozygous affected rd mice demonstrate a severe degeneration of photoreceptor cells, by apoptotic mechanisms, resulting in the complete loss of these cells (Farber, 1995). Furthermore, the rd
mutation has a direct homologue in one of the human ARRP (McLaughlin et al., 1995; McLaughlin et al., 1993). The rd mouse accumulates a high concentration of cGMP within the ROS that triggers retinal degeneration by an unknown mechanism (Farber, 1995).

The ROS apical surface is embedded in the RPE. In addition to being the site for the degradation of rod discs, the RPE is the site of regeneration of the light sensitive retinol derivative 11-cis-retinal. Defective synthesis of this compound may also cause retinitis pigmentosa. For example, retinol deficiency resulting from malnutrition or metabolic disorders results in a phenotype similar to retinitis pigmentosa (Seeliger et al., 1999). Furthermore, defects in genes expressed specifically within the RPE may disrupt retinol metabolism as exemplified by RPE protein (RPE65) and CRABP, both of which play a role in the metabolism and transport of vitamin A from the RPE to the photoreceptor cells in the retina (Gu et al., 1997; Maw et al., 1997). Mutations in CRABP have been identified in patients with atypical ARRP. Maw and co-workers have suggested that this mutation results in a loss of function that disturbs or blocks the regeneration of 11-cis-retinal and rhodopsin (Maw et al., 1997).

Direct study of human retinitis pigmentosa is slow owing to difficulty in obtaining the relevant tissues for biological study. As a consequence, animal models of retinal degeneration and retinitis pigmentosa have been used to gain insight into photoreceptor cell degeneration. At present a number of distinct retinal degeneration models exist including the rd murine and the progressive rod-cone degeneration (prcd) canine model (Acland et al., 1998). The latter has been proposed to be the canine model of RP17 (Acland et al., 1998). These models will undoubtedly provide further insight into the causative genes and mechanisms leading to retinitis pigmentosa.

With the aim of gaining an insight into MrdgBβ function and to investigate whether MrdgBβ plays any role in retinal degeneration diseases such as retinitis pigmentosa, the chromosomal localisation of the MrdgBβ gene was determined using two independent methods: fluorescence in situ hybridisation (FISH) and radiation hybrid mapping (RH mapping). FISH analysis allows genes to be mapped to a broad chromosome interval of around 40cM. In contrast, RH mapping analysis allows for the fine mapping of genes to a chromosome interval of around 1cM through statistical analysis (Gyapay et al., 1996).
RH mapping makes use of a panel of somatic cell hybrids, with each cell line containing a random set of fragments of irradiated human genomic DNA in a hamster background. Each rodent-human hybrid clone contains a unique set of fragments derived from a single human chromosome and each clone is typed for the presence or absence of known human polymorphic microsatellite sequence tagged sites (STS) DNA markers. Using this approach, a human gene map (the 1998 International Gene Map) of over 30,000 genetic markers has been produced (Deloukas et al., 1998). The basic premise of RH mapping is that the closer two loci occur on a chromosome the less likely it is that radiation will induce a break between them. Thus, proximal markers on a chromosome demonstrate correlated retention patterns in hybrid clones, while distant loci are retained nearly independently. The DNA of the gene under investigation is typed against an RH clone panel containing the whole genome. Sequence-specific PCR primers are used to amplify the DNA of each hybrid cell line within panel. The results are recorded as an RH score vector consisting of + and − signs to indicate the presence or absence of the gene under investigation in each hybrid. Computational analysis of the RH score vector relative to the International Gene Map identifies the framework markers that have a similar or identical RH vector score to the gene under investigation. Such analysis allows the chromosomal localisation of the gene, its optimal position on the framework and the framework markers that have the most similar RH score vector to be determined.

4.2 Results

4.2.1 Radiation hybrid mapping analysis

RH mapping using the GB4 mapping panel was performed in order to determine the chromosomal localisation of the human MrdgBβ gene. The GB4 mapping panel was provided by the UK Human Genome Mapping Project Resource Centre (HGMPRC). The panel of 86 hybrid-cell lines was screened by PCR using primers designed to DNA residues 717-1124 of the unique carboxy-terminal domain of MrdgBβ. As the intron and exon boundaries of the MrdgBβ gene were unknown, genomic databases were analysed for MrdgBβ sequences. Two genomic clones derived from chromosome 17 (GenBank accession numbers AC023123.3 and AC06534) were found to contain MrdgBβ sequences. Inspection of the sequence of these two clones suggested that residues 717-996 did not contain any intronic sequences. A PCR product of the predicted size of around 350bp was obtained in 63% of samples. For 14% of the clones, the results obtained from duplicate samples
<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position</th>
<th>Marker</th>
<th>LOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>26</td>
<td>AFM175xg3</td>
<td>2.036</td>
</tr>
<tr>
<td>17</td>
<td>27</td>
<td>AFM207vf4</td>
<td>1.605</td>
</tr>
<tr>
<td>17</td>
<td>25</td>
<td>AFMa051xg5</td>
<td>1.521</td>
</tr>
</tbody>
</table>

Table 4.3 RHmap 2-pt Analysis of MrdgBβ on all chromosomes. (A) 2-pt RHmap output. Chromosome 17 contains 33 framework markers; the position of the identified markers within these 33 markers is indicated. Position 25 is 13.56cR from position 26, which is 17.20cRs from position 27. The LOD score is the ratio of the likelihood of the framework marker and the gene under investigation being linked to the likelihood of them being totally unlinked. Two markers are considered linked if they have a LOD score greater 3.0. (B) RH score vectors for MrdgBβ and flanking markers. The reference interval refers to the genetic interval on the Human 1998 International Gene Map which defines the position of the framework marker on chromosome 17. (?: undetermined score).
**Table 4.4 Multipoint RHMAPPER Analysis of MrdgBβ on chromosome 17.** (A) Multipoint RHMAPPER output. The highest log likelihood is considered the best map of the interval in which the gene under investigation localises. The LOD score is the difference of the log likelihood's of two orders the best order, i.e. AF217yd10, and each subsequent order an interval. For example the interval defined by AF217yd10 interval is $10^{17.64}$ times more likely than that of AFMa247zg9. A LOD score greater than 3 is rejected by RHMAPPER. (B) RH score vectors for MrdgBβ and flanking markers. The reference interval defines the position of the marker on chromosome 17. (?) undertermined score.

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position</th>
<th>Marker</th>
<th>Log Likelihood</th>
<th>LOD</th>
<th>(cR1, cR2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>33</td>
<td>AFM217yd10</td>
<td>-442.21</td>
<td>0.00</td>
<td>(134.71, 0.00)</td>
</tr>
<tr>
<td>17</td>
<td>28</td>
<td>AFMa247zg9</td>
<td>-459.86</td>
<td>17.64</td>
<td>(11.99, 11.32)</td>
</tr>
<tr>
<td>17</td>
<td>25</td>
<td>AFM207vf4</td>
<td>-463.34</td>
<td>21.22</td>
<td>(8.23, 8.88)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reference Interval</th>
<th>Marker</th>
<th>RH Vector Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MrdgBβ</td>
<td>++++++----------+++++++-----------------++</td>
<td></td>
</tr>
<tr>
<td>D17S928</td>
<td>AFM217yd10</td>
<td>+++++++----------+++++++????????????????????????????????????????????????++</td>
</tr>
<tr>
<td>D17S1352</td>
<td>AFMa247zg9</td>
<td>++++++++----------+++++++????????????????????????????????????????????????++</td>
</tr>
<tr>
<td>D17S1874</td>
<td>AFMa051xg5</td>
<td>+??+------------++---------?++---------?+-----------------++</td>
</tr>
</tbody>
</table>

(A) Multipoint RHMAPPER output. The highest log likelihood is considered the best map of the interval in which the gene under investigation localises. The LOD score is the difference of the log likelihood’s of two orders the best order, i.e. AF217yd10, and each subsequent order an interval. For example the interval defined by AF217yd10 interval is $10^{17.64}$ times more likely than that of AFMa247zg9. A LOD score greater than 3 is rejected by RHMAPPER. (B) RH score vectors for MrdgBβ and flanking markers. The reference interval defines the to the genetic interval on the Human 1998 International Gene Map which defines the position of the marker on chromosome 17. (?) undertermined score.
were not consistent with one another. The screening of these 12 clones was therefore repeated. The results obtained from all duplicates were found to be consistent with one another. The results of the second PCR screen were used in further analysis.

The presence or absence of a 350bp product was scored as a positive or negative result. The results were converted into a GB4 vector score (Table 4.3 and 4.4) and submitted to the Sanger Centre radiation mapping service in which the MrdgB amplified vector was analysed in relation to the Human 1998 International Gene Map (http://www.sanger.ac.uk/Software/RHserver/). The MrdgB GB4 vector score was evaluated using RHmap 2-pt and RHMAPPER multipoint analysis. RHmap 2-pt analysis identifies the chromosome localisation by comparing the GB4 vector score of the gene under investigation with the GB4 vector scores of the framework markers (Boehnke et al., 1991). Two markers are considered to be linked if they have GB4 vector scores that are statistically significantly similar (defined as a LOD score). RHMAPPER multipoint analysis places the GB4 vector score of the gene of interest into each interval defined by the framework markers on a selected chromosome, thereby allowing the distance between the gene under investigation and any given marker to be established (Slonim et al., 1997). The distance between two markers is expressed in centirays (cR). cRs refer to the x-ray dosage used to construct the RH panel (i.e. cR_{3000} for the GB4 panel). A distance of 1 cR_{3000} between two markers corresponds to a 1% frequency of breakage between markers after exposure to 3000 rads of x-rays. One cR very roughly correlates to a distance of around 30 kb, although the conversion varies throughout the genome. The specific relationship between cR and kb can be found at http://www.sanger.ac.uk/Software/RHserver/

RHmap 2-pt analysis is shown in Table 4.3. Three framework markers were identified: AFM1755xg3, AFM207vf4 and AFMa051xg5. Although, none of the markers exhibited a LOD score greater than 3.00, suggesting that the linkage of each marker and the MrdgB gene were not significant, all three markers mapped to chromosome 17. This result was consistent with the chromosomal localisation of the genomic clones AC023123.3 and AC06534. Analysis of the physical mapping information of AFM1755xg3, AFM207vf4 and AFMa051xg5 (http://www.ncbi.nlm.nih.gov/gene map98) tentatively placed MrdgB within the interval 17q22–17q23.
RHMAPPER multipoint analysis of the MrdgB8 GB4 vector sequence on chromosome 17 is shown in Table 4.4. RHMAPPER multipoint analysis was carried out on all chromosomes and three markers AF217yd10, AFM207vf4 and AFMa247zg9 were identified on chromosome 17 (Table 4.3). However, marker AF217yd10 was rejected because the distance (134.71 cR) between this marker and the MrdgB8 gene was greater than 90 cR. When markers are this far apart, the distance between them can not be measured meaningfully or accurately by the equations used for the RHMAPPER multipoint analysis. An unmeasurable distance (gap) between two neighbouring markers is generally caused by a lack of framework markers within the local region between the two given markers. Alternatively, a gap can arise if the hybrid set being used failed to retain a particular region or if the data for the framework markers surrounding the gap is inherently wrong as discussed in Section 4.3.

In contrast to the RHmap 2-pt analysis, a LOD score greater than 3 is rejected by RHMAPPER multipoint analysis, therefore markers AFM207vf4 and AFMa247zg9 were also rejected. Hence, it is not possible to gain any information from the RHMAPPER multipoint analysis of the MrdgB8 GB4 vector score.

4.2.2 Analysis of chromosome 17q22-23

The results of the RH mapping analysis tentatively suggested that the MrdgB8 gene localises to chromosome 17q22-23. As the three human Nir genes have been linked to retinitis pigmentosa loci, the 17q21-23 region was analysed for similar disease markers using information provided by the Retinal Information Network (http://utsph.sph.uth.tmc.edu/www/utsph/RetNet/home.htm). Analysis of cloned and/or mapped disease genes revealed that the RP17 gene, which is linked to a dominant form of retinitis pigmentosa, maps to chromosome 17q22 (Bardien et al., 1995). This suggested that in common with other members of the rdgB family, the MrdgB8 gene may localise to a chromosomal region known to contain a dominant form of retinitis pigmentosa.

The gene responsible for RP17 remains to be determined. By combining linkage data and using haplotype analysis of recombinant chromosomes in the families of RP17 patients, the RP17 critical region has been mapped to a 1 cM interval on within chromosome 17q22 (Bardien-Kruger et al., 1999). Since the results obtained from the RH mapping analysis are not statistically significant, it is not possible to assign the chromosome localisation of the MrdgB8 gene to the
genetic interval defined by the markers AFM1755xg3 and AFMa051xg5. Therefore, it was necessary to use an additional method, FISH analysis, to map the chromosome localisation of the MrdgBβ.

4.2.3 FISH analysis

4.2.3.1 Isolation of genomic MrdgBβ clones

In order to isolate MrdgBβ-specific genomic clones, high-density gridded filters containing the human RPCI PAC library were obtained from the HGMPRC. A α32P-labelled cDNA fragment encoding residues 717-1124 of the MrdgBβ cDNA sequence was used to probe the PAC library. Hybridisation of the probe to the filters revealed eleven positive clones: 35D11, 44H13, 112G3, 190D1, 188E2, 211I3, 218N14, 287C7, 283C7, 306O9 and 312O9. The RPCI PAC library is known to contain non-recombinant clones; to date 30,000 such clones have been identified, however, only 353 of such clones are currently known to be present on the hybridisation filters supplied by the HGMPRC. Although these clones lack human DNA inserts, they retain a pUC linker sequence, which should have been removed during the construction of the library. Consequently, this sequence allows hybridisation with pUC-like sequences and are discounted as false positives. Of the eleven clones isolated, six were known to be pUC-positive clones, and thus false positives. The remaining five clones, 35D11, 112G3, 211I3, 218N14 and 312O9, were obtained from the HGMPRC.

Genomic clone DNA was screened by PCR using primers designed to amplify DNA residues 717-1124 of MrdgBβ. A 351bp PCR product was obtained from clones 112G3, 211I3 and 312O9 however, no product was obtained from clones 35D11 and 218N14. This suggested that clones 35D11 and 218N14 did not contain the MrdgBβ gene and, were not used for further studies.

