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The Diverse Biological Functions of Phosphatidylinositol Transfer Proteins in Eukaryotes

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ABSTRACT Phosphatidylinositol/phosphatidylcholine transfer proteins (PITPs) remain largely functionally uncharacterized, despite the fact that they are highly conserved and are found in all eukaryotic cells thus far examined by biochemical or sequence analysis approaches. The available data indicate a role for PITPs in regulating specific interfaces between lipid-signaling and cellular function. In this regard, a role for PITPs in controlling specific membrane trafficking events is emerging as a common functional theme. However, the mechanisms by which PITPs regulate lipid-signaling and membrane-trafficking functions remain unresolved. Specific PITP dysfunctions are now linked to neurodegenerative and intestinal malabsorbtion diseases in mammals, to stress response and developmental regulation in higher plants, and to previously uncharacterized pathways for regulating membrane trafficking in yeast and higher eukaryotes, making it clear that PITPs are integral parts of a highly conserved signal transduction strategy in eukaryotes. Herein, we review recent progress in deciphering the biological functions of PITPs, and discuss some of the open questions that remain.

KEYWORDS PITPs, lipids, signaling, genetics, membrane trafficking, cell polarity

INTRODUCTION

PITPs are approximately 35 kD MW phospholipid transfer proteins that facilitate the energy-independent transfer of either phosphatidylinositol (PtdIns) or phosphatidylcholine (PtdCho) between membrane bilayers *in vitro* (Cleves *et al.*, 1991a; Wirtz, 1991). Given their high level of conservation in eukaryotes, it is presumed that PITPs exhibit some important *in vivo* function. PITP conservation can be divided into two distinctive branches based upon their primary sequence homology: the fungal/plant PITPs and the metazoan PITPs. Plant and fungal PITPs display high primary sequence identity among themselves, as do PITPs from mammals, flies, fish, and worms (Bankaitis *et al.*, 1989; Dickeson *et al.*, 1989; Carmen-Lopez *et al.*, 1994). However, there is absolutely no sequence similarity between these two groups; the original mammalian homolog to the fungal/plant PITPs is the cellular retinaldehyde binding protein (CRALBP) (Salama *et al.*, 1990). Despite this lack of sequence identity between the two groups, these proteins are virtually indistinguishable in biochemical

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assays. While all PITPs can transfer both PtdIns and PtdCho *in vitro*, they do exhibit a 16-to 20-fold greater binding affinity for PtdIns (Szolderits *et al.*, 1989; Van Paridon *et al.*, 1987). Along with this preference for PtdIns, PITPs have the ability to stimulate the synthesis of phosphoinositides both *in vitro* and *in vivo*. It is assumed that PtdIns-binding/transfer is the critical activity of PITPs, and much less consideration is generally given to the PtdCho-binding/transfer activity of PITPs.

Since the discovery of PITPs, their function has been coupled to phospholipid metabolism, membrane trafficking, and polarized membrane growth. Despite these associations with essential biological processes and the abundance of PITPs throughout all eukaryotic organisms, there is little information about their precise physiological roles within cells. Even the most basic questions about the mechanism of action and cellular basis of function of individual PITP molecules are of large interest to cellular biology. This significance translates to the biomedical perspective. Deficiencies in PITP functions are associated with neurodegenerative, glucose homeostatic, and intestinal malabsorbtion disorders in mice. A more complete understanding of the exact molecular sites of action of PITPs will contribute to our understanding of such diseases. This review describes recent developments concerning the biological functions of PITPs in various eukaryotic species and identifies some of the major unresolved questions in this wide-open field. In this review, we limit our attention to recent advances in PITP structural biology, and to the contributions made by *in vivo* systems to our present understading concerning physiological functions for PITPs. Readers are encouraged to consult other recent PITP-centric reviews for alternative perspectives on the topic (Wirtz; 1997; Hsuan & Cockroft, 2001; Allen-Baume *et al.*, 2002).

YEAST PITPs

The *Saccharomyces Cerevisiae* Sec14p

The *SEC14* gene product (Sec14p) is the major PITP of the budding yeast *Saccharomyces cerevisiae*, and plays an essential role in protein transport from the *trans*-Golgi network (TGN) (Bankaitis *et al.*, 1990). Analyses of Sec14p function were made possible by the existence of a single temperature-sensitive allele of the *SEC14* structural gene (*sec14-1^{ts}*) (Cleves *et al.*, 1989). This allele was originally isolated from a genetic screen for mutants with thermosensitive defects in yeast secretory pathway function (Novick *et al.*, 1980). The various cell biological and biochemical data culled from analyses of the *sec14-1^{ts}* mutant indicate that Sec14p is essential for the biogenesis of secretory vesicles from the yeast TGN (Bankaitis *et al.*, 1989). Formation of these TGN-derived secretory vesicles entails a complex series of events, including the coordination of lipid signaling with the function of proteins involved in vesicle budding. It is this interface that is the site of regulation by Sec14p. The power of yeast genetics, in combination with solution of the three-dimensional structure of Sec14p, provides considerable insight into how this protein binds its phospholipid substrates and how it couples these binding reactions to biological function.

Crystal Structure of Sec14p

The Sec14p fold is a novel structure comprising 12 α-helices, 6 β-strands and 8 3_{10}-helices (Figure 1) (Sha *et al.*, 1998). These structural elements cooperate to form a large hydrophobic pocket (3000 Å^3) that is sufficient in size to accommodate a single phospholipid monomer. The available Sec14p structure describes an apo form of the protein where the phospholipid ligand is replaced by two detergent molecules. This substitution occurs during the crystallization reaction, a reaction that requires the presence of β-octylglucoside at concentrations in excess of its critical micellar concentration. As such, the crystallized form likely represents a transitional conformer where bound phospholipid has been unloaded, but Sec14p has not yet reloaded with a second phospholipid binding substrate. This apo-form is hypothesized to exist only transiently on the membrane surface during the course of the phospholipid exchange reaction.

Although a crystal structure of a Sec14p:: phospholipid complex is highly desirable, the available model provides considerable insight into how Sec14p may bind target membranes and individual phospholipids. With regard to substrate binding in the soluble Sec14p::phospholipid complex, bound lipid is predicted to orient with the acyl-chains packed into the hydrophobic interior of the pocket while the phospholipid headgroup is disposed towards solvent. Biochemical data demonstrating that the headgroup of PtdIns bound to Sec14p is accessible to the active site of a PtdIns 3-kinase are consistent with this model (Jones *et al.*, 1998).

How is access of substrate to the Sec14p hydrophobic pocket regulated? The current view is that an

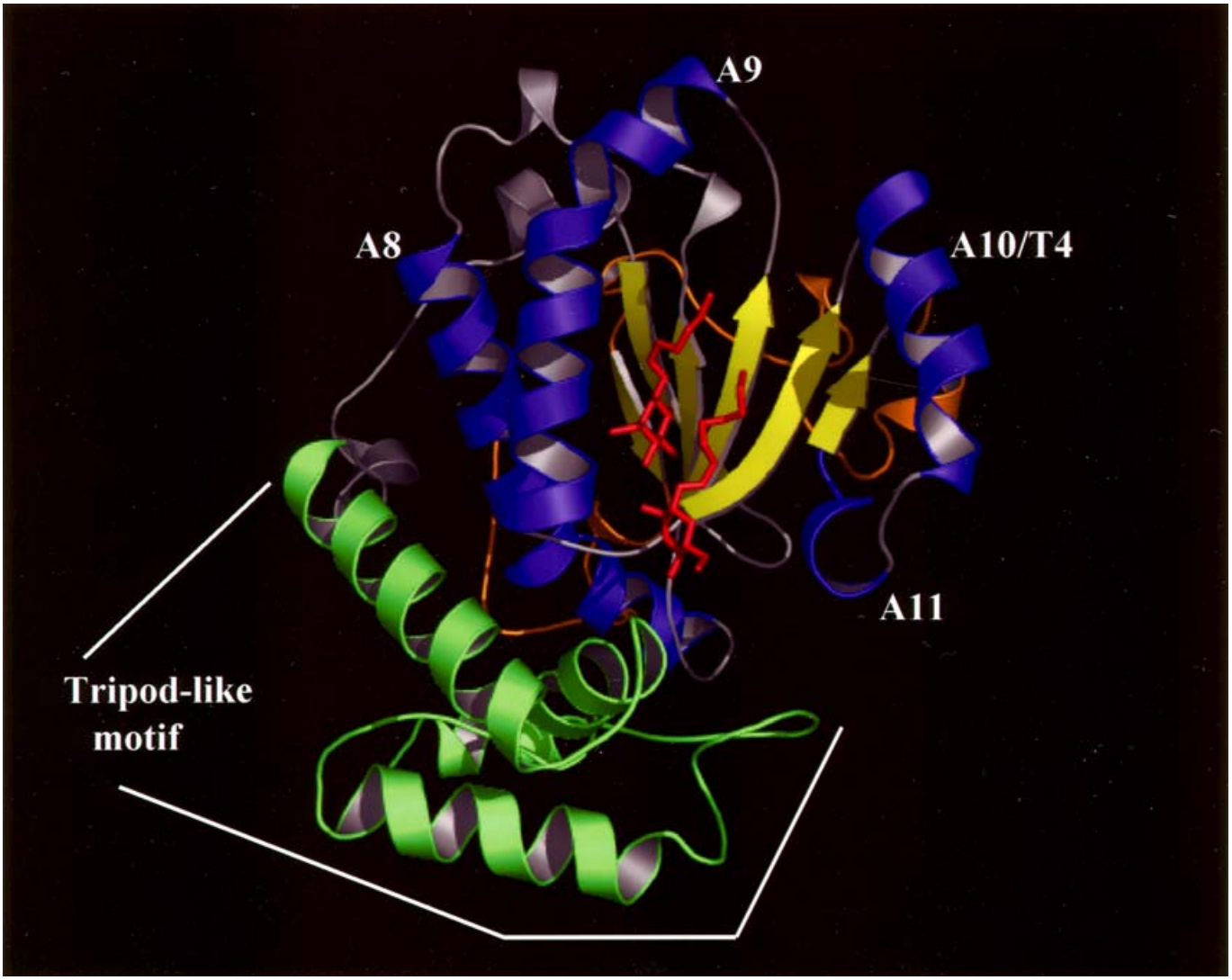

FIGURE 1 **Crystal structure of the major *Saccharomyces cerevisiae* PITP. The Sec14p fold consists of twelve α-helices, six β-strands, and eight 3_{10}-helices, and the available crystal structure is of the apo-form bound to two β-octylglucoside detergent molecules (shown in space fill mode). These detergent molecules reside in the single large hydrophobic pocket of the protein. This pocket's floor is formed by the six β-strands (in white), and its walls consist of the A8, A9, and A11 α-helices (in black; helices indicated). The pocket is gated by the A10/T4 helix (in black; helix labeled). The β-octylglucoside acyl-chains orient inward into the hydrophobic pocket while the detergent headgroup is disposed towards solvent and is bound with a hydrogen bonds network that also stabilizes binding of the inositol ring of PtdIns. The N-terminal A1, A2, A3, and A4 α-helices fold into the tripod motif (indicated) that consists of the bulk of the 129 amino acids that are sufficient for Sec14p targeting to Golgi membranes. Finally, the string motif stabilizes the hydrophobic pocket by wrapping behind the β-strand floor of the pocket. The 3_{10}-helices are not shown but are critical for protein stability.**

unusual hydrophobic helix (the A10/T4 helix), composed of both α-helical and 3_{10}-helical elements, gates the phospholipid binding pocket. The A10/T4 helix is positioned at the mouth of the pocket and represents the most hydrophobic patch on the surface of Sec14p. Based on these unusual structural features, A10/T4 is proposed to penetrate the membrane bilayer during the phospholipid transfer reaction (Sha *et al.*, 1998). This is an attractive proposition. An A10/T4-mediated penetration into the bilayer affords the dual advantage of lowering the free energy required for Sec14p-bound substrate to incorporate into a membrane, while the low dielectric constant of the unoccupied hydrophobic cavity reduces the free energy for partitioning of an individual phospholipid monomer from the membrane bilayer into the hydrophobic Sec14p phospholipid binding pocket.

One remaining question of major interest is how precisely Sec14p recognizes PtdIns- and PtdCho as *in vivo* binding substrates to the apparent exclusion of

other phospholipids. The three-dimensional structure of Sec14p speaks to the question of how the protein binds the inositol headgroup of PtdIns. Inferences gleaned from the structural data suggest that Sec14p binds the inositol headgroup via a hydrogen bonding network. This paradigm features Glu_{207} as a particularly important residue that hydrogen-bonds with the 4-OH of the inositol ring (Phillips *et al.*, 1999). Glu_{207} is itself held in position to affect such hydrogen bonding to the headgroup by a salt-bridge with Lys_{239}. Lys_{66} also projects into the immediate vicinity of the inositol ring. The fact that these three amino acids are relevant to PtdIns-binding is amply demonstrated by their specific requirement for PtdIns-binding/transfer, but not for PtdCho-binding/transfer, *in vitro*. Such analyses generate discerning tools for functional Sec14p studies in yeast. Interestingly, a mutant Sec14p that is manifestly defective in PtdIns-binding/transfer *in vitro* and in stimulation of phosphoinositide synthesis *in vivo*, yet retains the ability to transfer PtdCho, competently fulfills all the essential roles of Sec14p *in vivo* (Phillips *et al.*, 1999). This result underscores the importance of the PtdCho-binding/transfer activity of Sec14p to Golgi competence and cell viability.