4.2.3.2 FISH Analysis

Clones 112G3, 211I3 and 312O9 were labelled with Spectrum Green and hybridised to normal human metaphase chromosomes. The metaphase spreads were counterstained with DAPI and visualised using fluorescent microscopy. Around 5-10 metaphases were analysed in each experiment. Hybridisation with clone 112G3 resulted in specific labelling of chromosome 17 in all metaphase spreads analysed (Figure 4.2). In contrast no specific fluorescence was observed following the
Figure 4.2 Fluorescence in situ hybridisation of PAC 112G3 to human chromosomes. Metaphase spreads of normal blood lymphocytes were hybridised with PAC clone 112G3 as described in Section 2.2.10.c.
Figure 4.3 The MrdgBβ gene localises to chromosome 17q22-23. (A) DAPI binding patterns of a normal metaphase spread hybridised with PAC clone 112G3. Electronically overlaid DAPI and FITC images are shown. (B) Cytogenetic ideogram of chromosome 27.
hybridisation with clones 21113 and 31209 (data not shown). It is unknown why clones 21113 and 31209 did not hybridise to the metaphase spreads; one possibility is that these PAC clones lost their human genomic in culture during the preparation of DNA. The localisation on chromosome 17 was determined through visualisation of the banding pattern of the DAPI metaphase image, revealing that the MrdgBβ gene localises to 17q22-23 (Figure 4.3). It was not possible to define the genetic interval of the MrdgBβ gene more precisely within 17q22-23, due to the limitations of the technique. FISH analysis therefore identified MrdgBβ as a candidate gene for RP17.

4.3 Discussion

This chapter describes the chromosome mapping of the MrdgBβ gene by two independent methods: FISH analysis and RH analysis. FISH analysis allows for the mapping of the gene to a much broader chromosome interval of around 40cM. In contrast, RH analysis allows for the fine mapping of a gene to a chromosome interval of around 1 cM. However, this approach does not necessarily yield a statistically significant result, as was the case in the current study.

While FISH analysis revealed that the MrdgBβ gene localises to 17q22-23, the results obtained from the RH mapping analysis suggested that the MrdgBβ gene is not significantly linked to the framework markers AFM1755xg3 and AFMa051xg5 and hence, 17q22-23. The result obtained by FISH analysis demonstrates that the RH mapping analysis was incorrect. However, it was not possible to precisely assign the genetic locus of the MrdgBβ gene. It was predicted that the RHMAPPER multipoint analysis would identify the same markers as the RHmap 2-pt analysis. The discrepancy between the two results and the lack of correlation between any framework markers and the MrdgBβ gene, may have occurred because the framework markers within chromosome 17q22-23 are not comprehensive. Alternatively, the linkage data of the framework markers may be inherently wrong since an error rate of approximately 1.08% has been documented in the chromosome assignment of order of framework markers on the human 1998 International Gene Map (Deloukas et al., 1998).

Another possibility is that false positive or negative results obtained during the PCR screening of the GB4 mapping panel may account for the lack of a strong correlation. The existence of false positive and negative results is exemplified by the observation that 12% of the clones initially screened positive and subsequently
screened negative or vice versa (Section 4.2.1). Due to the nature of the analysis of the hybrid vector sequence, it is clear that the introduction of false positives and negatives will greatly influence the result. Since optimal cycling conditions of the primers were employed during the PCR screen of the GB4 clones (Section 2.2.7.d), it is unlikely that further screening with the primers used in this study would have allowed for the fine mapping of the MrdgBβ gene. Therefore, further studies, in which more suitable primer sequences are determined, will be required in order to determine whether RH mapping analysis is able to precisely identify the chromosome localisation of the MrdgBβ gene.

While the MrdgBβ gene localises to chromosome 17q22-23 (a region of around 40 cM), it was not possible to determine whether or not the MrdgBβ gene localises to the 1 cM interval on chromosome 17q22 that contains the RP17 locus. Further analysis is required to determine whether or not the MrdgBβ gene localises to the RP17 critical region. Nevertheless, the results presented in this chapter do identify MrdgBβ as a putative candidate gene for RP17.

RP17 is an autosomal dominant form of retinitis pigmentosa (Bardien et al., 1995). The RP17 locus was first described in two unrelated South African families and has subsequently been found in a large Dutch family (Bardien et al., 1997). Mutation and gene recombination analyses of affected patients have excluded several candidate genes found within the RP17 locus including, PDEγ (Bardien et al., 1997), tissue inhibitor of metalloproteinases-2 (TIM-2, (Bardien et al., 1997). PKCα (den Hollander et al., 1999), cone transducin-γ (den Hollander et al., 1999) and retinal specific amine oxidase (den Hollander et al., 1999).

The demonstration that, in common with other members of the rdgB family, the MrdgBβ gene localises to a chromosome region known to contain a dominant form of retinitis pigmentosa strengthens the hypotheses that mammalian rdgBs are candidate genes for retinitis pigmentosa, in particular ADRP. The co-localisation of gene and disease loci is yielding a number of candidate disease genes, particularly since more and more genes are being mapped to specific chromosome regions. Although positional candidates for various diseases have subsequently been identified as the causative gene in the predicted disease, such as the rhodopsin gene at an ADRP locus on chromosome 3q (Dryja and Li, 1995). A chromosome localisation within a disease locus is clearly insufficient to establish a disease gene. For example, the localisation of the type 1α-PtdIn4P 5-kinase to chromosome 13q13, suggested that this kinase was the key defect in Friedreich's ataxia (Carvajal et al.,
However, it was subsequently demonstrated the disease was caused by a mutation in an unrelated gene, named frataxin (Campuzano et al., 1996).

Nevertheless, the DrdgBα phenotype together with the findings that Nir1-3 and MrdgBB map to chromosome regions known to contain retinitis pigmentosa loci suggest that these proteins play a critical role in retinitis pigmentosa. Despite the key differences between vertebrate and invertebrate phototransduction discussed in Section 1.8.1, the retinal degenerative phenotype and defective ERGs exhibited by DrdgB mutants and other phototransduction mutants (Milligan et al., 1997b) are similar to those observed with the latter stages of various retinitis pigmentosa diseases (Birch, 1999; Fishman et al., 1985). Furthermore, the observation that many different genetic lesions cause photoreceptor degeneration and dysfunction in Drosophila is analogous to the heterogeneity of retinitis pigmentosa observed in human populations (Montell, 1999; van Soest et al., 1999).

Although Nir1-3 and MrdgBB exhibit different tissue distributions, these proteins are expressed within the eye (Section 1.8.5 and Figure 3.5). Likewise, a number of genes involved in retinal diseases do not show an expression that is restricted to the eye, for example, Sorsby’s fundus dystrophy (TIP-3, Weber et al., 1994) and RP2 (Weber et al., 1994).

It may be predicted that the pattern of histological abnormalities within retinitis pigmentosa retinas should reflect the pattern of expression of the causative gene within the retina. Confocal microscopy analysis of human retinas with retinitis pigmentosa, revealed abnormal neurite sprouting in amacrine and horizontal cells of the nuclear layers in addition to the rod cells (Fariss et al., 2000 and Figure 3.1). The cause and functional defects of these changes in the inner retina are unknown. However, it has been suggested that neurite outgrowth by these cells may alter existing synaptic connections and interfere with visual processing within the retina cells (Fariss et al., 2000). Although the cellular localisation of MrdgBB within the retina remains to be addressed, the Nir1-3 proteins have been shown to have both differential and overlapping patterns of expression within the retina (Section 1.9). Nir1-3 are expressed within the inner nuclear layer, containing the amacrine and horizontal cells (Chang et al., 1997; Lev et al., 1999). This localisation is consistent with the proposed role of the rdgB proteins in retinitis pigmentosa.

The hypothesis that all rdgB genes are positional candidates for different retinitis pigmentosa diseases suggests that these proteins may share a conserved function that is altered in retinitis pigmentosa. In agreement with this is the
observation that the expression of the cDNA encoding Nir2, and to a lesser extent
Nir3 and a Zebra fish Nir1-homologue, rescued rdgB\(^2\) null mutant flies (Chang et al.,
1997; Elagin et al., 2000; Lu et al., 1999). These observations suggest that the
members of the rdgB sub-family of PtdIns-TPs are functionally related through an
unknown activity that is critical to invertebrate phototransduction. Furthermore, the
expression of the PtdIns-TP domain of DrdgB\(\alpha\), but not PITPa, was sufficient to
completely rescue the mutant phenotype associated with rdgB\(^2\) null mutant flies
(Milligan et al., 1997b), suggesting that MrdgB\(\beta\) may also share this conserved
function despite the absence of any putative membrane-spanning and Pyk2-binding
domains. Mutations in different rdgB genes may have discrete effects on the diverse
cell populations within the retina, owing to their differential cellular localisation. It
would be predicted that this would result in slightly different phenotypes. For
example, a conserved rdgB function may be more critical in the photoreceptor cells
than in the inner nuclear cells, therefore, mutations in a rdgB protein that localises to
the photoreceptors will exhibit a more distinct phenotype than one that localises to
the inner segment. In agreement with this patients with different forms of ADRP
have distinct retinal histopathologies and ERG responses (Fishman et al., 1985).

How could mutations in rdgB genes lead to retinal degeneration? It is possible
that mutations in the rdgB genes give rise to retinitis pigmentosa by affecting any of
the functional mechanisms discussed in Section 4.1. However, the observation that
Nir1 and Nir3, and most probably Nir2 are not expressed in the RPE would suggest
that these proteins are not involved in an RPE-specific retinal function (Chang et al.,
1997; Lev et al., 1999).

From the evidence suggesting a function of rdgB proteins in membrane
traffic (Section 1.8.3), mutations in these genes may disrupt membrane flow within
the retina, particularly within photoreceptor cells where the balance of disc renewal
and disc shedding is critical. Disruption of membrane flow may result in retinal
degeneration. Alternatively, rdgB proteins may interact with proteins that play a
major structural role in the ROS, for example rhodopsin. In this regard, expression
of a dominant negative mutant form of rdgB in DrdgB\(^1\) \(\alpha\) flies resulted in a reduction of
rhodopsin levels within the fly photoreceptors and a degeneration morphology
similar to that observed with hypomorphic \textit{ninaE} alleles (Milligan et al., 1997b). In
this regard, whereas recessive forms of retinitis pigmentosa are often the result of
null-function mutations, usually in enzymes within the visual transduction cascade
dominant forms of retinitis pigmentosa are typically the result of dominant, negative-acting mutations (van Soest et al., 1999).

Finally, as discussed in Section 1.8.3, the rdgB genes may play a role in the phototransduction cascade, possibly by regulating Ca$^{2+}$ levels. Therefore, mutations in these genes may result in the inactivation or defective regulation of the phototransduction cascade, thereby precipitating retinal degeneration by the build up of toxic intermediates or inappropriate signalling. The retinal degeneration phenotypes, observed in the DrdgB mutants and the rd mouse, are rescued by treatment with the Ca$^{2+}$ channel blocker diltiazem, although the phototransduction cascades and the mutations are different in these two species (Frasson et al., 1999; Sahly et al., 1992). These observations suggest that Ca$^{2+}$ toxicity may be a primary or, more likely, a secondary effect in the retinitis pigmentosa degeneration process.

The roles of MrdgBβ and Nr1-3 in retinitis pigmentosa remain to be determined. The identification of MrdgBβ as the causative gene in RP17 will require analysis of the MrdgBβ gene in unrelated patients. The identification of any sequence variation would then have to be followed up with a clinical analysis of the relevant pedigrees to determine if it consistently cosegregated with the disorder. Likewise, analysis of the MrdgBβ gene in the canine model of RP17 (Acland et al., 1998) may provide evidence that MrdgBβ is the causative gene in RP17. If MrdgBβ is found to be the causative gene in RP17, studies will be required to determine whether the mutation results in a loss of or altered function. The subsequent generation of transgenic animals will provide a powerful method to assay a potential role of this gene in retinitis pigmentosa.
5.0 Biochemical and molecular characterisation of MrdgBβ

5.1 Introduction

The existence of multiple mammalian PtdIns-TP related proteins raises the question of functional diversity and specificity. In common with DrdgBα reconstitution studies suggest that PITPα participates in acute PLC signalling (Section 1.4.2). Although Nir3 does associate with a PtdIns 4-kinase and it has been proposed that Nir1-3 function in concert with Pyk2 in the regulation of Ca\(^{2+}\) and phosphoinositide-dependent pathways (Sections 1.9.4 and 1.9.5) the role of Nir1-3 and PITPβ in phosphoinositide metabolism remains unclear. This chapter examins MrdgBβ function at three levels: intracellular localisation, lipid binding specificity, and interaction with potential binding partners with the aim of providing insight into a possible role of MrdgBβ during PtdIns metabolism.

One problem in the study of PtdIns-TPs is that it is often difficult to reconcile the predicted functions of PtdIns-TPs inferred from \textit{in vitro} studies, with observations made from \textit{in vivo} studies. As an example, endogenous PITPα is predominantly distributed throughout the cytoplasm and within the nucleus, whereas endogenous PITPβ is preferentially associated with the Golgi membranes (Section 1.3.2). These observations suggest that despite the ability of both isoforms to facilitate diverse cellular processes such as PLC mediated signal transduction and constitutive vesicle transport in reconstitution studies, the physiological roles of the proteins do indeed differ.

Nevertheless, clear indications of the functions of the different PtdIns-TPs have been obtained by studying properties such as the specificity of phospholipid binding and transfer activity and the identity of binding partners. The ability of PITPβ to transfer SM \textit{in vitro}, a function that is not shared by PITPα, led to the suggestion that this isoform may have a specific role in the biosynthesis of SM (Westerman \textit{et al.}, 1995). Indeed, it was subsequently demonstrated that the over expression of PITPβ does influence SM biosynthesis in the NIH3T3 cell line (Van Tiel \textit{et al.}, 2000b). The different lipid transfer specificity of PITPα and PITPβ suggest that other PtdIns-TPs may also differ in the specificity of phospholipid transfer. \textit{In vitro} studies have demonstrated that the PtdIns-TP domain of DrdgBα does exhibit PtdCho and PtdIns transfer activity, however transfer activity still remains to be shown for the full length protein (Vihtelic \textit{et al.}, 1993). While Lev and co-workers have demonstrated that expression the PtdIns-TP domain of Nir3 can complement Sec14 temperature sensitive mutants (Lev \textit{et al.}, 1999), the
phospholipid transfer activity and specificity of Nir1-3 have yet to be addressed. The presence of a PtdIns-TP domain suggests that MrdgBβ should possess phospholipid transfer activity, the analysis of which could offer insight into the function of this PtdIns-TP in relation to what is known about other isoforms. In contrast to other members of the PtdIns-TP family, MrdgBβ contains a unique small carboxy-terminal domain (amino acid residues 248-333) (Section 3.2.1). It is possible that the presence of this domain may influence the phospholipid binding and transfer properties of MrdgBβ.

In agreement with the proposed role of PtdIns-TP during phosphoinositide metabolism, an agonist dependent association between PITPa and type II PtdIns 4-kinase in been demonstrated in A431 cell lysates (Kauffmann Zeh et al., 1995). Subsequent immunoprecipitation studies have also demonstrated that PITPa associates with human Vps34p (Volinia et al., 1995). Likewise, Nir2 associates with the type III PtdIns 4-kinase on the Golgi membranes in COS-7 cells (Aikawa et al., 1999). These observations suggest that the association of PtdIns-TPs with different PtdIns kinases may be a common function of this family of proteins. Furthermore, the phospholipid kinase binding activity and specificity may be related to the lipid binding specificity of a PtdIns-TP. In a manner analogous to PITPa and Nir2, MrdgBβ might also be predicted to play a role in phosphoinositide biosynthesis and may therefore be expected to associate with one or more phosphoinositide kinases.

While the expression of the isolated PtdIns-TP domain of DrdgBα was sufficient to fully complement DrdgBα null mutant flies, PITPa provided no detectable rescue (Milligan et al., 1997a). These observations suggest that the function of the PtdIns-TP domain of DrdgBα in phototransduction cannot simply be explained by the ability to bind and transfer PtdCho and PtdIns. Furthermore, PtdIns-TP domains appear to be functionally specific. Expression of the cDNA encoding Nir2, and to a lesser extent Nir3 and a Zebra fish Nir1-homologue, rescued DrdgBα null mutant flies (Chang et al., 1997; Elagin et al., 2000; Lu et al., 1999). These observations suggest that members of the rdgB sub-family of PtdIns-TPs are functionally related through an unknown activity that is independent of the ability to bind and transfer PtdCho and PtdIns. It remains to be seen whether or not MrdgBβ shares this apparently conserved function. In common with Nir1-3 and DrdgBα, MrdgBβ contains a small domain, rich in acidic amino acids. Although there is a lack of sequence homology, the acidic domains of Nir1-3 and DrdgBα all bind Ca2+ in vitro, suggesting that MrdgBβ may also bind Ca2+ (Lev et al., 1999; Vihtelic et al.,...
The physiological significance of the ability to bind Ca\(^{2+}\) remains to be shown, however it has been proposed that both DrdgB\(\alpha\) and Nir1-3 play a role in the control of cellular Ca\(^{2+}\) levels (Section 1.8.3 and Lev et al., 1999).

This chapter describes studies that explore the biochemical function of MrdgB\(\beta\) and the concept that different PtdIns-TPs possess distinct functions. The intracellular localisation of ectopic MrdgB\(\beta\) is defined and in a series of preliminary studies, association with several potential binding partners was investigated.

Given the putative role of the PtdIns-TP domain in PtdIns and PtdCho transfer, the phospholipid binding and transfer specificity of MrdgB\(\beta\) relative to PITP\(\alpha\), PITP\(\beta\) and the PtdIns-TP domain of Nir2 ([PITP]Nir2) was addressed using a radiolabelled and fluorescent-labelled phospholipid transfer assays.

PtdCho and SM transfer activity was assayed using fluorescent-labelled phospholipid transfer assay (van Paridon et al., 1988b). During this assay the change in the environment of a pyrene labelled phospholipid is continuously monitored as it is transferred from a donor (quenched) vesicle to an acceptor (unquenched) vesicle. The fluorescent-labelled transfer assay provides a sensitive method to analyse the initial rate of lipid transfer and substrate binding properties of the PtdIns-TP. Pyrene-labelled PtdIns is not yet available commercially hence it was not possible to investigate PtdIns transfer activity using this method. Therefore, PtdIns transfer activity was studied using a radiolabelled transfer assay in which the transfer of radiolabelled microsomal PtdIns to liposomes is determined. In contrast to the fluorescent-labelled transfer assay, the radiolabelled transfer assay is discontinuous. The acquisition of radiolabelled phospholipid in the acceptor liposomes is determined following the incubation of a PtdIns-TP with the donor/acceptor vesicle cocktail, allowing for the total amount of transferred phospholipid to be calculated.

### 5.2 Results

#### 5.2.1 Recombinant expression of PITP\(\alpha\), PITP\(\beta\), [PITP]Nir2 and [PITP]MrdgB\(\beta\)

In order to assess the transfer activity MrdgB\(\beta\) and the PtdIns-TP domain of MrdgB\(\beta\) ([PITP]MrdgB\(\beta\)) relative to other PtdIns-TP isoforms PITP\(\alpha\), PITP\(\beta\) and [PITP]Nir2 were expressed as recombinant proteins. Carboxy-terminal HIS\(_\varepsilon\)-tagged PITP\(\alpha\), PITP\(\beta\) and [PITP]Nir2 constructs were available in house (Sections 2.1 and 2.2.6.b). Sequence alignment of mammalian PtdIns-TPs revealed that amino acid residues 1-247 encoded [PITP]MrdgB\(\beta\). PCR was used to generate a fragment
containing residues 1-247 containing a 5' EcoRI and a 3' XhoI site. The PCR fragment was verified by sequence analysis and subcloned into the EcoRI and XhoI sites of pET21:GST, allowing for the expression of a GST fusion protein. The recombinant proteins were expressed in *E. coli* BL21(DE3)pLyS in the presence of IPTG and purified as described in Section 2.3.1 (Figure 5.1).

5.2.2 PtdIns transfer activity

The presence of a conserved PtdIns-TP domain suggests that MrdgBβ possesses PtdIns transfer activity. Consequently, the ability of recombinant MrdgBβ proteins to transfer rat liver microsomal [$^3$H]PtdIns to liposomes *in vitro* was compared with PITPa, PITPβ, [PITP]Nir2, and GST (Figure 5.2). MrdgBβ exhibited a similar level of PtdIns transfer activity to all of the other PtdIns-TP related proteins. Both, full-length MrdgBβ and [PITP]MrdgBβ mediated a robust transfer of PtdIns between the bilayers with 19.4% ± 3% and 16.6% ± 2% (n=3) respectively of the total counts transferred. Likewise, [PITP]Nir2, PITPa and PITPβ transferred between 16.7 ± 2% and 25% ± 2% of the total [$^3$H]PtdIns. In contrast, GST protein accounted for 4.2% ± 1% of the total [$^3$H]PtdIns (Figure 5.2).

5.2.3 Pyrene-transfer assays

The ability of MrdgBβ to transfer PtdCho and SM relative to PITPa, PITPβ and [PITP]Nir2 was determined using a continuous fluorescence assay using [I-hexadecanoyl(1-pyrenedecanoyl)-sn-glycero-3-phosphocholine (Pyr(10)PtdCho) or N-(1-pyrenedecanoyl)SM (Pyr(10)SM), respectively. This fluorimetric assay monitors changes in pyrene fluorescence upon the addition of a PtdIns-TP to a solution containing Pyr(10)PtdCho or SM in egg PtdCho:PtdOH:TNP-PtdEth donor vesicles and an excess of egg PtdCho:PtdOH acceptor vesicles (van Paridon et al., 1988b). In all the pyrene-labelled phospholipid experiments reported in this chapter, the fraction of pyrene lipid in the donor vesicles was small (10 mol%) to minimise perturbation of the vesicle membrane by the presence of the pyrene moieties. The relationship between fluorescence and pyrene-labelled phospholipid concentration was consistently found to be linear. Within the donor vesicles, the pyrene fluorescence is strongly quenched (>95%) due to energy transfer to the TNP-PtdEth acceptor. An increase in pyrene fluorescence is interpreted to result from the specific association of the PtdIns-TP with the pyrene-labelled lipid allowing the release of the
Figure 5.1 Recombinant expression of mammalian PtdIns-TPs. HIS$_6$-tagged PITPα, PITPβ, [PITP]Nir2 and MrdgBβ and thrombin-cleaved GST:MrdgBβ and GST:[PITP]MrdgBβ were expressed and purified as described in Section 2.3.1. Purified protein was analysed by SDS-PAGE using a 15% acrylamide gel (1µg protein/lane) and visualised by staining with Coomassie Blue.
Figure 5.2 PtdIns-TP isoform specific PtdIns transfer activity. The ability of the recombinant proteins to transfer radiolabelled microsomal PtdIns to unlabeled liposomes was compared to [PITP]Nir2, PITPa, PITPβ and GST (24μg/ml). Background-subtracted results are presented as the fraction of radiolabel transferred to liposomes relative to [PITP]Nir2. Background counts were typically in the range of 1000 dpm, while bona fide PtdIns transfer fell in the range of 6500-8000 dpm. Each data point represents the mean ±SEM of three independent experiments.
fluorescent phospholipid from the quenching effects of the donor vesicle and transfer into the acceptor vesicle (van Paridon et al., 1988b).

5.2.3.1 Pyrene-labelled PtdCho transfer assay

The changes in Pyr(10)PtdCho fluorescence emission upon the addition of 2-20 μg of the various PtdIns-TPs to the donor/acceptor vesicle cocktail were recorded as a function of time (Figure 5.3). The molar concentrations of the PtdIns-TPs were equal ± 6% of one another, 20 μg of each PtdIns-TP was equal to 58 ± 3.7 nmol. The addition of PITPα, PITPβ or [PITP]Nir2 resulted in an increase in pyrene fluorescence, indicating that all three proteins exhibit transfer activity (Figures 5.3a-c). The change in pyrene fluorescence varied with the amount of protein indicating that the PtdIns-TP concentrations were not saturating. The highest observed change in pyrene fluorescence upon the addition of PITPα was observably greater than that of [PITP]Nir2 and PITPβ, suggesting that PITPα exhibit greater Pyr(10)PtdCho transfer activity than Nir2 and PITPβ during 150 s (Figures 5.4a-c). The change in pyrene fluorescence upon the addition of GST was negligible (Figures 5.3d and 5.4d).

In contrast to the PITPα, PITPβ and [PITP]Nir2 results, no significant change in Pyr(10)PtdCho fluorescence was observed following the addition of 20μg MrdgBβ indicating that MrdgBβ does not transfer Pyr(10)PtdCho in vitro (Figures 5.3d and 5.4d). Titration of the amount (2-80 μg) of both MrdgBβ and MrdgBβ:His6 confirmed this result (data not shown).

It was possible that the carboxy-terminal domain exclusive to MrdgBβ inhibited Pyr(10)PtdCho binding and transfer activity. In order to address this possibility, Pyr(10)PtdCho transfer assays were repeated using the recombinant [PITP]MrdgBβ and [3']MrdgBβ fragments. No detectable change in pyrene fluorescence was observed upon the addition of either protein (data not shown). Consequently the apparent lack of Pyr(10)PtdCho transfer activity observed upon the addition of MrdgBβ was not due to an inhibitory effect of the carboxy-terminal domain.

5.2.3.2 Pyrene-labelled SM transfer assay

The ability of the various PtdIns-TPs (2-20 μg) to transfer SM were assayed as described in Section 5.2.3.1 using Pyr(10)SM instead of Pyr(10)SM (Figure 5.5).
Figure 5.3 PtdIns-TP isoform specific changes in Pyr(10)PtdCho fluorescence in the presence of donor and acceptor vesicles. The change in Pyr(10)PtdCho fluorescence upon the addition of 2-20 μg (A) PTPα, (B) PTPβ, (C) [PTP]Nir2 and (D) 20 μg MrdgBβ to a donor/acceptor vesicle cocktail. The change in Pyr(10)PtdCho fluorescence upon the addition of 20 μg GST is represented by the black trace on graphs A-D. An arrow indicates the addition of PtdIns-TP or GST. Each data point represents the mean of three independent experiments.
Figure 5.4 Maximal change in Pyr(10)PtdCho fluorescence. The maximal change in Pyr(10)PtdCho fluorescence at 150 s after the addition of 2-20 μg (A) PITPa, (B) PITPB, (C) [PITP]Nir2 and (D) 20 μg MrdgBβ or GST to a donor/acceptor vesicle cocktail. Each data point represents the mean ± SEM of three independent experiments. (*: a difference of P<0.0001 compared with the value obtained with 20 μg protein, **: maximal change in Pyr(10)PtdCho fluorescence at 113 s).
Figure 5.5 PtdIns-TP isoform specific changes in Pyr(10)SM fluorescence in the presence of donor and acceptor vesicles. The change in Pyr(10)SM fluorescence upon the addition of 2-20 μg (A) PITPa, (B) PITPb, (C) [PITP]Nir2 and (D) 20 μg MrdgBB to a donor/acceptor vesicle cocktail. The change in Pyr(10)SM fluorescence upon the addition of 20 μg GST is represented by the black trace on graphs A-D. An arrow indicates the addition of PtdIns-TP. Each data point represents the mean of three independent experiments.
Figure 5.6 Maximal change in Pyr(10)SM fluorescence. The maximal change in Pyr(10)SM fluorescence at 150 s after the addition of 2-20 μg (A) PITPa, (B) PITPβ, (C) [PITP]Nir2 and (D) 20 μg MrdBB or GST to a donor/acceptor vesicle cocktail. Each data point represents the mean ± SEM of three independent experiments. (* Signifies a difference of P<0.0001 compared with the value obtained with 20 μg protein).
In apparent contrast to previous reports (Rogers and Bankaitis, 2000; Westerman et al., 1995), the addition of PITPa, PITPb or [PITP]Nir2 resulted in an increase in Pyr(10)SM fluorescence, indicating that all three proteins are able to transfer SM in vitro (Figures 5.5a-c). The magnitude of the change of Pyr(10)SM fluorescence was PtdIns-TP isoform specific. At each concentration tested the maximal observed change in Pyr(10)SM fluorescence upon the addition of PITPb was significantly greater than that of [PITP]Nir2 and PITPa, suggesting that PITPb possesses greater Pyr(10)SM transfer activity than [PITP]Nir2 and PITPa (Figures 5.6a-c). The change in Pyr(10)SM fluorescence was negligible upon the addition GST (Figures 5.5 and 5.6d).

No significant change in Pyr(10)SM fluorescence was observed following the addition of 20 \( \mu \)g MrdgBB, indicating that MrdgBB does not transfer Pyr(10)SM, in vitro (Figures 5.5d and 5.6d). This result was confirmed by titrating the amount (2-80 \( \mu \)g) of MrdgBB, MrdgBB:HIS\(_6\), [PITP]MrdgBB and [3'-]MrdgBB added to the donor/acceptor vesicle mixture (data not shown).

5.2.3.3 Is the pyrene phospholipid assay measuring binding and transfer or just binding?

In contrast to the radiolabelled phospholipid transfer assay, the donor and acceptor vesicles are not separated during the pyrene-labelled phospholipid transfer assay. Whereas previous authors have relied on the assumption that this assay only measures transfer activity (van Paridon et al., 1988b; Westerman et al., 1995; Whatmore et al., 1999) it is clear that the assay could be measuring any of the following processes: (1) Binding of the fluorescent phospholipid followed by transfer of the bound fluorescent phospholipid to the acceptor vesicle, (2) Binding of the fluorescent phospholipid, and (3) Binding with the donor vesicle, that is the PtdIns-TP is sampling the fluorescent phospholipids present within the vesicles but does not remain bound to the same phospholipid molecule. The binding of a fluorescent phospholipid will result in a marked increase in pyrene fluorescence even if it is not subsequently transferred to the acceptor vesicle. This is because quenching by TNP-PtdEth is reduced by interaction from the membrane either into free solution or possibly by lifting away from the plane of the of the bilayer as suggested by the liftase model. Quenching is ablated when the distance between the protein-bound fluorophore and the membrane-embedded quencher exceeds the limits of energy transfer.
It is possible to determine which process the assay is measuring by comparing the changes in pyrene fluorescence, upon the addition of each PtdIns-TP in the presence and absence of acceptor vesicles. If the PtdIns-TP is binding and transferring the pyrene-labelled phospholipid to the acceptor it would be predicted that the maximum observed change in fluorescence in the absence of acceptors would be lower than that observed in the presence of acceptors. Furthermore a steady state may be achieved more slowly in the presence of acceptor vesicles since the rates of binding and exchange with acceptor vesicles would have to equilibrate.