The three-dimensional Sec14p model also provides a structural rationale for the *sec14-1^{ts}* mutation. The Sec14p carboxy-terminus defines a structural element termed the string motif. This motif is largely composed of a random coil that wraps around the back of the hydrophobic pocket and stabilizes it. A key feature of the string motif is the presence of four of the eight 3_{10}-helices of Sec14p, and these tightly wound helices are interpreted as wound springs that lend structural consequence to the string motif. These 3_{10}-helices are diagnosed in the primary sequence by the tandem GlyGly residues that permit formation of these particular structural elements. Sec14p residue Gly_{266}, which is mutated to Asp in the *sec14-1^{ts}* strain, is a component of one of these GlyGly pairs, in this case the GlyGly pair formed by residues 265 and 266. Interestingly, any side chain present at residue 265 is predicted to be disposed toward solvent, thereby suggesting that this position will prove tolerant to multiple amino acid substitutions. Site-directed mutageneses bear out this prediction. Position 266 is different in that any substantial side chain incorporated at that position will sterically clash in a 3_{10}-helix, thereby disqualifying formation of that structural element. Again, site-directed mutagenesis experiments bear out this prediction. Only Gly or Ala at residue 266 is compatible with thermostable Sec14p function. Even the small side-chain of Ser at position 266 is excluded. Thus, the Gly_{266}Asp substitution encoded by *sec14-1^{ts}* results in loss of one of the four 3_{10}-helical elements of the string motif, thereby causing destabilization of the hydrophobic phospholipid binding pocket (Sha *et al.*, 1998).

Finally, the novel Sec14p fold is itself a useful scaffold for modeling structures of proteins that contain the Sec14p lipid binding domain. Specific versions of this domain, *e.g.*, those found in the cellular retinaldehyde binding protein, the TRIO guanine nucleotide exchange factor, α-tocopherol transfer protein (α-TTP; mutations in α-TPP cause vitamin E-responsive ataxia), supernatant protein factor (SPF), and a novel gene caytaxin (mutations in caytaxin are linked to ataxia/dystonia in the jittery mouse) accommodate binding of small lipophilic molecules other than phospholipids (*e.g.*, retinal, vitamin E, squalene) (Bomar *et al.*, 2003; Panagabko *et al.*, 2003).

Life Without Sec14p

While the high-resolution Sec14p structure provides a detailed view of PITP architecture, it lends sparse insight into how this PITP regulates the biogenesis of TGN-derived vesicles destined for the plasma membrane. Taking advantage of the genetic system afforded by yeast, two different approaches are yielding surprising functional clues to how Sec14p executes biological function. First, individual mutations that permit cell viability and secretory pathway function in the total absence of Sec14p were identified (Cleves *et al.*, 1989, 1991b). Remarkably, each of these 'bypass Sec14p' mutations represent loss-of-function alleles residing within the coding sequences of a small set of non-essential genes. The principle behind analysis of these types of mutations is that these generate physiological conditions that mimic the consequences of Sec14p function *in vivo*. This class of mutations is expected to identify effectors that respond to Sec14p activity. Other genetic screens identified loss-of-function mutations that exacerbate defects associated with reduced Sec14p function. These synthetic mutations identify potential regulators of Sec14p and its effectors (Xie *et al.*, 1998; Yanagisawa *et al.*, 2002). We first consider what we are learning from 'bypass Sec14p' mutations.

The 'bypass Sec14p' mutations identify seven genes. Detailed analyses of six of these genes report a novel

and direct physiological coupling between vesicle biogenesis at the TGN and the metabolism of specific lipids. These lipids include PtdCho, diacylglycerol (DAG), and inositol-containing phospholipids. A seventh class of 'bypass Sec14p' mutations identifies a Golgi-associated phosphoinositide-binding protein of the enigmatic oxysterol-binding protein (OSBP) family (Fang *et al.*, 1996). Because the 'bypass Sec14p' gene products represent either enzymes of phospholipid metabolism or lipid binding proteins, the general conclusion is that Sec14p regulates a critical biological interface between phospholipid metabolism and Golgi secretory function. A model for how Sec14p utilizes its PtdIns- and PtdCho-binding/transfer activities to maintain an appropriate lipid environment in yeast TGN membranes conducive to vesicle formation is presented in Figure 2.

Sec14p and PtdCho Biosynthesis

That Sec14p function is tightly linked to PtdCho metabolism is demonstrated by the fact that five of the seven classes of 'bypass Sec14p' mutations represent structural genes for PtdCho biosynthesis (Cleves *et al.*, 1991b; Xie *et al.*, 2001), and that PtdCho levels rise in Golgi membranes upon inactivation of Sec14p (McGee

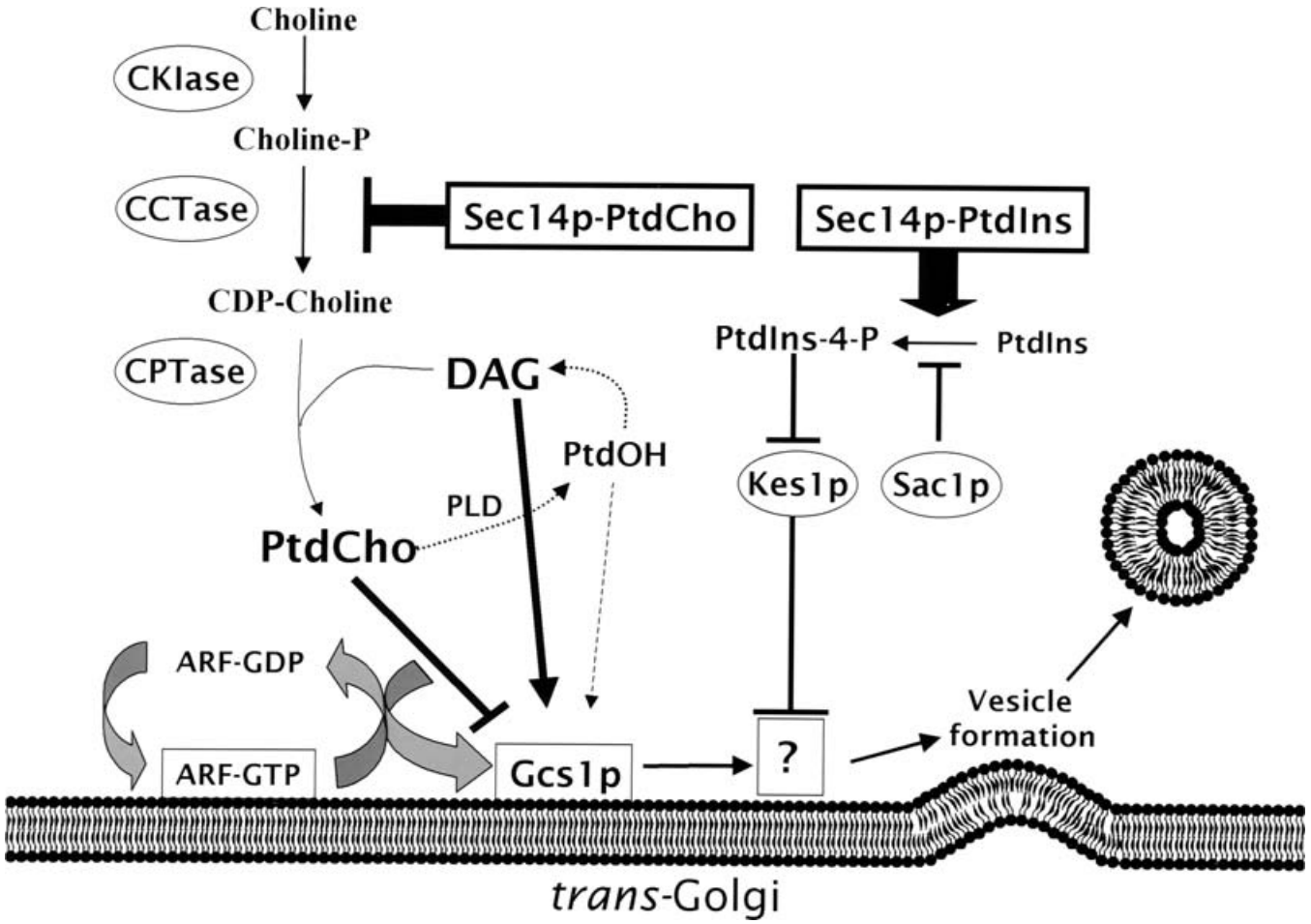

FIGURE 2 **The Sec14p pathway for Golgi secretory function. The relevant lipid metabolic pathways and principal lipids (PtdCho, DAG, PtdIns-4-P and PtdOH) are shown. Proteins encoded by 'bypass Sec14p' genes are circled. Three of the 'bypass Sec14p' genes encode for proteins of the CDP-choline pathway, choline kinase (CKIase), choline phosphate cytidylyltransferase (CCTase) and choline phosphotransferase (CPTase). This pathway consumes DAG to generate PtdCho and is downregulated by PtdCho-occupied Sec14p. Regulation of this pathway reduces levels of PtdCho while preserving DAG. Sec14p also stimulates the synthesis of PtdIns-4-P and inactivation of the polyphosphoinositide phosphatase Sac1p leads to a dramatic accumulation of PtdIns-4-P in the ER. Kes1p is a Golgi associated PtdIns-4-P binding protein that is mislocalized by the elevated PtdIns-4-P due to Sac1p dysfunction. The exact function of Kes1p remains unknown, but genetic data suggest it regulates the ARF-cycle on Golgi membranes. PLD acts to maintain an appropriate balance of PtdCho and DAG for Sec14p-independent Golgi function. Finally, the downstream effectors of Sec14p function that stimulate secretory vesicle biogenesis are two lipid-responsive ARFGAPs, one of which is Gcs1p. The ARFGAP activity of Gcs1p is modulated positively by DAG/PtdOH and negatively by PtdCho.**

et al., 1994). PtdCho is the major phospholipid in yeast, and its synthesis is catalyzed by the action of one of two distinct pathways; the cytidine diphosphate (CDP)-choline pathway and the phosphatidylethanolamine (PtdEtN)-methylation pathway (Carman & Zeimetz, 1996). Interestingly, only inactivation of the former results in 'bypass Sec14p' under normal laboratory conditions (Cleves *et al.*, 1991). The CDP-choline pathway consists of three individual enzymes that ultimately incorporate free choline into PtdCho. Functional ablation of any one of the three structural genes of the CDP-choline pathway (*CKI1*, *PCT1*, or *CPT1*) yields a 'bypass Sec14p' condition (Cleves *et al.*, 1991). This linkage between Sec14p and the CDP-choline pathway is bolstered by genetic and biochemical evidence demonstrating that Sec14p, specifically the PtdCho-bound form, reduces bulk levels of PtdCho by inhibiting the rate-determining enzyme of the CDP-choline pathway (McGee *et al.*, 1994; Skinner *et al.*, 1995; Phillips *et al.*, 1999).

Why does inactivation of the CDP-choline pathway effect 'bypass Sec14p' while inactivation of the PtdEtN-pathway does not? Evidence indicating CCTase to be a target of regulation by Sec14p suggests a very plausible scenario. Disparate intracellular localization for activity of these two pathways offers another possibility. Finally, the different biochemistries that fuel these two pathways is also informative. The CDP-choline pathway consumes 1 mole of DAG for every mole of PtdCho generated (Carman & Zeimetz, 1996). By contrast, the PtdEtN-methylation pathway does not consume DAG. Rather, it operates by modifying the headgroups of preformed lipids for which phosphatidic acid (PtdOH) is precursor. The data suggest some critical CDP-choline pathway precursor (*e.g.*, DAG) is consumed to the detriment of Golgi secretory function.

Interestingly, defects in the PtdEtN-methylation pathway do effect 'bypass Sec14p,' but only when choline salvage by the cell is prevented by either genetic or environmental intervention (Xie *et al.*, 2001). This complicated scenario results from a linkage of Sec14p dysfunction to a cycle of PtdCho-turnover, choline excretion, and choline reuptake by yeast cells (Xie *et al*, 2001). This choline reuptake and salvage pathway conceals 'bypass Sec14p' phenotypes associated with PtdEtN-methylation pathway defects. The collective data indicate that both PtdCho and DAG are regulatory lipids for TGN-secretory vesicle formation, and that these two lipids play inhibitory and stimulatory roles in this process, respectively (Cleves *et al.*, 1991; McGee *et al.*, 1994; Skinner *et al.*, 1995; Kearns *et al.*, 1997; Henneberry *et al.*, 2001; Xie *et al.*, 2001; Yanagisawa *et al.*, 2002).

The connection between Sec14p function and PtdCho metabolism is further emphasized by the requirement of the normally nonessential phospholipase D (PLD) for all mechanisms for 'bypass Sec14p' (Sreenivas *et al.*, 1998; Xie *et al.*, 1998). PLD is a PtdIns-4,5-bisphosphate (PtdIns(4,5)P_2)-activated enzyme that hydrolyzes PtdCho to choline and PtdOH. Deletion of the PLD structural gene has no discernable effect on Sec14p-dependent secretion from the Golgi, but PLD is obligatorily required for Sec14p-independent Golgi function. The requirement of PLD for Sec14p-independent Golgi secretory function can be interpreted as suggesting PtdOH rather than DAG is the key lipid effector. Several lines of evidence are counter to this possibility. Expression of DAG-kinase (an enzyme yeast do not express) in *sec14-1^{ts}* strains exacerbates Sec14p insufficiencies and compromises 'bypass Sec14p' (Kearns *et al.* 1997). Reciprocally, addition to cells of short chain DAG partially alleviates secretory defects associated with reduced Sec14p function (Kearns *et al.* 1997; Henneberry *et al.*, 2001). The various data are consistent with the idea that PLD-mediated PtdCho hydrolysis generates PtdOH, and this PtdOH is subsequently de-phosphorylated by lipid phosphatases to generate DAG (Xie *et al.*, 1998; Li *et al.*, 2000). In this manner, PLD activity generates a similar end to that proposed for Sec14p in maintaining a balance between PtdCho and DAG levels (Xie *et al.*, 1998; Xie *et al.*, 2001).

The Sac1p Phosphoinositide Phosphatase

Another class of 'bypass Sec14p' mutations identifies the *SAC1* gene. Sac1p is an integral membrane protein of the endoplasmic reticulum (ER) and Golgi complex, and a link between Sac1p activity and inositol lipid metabolism was noted early on (Cleves *et al.*, 1989; Whitters *et al.*, 1993). This linkage took direct form when it was demonstrated Sac1p is an enzyme with intrinsic phosphoinositide phosphatase activity (Guo *et al.*, 1999). Inactivation of this activity in cells results in accumulation of PtdIns-3-phosphate (PtdIns-3-P), PtdIns-4-phosphate (PtdIns-4-P) and PtdIns-3,5-bisphosphate (PtdIns(3,5)P_2); but the accumulation of

PtdIns-4-P is quantitatively by far the most impressive (Guo *et al.*, 1999; Rivas *et al.*, 1999; Hughes *et al.*, 2000; Nemoto *et al.*, 2000). The available data indicate it is loss of the PtdIns-4-P phosphatase activity that is particularly relevant to 'bypass Sec14p' (Nemoto *et al.*, 2000). The elevated levels of PtdIns-4-P alone are insufficient for 'bypass Sec14p,' however, as this *sac1*-associated phenotype requires PLD activity for its manifestation (Rivas *et al.*, 1999). Interestingly, it appears that the PtdIns-4-P accumulated in *sac1* mutants is synthesized predominantly, if not exclusively, via the Stt4p PtdIns-4-kinase pathway (Nemoto *et al.*, 2000; Foti *et al.*, 2001).

Two lines of evidence suggest accumulation of PtdIn-4-P in *sac1* mutations indirectly results in 'bypass Sec14p.' First, *sac1* deficiencies levy pleiotropic effects on lipid metabolism. These effects involve not only the metabolism of phosphoinositides, but of PtdCho and DAG as well (Rivas *et al.*, 1999). Second, accumulation of PtdIns-4-P in *sac1* mutants causes, in a dose-dependent fashion, mislocalization of phosphoinositide binding proteins that negatively regulate the Sec14p pathway (Li *et al.*, 2002; see Kes1p below).

The Oxysterol Binding Protein Family Member Kes1p

The final gene identified by 'bypass Sec14p' mutations is *KES1* (Fang *et al.*, 1996). *KES1* does not encode a known enzyme of lipid metabolism, but Kes1p exhibits specific lipid-binding properties that are relevant to *in vivo* function. Kes1p is one of seven yeast oxysterol binding protein homologs (OSBPs) and is unique among yeast OSBPs in its functional link to the cellular requirement for Sec14p function. The intimacy of this linkage is further demonstrated by the exquisite sensitivity of "bypass Sec14p" phenotypes to Kes1p levels (Fang *et al.*, 1996). Phenotypes associated with the loss of Kes1p function (*i.e.*, "bypass Sec14p"), and elevated Kes1p function (*i.e.*, loss of all pathways for "bypass Sec14p"), together firmly identify Kes1p as an antagonist of Sec14p-dependent Golgi secretory function. Indeed, *kes1* mutations represent the most frequent 'bypass Sec14p' mutation encountered (Fang *et al.*, 1996; Li *et al.*, 2002).

Kes1p consists of at least three functional domains as identified by analyses of spontaneous loss-of-function mutations that preserve protein expression and stability (Li *et al.*, 2002). The first domain is a pleckstrin homology (PH)-like domain that is required for Kes1p localization to the Golgi. This domain is termed a "PH-like" domain because various domain search programs fail to recognize this as a PH-domain, yet this domain binds phosphoinositides and presumptive binding sites were inferred productively from manual alignment to with other PH-domains (Li *et al.*, 2002). The PH-like domain is necessary for Kes1p localization to Golgi membranes, and it fulfills this function at least partly on the basis of its ability to bind PtdIns-4-P. Yet, this binding property is insufficient for targeting to Golgi membranes as mutations in a second domain that do not obviously compromise PtdIns-4-P binding also have the effect of compromising association of Kes1p with the Golgi complex (Li *et al.*, 2002). This second domain is a highly conserved bipartite motif present in all members of the eukaryotic OSBP family, and is designated the OSBP-domain on that basis. The OSBP-domain is predicted to bind to a second ligand on Golgi membranes (Li *et al.*, 2002), but the firm identity of this second ligand remains to be determined.

While the exact biological function of Kes1p remains to be determined, the genetic data suggest that Kes1p interfaces with the ARF-cycle (Li *et al.*, 2002). This does not seem to be at the level of regulating the GTPase-activating function of relevant ARFGAPs (Yanagisawa *et al.*, 2002; see below), nor does Kes1p exhibit detectable intrinsic ARF-GEF activity. Kes1p also exerts no measurable influence on PLD activity, which was once a formal possibility, given the phenotypic similarity of PLD dysfunction and increased Kes1p function (Li *et al.*, 2002).

Localization of Kes1p to the yeast Golgi complex is functionally important as demonstrated by loss-of-function mutations in the PH-like and OSBP domains. By extension, the PtdIns-4-P binding activity of the Kes1p PH-like domain renders Kes1p localization susceptible to disturbances in PtdIns-4-P homeostasis (Li *et al.*, 2002). Indeed, such a mechanism appears to be at play in the case of *sac1* mutants. The nearly 10-fold increase in steady-state PtdIns-4-P levels in *sac1* mutants effects mislocalization of wild-type Kes1p from Golgi membranes, thereby phenocopying loss of Kes1p function at the Golgi complex and a 'bypass Sec14p' phenotype. That the dose of Kes1p required to overcome *sac1*-mediated 'bypass Sec14p' is directly related to PtdIns-4-P mass provides further support for this concept (Li *et al.*, 2002).

ARFGAPs and Sec14p Function

The idea that Sec14p function promotes the secretory competence of yeast TGN membranes by regulating the lipid composition of these membranes raises several fundamental questions. Among these unresolved questions is whether lipids provide some biophysical environment that supports vesicle budding, do lipids exert their regulatory effects solely through the action of protein effectors, or do both mechanisms contribute significantly? The first clues that protein effectors respond to a Sec14p-regulated lipid environment came from the finding that "bypass Sec14p" mutations require a functional ARF cycle to levy Sec14p-independence to yeast Golgi secretory function (Li *et al.*, 2002). This insight was further refined, and the available data suggest two lipid-regulated ARF-GTPase-activating proteins (ARFGAPs; Gcs1p and Age2p) are effectors that respond to a Sec14p-dependent lipid environment (Yanagisawa *et al.*, 2002).

Gcs1p and Age2p are imperfectly redundant proteins that function downstream of Sec14p and PLD in both Sec14p-dependent and Sec14p-independent pathways for protein secretion from the Golgi, respectively. Biochemical data demonstrate that the ARFGAP activity is essential to the function of these proteins (primarily Gcs1p) in the Sec14p-pathway and that this activity is itself modulated negatively by PtdCho and positively by DAG/PtdOH (Yanagisawa *et al.*, 2002). Interestingly, the ARFGAP activities are also stimulated by PtdIns-4,5-P_2 *in vitro*–a tantalizing result, given that Gcs1p is a phosphoinositide binding protein with a C-terminal PH-domain. However, a truncated Gcs1p lacking the PH-domain retains ARFGAP activity and is fully functional *in vivo* (Yanagisawa *et al.*, 2002). These data suggest phosphoinositides do not play an essential role in regulating Gcs1p/Age2p ARFGAP activity in cells, but that activity of these ARFGAPs is sensitive to DAG and PtdCho.

The hypothesis that ARFGAPs respond to Sec14p function in a fashion that activates these proteins has both satisfying and controversial aspects. The satisfying aspect is that it accounts for the various genetic and metabolic data culled from studies of the Sec14p pathway that implicate DAG and PtdCho as positive and negative modulators of the Sec14p pathway, respectively. The controversial aspect is the departure from prevailing models for how ARFGAPs regulate the vesicle cycle. The present dogma is that ARF regulates recruitment of coat proteins of the COP class to Golgi membranes, and that self-polymerization of COPs drives formation of transport vesicles (Rothman, 1996; Schekman & Orci, 1996). By this view, positive regulation of the guanine nucleotide exchange factor (GEF) arm of the ARF cycle, or negative regulation of the ARFGAP arm of the cycle, is required for vesicle budding. Once a vesicle is formed, ARFGAPs are posited to stimulate GTP hydrolysis in a manner that couples this hydrolysis to subsequent dissociation of the protein coat from the vesicle membrane as a preparatory step for fusion (Rothman, 1996; Schekman & Orci, 1996).

While these discordant issues await resolution, it is becoming clear that ARFGAPs do play previously unrecognized roles in vesicle biogenesis. Recent evidence from mammalian reconstitution systems also supports a role for ARFGAPs in the coupling of cargo sorting to the assembly of the transport vesicle (Rein, 2002; Yang *et al.*, 2002; Lewis *et al.*, 2004; Lee *et al.*, 2005). Similarly, the yeast ARFGAP Glo3p is required for formation of retrograde transport vesicles *in vitro* and *in vivo* (Lewis *et al.*, 2004).

Sec14p and Developmentally Regulated Membrane Trafficking in *Saccharomyces Cerevisiae*

Diploid yeast challenged with carbon or nitrogen starvation initiate a complex developmental signaling program that activates switch from a mitotic mode of vegetative growth to one that couples meiosis to sporulation (reviewed by Engebrecht, 2003). This developmental switch is accompanied by a redirection of post-Golgi vesicle trafficking so that it is no longer the plasma membrane that accepts membrane flow. Rather, post-Golgi secretory vesicles target to the forespore membrane, which is itself assembled around the forming nuclei produced by meiosis. That remarkable reconfiguration of the secretory pathway reports an outstanding confirmation of a fundamental prediction of the SNARE hypothesis proposed by Rothman and colleagues (1994). Namely, that the v-SNARE/t-SNARE combination determines the site of vesicle fusion. In the case of sporulating yeast, a sporulation-specific t-SNARE (Spo20p) substitutes for the vegetative Sec9p plasma membrane t-SNARE and is localized to the site where the forespore membrane is formed (Neiman *et al.*,

2000; Nakanishi *et al.*, 2004). Forespore membrane assembly is important, as this structure will mature to the spore coat which ultimately encases the haploid meiotic progeny.

Sec14p redistributes to forespore membranes during sporulation in *S. cerevisiae*, and its function is required for forespore membrane biogenesis (Rose *et al.*, 1995; Rudge *et al.*, 2004). In this regard, sporulation-specific functions for Sec14p are conserved as Sec14p is also essential for sporulation in the highly divergent fission yeast *Schizosaccharomyces pombe* (Nakase *et al.*, 2001; see below). There are two general possibilities for how Sec14p may be involved. First, Sec14p may be required for the biogenesis of Golgi-derived vesicles for trafficking to the site of forespore membrane assembly. This involvement is directly analogous to the role of Sec14p in vegetative cells. An alternative, and not mutually exclusive possibility, is that Sec14p plays a distinct signaling function at the forespore membrane. There is evidence for the latter possibility. This membrane biogenesis program requires the action of PLD whose biochemical function is to generate PtdOH. The PLD-dependent PtdOH pool, in turn, recruits the sporulation-specific t-SNARE Spo20p to nucleate and sustain membrane delivery to the forming forespore membrane (Neiman *et al.*, 2000; Nakanishi *et al.*, 2004). The available data indicate Sec14p is directly integrated into the PLD-signaling circuit. Sec14p collaborates with a specific PtdIns 4-OH-kinase, Pik1p, to generate a pool of PtdIns-4-P essential for forespore membrane assembly. The ultimate fate of this pool is conversion to PtdIns(4,5)P_2 (Rudge *et al.*, 2004), and this phosphoinositide is essential for PLD enzymatic activity (Sciorra *et al.*, 2002). Thus, Sec14p, Pik1p, and PLD coordinate the synthesis of a PtdOH 'platform' for recruitment of an essential sporulation-specific t-SNARE.

There is one point regarding sporulation-specific functions for Sec14p that is of particular interest from the PITP perspective. While Sec14p and the Pik1p PtdIns 4-OH-kinase are functionally engaged in satisfying the phosphoinositide requirement for sporulation in budding yeast (Rudge *et al.*, 2004), the face of the PITP/PLD interface is very different in vegetative cells. In that case, decreased Sec14p activity coupled with the action of the nonclassical *SFH* PITPs is required for optimal stimulation of PLD activity (Sreenivas *et al.*, 1998; Xie *et al.*, 1998; Li *et al.*, 2000). The *SFH* PITPs are treated in detail in the following section. The rationale for why yeast cells reorient the coupling of PITP/PtdIns 4-OH kinase/PLD function during the vegetative cell/sporulation developmental switch remains unknown.

Sec14p Homologs (*SFH*): Non-Classical PITPs in *Saccharomyces Cerevisiae*

Along with *SEC14*, the genome of *S. cerevisiae* encodes five additional Sec14p homologs: Sfh1p, Sfh2p, Sfh3p, Sfh4p, and Sfh5p (Li *et al.*, 2000; Schnabl *et al.*, 2003). Sfh1p shares the highest homology to Sec14p (64% primary sequence identity), yet retains no *in vitro* transfer activity of PtdIns or PtdCho and is unable to rescue the defects associated with Sec14p dysfunction. The other *SFH* proteins share roughly 45% similarity and 25% identity to Sec14p and posses the ability to transfer PtdIns but not PtdCho. Since PITPs are classically defined by their ability to transfer both PtdIns and PtdCho, Sfhps represent a class of non-classical PITPs capable of only PtdIns-transfer activity. Furthermore, unlike *SEC14*, the *SFH* proteins are neither individually nor collectively required for cell viability (Li *et al.*, 2000).

Individual overexpression of some *SFH* proteins rescues (to varying extents) the growth and secretory defects of *sec14-1^{ts}* mutants, but all accumulating evidence indicates that these proteins do not exhibit functional redundancy with Sec14p. Some *SFH* functions may be common to this class of nonclassical PITPs. For example, *SFH* protein function contributes collectively and significantly to optimal stimulation of PLD activity in yeast (Li *et al.*, 2000; Schnabl *et al.*, 2003). PLD requires PtdIns(4,5)P_2 for activity and the *SFH* proteins appear to activate PLD by increasing the levels of PtdIns(4,5)P_2. Present evidence suggests that these proteins act primarily, if not exclusively via the Stt4p PtdIns 4-OH-kinase and not the Pik1p PtdIns 4-OH-kinase (Routt *et al.*, 2005).

A Role for Budding Yeast *SFH* Proteins in Lipid Trafficking via Intermembrane Contact Sites?