If however, the PtdIns-TP is binding the fluorescent phospholipid rather than transferring the fluorescent phospholipid to the acceptor, it would be predicted that the change in pyrene fluorescence would be the same in the presence and the absence of acceptor vesicles.

Finally, if the PtdIns-TP binds the fluorescent phospholipid and exchanges it with another fluorescent phospholipid within the donor vesicles, it would also be predicted that there would be no net change in fluorescence in the presence and absence of acceptor vesicles.

In order to investigate whether the changes in pyrene fluorescence observed upon the addition of PITPα, PITPβ and [PITP]Nir2 were due to binding and transfer of Pyr(10)SM and Pyr(10)PtdCho or binding alone, fluorescence was recorded following the addition of 10 μg of PtdIns-TP to donor vesicles only. The maximum observed change in Pyr(10)PtdCho fluorescence upon the addition of either PtdIns-TP in the absence of acceptors was clearly lower than that observed in the presence of acceptor vesicles (Figure 5.7). Furthermore, in each case the changes in fluorescence appeared to approach equilibrium faster in the absence of acceptor vesicles. These observations suggest that, in the presence of acceptor vesicles, the changes in Pyr(10)PtdCho fluorescence observed upon the addition of PITPα, PITPβ or [PITP]Nir2 was due to both the binding and transfer of Pyr(10)PtdCho. Likewise, the change in Pyr(10)SM fluorescence upon the addition of PITPβ in the presence of acceptors vesicles was the result of binding and transfer of Pyr(10)SM (Figure 5.8b).

In contrast, the changes in Pyr(10)SM fluorescence observed upon the addition of PITPα and [PITP]Nir2 were very similar in the presence and absence of acceptor vesicles (Figures 5.8a and c). These observations suggest that the change in Pyr(10)SM fluorescence observed upon the addition of PITPα or [PITP]Nir2, in the presence of acceptor vesicles, was the result of binding Pyr(10)SM, consequently, these PtdIns-TPs do not transfer SM.
The changes in Pyr(10)PtdCho and Pyr(10)SM fluorescence in the absence of acceptor vesicles following the addition of 20 µg MrdgBβ, [3’]MrdgBβ fragment, [PITP]MrdgBβ fragment, or GST were not significantly different from those observed in the presence of acceptor vesicles (data not shown). These data demonstrate that in contrast to PITPa, PITPβ and [PITP]Nir2, MrdgBβ does not bind and transfer Pyr(10)PtdCho. While PITPa, PITPβ and [PITP]Nir2 all bind Pyr(10)SM, PITPβ was unique in its ability to transfer this phospholipid and MrdgBβ was unique in its inability to bind Pyr(10)SM.

5.2.3.4 Initial rates of transfer of Pyr(10)PtdCho and Pyr(10)SM

Figure 5.9 shows the rates of change in Pyr(10)PtdCho and Pyr(10)SM fluorescence during the initial 10 s after the addition of PITPa, PITPβ or [PITP]Nir2, in the presence and absence of acceptor vesicles. This analysis allows an initial rate of transfer of the pyrene phospholipid by each PtdIns-TP to be determined. While PITPa, PITPβ or [PITP]Nir2 mediated transfer of Pyr(10)PtdCho to the acceptor vesicles did contribute to the initial change in Pyr(10)PtdCho fluorescence, it is clear that the extent of this contribution was isoform specific. In two independent experiments [PITP]Nir2, PITPβ and PITPa mediated Pyr(10)PtdCho transfer accounted for on average 29%, 17% and 5%, respectively, of the total rate of change in pyrene fluorescence in the presence of acceptor vesicles (Figure 5.9a and Table 5.1). Likewise the transfer of Pyr(10)SM by PITPβ accounted for approximately 18% of the initial rate of change in pyrene fluorescence (Figure 5.9b and table 5.1).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Pyr(10)PtdCho + acceptors</th>
<th>Pyr(10)PtdCho - acceptors</th>
<th>Pyr(10)SM + acceptors</th>
<th>Pyr(10)SM - acceptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>PITPa</td>
<td>4.0</td>
<td>3.8</td>
<td>2.1</td>
<td>2.1</td>
</tr>
<tr>
<td>PITPβ</td>
<td>3.3</td>
<td>2.8</td>
<td>2.7</td>
<td>2.2</td>
</tr>
<tr>
<td>[PITP]Nir2</td>
<td>5.1</td>
<td>4.2</td>
<td>2.1</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Table 5.1 Initial rate of change in pyrene fluorescence in the presence and absence of acceptor vesicles following the addition of 10 µg PITPa, PITPβ or [PITP]Nir2.
Figure 5.7 PtdIns-TP isoform specific changes in Pyr(10)PtdCho fluorescence in the presence and absence of acceptor vesicles. The change in Pyr(10)PtdCho fluorescence upon the addition of 10 μg (A) PITPa, (B) PITPβ and (C) [PITP]Nir2 to donor vesicles in the presence or absence of acceptor vesicles. An arrow indicates the addition of PtdIns-TP. Each data point represents the mean of two independent experiments.
Figure 5.8 PtdIns-TP isoform specific changes in Pyr(10)SM fluorescence in the presence and absence of acceptor vesicles. The change in Pyr(10)SM fluorescence upon the addition of 10 µg (A) PITPa, (B) PITPb and (C) [PITP]nir2 to donor vesicles in the presence or absence of acceptor vesicles. An arrow indicates the addition of PtdIns-TP. Each data point represents the mean of two independent experiments.
Figure 5.9 Initial rate of change in Pyr(10)PtdCho and Pyr(10)SM fluorescence in the presence or absence of acceptor vesicles. The slope of the curve during the initial 10 s after the addition of 10μg PITPα, PITPβ or [PITP]Nir2 to donor vesicles in the presence or absence of acceptor vesicles was taken to measure the initial rate in change of (A) Pyr(10)PtdCho and (B) Pyr(10)SM fluorescence upon the addition. Each data point represents the mean of two independent experiments.
Figure 5.10 Initial rates of change in Pyr(10)PtdCho and Pyr(10)SM fluorescence upon the addition of 2-20 µg PtdIns-TP. The slope of the curve during the initial 10 s after the addition of 2-20 µg PtdIns-TP was taken as the initial rate in change of (A) Pyr(10)PtdCho and (B) Pyr(10)SM fluorescence in the presence of donor and acceptor vesicles. Each data point represents the mean ± SEM of three independent experiments.
In agreement with the apparent lack of transfer of Pyr(10)SM by PITPα and [PITP]Nir2, the corresponding initial rates of change in Pyr(10)SM fluorescence were not significantly different in the presence and absence of acceptor vesicles (Figure 5.9b and Table 5.1). Thus, the initial rate of change of fluorescence in the presence of acceptor vesicles is caused by binding of Pyr(10)SM, rather than Pyr(10)SM transfer by these PtdIns-TPs.

As expected, the initial rate of change in Pyr(10)PtdCho and Pyr(10)SM fluorescence in the presence and absence of acceptor vesicles were not significantly different following the addition of MrdgBβ or GST (data not shown).

In addition to being specific to the isoform of PtdIns-TP employed in the assay, the initial rate of change in fluorescence in the presence of acceptor vesicles was found to be concentration dependent for the PtdIns-TPs that were investigated (Figure 5.10).

**5.2.4 Mammalian expression of MrdgBβ in HEK293 cells**

In order to study the in vivo properties of MrdgBβ, including its subcellular localisation and intracellular binding partners, the MrdgBβ ORF was subcloned into a modified pEFpLink2 vector. The carboxy-terminally FLAG-tagged protein was then transiently expressed in HEK293 cells. Although several cell lines were transfected, including the rat adrenal phaeochromocytoma PC12 and human neuroblastoma SH-SY5Y cells, efficient transfection levels were obtained only with HEK293 (data not shown). Transient expression was assessed using immunoprecipitation with an anti-FLAG monoclonal antibody, followed by Western blot analysis of the precipitates using the same antibody. Two proteins of similar size (approx. 40 kDa and 38 kDa) were detected, possibly due to degradation and/or post-translational modification (Figure 5.11a). Chang and co-workers demonstrated that a Nir2-specific antibody also recognised two proteins of similar size using Western blot analysis of retinal samples (Chang et al., 1997).

**5.2.5 Subcellular localisation of MrdgBβ in HEK 293 cells**

Another aspect to be considered in the understanding of the function of a protein is to investigate its intracellular localisation. In order to determine the subcellular localisation of MrdgBβ, HEK293 cells were transiently transfected with the FLAG-tagged MrdgBβ expression vector. Transfected cells were stained with anti-FLAG antibody and analysed by confocal immunofluorescence microscopy. The
Figure 5.11 Intracellular localisation of MrdgBβ. The expression of carboxy-terminally FLAG-tagged MrdgBβ in HEK293 cells was analysed using (A) immunoprecipitation and western blotting with M2 anti-FLAG antibody and (B, C) confocal scanning microscopy of cells stained with M2 anti-FLAG antibody followed by FITC-coupled anti-mouse IgG antibodies and TRITC-labelled phalloidin. Green and red emissions from a single field are shown in panels B and C, respectively. Bar = 20 μm.
FLAG-tagged protein was diffusely present throughout the cytoplasm of Immunoreactive cells and appeared to be completely absent from the nucleus (Figures 5.11b and c). Immunoreactive cells accounted for approximately 20% of all cells and no detectable staining of non-expressing cells was observed (Figures 5.11b and c).

5.2.6 Association of MrdgBβ with Type Iα and Type IIα PtdInsP kinases

The ability of MrdgBβ to bind PtdIns, which is a substrate for several phosphoinositides suggests that this protein may play a role in the biosynthesis of phosphoinositides. In order to explore this possibility, the ability of MrdgBβ to interact with phosphoinositide kinases was investigated in a series of pilot experiments. Reagents required to investigate an interaction between MrdgBβ and PtdIns 4-kinases were not available in house. However, Myc-Tagged Type Iα and Type IIα PtdInsP kinases were readily available, therefore association between each of these proteins and MrdgBβ was investigated using co-transfection and immunoprecipitation studies. Furthermore, although several groups have reported that type Iα PtdInsP kinase localises to the plasma membrane, others have demonstrated that, in common with MrdgBβ, this protein exhibits a cytoplasmic localisation (Shibasaki et al., 1997; and M. dos Santos, unpublished work). Type IIα PtdInsP kinase also localises to the cytosol in addition to the nucleus (S. Minogue, 1999).

HEK293 cells were transiently transfected with expression vectors for FLAG-tagged MrdgBβ and carboxy-terminally Myc-tagged type Iα or Type IIα PtdInsP kinases. The transfected cells were solubilised with 0.05% Triton X-100, and the lysates were immunoprecipitated with anti-FLAG monoclonal antibody. The immunoprecipitates were then immunoblotted with anti-N-Myc or anti-FLAG monoclonal antibodies. Expression of the Myc-tagged proteins was confirmed by Western blot analysis of the total cell lysates. Preliminary results demonstrated that Myc-tagged proteins were not immunoprecipitated with the anti-FLAG antibody (data not shown), suggesting that MrdgBβ does not associate with type Iα or type IIα PtdInsP kinases. Furthermore, the anti-FLAG immunoprecipitates exhibited no PtdInsP 4- or 5-kinase activity when subjected to PtdInsP kinase assays (data not shown).
Figure 5.12 Evidence that MrdgBβ does not bind Ca\(^{2+}\) \textit{in vitro}.

25 μg of each recombinant protein (GST, HIS\(_{6}\) tagged MrdgBβ and thrombin-cleaved GST:MrdgBβ, GST:[PITP]MrdgBβ and GST:[3']MrdgBβ and calmodulin were resolved by SDS-PAGE using a 15% acrylamide gel, transferred to a PVDF filter and incubated with \(^{45}\)Ca\(^{2+}\) as described Section 2.6.6
5.2.7 Investigation of the presence of a Ca\(^{2+}\)-binding domain

MrdgBB contains an acidic carboxy-terminal region that may be predicted to act as a Ca\(^{2+}\)-binding domain. Moreover, it has been demonstrated that DrdgB\(\alpha\) and Nir1-3 all bind Ca\(^{2+}\) (Lev et al., 1999; Vihtelic et al., 1993). To examine the possibility that the carboxy-terminal domain of MrdgBB is able to bind Ca\(^{2+}\), [3']MrdgBB, [PITP]MrdgBB, MrdgBB, GST and calmodulin were tested for the ability to bind \(^{45}\)Ca\(^{2+}\) in an overlay assay. The latter two proteins were used as a negative and a positive control respectively. Following exposure to X-ray film, the PVDF filter was stained with Ponceau S to confirm the presence of each protein (data not shown). The experiment presented in Figure 5.12 shows specific binding of Ca\(^{2+}\) to calmodulin but not to any of the other recombinant proteins. Therefore, unlike other members of the rdgB family, MrdgBB does not bind Ca\(^{2+}\) \textit{in vitro}. However, further tests will be required to confirm this preliminary result and to determine what function, if any, this carboxy-terminal might have.

5.3 Discussion

This chapter describes the biochemical and molecular characterisation of MrdgBB. The intracellular localisation of ectopic MrdgBB and the ability of MrdgBB to bind and transfer PtdIns, PtdCho and SM species relative to other PtdIns-TPs has been determined \textit{in vitro}. In a series of preliminary experiments the association of MrdgBB with several binding partners has been investigated. This work aimed to provide insight into the physiological function of MrdgBB and how this function may differ from the other members of the PtdIns-TP family. Ectopically expressed MrdgBB was found to be mainly cytosolic. Furthermore, MrdgBB binds and transfers PtdIns but not PtdCho and SM and does not bind Ca\(^{2+}\) or type I\(\alpha\) and type II\(\alpha\) PtdInsP kinases. In this respect, the results presented in this chapter supports the hypothesis that different PtdIns-TPs are not functionally degenerate.