Individually, the *SFH* proteins connect to identifiable physiological processes. For example, transcription of *SFH3* and *SFH4* is induced when cells are challenged with inhibitors of sterol synthesis. Deletion of both *SFH3* and *SFH4* (also known as *PDR16* and *PDR17*, respectively) sensitizes yeast to many drugs, while Sfh3p

dysfunction results in yeast cells with heightened sensitivities to ergosterol-directed pharmacologs (Van den Hazel *et al.*, 1999). Sfh4p is required for efficient decarboxylation of phosphatidylserine (PtdSer) to PtdEtn via the activity of a specific non-mitochondrial PtdSer decarboxylase (Wu *et al.*, 2000). Finally, Sfh5p appears to play a significant role in modulation of PtdIns(4,5)P_2 homeostasis at the plasma membrane (Routt *et al.*, 2005). These data implicate *SFH* gene products in a wide array of cellular functions, some of which are similar to Sec14p in their ability to regulate lipid metabolism.

An interesting point of discussion that is emerging from the study of *SFH* proteins is that specific versions of these proteins may function in promoting lipid trafficking via intermembrane contact sites. The concept that lipids can traffic between closely apposed membranes via a hemifusion intermediate enjoys a long and frustrated history. Evidence consistent with the existence of mitochondrion-ER, trans-Golgi-ER and plasma membrane-ER inter-membrane contact sites has been described (Shiao *et al.*, 1995; Achleitner *et al.*, 1999; Marsh *et al.*, 2001; Pichler *et al.*, 2001; Wu and Voelker, 2002). However, a demonstration that such sites represent portals for lipid trafficking remains unproven, and the molecular components that functionally define such contact sites are equally elusive.

Recently, more compelling evidence that such sites exist has come from several directions. First, functional studies of the mammalian ceramide transfer protein (CERT) are suggestive. CERT stimulates ceramide trafficking between ER and Golgi membranes, and this trafficking protein exhibits domains dedicated both to ER- and Golgi-binding (Hanada *et al.*, 2003). That is, CERT is constructed in a fashion that is ideally suited for a protein that forms ER-Golgi contact sites. One of the major reasons for entertaining the idea that *SFH* proteins may be involved with such contact sites is that these proteins are firmly bound to intracellular membranes. Release requires strip with 0.5 M salt (Li *et al.*, 2000). Other supportive evidence derives from more detailed functional study of individual *SFH* proteins. In this regard, the functional data collected for Sfh4p and Sfh5p make a case that these proteins too may be involved in lipid trafficking via membrane contact site mechanisms.

Sfh4p (also described by the nonconventional designation PstB2p; Wu *et al.*, 2000) was identified in a genetic screen for components of a non-mitochondrial pathway for PtdSer decarboxylation to phosphatidylethanolamine (PtdEtN). It is currently thought that this Sfh4p-dependent pathway reports the trafficking of PtdSer from the yeast ER to an endosomal compartment that houses a PtdSer decarboxylase isoform (Psd2p) (Wu & Voelker, 2002). It is unlikely that Sfh4p delivers PtdSer monomers to Psd2p, given that Sfh4p exhibits no detectable PtdSer binding or transfer activity *in vitro* (Wu *et al.*, 2000). *In vitro* reconstitution of an Sfh4p- and Psd2p-dependent decarboxylation of PtdSer provides further details. In a system where PtdSer is provided on a chemically defined donor vesicle, and where Psd2p is located on microsomal membranes, Wu and Voelker (2004) found that transport of PtdSer from donor vesicle to Psd2p is sensitive to phospholipid composition and curvature of the donor membrane. High concentrations of PtdSer are required in the donor, and the transfer reaction is sensitive to surface dilution of the PtdSer substrate by other phospholipids (including acidic phospholipids). Strikingly, the transfer reaction operates much more efficiently when the donor membrane is not highly curved (vesicle diameter $\geq$400 nm) (Wu & Voelker, 2004). Validation of such reconstituted systems is always a critical element, and this cell-free assay satisfies two key features of the *in vivo* correlate. First, the assay faithfully reconstitutes the Sfh4p-dependence of the reaction. Second, the *in vivo* requirement of the Ca^{2+}-dependent phospholipid binding domain of Psd2p for this specific metabolic channeling event is also reproduced in the assay (Wu & Voelker, 2004). Further resolution and reconstitution of this system promises to be a most fruitful avenue for future research.

The concept of an involvement for Sfh4p in contact-site formation or function may well extend to other members of the *SFH* protein family, particularly Sfh5p. As described above, Sfh5p couples to the Stt4p PtdIns 4-OH kinase to generate a PtdIns-4-P pool that contributes to PtdIns(4,5)P_2 homeostasis in the yeast plasma membrane (Routt *et al.*, 2005). This plasma membrane PtdIns(4,5)P_2 pool likely contributes not only to activation of PLD under appropriate conditions, but also to the activity of the post-Golgi vesicle docking/fusion machinery–likely at the level of the functional interaction of specific subunits of the yeast exocyst complex with SNAP-25-like t-SNARE Sec9p (Routt *et al.*, 2005). The physiological coupling of Sfh5p and Stt4p notwithstanding, these two proteins are not localized to the same compartment. Rather, Sfh4p is

unevenly distributed along the peripheral ER system that lies closely apposed to the plasma membrane where Stt4p resides (Routt *et al.*, 2005). The simplest (but not the only) interpretation of the various data is that Sfh5p regulates a direct communication between ER subdomains and the plasma membrane. A contact-site–mediated mechanism for such an interface is an attractive and reasonable possibility.

Regardless of the gathering momentum, the contact site concept remains speculative. A role for *SFH* proteins in the activity of such sites is even more speculative. However, we believe that consideration of the possibilities is worthwhile, as a role for *SFH* proteins in regulating membrane contact sites raises several intriguing issues. Sfh4p and Sfh5p both couple to the Stt4p PtdIns 4-OH kinase in executing their respective biological functions (Trotter *et al.*, 1998; Routt *et al.*, 2005). One attractive (and potentially shared) mechanism of function is in the delivery of PtdIns to Stt4p on plasma membrane, and perhaps endosomal membranes, so that a critical pool of phosphoinositide can be generated in a spatially appropriate manner. However, Sfh4p stimulates PtdSer transfer between liposomes *in vitro* without an obligate involvement of either PtdIns or phosphoinositide (Wu & Voelker, 2004). This raises the possibility that the PtdIns-transfer activity of Sfh4p is dispensible for biological function. It is also possible that the *in vitro* reconstitution for PtdSer transfer is not faithfully reporting all requirements.

An obligate role for *SFH* proteins raises two other major mechanistic questions. First, there is the question of how any specificity can be imposed on which lipids are permitted to pass through a contact site. One might expect that contact sites are intrinsically promiscuous from the standpoint of permitting lipid movement between engaged membranes. If there is a strong trafficking specificity, however, how is it imposed? It seems the simplest way to gate the contact site is via the lipid binding specificity of the *SFH* protein. That basic idea is not obviously supported by the finding that a PtdIns-binding/transfer protein (Sfh4p) effects PtdSer transfer, although it remains formally possible that the PtdSer-specific 'gating' component has not yet been identified. This principle raises a number of interesting experimental possibilities. Second, there is the related question of whether *SFH* proteins are general components of distinct families of contact sites, or whether *SFH* proteins dictate specificity of a particular contact site. The striking lack of functional redundancy among these proteins *in vivo* suggests that *SFH* proteins are more likely to be dedicated components of specific contact site populations (Routt *et al.*, 2005). Again, this idea is more congruent with the case of Sfh5p-mediated control of plasma membrane PtdIns(4,5)P_2 homeostasis, rather than the case of Sfh4p-mediated PtdSer transfer.

Sec14p-like PITPs, Dimorphism and Cytokinesis in Other Yeast Species

An increasingly familiar theme in functional analyses of Sec14p-like PITPs is their involvement in controlling developmentally regulated, polarized membrane trafficking pathways. Several examples from yeast and plant systems are described in this review. One example of a Sec14p-like PITP-regulating developmental pathway for membrane biogenesis is the involvement of these proteins in yeast dimorphism. In two yeast model systems (*Yarrowia lipolytica* and *Candida albicans*), Sec14p-like PITPs are essential for maintenance of dimorphic growth. In *Yarrowia*, a Golgi membrane-associated Sec14p isoform (Sec14p^{YL}) regulates the developmental switch from a budding mode of cell division to a mycelial growth mode (Carmen-Lopez *et al.*, 1994). Unlike the *Saccharomyces* Sec14p, Sec14p^{YL} is a nonessential protein whose functional ablation levies no obvious effect on cell viability. However, loss of *SEC14*^{YL} is incompatible with sustained mycelial growth. Interestingly, Sec14p^{YL}-deficient cells appear to initiate a program of mycelial growth, but this collapses into an unusual and apparently abortive pathway that exhibits properties of both budding and mycelial growth (Carmen-Lopez *et al.*, 1994).

In the fungal pathogen *Candida albicans*, not only are Sec14p-like PITP isoforms engaged in the regulation of mycelial growth, but their involvement in dimorphism exhibits isoform-specificity. The *C. albicans SEC14* gene is an essential gene, but, as in the case of Sec14p^{YL}, hypomorphic alleles elicit the striking phenotype of blocking filamentous growth under conditions that induce the mycelial growth mode (Monteoliva *et al.*, 1996). As mycelia are the product of a highly invasive form of growth, and have the effect of embedding cells in the substratum, the ability to induce and sustain mycelial growth is a determining factor in the infectivity and pathogenicity of fungal parasites. Thus, the Sec14p class of proteins represents a potential target for pharmacological intervention in the treatment of fungal diseases.

Other developmentally regulated pathways for membrane biogenesis involve Sec14p-like PITPs as well. The Sec14p analog in the fission yeast *Schizosaccharomyces pombe* (Sec14p^{SP}) is encoded by the *spo20*$^{+}$ gene. Sec14p^{SP} exhibits both PtdIns- and PtdCho-transfer activity, and deficiencies in its activity evoke two clear phenotypes. First, Sec14p^{SP} is essential for Golgi secretory function in vegetative cells, an expected constitutive and essential household cellular function, given the precedent provided by budding yeast Sec14p. Under conditions of Sec14p^{SP} insufficiency, distended Golgi membranes become apparent, and secretory protein transport to the cell surface is compromised. Second, Sec14p^{SP} is also required for completion of cytokinesis, where a membraneous cell plate must be laid down to separate mother and daughter cells at the end of cell division (Nakase *et al.*, 2001). Consistent with this function in the vegetative cell cycle, Sec14p^{SP} is enriched at the cell poles and distributes to sites of septation in a cell-cycle-dependent manner. Interestingly, Sec14p^{SP} localization is dependent on the nutrient status of the cell. Under nitrogen starvation conditions, Sec14p^{SP} translocates to the nucleus. It is unknown whether Sec14p^{SP} plays any role in regulating nuclear signaling under such conditions, or whether this redistribution sequesters Sec14p^{SP} in a non-secretory compartment as a means of inhibiting secretory pathway function under adverse nutritional conditions.

Sec14p^{SP} also redistributes in a developmentally-regulated manner. During meiosis, Sec14p^{SP} translocates to forespore membranes that are formed at the nuclear envelope. The functional significance of this redistribution is demonstrated by the fact that biogenesis of forespore membranes is defective in Sec14p^{SP}-deficient cells (Nakase *et al.*, 2001).

PITPs IN HIGHER PLANTS

The plant PITP family is large and unstudied, yet there are already concrete indications that plant PITPs execute intriguing biochemical and biological functions. Emerging data from higher plant systems are broadening the menu of signaling pathways that involve PITPs, and it is now clear that plant systems, such as *Arabidopsis*, will assume ever-increasing significance as fruitful genetic models for elucidating functional roles for individual PITPs. We devote significant attention in this review to plant PITPs, simply because so little is known about them, and because we are convinced that plants will prove to be useful model systems for the study of how novel Sec14p-like PITPs interface with biologically essential cellular functions.

Novel Sec14p-nodulin PITPs in *Arabidopsis thaliana*

Bioinformatic mining of genome sequence databases indicates the family of Sec14p-like proteins is large in every eukaryote from flies to mammals. In this regard, plants have expanded the Sec14p protein family in an impressive way. While some members of the plant Sec14p family represent proteins consisting of the Sec14p-domain only, others proteins exhibit a Sec14p-domain joined to either a nodulin-domain or a GOLD-domain (see below). As case in point, *Arabidopsis thaliana* encodes at least 31 potential Sec14p-like proteins in its genome. Of these, 11 consist of a Sec14p-nodulin two-domain arrangement, while the remaining 20 members harbor GOLD-domains or consist entirely of a Sec14p domain (Vincent *et al.*, 2005). Because functional analyses of plant Sec14ps are essentially nonexistent, the genetically tractable plant *Arabidopsis* model offers a unique opportunity for functional analyses of Sec14p-like PITPs in higher organisms. The early returns for such functional analyses are promising. Testimony to this effect comes from the study of AtSfh1p, a novel Sec14p-nodulin two domain protein. As reviewed in more detail below, AtSfh1p regulates a complex developmental membrane morphogenetic pathway that requires coordination of membrane trafficking, phosphoinositide signaling, Ca^{2+} signaling, and regulation of the actin and microtubule cytoskeletal systems.

AtSfh1p function is essential for proper development of root hairs in *Arabidopsis* (Vincent *et al.*, 2005). Plant root hair biogenesis involves a complex and developmentally regulated morphogenetic program requiring polarized membrane growth from a precise position on the root epidermal cell plasma membrane. A molecular understanding of the critical components required for establishment and maintenance of such a polarized membrane trafficking pathway remains elusive. As indicated above, AtSfh1p is a member of a large and novel *Arabidopsis* two-domain Sec14p-nodulin family, a protein family whose basic domain arrangement of physically linking a Sec14p-domain with a nodulin-domain is itself intriguing (see below). Functions to each domain have been assigned. The Sec14p-domain exhibits intrinsic PtdIns/PtdCho-transfer activity and is able to

stimulate phosphoinositide synthesis, particularly synthesis of phosphoinositides modified at the 4-OH position; i.e., PtdIns-4-P and PtdIns(4,5)P_2. The C-terminal nodulin-domain is a membrane targeting motif, and AtSfh1p is localized to intracellular compartments of the late stages of the secretory pathway and the plasma membrane. Integrity of both the Sec14p- and nodulin-domains is required for AtSfh1p function in the plant (Vincent *et al.*, 2005).