5.3.1 Intracellular localisation of MrdgBB

In addition to tissue distribution, the physiological roles of different PtdIns-TP isoforms are likely to be defined by their intracellular localisation and cognate binding partners. Although the intracellular localisation of the Nir1 and 3 proteins has yet to be addressed, Nir2 has been found on the Golgi and ER membranes (Aikawa et al., 1999). DrdgB\(\alpha\) is also localised to the retinal endoplasmic reticulum (SRC; Suzuki and Hirosawa, 1994). Ectopically expressed MrdgBB is present
throughout the cytoplasm, which is consistent with the absence of any putative membrane spanning or hydrophobic segments (Section 3.3). Microinjection of the same MrdgBβ:FLAG construct into Madine canine kidney (MDCK) cells also revealed a cytoplasmic localisation (M. dos Santos, unpublished work). Furthermore, no visible changes to the morphology of these cells were observed following microinjection (M. dos Santos, unpublished work). It is possible that ectopic expression influences the intracellular localisation of a protein, for example if a binding partner such as Nir1 is required for appropriate localisation. In this case binding may be saturated by over expression or masked by the carboxy-terminal epitope tag. It remains to be determined whether or not ectopic MrdgBβ localises similarly to its endogenous counterpart. It is noteworthy that endogenous PITPa also localises to the cytoplasm, whether or not MrdgBβ and PITPa proteins have overlapping function in vivo requires study.

5.3.2 PtdIns transfer activity

PtdIns-TPs are classically defined by the ability to transfer PtdIns and PtdCho between membrane compartments in vitro. The ability of MrdgBβ to bind and transfer PtdIns, was compared to PITPa, PITPβ and [PITP]Nir2 using a discontinuous radiolabelled transfer assay. Recombinant MrdgBβ exhibited a level of PtdIns transfer activity in vitro that was similar to other members of the PtdIns-TP family, suggesting that the function of MrdgBβ may involve the mobilisation of PtdIns. Studies on the lipid-binding properties of PITPa have indicated a single lipid-binding site (Van Paridon et al., 1987a) and no naturally occurring apo-forms of PITPa or PITPβ have yet been identified. It remains to be determined whether or not MrdgBβ and indeed PITPβ, Nir2 and Nir3 contain a single or multiple lipid binding sites and more detailed binding studies may provide an insight. Based on the high level of sequence identity within the PtdIns-TP domain, it would be predicted that all members of the PtdIns-TP family contain a single lipid-binding site.

It is possible that phospholipid binding, rather than transfer activity, is critical to MrdgBβ function. In this regard, mutation studies suggest that PtdIns transfer activity is not the only essential activity of DrdgBα (Milligan et al., 1997a). The binding of PtdIns to MrdgBβ may function as a molecular switch through which some second effector activity of the protein is regulated.
5.3.3 MrdgBβ does not exhibit Pyr(10)PtdCho and Pyr(10)SM transfer activity

The ability of MrdgBβ to bind and transfer PtdCho and SM, relative to other PtdIns-TPs was determined using a continuous assay, using the fluorescent substrates Pyr(10)PtdCho and Pyr(10)SM. In the presence of acceptor vesicles this assay had previously been reported to measure transfer of the respective fluorescent phospholipid by a PtdIns-TP (van Paridon et al., 1988b). However, it is clear from the results presented in this chapter that the initial assumption is incorrect, since an increase in fluorescence occurred as a consequence of binding. Comparisons of the changes in pyrene fluorescence in the presence and absence of acceptor vesicles therefore allowed the binding and transfer of a phospholipid to be considered independently.

In contrast to other members of the PtdIns-TP family, MrdgBβ does not bind and transfer Pyr(10)PtdCho in vitro, suggesting that the physiological function of MrdgBβ does not involve the binding and transfer of PtdCho. Similarly, neither does the physiological function of MrdgBβ involve SM. Furthermore, while the PtdIns transfer assay demonstrated that MrdgBβ does bind and transfer PtdIns, the observations that MrdgBβ exhibits no PtdCho transfer activity suggests that this protein is not a classical PtdIns-TP. Consequently MrdgBβ may also bind and transfer alternative phospholipids. Indeed, since MrdgBβ and PITPα are both expressed ubiquitously and within the cytoplasm, their lipid binding and transfer functions may be expected to differ. Alternatively, MrdgBβ may bind polyphosphoinositides or other lipids. It is possible that MrdgBβ is an oligo-specific PLTP that also exists in an apo form, a property that would allow for the vectorial transfer of PtdIns to different membrane compartments.

Since the structures of the phospholipid binding sites of PITPα, PITPβ and Nir2 are unknown, it is not clear from sequence analysis, why MrdgBβ binds and transfers PtdIns but not PtdCho. Furthermore, all residues that have been implicated in transfer activity (Section1.6), including T59 and S166, are conserved. Definition of the crystal structure of MrdgBβ and other members of the PtdIns-TP family will be necessary to provide insight into apparent differential phospholipid binding and transfer specificity.

The conclusion that the physiological function of MrdgBβ does not involve the binding and/or transfer of PtdCho or SM is based upon the assumption that the relative binding affinities of MrdgBβ for the corresponding fluorescent and physiological phospholipids are similar. Several groups have determined the relative
binding affinities of PITPα and PITPβ for a variety of natural and related PtdCho, PtdIns and SM species by employing fluorimetric competition assays using pyrene-labelled and unlabelled phospholipids (Kasurinen et al., 1990; van Paridon et al., 1988b; Westerman et al., 1995). Such studies demonstrated that there are marked differences in binding affinities between different chemical forms of PtdIns, PtdCho or SM, indicating that PtdIns-TPs have a preference for certain molecular species of PtdIns, PtdCho and SM. Although the binding affinity of PITPα for Pyr(10)PtdCho compares well with egg PtdCho in vitro (Van Paridon et al., 1987b), variation of the acyl chain structure of a phospholipid was found to strongly influence the binding and transfer by PITPα (van Paridon et al., 1988b). In addition to labelled and unlabeled chain length, the overall lipid hydrophobicity and steric properties of the acyl chains contributed to the apparent binding affinity of PITPα for various pyrene-labelled phospholipid species (Section 1.3.1.a).

While MrdgBβ did not bind Pyr(10)PtdCho and Pyr(10)SM, the possibility that this protein binds and transfers other species of pyrene-labelled PtdCho or SM cannot be excluded. Consequently, it is necessary to carry out assays to determine the binding of pyrene-labelled PtdCho and SM species that are structurally more similar to naturally occurring PtdCho and SM species. Alternatively, PtdCho and SM transfer activity could be investigated by measuring the transfer of radiolabelled PtdCho and SM from microsomes to liposomes, since such assays utilise near natural species of PtdCho or SM.

In addition to the possible interference due to the structure of the pyrene labelled phospholipid, there are other experimental factors that may account for the lack of PtdCho and SM binding and transfer activity, most notably is competition between the lipid endogenously bound to MrdgBβ and other lipids present in the assay. The biophysical properties of the vesicles used in the assay could also affect binding and transfer activity.

The binding of a phospholipid to a PtdIns-TP is competitive since one phospholipid is displaced by another one binding. To date, naturally occurring apo forms of PtdIns-TPs have not been identified therefore, the apparent binding of a phospholipid is a two step process involving the exchange of the endogenous phospholipid for another phospholipid. Although Hara et al., have shown that recombinant PITPα is preferentially loaded with PtdGly (Hara et al., 1997), the identity of the endogenous lipids bound to each of the recombinant proteins used in the current transfer assays is unknown. The initial and subsequent exchanges of the
endogenous phospholipid molecule for a pyrene-labelled molecule from the donor vesicle is determined by the relative binding affinity of the PtdIns-TP for these two phospholipids, and any other transferable phospholipid species present within both the donor and acceptor vesicles. For example, PITPα exhibits a 15 fold higher binding affinity for PtdIns than for PtdCho (Van Paridon et al., 1987b). From kinetic analysis it was estimated that PITPα exchanges its bound PtdIns 20-times faster for another PtdIns than for PtdCho, and its bound PtdCho five-times faster for PtdIns than for PtdCho (Van Paridon et al., 1987b).

The binding of a phospholipid to a PtdIns-TP is also dependent on the structure and physical properties of the membrane (Section 1.3.1.b). For example PtdIns-TPs appears to be very sensitive towards the fluidity, curvature and pH of a membrane. Additionally non-specific ionic interactions with phospholipids strongly influence the association of a PtdIns-TP with a vesicle (Section 1.3.1.b). Several studies have demonstrated that the inclusion of negatively charged phospholipids into the acceptor liposomes leads to an inhibition of transfer of phospholipid from the donor vesicle to the acceptor vesicles. For example, Van Paridon and co-workers have demonstrated that increasing the PtdOH content of acceptor vesicles from 8 to 17% greatly decreases PtdCho transfer activity despite the observation that PITPα does not transfer or specifically bind PtdOH (Van Paridon et al., 1988c).

It is possible that MrdgBβ does not bind and transfer Pyr(10)PtdCho or Pyr(10)SM due to competition with endogenously bound lipid, egg PtdCho or PtdOH present in the donor and acceptor vesicles. The identity of the endogenously bound phospholipid is particularly important since this lipid contributes to the rate of exchange with labelled phospholipid and therefore binding and transfer. Furthermore, there are clear differences in the binding ad transfer rates of different PtdIns-TPs

Alternatively, MrdgBβ may bind and transfer PtdCho and SM in a different membrane environment to the one used in the current study. Determining the identity of the phospholipid(s) bound endogenously and the relative binding affinities of MrdgBβ for PtdOH and PtdCho using vesicles with various phospholipid compositions will address these possibilities.

5.3.4 PtdCho and SM transfer activity of PITPα, PITPβ and [PITP]Nir2

While PITPα, PITPβ and [PITP]Nir2 all exhibit Pyr(10)PtdCho transfer activity, albeit with different rates, only PITPβ exhibited Pyr(10)SM transfer activity
Likewise, several groups have independently shown that PITPB can transfer radiolabelled and pyrene-labelled SM in vitro (Milligan et al., 1997b; Westerman et al., 1995). These observations are in agreement with the proposed role of PITPB in SM biosynthesis (Van Tiel et al., 2000b).

Westerman et al. (1995) have reported that the initial rate of change of Pyr(10)SM fluorescence, in the presence of acceptor vesicles, was negligible upon the addition of 1-10 μg PITPa suggesting that PITPa does not bind Pyr(10)SM. The present results are in contradiction with these findings. Comparison of the change in Pyr(10)SM fluorescence in the presence and absence of acceptors vesicles reveal that both PITPa and [PITP]Nir2 do bind SM (Figure 5.8). The discrepancies between the results presented here and those of Westerman et al. (1995) are unclear since an identical acceptor/donor vesicle mixture was used in both studies. Whether or not PITPa and [PITP]Nir2 bind physiological species of SM remains to be determined as does the significance of the ability to bind yet not transfer SM. One possibility is that competition with transferable PtdIns and PtdCho will prevent an association of PITPa and [PITP]Nir2 with SM in vivo. Alternatively, the binding of SM may initiate an allosteric switch into an inactive form of PITPa and [PITP]Nir2.

The results presented in this chapter, together with those of other groups, indicate that PITPa and [PITP]Nir2 bind PtdCho binding more avidly than SM (Figure 5.9) while the reverse is true for PITPB (Figure 5.9). Furthermore, MrdgBβ only binds and transfers PtdIns. It has previously been established that binding affinity of PITPa for PtdIns is greater than for PtdCho (Figures 5.2, 5.3 and 5.5). Westerman et al. (1995) have demonstrated that the binding activity of PITPB for several pyrene-labelled SM species is greater than that of pyrene-labelled PtdCho or PtdIns species. The physiological significance of the ability of these PtdIns-TPs to bind several phospholipid species remains uncertain. The possibilities are that competition between various phospholipid species will result in the vectorial transfer of those species with the highest binding affinity for the PtdIns-TP. Alternatively, the binding of different phospholipids may regulate the activity of the PtdIns-TP or in a manner analogous to Sec14p, may regulate a cellular activity. Additionally, the different binding specificities of the various PtdIns-TP isoforms may influence the intracellular localisation of these proteins. As such the localisation of a PtdIns-TP may be driven by the different lipid compositions at different membranes. For example, the ability of MrdgBβ to bind PtdIns but not PtdCho or SM may target this
protein to membrane compartments that exhibit a high concentration of PtdIns, for example membrane rafts (Waugh et al., 1998).

5.3.5 Lipid transfer assays: radiolabelled versus pyrene-labelled transfer assays

The different methods of transfer activity determination employed in this study clearly have various advantages and disadvantages. The phospholipid composition and concentration of these vesicles can direct protein catalysed movements of phospholipids into and out of biological and artificial membranes. The use of artificial donor and acceptor vesicles in the pyrene labelled phospholipid transfer assay leads to clearly defined test conditions. However, whereas such artificial membrane systems are better defined, the biological membrane employed in the radiolabelled assay come closer to the in vivo situation. In this regard, the pyrene-labelled phospholipid transfer assay could be improved by utilising purified membranes. Furthermore, since the phospholipids employed in these assays contain a bulky pyrene group and short acyl chains, the extent of how the binding and transfer of such species relates to their physiological counterparts are not always clear. Nevertheless, the pyrene labelled phospholipid assay provides an extremely sensitive method that allows the characterisation of the phospholipid binding and transfer properties of a PtdIns-TP to be determined in addition to transfer activity. For example, in the current study, the pyrene-labelled phospholipid transfer assay demonstrated while PITPα, PITPβ and [PITP]Nir2 do bind and transfer PtdCho, the initial rates of transfer varied with each isoform. Analysis of phospholipid transfer activity using the radiolabelled assay may not necessarily reveal isoform difference in transfer activity since the assay is discontinuous and measurements are made after 30 min incubation. Furthermore, by varying the structure of the pyrene-labelled phospholipid, this method allows the properties of the phospholipid-binding site to be investigated. Such analyses may be important into elucidating the mechanism of action of PtdIns-TPs.

5.3.6 MrdGBβ binding partners

The ability of MrdGBβ to bind PtdIns, which is a substrate for several phosphoinositide kinases, suggests that this protein may play a role in the biosynthesis of PtdIns(4,5)P₂ in a manner analogous to PITPα and Nir2. Based on the observations that PITPα and Nir2 associate with PtdIns 3- and 4-kinases, it has been suggested that PtdIns-TPs allow substrates to be specifically presented PtdIns to
PtdIns and PtdInsP kinases (Aikawa et al., 1999; Cockcroft, 1998). However, the ability of MrdgBβ to associate with PtdIns kinases was not addressed in this study and it was not possible to demonstrate an association of MrdgBβ with type Iα or type IIα PtdInsP kinases using co-expression and immunoprecipitation studies. Nevertheless, failure to detect any association under the experimental conditions employed does not rule out an association between either of these two enzymes and MrdgBβ in vivo. For example, association of MrdgBβ with either isoform may be subject to regulation. In this regard, strong association of PITPα with type II PtdIns 4-kinase in A431 cells was agonist dependent, with only a very weak association being apparent in unstimulated cells (Kauffmann Zeh et al., 1995). Studies using a variety of agonists may reveal an association between MrdgBβ and with type Iα or type IIα PtdInsP kinases. Alternatively, MrdgBβ may preferentially associate with other phosphoinositide kinases. Investigation into the ability of MrdgBβ to bind various polyphosphoinositides may provide an insight into kinases with which this protein could associate. Finally, as discussed previously, ectopic expression of FLAG-tagged MrdgBβ may disrupt association with binding partners (Section 5.3.1), therefore the study of the endogenous protein may allow characterisation of any associating phosphoinositide kinases or any other proteins.

In addition to the PtdIns-TP-like domain, MrdgBβ contains a small carboxy-terminal domain, which had no apparent affect on the ability of MrdgBβ to transfer PtdIns (Section 5.2.2). Since the sequence of MrdgBβ diverges from all other PtdIns-TPs, including DrdgBα, at the start of this domain, the function of this unique carboxy-terminal sequence is of particular interest. While this domain contains acidic residues, it exhibits no homology to the acidic, Ca^{2+}-binding domains of Nir1-2 and DrdgBα. Furthermore, in contrast to other rdgB proteins, this acidic domain does not bind Ca^{2+} in vitro. Consequently, the function of this unique sequence may be to interact with other proteins. As discussed earlier (Section 3.3) one such candidate binding partner is Nir1. In contrast to Nir1-3, MrdgBβ does not contain a conserved Pyk2-binding domain, suggesting that this isoform does not associate with Pyk2. Indeed, Pyk2 has not been detected in immunoprecipitates of FLAG-tagged MrdgBβ from HEK293 cells (data not shown). It is possible that complex formation with Nir1 would allow MrdgBβ to form an indirect association with Pyk2. Additionally, MrdgBβ may associate with an alternative protein that does not associate with any other isoform of the PtdIns-TP family.
5.3.7 Summary

In conclusion, the results presented in this chapter support the hypothesis that different PtdIns-TP domains exhibit distinct functions. While both PITPα and ectopic MrdgBβ both localise to the cytoplasm, the phospholipid binding and transfer properties of these PtdIns-TPs differ. Furthermore, although the PtdIns, PtdCho and SM transfer ability of Nir1, Nir3 and full length Nir2 have yet to be addressed, MrdgBβ is unique in its inability to transfer PtdCho. In contrast to other rdgB proteins, MrdgBβ does not appear to bind Ca\(^{2+}\) in vitro, despite the presence of a small acidic domain. Preliminary experiments suggest that MrdgBβ does not bind to polyphosphoinositide kinases, however further experiments are required to test for any association of this isoform with PtdIns or PtdIns P kinases and likewise, a role in phosphoinositide metabolism and Pyk2 signalling cascades.
6.0 Discussion

6.1 Overview

This thesis describes the identification and characterisation of MrdgBB, a novel member of the PtdIns-TP family. At the end of Chapters 3, 4 and 5, the conclusions of the results presented in each of those chapters were discussed. In this chapter all of these results are brought together in order to discuss more general conclusions that can be drawn about MrdgBB, as well as the relevance of these conclusions to the wider understanding of metazoan PtdIns-TPs. Finally, in order to place this thesis in a broader context, current problems and contradictions within the field of PtdIns-TPs are identified and discussed.

6.2 MrdgBB: a novel member of the PtdIns-TP family

Assignment of MrdgBB as a member of the multidomain rdgB subfamily of PtdIns-TPs was based on multiple primary sequence alignments of the PtdIns-TP domain of all known members of the metazoan PtdIns-TP family. However, since MrdgBB defines a novel structural isoform that differs from the generic PITP and rdgB proteins and exhibits the greatest primary sequence identity with a novel Drosophila rdgB protein (DrdgBB), the rdgBB isoform may define a new group of metazoan PtdIns-TPs. Indeed, the absence of a Ca$^{2+}$-binding domain, putative membrane spanning domains and a conserved Pyk2-binding domain (Figures 3.2 and 5.12) suggests that rdgBB isoforms possess physiological functions that are distinct to other members from the rdgB subfamily.

In common with other members of the PtdIns-TP family, MrdgBB is ubiquitously expressed (Figure 3.5). Ectopic expression studies have indicated that, like PITPα, MrdgBB is present throughout the cytoplasm (Figure 5.11), but whether or not MrdgBB and PITPα proteins have any overlapping function in vivo requires further study.

While MrdgBB does exhibit PtdIns transfer activity, pyrene-labelled phospholipid transfer assays suggested that this protein does not transfer PtdCho and SM (Figures 5.2, 5.3 and 5.5). As such, MrdgBB represents the first mammalian PtdIns-TP that does not bind and transfer PtdCho in vitro, suggesting that this protein is a non-classical PtdIns-TP. Definition of the crystal structure of MrdgBB and other members of the PtdIns-TP family will help rationalise differences in phospholipid binding specificity. Furthermore, elucidation of the functions of different PtdCho-bound PtdIns-TP isoforms may provide insight in the physiological relevance of the
inability of MrdgBβ to bind and transfer PtdCho. Future studies including the identification of which phospholipids are naturally bound to MrdgBβ in mammalian cells, will be required to address whether or not MrdgBβ is a mono-specific or oligo-specific PLTP.

In addition to determining phospholipid-binding specificity, the T59 residue of PITPa is a potential substrate for PKC and phosphorylation has been implicated in the regulation of PtdIns transfer activity (Alb et al., 1995; van Tiel et al., 2000a and Section 1.6). The conservation of this residue, together with others implicated in the regulation of PITPa, specifically S166, within the primary sequence of MrdgBβ suggests that the function of this protein may also be regulated by PKC. Alternatively, since Nir 1-3 are phosphorylated in vivo in response to stimuli known to act via Pyk2 (Lev et al., 1999), MrdgBβ may be regulated directly or indirectly by Pyk2 even though this protein lacks the domain required for Pyk2 interaction.

A novel partial cDNA sequence (MrdgBβ') which exhibits a high level of identity with MrdgBβ but lacks a six amino acid region, was identified during analysis of the tissue distribution of MrdgBβ in various murine tissues (Figures 3.9 and 3.10). The deletion in MrdgBβ' removes the putative conserved PKC phosphorylation site T59. Identification of the complete cDNA sequence is required to establish whether or not MrdgBβ' represents a MrdgBβ splice variant or a novel rdgB. If MrdgBβ' does indeed represent a rdgB splice variant or novel rdgB isoform, it would be predicted that the encoded protein would not exhibit PtdIns transfer activity, since mutation of the T59 residue of PITPa abolishes PtdIns transfer activity. This would be particularly intriguing if MrdgBβ' does not transfer PtdCho and SM.

Identification of the physiological function of MrdgBβ awaits further studies, for which the availability of specific immunological reagents will be critical. Although initial attempts to generate MrdgBβ-specific antibodies to recombinant proteins were unsuccessful, MrdgBβ-specific anti-peptide antibodies are currently being generated. A distinct approach to characterising the specific cellular functions of MrdgBβ will be to identify any interacting proteins. The ability of MrdgBβ to bind and transfer PtdIns suggests that, in a manner analogous to PITPa and Nir2, this protein may associate with phosphoinositide kinases. Although no association between MrdgBβ and type Ia and type Ila PtdInsP kinases was detected (Section 5.2.6), MrdgBβ may associate with alternative PtdIns and/or PtdInsP kinase isoforms. Identification of PtdInsP or PtdInsP_2-bound forms of MrdgBβ in
stimulated and unstimulated mammalian cells will provide an insight into which, if any, phosphoinositide kinases could be associated with MrdgBβ. Compared with other members of the PtdIns-TP family, MrdgBβ contains a unique carboxy-terminal extension. The function of this domain may be to specifically interact with other proteins, identification of any such proteins will provide insight into how, if at all, the physiological function of this protein differs from other members of the PtdIns-TP family. Characterisation of the physiological function of MrdgBβ will allow the mechanism of action and regulation of this protein to be accessed.

Finally, MrdgBβ maps to chromosome 17q21-23 a region containing the retinitis pigmentosa RP17 locus (Figures 4.2 and 4.3). Furthermore, Nir1-3 all map to other chromosome regions known to contain distinct retinitis pigmentosa gene loci (Chang et al., 1997; Lev et al., 1999; Lu et al., 1999). The observation that all known mammalian rdgB proteins map to distinct retinitis pigmentosa loci suggests that the rdgB genes are candidates for various forms of this neurodegenerative disease. The established requirement for DrdgBα and PITPa in the protection of neurons from degenerative processes supports this hypothesis (Hamilton et al., 1997).

The hypothesis that different PtdIns-TP domains possess isoform-specific functions in vivo gains additional support from the cloning and characterisation of MrdgBβ described in this thesis. The distinct domain structure, in vitro phospholipid transfer specificity and apparent lack of Ca²⁺ and Pyk2-binding activity suggests that rdgBβ has a physiological function that is distinct from other members of the PtdIns-TP family.

### 6.3 Functions of the PtdIns-TP family

Over the past 7 years numerous studies have identified roles for PITPa and PITPβ during PLC-mediated signal transduction, regulated exocytosis and the biogenesis of both constitutive and regulated secretory vesicles and granules from Golgi membranes (Section 1.4). The accepted consensus from these in vitro systems is that PITPa and β act to supply PtdIns substrate to various PtdIns kinases so that specific pools of PtdIns(4,5)P₂ can be generated and maintained for use in diverse cellular processes. As such PtdIns-TP function may be implicated in a variety of other cellular processes that utilise PtdIns(4,5)P₂. Furthermore, several studies have suggested a role for PITP in the delivery of substrate to PtdIns 3-kinase (Jones et al., 1998; Kular et al., 1997; Volinia et al., 1995).
The physiological functions of the different PtdIns-TPs are likely to be defined in part by their intracellular localisation. One of the most striking anomalies that is apparent in the cited literature concerning the function of PtdIns-TPs is the similar ability of ectopic PITPa and PITPB to reconstitute PLC mediated signalling at the plasma membrane, given that neither of these proteins have been detected at the plasma membrane (Section 1.4.2). It is possible that upon reconstitution of PLC signalling in permeabilised cells, PITPa and PITPB do not assume their normal intracellular localisation, allowing the proteins to act at the plasma membrane.

Studies using permeabilised cells have undoubtedly provided insight into the function of PtdIns-TPs and provide a biological read out for PtdIns-TP activity; however, it is now critical that these findings are reinforced by studies in intact cells and intact organisms.

The studies describing a role for PITPa and β during PLC-mediated signal transduction and vesicle trafficking have employed permeabilised cells and cell-free systems (Hay and Martin, 1993; Ohashi et al., 1995; Thomas et al., 1993). In order to investigate PtdIns-TP function using an intact cell system, Wirtz and co-workers have adopted an alternative approach in which the level of PITPa or PITPB is altered (Monaco et al., 1998; Snoek et al., 1999; Van Tiel et al., 2000b). Surprisingly, these studies did not identify a direct role for PtdIns-TPs in the biosynthesis of PtdIns(4,5)P2 but implicated PITPa and PITPB in the regulation of PtdCho metabolism, PLA activity and SM metabolism, respectively. In contrast, using a intact cells in which the level of Ins(1,4,5)P3 5-phosphatase was decreased by the transfection of antisense DNA, Speed and Mitchell have demonstrated a role for PtdIns-TPs during the biosynthesis of PtdIns(4,5)P2 in plasma membrane microdomains during agonist stimulation (Speed and Mitchell, 2000). Similarly studies using endogenous Nir2 have demonstrated a role for Nir2 during PtdIns(4,5)P2 biosynthesis at the Golgi membranes (Aikawa et al., 1999).

Although several roles have been proposed for PITPa and PITPB, the mechanism of action still remains contentious and has been complicated by the fact that different empirical approaches have been used to study mechanism of PtdIns-TP. Furthermore, all of these studies have employed compromised types of analysis and empirical approaches that offer additional insight have yet to be carried out. The observation that the DrdgBa PITP domain but not mammalian PITPa, were able to rescue DrdgBa mutant flies (Milligan et al., 1997b) emphasises the difficulties

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associated with inferring \textit{in vivo} function for \textit{PtdIns-TP}s from \textit{in vitro} studies that show no discrimination with regard to the type of \textit{PtdIns-TP}.

The isolation and characterisation of mutant \textit{PtdIns-TP}s with selective functional defects represent a very important step forward in the analysis of \textit{PtdIns-TP} function. Such mutants will hopefully prove very useful in a detailed dissection of what \textit{PtdIns-TP}s activities are critical for \textit{in vivo} function. For example, mutation studies have already demonstrated that the \textit{PtdIns} binding and transfer activities of \textit{PITP}\textsubscript{\textalpha} can be uncoupled and that transfer activity is not critical to Drdg\textsubscript{Ba} function in \textit{Drosophila} (Milligan \textit{et al.}, 1997b). Furthermore the identification of mutant \textit{PtdIns-TP}s in mammalian cells may generate dominant negative phenotypes that could prove informative in revealing binding partners and \textit{in vivo} execution points.

While the identification of the Drdg\textsubscript{Ba} and \textit{vb} mutants in \textit{Drosophila} and mouse, respectively, demonstrates an essential requirement for \textit{PtdIns-TP}s in eukaryotic cells, to date these mutants have not clarified the \textit{in vivo} function of these \textit{PtdIns-TP}s. Nevertheless, the identification of these model systems provides valuable tools for the analysis of the function \textit{PtdIns-TP}s by genetic approaches. For example functional analysis of DrdgB have yielded entirely unanticipated results that are difficult to interpret in the context of phospholipid transfer, reinforcing the concept that cells utilise different \textit{PtdIns-TP}s in diverse ways. For example, \textit{Drosophila} rdg\textsubscript{B} rescue studies have demonstrated that the \textit{PtdIns-TP} domain of Drdg\textsubscript{Ba} is functionally distinct from \textit{PITP}\textsubscript{\textalpha}, and that the carboxy-terminal domains of the protein are not required for phototransduction (Milligan \textit{et al.}, 1997b).

The identification of mammalian Nir2 and Nir3 proteins has further complicated interpretation of the function of \textit{PtdIns-TP}s, particularly since Nir2 can associate with \textit{PtdIns} 4-kinase and Pyk2 via two distinct domains \textit{in vivo} (Aikawa \textit{et al.}, 1999; Lev \textit{et al.}, 1999). The characterisation of Nir proteins has only just begun; no doubt analysis of the function of these proteins will provide a alternative twists to the function of \textit{PtdIns-TP}s \textit{in vivo}. Although Nir1 is clearly not a \textit{PtdIns-TP}, at least in some cell types this protein may associate with Mrdg\textsubscript{B} to form a heterodimer containing all of the domains present in other members of the rdg\textsubscript{B} family.