AtSfh1p expression is highly root-specific, and the subcellular localization of this protein in the growing root hair recapitulates the organization of discrete PtdIns(4,5)P_2-enriched root hair plasma membrane domains and PtdIns(4,5)P_2-enriched secretory compartments (TGN or vesicles; Vincent *et al.*, 2005). Because the AtSfh1p Sec14p-domain exhibits intrinsic PtdIns/PtdCho-binding capability, and the ability to stimulate PtdIns-4-P and PtdIns(4,5)P_2 synthesis, the current view is that the nodulin-domain positions the Sec14p-domain appropriately such that localized phosphoinositide synthesis can occur (Vincent *et al.*, 2005). In support of this idea, AtSfh1p-deficiency compromises polarized root hair growth by evoking loss of intracellular PtdIns(4,5)P_2 pools associated with late secretory pathway membranes, compromises plasma membrane tip-directed PtdIns(4,5)P_2 gradients, leads to derangement of root hair Ca^{2+}-signaling, and collapse of both the tip *f*-actin network and root hair microtubule organization. Taken together, the available data suggest AtSfh1p regulates both intracellular and cell-surface phosphoinositide landmarks that focus membrane trafficking, Ca^{2+}-signaling and cytoskeletal functions to the growing root hair apex (Vincent *et al.*, 2005). Because of the plethora of AtSfh1p-like proteins encoded in the *Arabidopsis* genome, the Sec14p-nodulin two domain proteins may well define a novel PITP family whose individual members are dedicated to the regulation of tissue-specific pathways of polarized membrane growth in plants (Vincent *et al.*, 2005). In this model, the nodulin-domains identify specific sites of lipid signaling while the Sec14p-domains facilitate the lipid enzymology that drives signaling.

Novel Transcriptional Regulation for an AtSfh1p Homolog in a Leguminous Plant

The insights derived from analyses of AtSfh1p function in regulating polarized membrane trafficking during *Arabidopsis* root hair development, when coupled with predictions of the individual functional contributions of Sec14p- and nodulin-domains, provide interesting perspectives regarding the nature of specific nodulin proteins. Broadly defined, nodulins are proteins selectively expressed in root tissues of leguminous plants infected by *Rhizobium* bacteria. There are many classes of nodulins based on expression profile and primary sequence homology, and the temporal association of their expression with defined stages of the developmental program of nodulation has given rise to the thought that nodulins promote the establishment and/or maintenance of the *Rhizobium*/legume symbiosis that drives nitrogen fixation (for a review, see Long, 2001). Because nitrogen fixation is a biological process of planetary significance, the physical association of nodulins with Sec14p-domains is a remarkable one. This raises the question of what relationship exists between nodulins and the cognate nodulin-domain proteins. Recent work on the regulation of nodulin expression in leguminous plants offers interesting possibilities in this regard.

The model legume *Lotus japonicus* expresses an *LjNOD16* cDNA that programs translation of a 15.5 kDa nodulin termed Nlj16 (Kapranov *et al.*, 1997). The Nlj16-nodulin is not encoded as a free standing open reading frame in the Lotus genome, however. It is derived from a gene that encodes the *Lotus* homolog of *Arabidopsis AtSFH1*, and this gene is designated *LjPLP-IV* (Kapranov *et al.*, 2001). Interestingly, LjPLP-IVp expression is subject to substantial transcriptional regulation. In nodulating plants, the promoter driving expression of full-length *LjPLP-IV* is silenced, eight nodule-specific antisense mRNAs complementary to coding sequences of the Sec14p-like domain of *LjPLP-IV* are expressed, and the C-terminal nodulin-domain is also highly expressed as the Nlj16-nodulin. Transcription of both the antisense transcripts and Nlj16 is driven by a bidirectional promoter located within an intron that separates Sec14p-domain coding sequences from those of Nlj16 (Kapranov *et al.*, 2001).

The physiological foundation for this complex reorganization of the *LjPLP-IV* transcriptional program can now be rationalized as a combinatorial strategy to inactivate a major polarity regulator of *Lotus* root hair tip growth, *i.e.*, LjPLP-IVp. This is accomplished by a two-pronged downregulation of *LjPLP-IV* gene expression that involves silencing of the promoter for the full-length gene, and production of antisense transcripts

that interfere with expression of any mRNAs that contain Sec14p-domain coding information. The third component is high-level synthesis of the Nlj16 nodulin, an outstanding candidate for a dominant-negative interaction domain that sequesters LjPLP-IVp plasma membrane binding sites. Based on the concepts gleaned from study of AtSfh1p in *Arabidopsis*, it is tempting to speculate such an event breaks the polarity program so that *Rhizobium* bacteria can effectively infect the root hair tip (Vincent *et al.*, 2005). Finally, it is interesting to note that the internal bidirectional promoter of the *Lotus LjPLP-IV* is activated during nodulation because it harbors so-called 'nodulin-box' enhancer sequences (Kapranov *et al.*, 1997; Kapranov *et al.*, 2001). *Arabidopsis*, a non-leguminous plant does not exhibit obvious 'nodulin-boxes' in the intronic region that demarcates Sec14p-domain coding sequences from nodulin-domain coding sequences. *Lotus* may have evolved such sequences as a natural means for inactivating a specific Sec14p-nodulin two-domain protein so as to potentiate bacterial/plant symbiosis.

A Family of Sec14p-GOLD-Domain Proteins

For the purpose of this review, we employ the term "Sec14p-like" to describe proteins with structural similarity with, but unconfirmed functional homology to, the prototypical *Saccharomyces cerevisiae* Sec14p. A particularly interesting set of eukaryotic Sec14p-like proteins are represented by a conserved family of multi-domain proteins in which an N-terminal Sec14p homology domain is aligned in tandem with a C-terminal GOLD-domain (Figure 3). While these Sec14p-GOLD proteins are widely conserved among multicellular eukaryotes, we discuss these in the context of plant PITPs simply because this protein family is well represented in plants (see below).

What makes the Sec14p-GOLD family interesting is the ontogeny of the GOLD-domain itself. The GOLD (Golgi dynamic) domain proteins were first identified in opisthokonts, animals, and fungi, based on sequence homology to the luminal portion of K08E4.6, a member of the p24 family of proteins in *Caenorhabditis elegans* (Anantharaman *et al.*, 2002). The members of the p24 protein superfamily are type I membrane proteins involved in secretory cargo selection and concentration during the biogenesis of vesicles that traffic between the ER and the *cis*-Golgi system (see Springer *et al.*, 2000 for a discussion). Given the role of Sec14p in regulating membrane trafficking, and the role of p24 proteins in cargo sorting, the conserved physical linkage of Sec14p and GOLD domains must be of functional consequence.

Presently, six structurally distinct families of GOLD-domain–containing proteins are recognized on the basis of a complicated bioinformatics-based definition: animal and fungal p24 proteins; mammalian Sec14p-GOLD proteins; human GCP60; yeast Osh3p; human FYCO1; and plant Sec14p-GOLD proteins (Anantharaman *et al.*, 2002). The single luminal GOLD-domain structurally distinguishes the p24 family from the other families. The remaining five GOLD-domain protein families are characterized by the presence of additional lipid/membrane binding domains: Sec14p; acyl-CoA-binding (Aco); pleckstrin-homology (PH) and oxysterol binding (OSBP); and the PtdIns-3-phosphoinositide binding module FYVE (Anantharaman *et al.*, 2002). Thus, the Sec14p-like proteins represent one family of GOLD-domain proteins, in which the N-terminal lipid binding domain of Sec14p precedes the C-terminal GOLD-domain.

The recognized *Arabidopsis* Sec14p-GOLD proteins belong to the six Patellins (PATL1-6; Peterman *et al.*, 2004). The Patellin Sec14p domains share approximately 26% primary sequence identity and 48% similarity to the yeast Sec14p. This is much lower than what is scored for the Sec14p domains of Sec14p-nodulin two domain proteins. However, amongst themselves, Patellin Sec14p-domains exhibit more extensive homologies; ranging from 40% to 88% primary sequence identity and 68% to 95% similarity.

The three amino acids of the Sec14p lipid binding domain essential for PtdIns binding/transfer, Glu_{207}, Lys_{239}, and Gly_{266}, are conserved in all six *Arabidopsis* Patellins; suggesting the corresponding Sec14p domains may intrinsically harbor Sec14p-like activities. In this regard, vesicle cosedimentation assays indicate preferential binding of PATL1 for phosphoinositides over PtdEtN and PtdCho (Peterman *et al.*, 2004). The true significance of such binding activities is yet to be established. In addition, the Patellin GOLD-domains, and to a lesser the yeast Osh3p, exhibit a unique lysine-rich terminal region that is not found in animal Sec14p-like GOLD-domains. The conserved $Lys(X_{10})$-(Lys/Arg)LysLysLys/Met(Gln_{2-3})TyrArg motif is similar to the PtdIns(4,5)P_2 binding motif of AP160 and U2-adaptin. This raises the possibility that

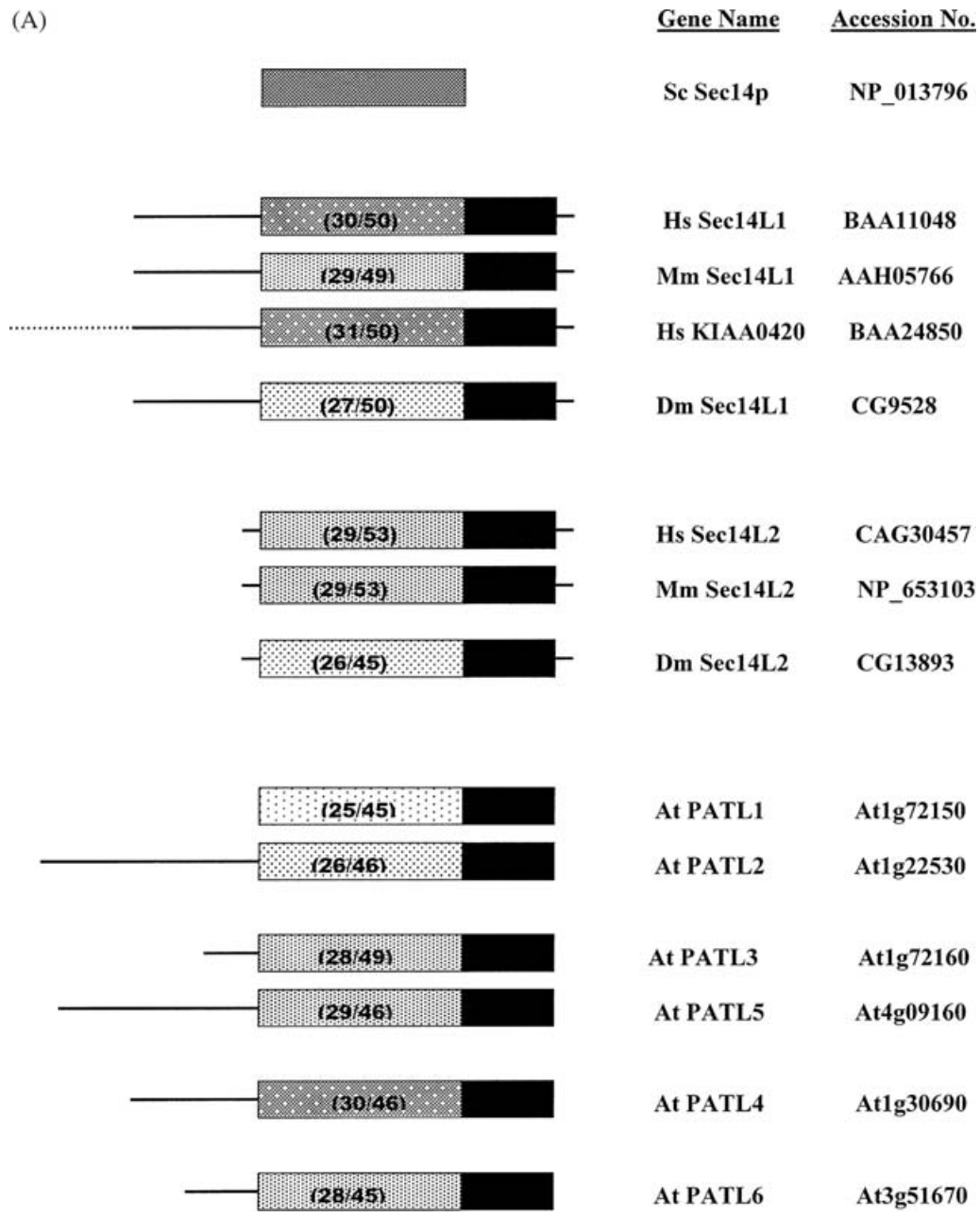

FIGURE 3 **The Sec14p-GOLD family. (*A*) Protein sequences were aligned and analyzed using ClustalX and BLAST (http://www.ncbi.nlm.nih.gov/BLAST; http://arabidopsis.org/BLAST). The Sec14p and GOLD domain containing proteins cluster into three phylogenetic groups: Sec14L1 (*top*); Sec14L2 (*middle*); and the Patellins (*bottom*). The *Drosophila* proteins within the Sec14L1 and Sec14L2 groups exhibit further divergence from their mammalian counterparts, and this is schematically represented by spatial separation between the *Drosophila* and mammalian proteins. All of the proteins, with the exception of Patellin 1, have an N-terminal extension of variable length with the Sec14L2 group having uniformly the shortest. The Sec14L1 and Sec14L2 are further distinguished by a small C-terminal tail. Percent primary sequence identity and similarity of each Sec14p domain to the *Saccharomyces cerevisiae* Sec14p are shown in parentheses, respectively, and are coded as follows: 30–31.99, 28–29.99, 26–27.99, 24–25.99 percent identity. (*B*) Cladogram of the Sec14p family. This represents a partial listing of known members of the eukaryotic Sec14p family. When known, common designations are given along with the corresponding accession numbers. The budding yeast Sec14p and *SFH* proteins discussed in the text are boxed in black. Other groups of Sec14p-like proteins discussed are boxed in gray and are identified by the descriptions provided in the corresponding gray boxes. Topology searches were conducting using the Minimum Evolution distance algorithm in PAUP 4.0* (beta version 10) (Swofford, 2001). For each data set, branch-swapping was conducted using tree-bisection-reconnection with random sequence addition of 10 and 1000 for the Sec14p dataset. Bootstrap analyses used the conditions stated above with a fast-heuristic search and 1000 replicates for each dataset. Cladograms were generated with Treeview (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html, version 1.6.6). Species abbreviations: At, *Arabidopsis thaliana*; Ce, *Caenorhabditis elegans*; Dd, *Dictyostelium discoideum*; Dm, *Drosophila melanogaster*; Dr, *Danio rerio*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Pf, *Plasmodium falciparum*.; Sc, *Saccharomyces cerevisiae*; Tc, *Trypanosoma cruzi*. Other abbreviations: CDC42 GAP, CDC42 GTPase activating protein; PTP MEG2, MEG2 protein tyrosine phosphatase.** ***(Continued)***