While the yeast Sec14p has not provided direct information with regard to \textit{PtdIns-TP} function in mammals, analysis of this protein has offered an alternative interpretation of how a \textit{PtdIns-TP} might employ its phospholipid exchange activities (Section 1.2.1). In the case of Sec14p, this activity may represent a mechanism for
the regulation of Sec14p effector functions. Consequently, the PtdIns-TP domains of DrdgBα, Nir2 and Nir3 may represent regulatory subunits that, when bound to a specific phospholipid, regulates some effector function of these proteins.

The larger number of metazoan PtdIns-TP isoforms compared with those found in yeast most probably reflect greater variety and complexity of PtdIns-dependant products and pathways in metazoan organisms. As such, the distinct subcellular localisations and lipid binding and transfer specificities of different PtdIns-TP isoforms observed in mammalian cells may facilitate the compartmentation of phosphoinositide metabolism (Section 1.4.4).

In conclusion the identification of multiple mammalian PtdIns-TP proteins with distinct biochemical properties supports the hypothesis that cells utilise different PtdIns-TPs in diverse ways and that PtdIns-TPs can no longer be considered to simply play housekeeping roles within the cell. While reconstitution studies do indicate functional degeneracy, in vivo data including the inability of PITPβ to rescue the mouse vibrator mutant, the specificity of rdgB rescue in Drosophila and the distinct subcellular localisation of PITPa and PITPβ (De Vries et al., 1996; Hamilton et al., 1997; Milligan et al., 1997b) strengthens the argument for functional specificity of the different PITP isoforms. Hopefully, key issues including subcellular context and functional specificity of different PtdIns-TP isoforms will be considered in future studies by various groups investigating the function and mechanism of action of PtdIns-TPs.
### 7.0 Abbreviations

The abbreviations used in this thesis follow the guidelines of the IUPAC-IUBMB Joint Commission on Biochemical Nomenclature (summarised in *Biochemical Journal*, **321**, 1-16, 1997). In addition the following abbreviations are used:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>APMSF</td>
<td>(p-amidino)phenylmethylsulphonylfluoride</td>
</tr>
<tr>
<td>ARRP</td>
<td>autosomal recessive retinitis pigmentosa</td>
</tr>
<tr>
<td>BLAST</td>
<td>basic local alignment search tool</td>
</tr>
<tr>
<td>CCT</td>
<td>choline phosphate cytidyltransferase</td>
</tr>
<tr>
<td>cGMP</td>
<td>3'-5' cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>ddH2O</td>
<td>double distilled water</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DrdgB</td>
<td>Drosophila retinal degeneration B</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>ERG</td>
<td>electroretinogram</td>
</tr>
<tr>
<td>EST</td>
<td>expressed sequence tag</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescence <em>in situ</em> hybridisation</td>
</tr>
<tr>
<td>GTPγS</td>
<td>guanosine 5'-o-(3'thiotrisphosphate)</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione-S-transferase (GST:[protein] denotes an aminoterminal GST protein)</td>
</tr>
<tr>
<td>HIS₆</td>
<td>hexahistidine affinity purification tag ([protein]:HIS₆ denotes a carboxyl-terminal HIS₆ protein)</td>
</tr>
<tr>
<td>MrdgB</td>
<td>mammalian retinal degeneration B</td>
</tr>
<tr>
<td>Nir</td>
<td>Pyk2 amino-terminal domain interacting receptor proteins</td>
</tr>
<tr>
<td>Ni-NTA-(agarose)</td>
<td>nickel nitrilotriacetic acid (agarose)</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PDE</td>
<td>phosphodiesterase</td>
</tr>
<tr>
<td>PH</td>
<td>pleckstrin homology</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLD</td>
<td>phospholipase D</td>
</tr>
<tr>
<td>PLTP</td>
<td>phospholipid transfer protein</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol myristate</td>
</tr>
<tr>
<td>PtdCho</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>PtdEth</td>
<td>phosphatidylethanolamine</td>
</tr>
<tr>
<td>PtdGly</td>
<td>phosphatidylglycerol</td>
</tr>
<tr>
<td>PtdIns</td>
<td>phosphatidylinositol</td>
</tr>
<tr>
<td>PtdIns-TP</td>
<td>phosphatidylinositol transfer protein</td>
</tr>
<tr>
<td>PtdInsP</td>
<td>phosphatidylinositol monophosphate</td>
</tr>
<tr>
<td>PtdInsP₂</td>
<td>phosphatidylinositol bisphosphate</td>
</tr>
<tr>
<td>PtdOH</td>
<td>phosphatidic acid</td>
</tr>
<tr>
<td>PtdSer</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Pyr(10)PtdCho</td>
<td>1-heaxadecanoyl(1-pyrene-decanoyl)-sn-glycero-3-phosphatidylcholine</td>
</tr>
<tr>
<td>Pyr(10)SM</td>
<td>N-(1-pyrene-decanoyl)sphingomyelin</td>
</tr>
<tr>
<td>RH</td>
<td>radiation hybrid</td>
</tr>
<tr>
<td>RACE</td>
<td>rapid amplification of cDNA ends</td>
</tr>
<tr>
<td>ROS</td>
<td>rod outer segment</td>
</tr>
<tr>
<td>RPE</td>
<td>retinal pigment epithelium</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>SH3</td>
<td>Src homology 3</td>
</tr>
<tr>
<td>SM</td>
<td>sphingomyelin</td>
</tr>
<tr>
<td>SRC</td>
<td>subrhabdomeric cisternae</td>
</tr>
<tr>
<td>SSC</td>
<td>standard sodium citrate buffer</td>
</tr>
<tr>
<td>SSPE</td>
<td>standard sodium phosphate-EDTA buffer</td>
</tr>
<tr>
<td>TNP-PtdEth</td>
<td>trinitrophenyl dioleyl-phosphatidylethanolamine</td>
</tr>
</tbody>
</table>
8.0 References


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Cloning and Characterization of a Novel Human Phosphatidylinositol Transfer Protein, rdgBβ*

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The various PITP, retinal degeneration B (rdgB), and amino-terminal domain interacting receptor (Nir) phosphatidylinositol transfer proteins can be divided into two structural families. The small, soluble PITP isoforms contain only a phosphatidylinositol transfer domain and have been implicated in phosphoinositide signaling and vesicle trafficking. In contrast, the rdgB proteins, which include Nir2 and Nir3, contain an amino-terminal PITP-like domain, an acidic, Ca2+-binding domain, six putative transmembrane domains, and a conserved carboxyl-terminal domain. However, the biological function of rdgB proteins is unclear. Here, we report the isolation of a cDNA encoding a novel rdgB protein, mammalian rdgBβ (MrdgBβ). The 38-kDa MrdgBβ protein contains an amino-terminal PITP-like domain and a short carboxyl-terminal domain. In contrast to other rdgB-like proteins, MrdgBβ contains no transmembrane motifs or the conserved carboxyl-terminal domain. Using Northern and reverse transcription-polymerase chain reaction analysis, we demonstrate that MrdgBβ mRNA is ubiquitously expressed. Immunofluorescence analysis of ectopic MrdgBβ showed cytoplasmic staining, and the ability of recombinant MrdgBβ to transfer phosphatidylinositol in vitro was similar to other PITP-like domains. Although early reports found functional degeneracy in vitro, the identification of a fifth mammalian PITP-like protein with a unique domain organization and widespread expression supports more recent results that suggest that different PITP-like domains have distinct functions in vivo.

Metazoan PITPs are ubiquitous, 35-kDa soluble proteins that catalyze the transfer of phosphatidylinositol (PtdIns)3 and phosphatidylinositol diphosphate between membrane bilayers (1, 2). Human cells contain two closely related PITP isoforms, PITPa and PITPβ (77% identity). Reconstitution studies indicated that PITPs play an essential role in the biosynthesis of PtdIns 4,5-bisphosphate during phospholipase C and phosphoinositide 3-kinase mediated signal transduction and exocytosis (3-9). The PITP requirement revealed by these experiments is satisfied by either isoform (8, 10), although the intracellular distributions of endogenous PITPs differ (11). Thus the physiological relevance of different PITP isoforms remains unclear. Hamilton et al. (12) reported that the murine vibrator (vb) mutation, which causes neuronal degeneration, is due to a hypomorphic mutation in the PITPa gene. Their studies have provided evidence for an essential requirement for PITPa in mammalian cells.

Further support for an important in vivo role for specific PtdIns transfer proteins came from the characterization of Drosophila rdgB (DrdgB) mutations. The DrdgB protein has been reported to be a membrane-bound PtdIns transfer protein, which has been exclusively implicated in retinal and olfactory neurosensory signaling (13, 14). DrdgB is a 160-kDa protein containing an acidic, Ca2+-binding domain, six putative membrane-spanning regions, and a carboxyl-terminal domain. The amino-terminal 281 amino acids of DrdgB share >40% identity with PITPs. Although transfer activity still remains to be demonstrated for the full-length protein, the PITP-like domain of DrdgB does possess PtdIns and phosphatidylinositol transfer activity in vitro (15).

DrdgB mutations were originally identified by defects in the compound eye: null mutations cause light-induced retinal degeneration and abnormal termination of the light response (16-18). A combination of genetic, biochemical, and electrophysiological evidence indicates that DrdgB plays a critical role in the phospholipase C-dependent phototransduction cascade in Drosophila, both downstream of phospholipase C and in the recovery phase of the light response (19-23). Nevertheless, the exact biochemical role of DrdgB remains to be determined. Interestingly, although the expression of the PITP-like domain of DrdgB was sufficient for complete rescue of specific DrdgB mutants, PtdIns transfer activity alone appears not to be sufficient, as PITPs was unable to rescue the same mutants (24).

A mammalian homologue of DrdgB has recently been cloned and termed mammalian rdgBα (MrdgBα) (27-29). Unlike PITPa, expression of MrdgBα in DrdgB mutant flies was sufficient to completely restore the wild type phenotype, suggesting that a biochemical activity required for invertebrate phototransduction has been conserved by the Drosophila and mammalian proteins (27). Using a yeast two-hybrid approach to screen for proteins interacting with the protein tyrosine kinase PYK2, Lev et al. (30) identified MrdgBα and two novel human rdgB proteins. Because all three proteins bound the amino-terminal domain of PYK2, they have been designated PYK2 amino-terminal domain interacting receptor (Nir) proteins. According to this nomenclature, which we employ in this report, Nir2 corresponds to MrdgBα.

Like DrdgB, the Nir proteins have a multiple domain structure, containing an acidic, Ca2+-binding domain, six putative

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† Supported by the Biotechnology and Biological Sciences Research Council and Praxis XXI (Portugal).

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF171102.

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† Supported by the Biotechnology and Biological Sciences Research Council and Praxis XXI (Portugal).

The abbreviations used are: PtdIns, phosphatidylinositol; rdgB, retinal degeneration B; DrdgB, C. elegans rdgB; DrgB, Drosophila rdgB; MrdgB, mammalian rdgB; EST, expressed sequence tag; GST, glutathione S-transferase; Nir, PYK2 amino-terminal domain interacting receptor; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RT, reverse transcription.
Cloning and Characterization of Human rdbB

transmembrane domains, and a carboxyl-terminal domain. Both Nir2 and Nir3 contain an amino-terminal FITP-like domain; however, this domain is absent in Nir1. Although Nir1–3 mRNAs possess different tissue expression patterns, all these genes are abundantly expressed in the brain and retina. Leveille et al. (30) also demonstrated that Nir proteins form a complex with PYK2 via their carboxyl-terminal domain, leading to their tyrosine phosphorylation in brain tissue and cultured cell lysates. The authors therefore postulated that Nir proteins function in concert with PYK2 in the regulation of Ca2+ and phosphoinositide-dependent pathways.

Here, we report the identification of a cDNA encoding a novel human rdbB protein, which we have provisionally termed MrdgBβ. Interestingly, unlike Drdβ and the Nir proteins, the predicted amino acid sequence of MrdgBβ contains no recognizable transmembrane motifs. Furthermore, the absence of the carboxyl-terminal domain, which is present in the Drdβ and Nir proteins, suggests that MrdgBβ does not interact with PYK2. We show that Mrdgβ is ubiquitously expressed, cytoplasmic protein that possesses a similar ability to transfer PtdIns compared with other FITP-related proteins.

**EXPERIMENTAL PROCEDURES**

**Isolation of the MrdgBβ cDNA**—MrdgBβ was originally detected as a human brain expressed sequence tag (EST) sequence (GenBank™ accession number R23454) in BLAST searches against the Drdβ sequence. The clone containing R23454 was obtained (IMAGE Consortium, Livermore, CA) and sequenced. To isolate sequences encompassing the initiation codon, 5'-rapid amplification of cDNA ends (RACE) PCR was performed using an ADR2 fetal brain cDNA library (approximately 105 plaques) and pituitary tissue complementary to the 5'-region of R23454 (primary primer, 5'-AAAACATATGCTCATCAAGGAATCTGAGAGC-3'; nested primer, 5'-AAAAATCCAGGCTGCTGATTCTCGC-3') and subcloned into pGEM-T Easy (Promega, South Hampton, UK). A 700-base pair race PCR product was isolated, subcloned into pGEM-T Easy (Promega) and sequenced. In order to confirm the integrity of the complete cDNA, the full-length cDNA, termed PCR-1, was amplified from the ADR2 library (forward primer, 5'-ATATGGAATCTTCAATGCTGCTGAAAGAGTACCG-3'; reverse primer, 5'-ATATCTCGACCTCAGATTTGGGCCGACATGG-3') was subcloned into pGEM-T Easy using the XhoI and EcoRI restriction sites. Several independent clones were isolated and sequenced in full to confirm the full-length MrdgBβ cDNA sequence. (The nucleotide sequence for MrdgBβ has been deposited in GenBank™ under accession number AF191450). The predicted amino acid sequence of this EST is identical to residues 1–91 of MrdgBβ. The PCR products were cloned into pGEM-T Easy and verified by sequence analysis.

**Mammalian Expression Plasmids**—MrdgBβ was expressed with a carboxyl-terminal FLAG tag. The FLAG epitope was hybridized by complementary oligonucleotides (5'-ATATCCGAGATAAAATTTTCCGGCCACAGAATGAAA-3'; reverse primer, 5'-GGCCGATTCGCGGTCTC-3') and subcloned into the mammalian expression vector pEFPIink2 (31) at the EcoRI and SpeI sites. A PCR fragment encoding the MrdgBβ open reading frame with terminal EcoRI and EcoRI restriction sites (forward primer, 5'-ATATGATATCCGCTGAAGAAGATCAGG-3'; reverse primer, 5'-ATATGATATCCGCTGAAGAAGATCAGG-3') was ligated into the NotI and EcoRI sites of the pEFPIink2-FLAG construct. The final open reading frame was verified by sequence analysis.

**Cell Culture and Transfection**—Human embryonic kidney HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium containing Glutamax (Life Technologies) and 10% fetal calf serum, 50 IU/ml penicillin and 50 µg/ml streptomycin. Cells were transfected with expression plasmid in 100-mm dishes containing glass coverslips using Superfect (Qiagen) according to the manufacturer’s instructions. Cells were harvested 48 h after the addition of DNA.

**Immunofluorescence Analysis**—Transfected HEK293 cells grown on glass cover slips were fixed with 4% paraformaldehyde in phosphate-buffered saline for 20 min at room temperature. Fixed cells were permeabilized in phosphate-buffered saline containing 0.2% Triton X-100 for 5 min. Cells were incubated for 1 h at room temperature with M2 anti-FLAG monoclonal antibody (Sigma) diluted 1:360 in phosphate-buffered saline containing 0.1% bovine serum albumin. Secondary staining was performed using FITC-labeled anti-mouse antibodies (Jackson Immuno-Research Laboratories, West Grove, PA) diluted 1:200 in phosphate-buffered saline. Actin filaments were detected by incubation with 0.8 µM TRITC-labeled phalloidin (Sigma) in order to identify untransfected cells. Stained samples were mounted in Mowiol (Calbiochem) and analyzed by confocal laser scanning microscopy (Zeiss LSM 510).

Expression and Purification of Recombinant Protein—A PCR fragment from pOGX-EC2 (32) encoding glutathione S-transferase (GST) and containing the Ndel site at the beginning of the open reading frame, was subcloned into the Ndel and XhoI sites of pET21b (Novagen, Cambridge, UK) and designated pET21-GST. The PCR cDNA fragment was subcloned into the EcoRI and XhoI sites of pET21-GST and sequenced. Recombinant protein expression was induced in Echerichia coli strain BL21(DE3)pLyseS (Novagen) using 0.1 mM isopropyl-β-D-thiogalactopyranoside for 3 h at room temperature. Bacteria expressing the GST-MrdgBβ fusion protein were sonicated in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM KCl, 0.5% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.1% β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 10 µM benzamidine, 1 mM leupeptin, 1 mM aprotinin) and centrifuged at 20,000 × g for 20 min. Supernatants were incubated for 30 min at 20°C with glutathione-Sepharose (Amersham Pharmacia Biotech), washed extensively with lysis buffer without protease inhibitors, and resuspended in 80 mM Tris-HCl, pH 7.9, 2 mM NaCl. Bovine thymon was added at a concentration of 25units/mg of fusion protein, and cleaved protein was collected following centrifugation. In order to obtain a similar buffer composition to His-tagged protein preparations, EDTA was added to a final concentration of 400 mM. Recombinant GST was purified essentially as above, with the exception that GST was eluted from the Sepharose using reduced glutathione.

**PITPα and PITPβ isoforms** were cloned and expressed as His-tagged fusion proteins in bacteria as described previously (10). The FITP-like domain of the human Mrdnβ was obtained from a partial murine mRNAs by a combination of screening in a Ph.D.2 human infant brain cDNA library (forward primer, 5'-AAAAACATATGCTCATCAAGGAATCTGAGAGC-3'; reverse primer, 5'-AAAAATCCAGGCTGCTGATTCTCGC-3') and subcloned into pGEM-T Easy. Several independent clones were sequenced to the XhoI site at the beginning of the open reading frame, and the final open reading frame was verified by sequence analysis.

**Sequence Determination and Analysis**—All DNA sequencing was carried out using the dye-deoxy chain termination reaction (PRISM Dye Deoxy Terminator Cycle Kit; Perkin-Elmer Biosystems) and an automated DNA sequencer (PRISM 317; Perkin-Elmer Biosystems). Sequence comparisons and multiple sequence alignments were performed using the BESTFIT and the PILEUP programs, respectively (version 7, Genetics Computer Group).
Fig. 1. The nucleotide and predicted amino acid sequence of human rdgBβ. The translation initiation methionine is at nucleotide and amino acid position 1, and the stop codon (TAA) is marked by an asterisk. The 5'-untranslated region was derived from four independent ESTs (GenBank™ accession numbers AI652942, AI554136, AI378137, and AI523595).

10 min at 4°C. Supernatants were incubated with M2 anti-FLAG immunoaffinity beads (Sigma) for 2 h at 4°C. After extensively washing with lysis buffer, FLAG fusion protein was competitively eluted using FLAG peptide (Sigma). Eluates were mixed with an equal volume of 2x sample buffer and separated by SDS-PAGE. Proteins were transferred with lysis buffer, FLAG fusion protein was competitively eluted using FLAG monoclonal antibody (Sigma). Bound antibody was detected using the ECL system (Amersham Pharmacia Biotech).

**Transfer Assays**—PdIns transfer activity was assayed using rat liver microsomes and [3H]PtdIns as described previously (3) and either equal concentrations of each protein (24 µg/ml) or corresponding buffer (24 µg/ml) or corresponding buffer.

**RESULTS**

**Cloning of MrdgBβ**—In order to isolate novel human homologues of DrdgB, EST data bases were screened for human sequences showing similarity to DrdgB. A human brain EST fragment (R24545) was found, the complete sequence of which encoded amino acid residues 210–333 of the open reading frame, as well as 1782 base pairs of the 3'-untranslated region. Using PCR primers designed to anneal either side of the stop site in R24545, a fragment of the expected size was amplified from an infant brain cDNA library. The sequence of this fragment confirmed the position of the stop site in R24545. Furthermore, several EST sequences (GenBank™ accession numbers AA021507, H86340, and AA080929) identical to the 3'-region of R24545 were identified by performing additional BLAST searches. The position of the stop site in these ESTs was identical to that observed in R24545. In order to obtain the sequence encoding amino acid residues 4–209, 5'-RACE PCR was used to screen the infant brain cDNA library. The remaining cDNA sequence encoding residues 1–3 was derived from the EST data base by screening for sequences homologous to the cDNA sequence encoding residues 4–8. Three partial cDNA sequences (GenBank™ accession numbers AA021507, H86340, and AA080929) were identified from human tissue. The complete open reading frame was verified by PCR using the infant brain cDNA library. The closer similarity of the predicted amino acid sequence to rdgB compared with PTP isoforms (see below) suggested that this sequence defined a novel human rdgB protein, which we have therefore termed mammalian rdgBβ (MrddgBβ).

MrddgBβ has an open reading frame of 999 base pairs, which encodes a 333-amino acid polypeptide of molecular mass 38.2 kDa (Fig. 1). MrddgBβ contains a PTP-like amino-terminal domain and a small carboxyl-terminal domain that exhibits no sequence homology to the Ca2+-binding and the conserved carboxyl-terminal domains of the Nir1–3 (Fig. 2A). Interestingly, unlike the DrdgB and Nir proteins, the predicted protein sequence of MrddgBβ contains no recognizable transmembrane regions.

Sequence alignments of MrddgBβ, Nir1–3, PTPα, PTPβ, and DrdgBβ are shown in Fig. 2. The amino-terminal domain of MrddgBδ exhibits 47 and 42% amino acid sequence identity with DrdgB and Nir2, respectively. These levels of amino acid sequence identity are higher than between MrddgBβ and either PTPβ (41%) or PTPβ (39%). These data are represented as a dendrogram in Fig. 2B.

MrddgBβ exhibits 58 and 40% identity with two Drosophila ESTs (GenBank™ accession numbers AA439582 and AA698247), both of which are distinct from DrdgB. Consequently, from here onward, we refer to DrdgB as DrdgBα. The predicted amino acid sequence of the first EST shows a greater level of identity with MrddgBβ than with DrdgBα, Nir1, or Nir2, thereby indicating the existence of DrdgBα. We therefore suggest that rdgBβ proteins occur in mammals and insects, al-
Fig. 2. Comparisons of amino acid sequences of PITP-related proteins. Amino acid sequences of the PITP-like domains of the five human and three Drosophila (DrdgBa, DrdgBβ, and PITP-Dm) PITP-related proteins are compared using multiple sequence alignment (A) and a dendrogram (B). *, DrdgB^ and PITP-Dm are incomplete sequences derived from EST database (see text for details); consequently, complete sequence information on these two proteins is required to confirm this analysis. Positions containing identities between >4 sequences are shown in black boxes, and remaining positions containing similarities between >3 sequences are shown in gray boxes. The consensus sequence is derived from identities in all 5 sequences (uppercase) or similarity in >3 sequences (lowercase).

though further analysis is required to establish the full-length DrdgBβ sequence. The second Drosophila EST sequence is more similar to mammalian PITPs than to any protein in the rdgB family, and is therefore termed Drosophila PITP (PITP-Dm in Fig. 2B). The genome of Caenorhabditis elegans appears to contain only one PITP-like (Wormpep accession number Y71G12A_205.C (produced by the C. elegans Sequencing Group at the Sanger Center)) and one rdgB-like gene (GenBank™ accession number Z77131). The probable functions of the Drosophila and C. elegans PITPs are unclear from sequence comparisons, as they are less similar to either mammalian PITP isoform than the latter are to each other (Fig. 2B). The predicted C. elegans rdgB protein (CrdgB) has a similar domain organization to DrdgBα, Nir2, and Nir3 and may therefore bind a PYK2-like protein.

Analysis of the deduced MrdgBβ protein sequence using the MOTIFS algorithm (GGG) revealed several potential protein kinase A and protein kinase C phosphorylation sites, suggesting possible mechanisms of functional regulation. Furthermore, in common with all proteins containing a PITP-like domain, rdgBβ contains a threonine residue corresponding to residue 59 of PITPa, which has been suggested to allow protein kinase C to regulate the PtdIns transfer activity of PITPa (33).
ple human tissues indicated that MrdgBβ is ubiquitously expressed (Fig. 3A). The 2.0-kilobase MrdgBβ transcript was expressed strongly in heart, muscle, kidney, liver, and peripheral blood leukocytes and weakly expressed in all other tissues. In comparison, DrdgBa shows multiple transcripts ranging from 3.9 to 9.5 kilobases with expression limited to the brain and retina, and Nir2 is ubiquitously expressed as a transcript of 4.5 kilobases, whereas Nir1 and Nir3 exhibit more limited expression patterns with transcripts of around 7.5 kilobases (15, 30). The size of the MrdgBβ transcript is consistent with the absence of sequence encoding the transmembrane domain, which is present in the DrdgBa, CrdgB, and Nir proteins. RT-PCR analysis of cDNA from various mouse tissues confirmed the ubiquitous expression of MrdgBβ (Fig. 3B). The detection of MrdgBβ transcripts in all tissues analyzed is consistent with the presence of MrdgBβ ESTs derived from a variety of human tissues (data not shown).

**Subcellular Localization of MrdgBβ—**In order to define the intracellular localization of MrdgBβ, transient expression of FLAG-tagged MrdgBβ in HEK293 cells was assessed using immunoprecipitation with an anti-FLAG monoclonal antibody followed by Western blot analysis of the precipitates using the same anti-FLAG antibody. Two proteins of similar size (approximately 40 and 48 kDa) were detected, possibly due to degradation and/or posttranslational modification (Fig. 4A). Chang et al. (27) demonstrated that a Nir2-specific antibody also recognized two proteins of similar size using Western blot analysis of retinal samples. Transfected cells were stained with anti-FLAG antibody and analyzed by confocal immunofluorescence microscopy. Immunoreactive cells revealed that the FLAG-tagged protein was diffusely present throughout the cytoplasm (Fig. 4B). No detectable staining of nontransfected cells was observed (Fig. 4C).

**Transfer Activity—**The presence of a conserved PTP-like domain suggested that MrdgBβ may possess PtdIns transfer activity. To assess the PtdIns transfer activity of MrdgBβ, we expressed the full-length protein and the PTP-like domain in bacteria (Fig. 5A). The ability of the recombinant proteins to transfer rat liver microsomal [3H]PtdIns to liposomes was compared with the PTP-like domain of Nir2 (PTTP/Nir2), PTTPα, PTTPβ, and GST (24 μg/ml). Background-subtracted results are presented as the fraction of radiolabel transferred to liposomes relative to the PTP-like domain of Nir2. Background counts were typically in the range of 1000 dpm, whereas bona fide PtdIns transfer fell in the range of 6500–8000 dpm.

**Discussion**

Although the first rdgB protein to be identified was an invertebrate phototransduction protein, the conservation of amino acid sequence and domain topology between DrdgBa, CrdgB, Nir2, and Nir3 suggests that the functions of the rdgB family, like the PTP family, have been conserved during metazoan evolution. These functions have so far been only partially characterized and are discussed later.

We have isolated the cDNA of a novel PtdIns transfer pro-
protein. Sequence alignment of its conserved PITP-like domain revealed that, although it is a member of the rdgB family, it contains no apparent membrane-spanning domains, nor the domain required for interaction with PYK2. Accordingly, we have provisionally adopted the name rdgB. The existence of five mammalian proteins with PITP-like domains suggests that there are differences in their cellular functions. Furthermore, as the MrdgB protein defines a novel structural form, it may have a novel biological function. The differential subcellular distribution of PITP isoforms (11, 34), the properties of the mouse vibrator mutant (12), DrdgBo rescue studies (26), and the co-expression of Nir and PITP isoforms within mammalian tissues (1, 30) suggest that different PITP domains are not functionally degenerate in vivo, although recombinant PITP proteins can behave similarly in vitro (8, 10, 35).

In addition to tissue distribution, the roles of different rdgB isoforms in vivo are likely to be defined by their intracellular localization and cognate binding partners. Although the intracellular localization of the Nir1 and 3 proteins has yet to be addressed, Nir2 has been found in Golgi and endoplasmic reticulum membranes (36). DrdgBo is also localized to the retinal endoplasmic reticulum (subrhabdomeric cisternae) (15, 37). We show that ectopically expressed MrdgB is present throughout the cytoplasm, although it is possible that ectopic expression influences the intracellular localization of a protein, for example, if a binding partner such as Nir1 is required for appropriate localization. In this case, binding may be saturated by overexpression or masked by the carboxyl-terminal epitope tag. Thus, the intracellular localization of endogenous MrdgB will be addressed in future studies. An association between Nir1, which lacks a PITP-like domain, and MrdgB would form a heterodimer containing all of the domains present in other rdgB proteins. However, the restricted tissue distribution of Nir1 suggests that if such an association occurs in vivo, it would not be ubiquitous.

The demonstration that the conserved carboxyl-terminal domain of each Nir protein forms a complex with PYK2 and is tyrosine-phosphorylated in response to PYK2 activation led to the suggestion that PYK2 is an upstream regulator of Nir functions specific to rapid neurosensory transmission in invertebrates (15, 26), the occurrence and expression of Nir proteins throughout the central nervous system and other tissues suggests that additional roles exist for these proteins in vivo. We have demonstrated here that a novel member of the rdgB family is expressed ubiquitously, an observation that extends the role of this family toward more fundamental cellular processes. Future work is aimed at identifying specific in vivo roles for MrdgB.

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