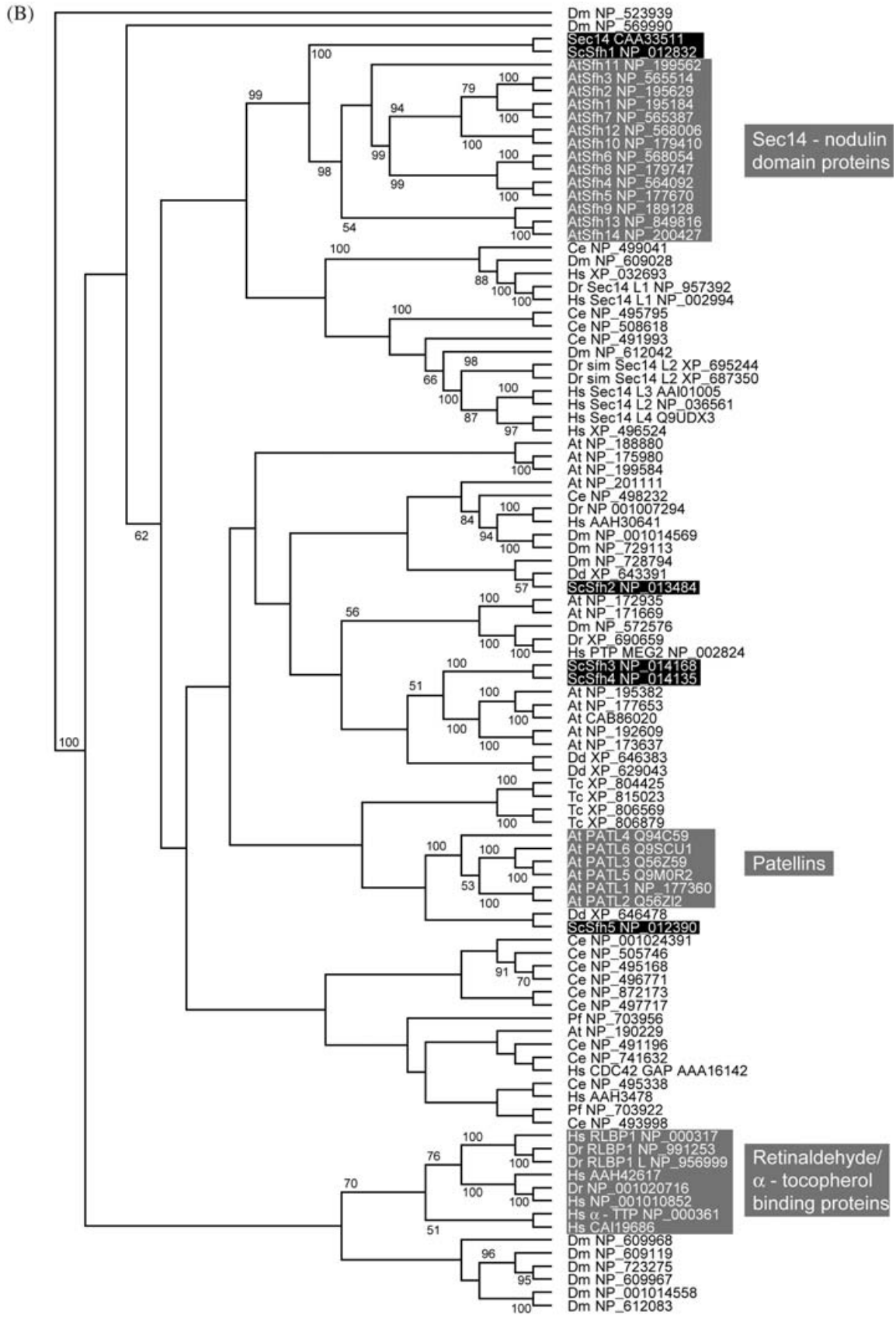

FIGURE 3 **(Continued)**

GOLD-domains, as well as Sec14p-domains, bind specific lipids.

What are the functions of Patellins? PATL1 is suggested to be involved in plant cell cytokinesis, specifically with the formation and maturation of the cell plate during late telophase (Petermann *et al.*, 2004). This inference is based on localization data, however, and no functional information from analyses of mutant plants is yet available.

Other Plant PITPs

Two Sec14p-like soybean proteins (Ssh1p and Ssh2p), each exhibiting approximately 25% primary sequence identity with Sec14p and consisting solely of a Sec14p-domain, have been characterized. Both Ssh1p and Ssh2p bind phosphoinositides with reasonable affinity and specificity. For example, Ssh1p exhibits a binding preference for PtdIns(3,5)P_2 (Kearns *et al.*, 1998), a phosphoinositide whose synthesis is strongly enhanced under conditions of hyperosmotic stress in yeast, plants and mammals (Dove *et al.*, 1997). In this regard, Ssh1p is itself rapidly phosphorylated in specific response to hyperosmotic stress in plants, and when expressed in *S. cerevisiae* is similarly phosphorylated after being subjected to hyperosmotic challenge (Kearns *et al.*, 1998; Monks *et al.*, 2001). This modification is spatially restricted to the site of stress application in plants, thereby indicating a cell autonomous regulation (Monks *et al.*, 2001).

The signaling pathway involved in Ssh1p phosphorylation in yeast (and presumably in plants) is not via an osmotic stress-responsive MAP kinase pathway (Kearns *et al.*, 1998) but, rather, via the hyperosmotic stress response kinases SPK1 and SPK2 (Monks *et al.*, 2001). In yeast, subcellular fractionation experiments indicate stress-regulated Ssh1p phosphorylation mobilizes Ssh1p from a predominantly membrane associated profile to a predominantly cytosolic one (Kearns *et al.*, 1998). Taken together, these data suggest phosphorylated Ssh1p functions as a signaling component of a stress response pathway that protects plants from osmotic insult, and that its suggested role in such a pathway is potentiated by Ssh1p mobilization from membranes (Kearns *et al.*, 1998; Monks *et al.*, 2001). Whether, the SPK1/SPK2/Ssh1p signal transduction pathway functions in plant hyperosmotic stress responses, how phosphorylation affects the phosphoinositide binding properties of Ssh1p, and how Ssh1p might couple its phosphoinositide binding properties to promoting stress signaling, remain fruitful areas for further investigation.

METAZOAN PITPs

Mammalian PITPs

As discussed in greater detail below, mammals express three known soluble PITPs (PITPα, RdgBβ, PITPβ). While PITPα and PITPβ are highly related at the primary sequence level, RdgBβ is a more distant member of this family (see Figure 4). In addition, mammals express two large insoluble members of the PITP family where an N-terminal PITP-domain is followed by long C-terminal extensions which themselves exhibit recognizable domain motifs. The PITP-domains of that class of proteins also share a large degree of primary sequence homology to the soluble mammalian PITPs. The conservation of primary sequence between members of the metazoan PITP family is obvious from mammals down to flies, worms (Figure 4), and even slime molds. It is therefore clear that structural insights gathered for one member of the metazoan PITP family will translate to most, if not all, members of this protein family. In that regard, considerable progress has recently been made in elucidating the structure of PITPα.

Structural Studies

Three distinct forms of PITPα have been crystallized and the structures solved to high resolution: a PtdCho-occupied form (Yoder *et al.*, 2001), a PtdIns-occupied form (Tilley *et al.*, 2004), and a form devoid of bound phospholipid (Schouten *et al.*, 2002). While lack of primary sequence homology between fungal/plant PITPs and metazoan PITPs does not formally exclude the possibility of convergent structural evolution, the high resolution structural data now indicate the compact Sec14p fold is completely unrelated to the metazoan PITP fold. Mammalian PITPα conforms to a START structural fold (StAR-related lipid transfer). PITPα consists of seven α-helices and 8 β-strands that define two major structural features; a large concave β-sheet motif and three long α-helices. Two of these long α-helices (A and F) face the interior of the β-sheet and form a lipid-binding cavity (Figure 5; Yoder *et al.*, 2001).

Interestingly, the structures of PtdIns- and PtdCho-bound PITPα are virtually indistinguishable and, in

(A)

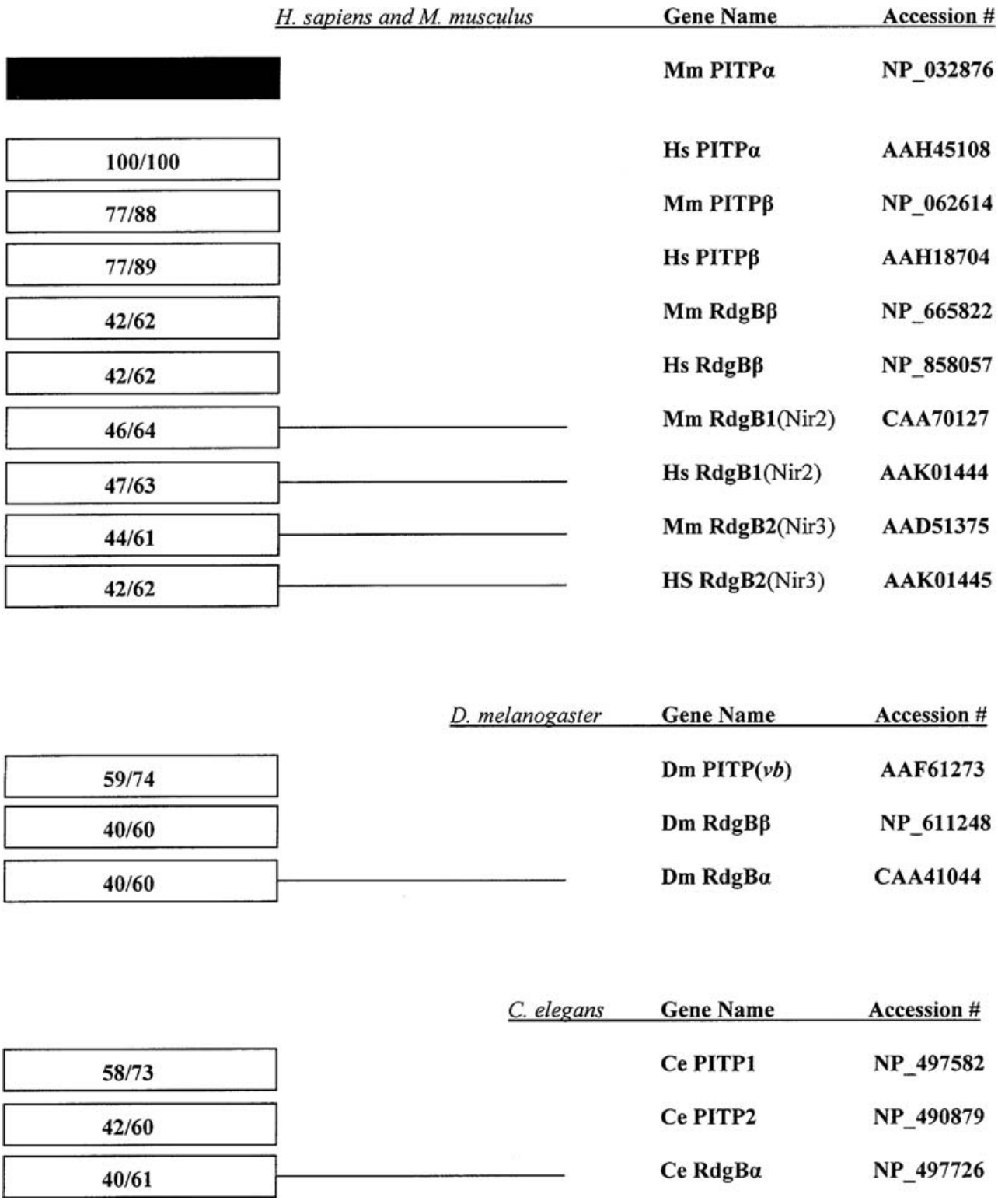

FIGURE 4 **Metazoan PITPs. (*A*) The protein sequences were analyzed by BLAST (http://www.ncbi.nlm.nih.gov/BLAST). The homology of each corresponding PITP domain to *M. musculus* PITPα is shown, and corresponding percent primary sequence identity and similarity of each PITP domain to murine PITPα is given. (*B*) Cladogram of the metazoan PITP family. This represents a partial listing of known members of the metazoan PITP family. When known, common designations are given along with the corresponding accession numbers. Proteins discussed in the text are highlighted in black. Unrooted minimum evolution distance-based topology for human PITPα and its homologous sequences. Values represent bootstrap support when greater than or equal to 50% based on 1000 replicates. Species abbreviations: Ce, *Caenorhabditis elegans*; Dd, *Dictyostelium discoideum*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Pf, *Plasmodium falciparum. (Continued)***

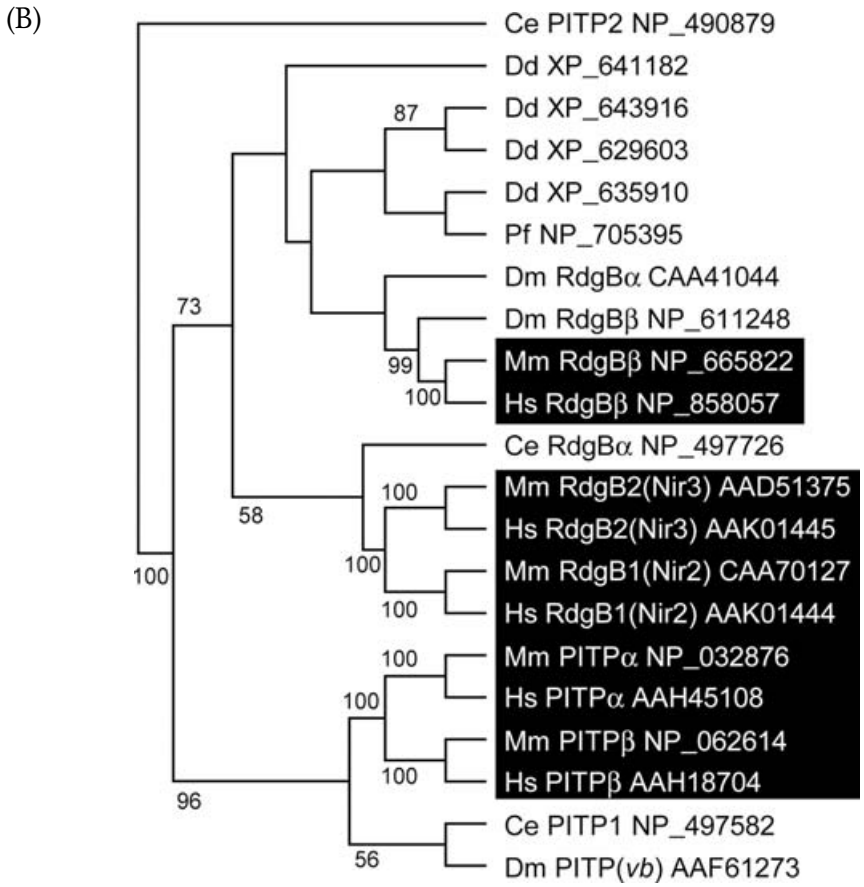

FIGURE 4 **(Continued)**

each case, the bound phospholipid ligand is disposed such that the headgroup is buried deep in the lipid-binding cavity with the acyl-chains oriented outward toward the solvent face of the binding cavity (Yoder *et al.*, 2001; Tilley *et al.*, 2004). This orientation sequesters the headgroup from any potential interaction with an active site of a PtdIns kinase, thereby arguing against models where metazoan PITPs directly 'present' bound phospholipid to an enzyme for modification. These structures describe the solution forms of the PITPα-phospholipid complex, however. It remains to be determined whether the conformation of the PITPα-ligand complex transiently exposes the headgroup for enzymatic modification when engaged with a membrane surface.

The PITPα structure allows incisive interpretation of earlier mutagenesis studies that identified four residues that, when individually altered, resulted in specific ablation of PtdIns-transfer activity. These residues include Thr_{59}, Ser_{25}, Pro_{78} and Glu_{248}, and it was proposed that these residues contact the inositol headgroup of bound PtdIns (Alb *et al.*, 1995). This proposal was justified on the grounds that alteration of these residues evoked essentially complete loss of PtdIns-binding/transfer activity without any significant diminution of PtdCho-transfer activity.

The structural studies of Yoder *et al.* (2001), and subsequently Tilley *et al.* (2004), confirm some of these predictions and raise interesting issues regarding others. It is clear that Thr_{59} lies directly adjacent to the inositol ring and, in cooperation with Lys_{61} and Asn_{90}, forms a hydrogen bond network that constitutes the inositol headgroup binding site of PITPα. Glu_{248} forms a salt-bridge with Lys_{61} and thereby positions Lys_{61} properly so that it can interact with the inositol headgroup. Thus, in principle, missense substitutions for Thr_{59}, Lys_{61} and Glu_{248} cause PtdIns-binding/transfer defects by destabilizing binding of the inositol headgroup. The reason missense substitutions for residues Ser_{25} and Pro_{78} produce such specific PtdIns-binding defects is less clear, as these residues do not reside in the immediate vicinity of the inositol headgroup nor do these obviously stabilize the association of contact residues with the inositol headgroup (Yoder *et al.*, 2001). Perhaps these residues

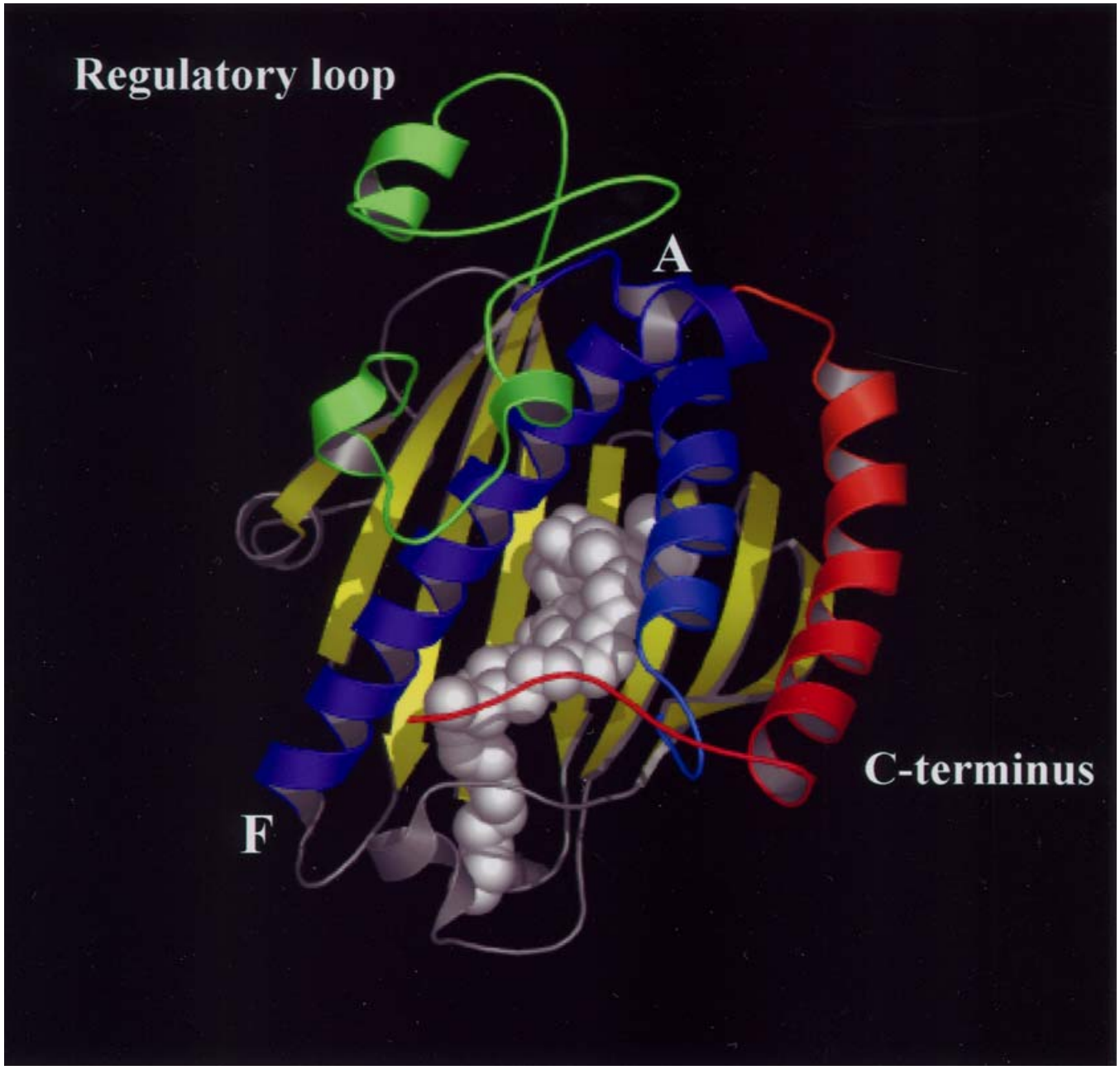

FIGURE 5 **Three-dimensional structure of mammalian PITPα occupied with PtdCho. PITPα exhibits a START-domain fold comprised of seven α-helices and eight β-sheets. The PITPα lipid-binding structural element consists of an eight-stranded concave β-sheet flanked by α-helices A and F (*in black; helices indicated*) that encloses the bound PtdCho monomer (indicated in space-fill mode). A regulatory loop harbors a potential PKC-phosphorylation site at residue Ser166. The C-terminal helix (*dark grey*) and remaining C-terminal random coil constitute the most highly divergent stretch of amino acids between PITPα and PITPβ.**

play a specific role in the recognition of PtdIns prior to its loading into the binding cavity, or these residues are involved in conformational changes somehow unique to loading of PITPα with PtdIns. In that regard, Pro_{78} (like Thr_{59}, Lys_{61}, Asn_{90}, and Glu_{248}) is absolutely conserved amongst metazoan PITPs while Ser_{25} is not.

There are two other insights the structural information provides regarding how metazoan PITPs may be regulated *in vivo* and how these engage membrane surfaces. With regard to regulation, Alb and colleagues (1995) proposed a model where a PITP-kinase/phosphatase cycle could function in imposing a vector to phospholipid-transfer between membranes *in vivo*. In that model, Thr_{59} was suggested as an attractive site for regulation given the specific biochemical consequences associated with missense substitutions at

the residue. The available structural data indicate Thr_{59} is solvent inaccessible; at least in the solution complex (Yoder *et al.*, 2001; Tilley *et al.*, 2004). Thus, a significant conformational change is required to expose Thr_{59} to a protein kinase for phosphorylation, and there is no evidence that Thr_{59} is ever phosphorylated. An analogous proposal was subsequently suggested on the basis of mapping of protein kinase C phosphorylation sites utilized *in vitro* (Van Tiel *et al.*, 2000). The one modification of the hypothesis of Alb and colleagues (1995) was that Ser_{166} is the operant regulatory site. This proposal is supported by data indicating missense substitutions for Ser_{166} inactivate both PtdIns- and PtdCho-transfer activity (Van Tiel *et al.*, 2000). In support, Ser_{166} is present in most (but not all) metazoan PITPs and resides on a large solvent-accessible loop (Yoder *et al.*, 2001). Thus, Ser_{166} may, under certain conditions, be accessible to a protein kinase when PITPα is in solution. Whether Ser_{166} has any role as regulatory residue *in vivo* remains to be determined.

Structural studies also suggest how metazoan PITPs may initially engage membranes. Schouten and colleagues (2002) crystallized an apo-form of PITPα. That is, a form that has no lipid bound in the hydrophobic cavity, and the crystallizing unit is a dimer where one putative membrane insertion domain of each monomer inserts into the hydrophobic pocket of the other. This apo-structure is proposed to represent the configuration of PITPα as its sits on the membrane surface. While the basic form of the apo- and holo-PITPα structures are similar, there are several conformational changes in the apo form that are suggestive. Primarily, an open channel surrounded by residues 48 to 56 is present in the apo-form, and this channel is positioned adjacent to the headgroup binding region (Schouten *et al.*, 2002). The channel suggests a mechanism by which a lipid kinase could potentially access the bound headgroup that is otherwise buried deep within the PITP interior. In this model, any productive association of holo-PITP with lipid kinase should occur during the lipid exchange reaction on the membrane interface.

The structural description of PITPα is completed by solution of the structure of the PtdIns-bound form. The asymmetric unit of the PtdIns-bound PITPα crystal contains two PITPα molecules arranged as an 'end-to-end' dimer. The dimer interface is defined by a loop that contains residues Trp_{203} and Trp_{204}, and a $Trp_{203,204}$Ala PITPα is purportedly unable to transfer either PtdIns or PtdCho *in vitro* (Tilley *et al.*, 2004). These data are interpreted to indicate that Trp_{203} and Trp_{204} may be responsible for PITP-membrane interaction and are in agreement with the predictions of Schouten and coworkers (2002) that the $Trp_{203}Trp_{204}$ motif is a constituent of a PITP membrane binding interface. However, there is as yet no information to indicate the $Trp_{203,204}$Ala missense substitutions genuinely compromise physiologically relevant membrane interactions, or even whether these mutations are structurally benign.

In Vitro Models of Mammalian PITP Function

Mammals express three known soluble PITPs (PITPα, RdgBβ, and PITPβ). PITPα and PITPβ share 77% primary sequence identity (Figure 4) (Dickeson *et al.*, 1989; Tanaka & Hosaka, 1994), while RdgBβ shares approximately 40% sequence identity with PITPα and PITPβ (Fullwood *et al.*, 1999). PITPβ is distinct from PITPα in that it catalyzes the *in vitro* transfer of PtdIns, PtdCho and sphingomyelin (SM) (De Vries *et al.*, 1995). Moreover, PITPα localizes to the cytoplasm and nucleus while PITPβ localizes to the Golgi complex (De Vries *et al.*, 1996). PITPα is required for the Ca^{2+}-activated exocytosis of secretory granules in permeabilized neuroendocrine cells (Hay & Martin, 1993), budding of secretory vesicles and immature granules from neuroendocrine and hepatocyte TGN (Jones *et al.*, 1998; Ohashi *et al.*, 1995), and for plasma membrane receptor/G-protein-coupled hydrolysis of PtdIns(4,5)P_2 by phospholipase C (PLC) (Cunningham *et al.*, 1996). The former two systems implicate a role for PITPα in vesicle trafficking, an activity analogous to yeast PITP function. The PITP requirement for agonist-stimulated PtdIns(4,5)P_2 synthesis is recorded, regardless of whether the plasma membrane receptor signals through receptor or non-receptor tyrosine kinases, or through PLCβ or PLCγ1 (Cunningham *et al.*, 1996). The idea that PITP function is conserved between yeast and mammals is supported by the ability of high-level expression of PITPα and PITPβ to rescue PITP defects in yeast (Skinner *et al.*, 1993; Tanaka & Hosaka, 1994), and the ability of Sec14p to substitute for PITP in each of these reconstitutions (Cunningham *et al.*, 1996; Hay & Martin, 1993; Jones *et al.*, 1998; Ohashi *et al.*, 1995).

What is the mechanism of PITP function in the *in vitro* systems? In all PITP-dependent reactions reconstituted to date, PITP-stimulated synthesis of phosphoinositides is a common denominator. One

interpretation of the biochemical reconstitution data that has been offered is that PITPs represent a subunit of a phosphoinositide-driven signaling machine (Cunningham *et al.*, 1996). This is not likely the case, and the arguments against this proposal are powerful ones. First, the PITPα crystal structure indicates the phospholipid headgroup is sequestered deep within the protein interior where it cannot be available to the active site of lipid kinases (Yoder *et al.*, 2001). As noted above, however, the apo-PITPα structure does suggest the possibility that the inositol headgroup may become accessible to a lipid kinase active site, but this exposure will be limited to the membrane interface during a lipid loading/unloading reaction. Second, and perhaps most significantly, yeast PITP (Sec14p) substitutes for PITPα in the priming and the TGN-derived vesicle biogenesis assays as well as the plasma membrane receptor/PLC-regulated hydrolysis of PtdIns(4,5)P_2 (Hay & Martin, 1993; Jones *et al.*, 1998; Ohashi *et al.*, 1995, Phillips *et al.*, 1999). Given that the mammalian PITP three-dimensional protein fold is completely unrelated to that of yeast/plant PITPs, it is difficult to envision how these two disparate PITP species enter into a dedicated physical interaction with mammalian phosphoinositide metabolic machines.

Other *in vitro* data suggest that PITPs do not act catalytically to stimulate phosphoinositide synthesis (Currie *et al.*, 1997). The lack of PITP specificity in these reconstitution assays, when coupled with the deep sequestration of the inositol ring of bound PtdIns in the metazoan PITP lipid binding pocket (at least in the soluble complex), are most consistent with PITPs acting indirectly to render PtdIns monomers better substrates for PtdIns-kinases.

In Vivo Models of Mammalian PITP Function

An initial clue into mammalian PITP function came from the finding that the *vibrator* (*vb*) allele is a recessive mutation that, when homozygous, reduces synthesis of wild-type PITPα protein to 18% of normal levels and results in a progressive whole-body action tremor in mice. *vb/vb* mice die between postnatal day 30 to 160, depending on genetic background (Weimar *et al.*, 1982; Hamilton *et al.*, 1997). Because it is difficult to derive mechanistic interpretations from hypomorphic alleles such as *vb*, PITPα-nullizygous cells and mice were generated. Contrary to expectations, ablation of PITPα function in murine cells evokes no deleterious consequences for growth and bulk phospholipid metabolism. Moreover, PITPα does not play an obvious role in protein trafficking through the constitutive secretory pathway, endocytic pathway function, biogenesis of mast cell dense core secretory granules or the agonist-induced fusion of dense core secretory granules to the mast cell plasma membrane; *i.e.*, reactions where PITPα was reconstituted as an essential stimulatory factor *in vitro* (Alb *et al.*, 2002).

Finally, PITPα-nullizygous cells preserve full responsiveness to bulk growth factor stimulation, and retain their pluripotency. Thus, PITPα plays a more dedicated function in mammals than that indicated by *in vitro* systems that show PITP-dependence. By contrast, PITPβ deficiency results in an apparently catastrophic failure early in murine embryonic development, and the data suggest that PITPβ is an essential housekeeping PITP in murine cells (Alb *et al.*, 2002).

The elusive cellular function of PITPα notwithstanding, ablation of PITPα function is devastating to the neonatal mouse. PITPα nullizygosity is fully tolerated throughout fetal development, an outcome consistent with the nullizygous ES cell data indicating a highly specialized function for PITPα. However, *PITP*$\alpha^{-/-}$ mice succumb to a perinatal lethality, usually within 8 days of birth, stemming from a complex course of aponecrotic spinocerebellar disease, hypoglycemia, and intestinal and hepatic steatosis. The intestinal and hepatic steatosis results from the intracellular accumulation of neutral lipid and free fatty acid mass in these organs suggesting defective trafficking of triglycerides and DAG from the ER. The hypoglycemia is associated with inefficient glycogenolysis, pancreatic islet cell defects, and reduced proglucagon gene expression. It is proposed that deranged intestinal and hepatic lipid metabolism and defective proglucagon gene expression cause hypoglycemia in *PITP*$\alpha^{-/-}$ mice, and that hypoglycemia is a significant factor in onset of spinocerebellar disease. The collective data suggest an unanticipated role for PITPα in glucose homeostasis and in mammalian ER functions that interface with transport of specific lumenal lipid cargos (Alb *et al.*, 2003).

Drosophila RdgBp

The *Drosophila rdgB* protein (RdgBp) is a large, conserved protein with an amino-terminal domain that

exhibits 42% primary sequence identity to the full-length mammalian PITPα and PITPβ (Figure 4). This PITP-domain exhibits PtdIns- and PtdCho-transfer *in vitro*, and lies adjacent to a proposed Ca^{2+}-binding domain that is positioned immediately carboxy-terminal to the PITP-domain (Vihtelic *et al.*, 1991; Vihtelic *et al.*, 1993; Milligan *et al.*, 1997). That RdgBp regulates phototransduction is amply demonstrated by the rapid and light-accelerated retinal degeneration suffered by RdgBp-nullizygous flies (Vihtelic *et al.*, 1991; Vihtelic *et al.*, 1993; Milligan *et al.*, 1997). In a wild-type photoreceptor, RdgBp is required for proper termination of phototransduction upon removal of a light stimulus and in establishment/maintenance of rhodopsin levels in photoreceptor cells. RdgBp-nullizygous flies, while exhibiting a normal initial light response, fail to rapidly repolarize photoreceptors upon cessation of light signal and are deficient in dark recovery of the photoreceptor cells (Milligan *et al.*, 1997). Interestingly, RdgBp is associated with the subrhabdomeric cisternae and does not reside in the rhabdomeres themselves (*i.e.*, the terminal structures that house rhodopsin and represent the site of the phototransduction cascade; Vihtelic *et al.*, 1993). As such, RdgBp is unique in its involvement in regulation of phototransduction while failing to reside in the rhabdomere itself.

While the PITP-domain of RdgBp (RdgB-PITP) comprises only 27% of the full-length RdgBp, this domain is both necessary and sufficient for fulfillment of all RdgBp functions in regulating the fly visual cascade. Strikingly, expression of the isolated RdgB-PITP-domain in *rdgB*-nullizygous flies rescues the retinal degeneration fully and restores a normal electrophysiological light response to photoreceptor cells (Milligan *et al.*, 1997). Two lines of evidence indicate the phospholipid-binding/transfer activity of the RdgB-PITP-domain is not the sole functional activity of this domain. First, RdgBp function in phototransduction can be compromised without affecting the phospholipid-transfer activities of RdgBp-PITP. Second, domain swapping experiments indicate that the PITP-domain of RdgBp and mammalian PITPα are not functionally interchangeable. Substitution of mammalian PITPα for the RdgB-PITP-domain in the context of a full-length RdgBp results in a chimera that, when expressed in the fly retina, evokes a robust and genetically dominant retinal neurodegenerative disease (Milligan *et al.*, 1997). This is a remarkable result on several counts, but the fact that such a chimera so potently interferes with activity of a wild-type RdgBp is particularly striking.

Mammalian Versions of RdgB and Golgi Secretory Function

The *Drosophila* RdgBp is the founding member of a larger protein family with readily identifiable homologs in worms, fish, and mammals (Figure 4). Information to this effect comes from two different avenues of investigation. First, based on the body of information generated for fly RdgBp, murine homologs were originally sought and isolated as candidate proteins affected in mammalian retinal degenerative diseases. Two such murine homologs, M-RdgB1 and M-RdgB2, were isolated, and at least M-RdgB1 does indeed exhibit some functional relatedness to RdgBp, as its expression in *rdgB* nullizygous flies fully restores the ERG light response in these *rdgB* mutants (Chang *et al.*, 1997; Lu *et al.*, 1999). Interestingly, the high degree of homology (72% sequence identity) between M-RdgB1 and M-RdgB2, notwithstanding, M-RdgB2 expression does not result such an efficient rescue. Second, RdgBp homologs were subsequently recovered from protein interaction screens searching for binding partners of the PYK2 protein tyrosine kinase. This interaction led to the identification of three M-RdgBs or Nirs (PYK2 N-terminal domain-interacting receptors) that are tyrosine phosphorylated by PYK2 (Lev *et al.*, 1999). Nir2 and Nir3 correspond to M-RdgB1 and M-RdgB2 respectively, while Nir1 lacks the amino-terminal PITP-domain (Figure 4). As our focus is on PITPs; Nir1 will not be discussed further.

Mice individually nullizygous for M-RdgB1 or M-RdgB2 were generated with the expectation that either individual or combinatorial deficiencies might evoke murine retinal degenerative disease. That expectation was not met. Ablation of M-RdgB2 yields viable mice with no noticeable compromise of photoreceptor function, mutant survival, or overall development of the nullizygous animal (Lu *et al.*, 2001). By contrast, M-RdgB1-nullizygosity results in embryonic lethality, with *M-RdgB1*$^{-/-}$ embryos expiring before day E7.5 (Lu *et al.*, 2001). These data suggest either M-RdgB1 and M-RdgB2 play distinct functions in the mouse, or that M-RdgB1 is the major isoform of two functionally redundant M-RdgB isoforms. It also remains possible that M-RdgB1 and M-RdgB2 are functionally redundant with respect to retinal function/maintenance, but that

M-RdgB1 plays other essential cellular housekeeping roles.

Although mouse gene ablation strategies failed to provide much insight into M-RdgB function *in vivo*, progress is coming from cell biological analyses. M-RdgB1/Nir2 localizes to the mammalian Golgi complex (Aikawa *et al.*, 1999; Litvak *et al.*, 2005), where it is required for efficient membrane and protein trafficking from the TGN. Depletion of function by M-RdgB1/Nir2 siRNA evokes dramatic changes in Golgi structure. Morphological analyses of the affected Golgi by electron microscopy reveals swollen Golgi cisternae and loss of the typical compact perinuclear Golgi structure (Litvak *et al.*, 2005). These morphological defects correlate with strikingly reduced efficiencies of vesicular stomatitis virus coat glycoprotein (VSV-G) trafficking from the TGN to the plasma membrane. Furthermore, YFP-tagged VSV-G in M-RdgB1/Nir2 knockdown cells was localized to tubules emanating from the Golgi. These structures are most likely due to impaired fission of transport carriers from the TGN and are comparable to Golgi tubular structures generated from the overexpression of a kinase-deficient protein kinase D (PKD) that is required for vesicle trafficking from the TGN (Liljedahl *et al.*, 2001; Baron *et al.*, 2002). This membrane trafficking defect is specific, as there are no discernable effects on ER to Golgi or intra-Golgi transport (Litvak *et al.*, 2005).

How does M-RdgB1/Nir2 regulate vesicle trafficking from the TGN? Clearly, as is the case with the *Drosophila* RdgBp, the PITP-domain is the key functional domain. Expression of the PITP-domain of M-RdgB1/Nir2 alone is sufficient for efficient rescue of VSV-G transport from TGN depleted of full-length endogenous M-RdgB1/Nir2. Strikingly, the role of the M-RdgB1/Nir2 PITP-domain in facilitating vesicle biogenesis at the TGN surface appears to be very similar to that executed by Sec14p in budding yeast; *i.e.*, control of DAG homeostasis. Several lines of evidence indicate M-RdgB1/Nir2 controls the consumption of a Golgi DAG pool that is sensitive to consumption via the CDP-choline pathway for PtdCho biosynthesis. Indeed, pharmacological inhibition of CDP-choline pathway activity rescues the TGN membrane trafficking defects associated with reduced M-RdgB1/Nir2 function (Litvak *et al.*, 2005).

Whether M-RdgB1/Nir2 effects direct or indirect regulation of the CDP-choline pathway is unclear. However, these findings are remarkably congruent with the demonstration that the essential Sec14p requirement in yeast is bypassed by genetic ablation of the CDP-choline pathway (Cleves *et al.*, 1991b). The similar derangements of the Golgi system recorded upon depletion of M-RdgB1/Nir2 or PKD function, when coupled with the DAG requirement for PKD recruitment to the mammalian TGN, suggests the attractive possibility that M-RdgB1/Nir2 regulates DAG-mediated recruitment of PKD to the TGN surface (Litvak *et al.*, 2005).

Other Functions for M-RdgB1/Nir2

M-RdgB1/Nir2 is a multifunctional protein whose site of action does not appear to be limited to the TGN. As one example, both M-RdgB1/Nir2 and M-RdgB2/Nir3 interact with the integral ER-membrane protein VAP-B via a conserved acidic region containing the FFAT motif (Amarilio *et al.*, 2005). These two VAP-B interactions induce stacked ER membrane arrays and gross ER rearrangements, suggesting novel possibilities for the involvement of M-RdgB1/Nir2 and/or M-RdgB2/Nir3 in regulating membrane biogenesis. These effects are only observed when M-RdgB1/Nir2 or M-RdgB2/Nir3 proteins are overexpressed, however, raising questions regarding the physiological significance of these effects (Amarilio *et al.*, 2005).

M-RdgB1/Nir2 also engages lipid droplets which are themselves sites of storage for neutral lipids such as triacyglycerol, DAG and cholesteryl esters. The neutral lipid core of these droplets is surrounded by a shell of phospholipids and proteins. When cells are grown in the presence of excess oleic acid, lipid droplets accumulate within the cell and M-RdgB1/Nir2 localizes on the surface of these lipid droplets. This localization is mediated by a proposed phosphorylation of Thr_{59} in the PITP-domain, a regulation particularly apparent in the context of localization of the PITP-domain alone. Truncated M-RdgB1/Nir2 consisting of only the PITP-domain localizes to the cytoplasm, while the M-RdgB1/Nir2^{T59E} truncation mutant localizes to lipid droplet surfaces (Litvak *et al.*, 2002a).

The fact that M-RdgB1/Nir2 localizes to the lipid droplet surface at all raises interesting questions regarding the mechanism of such localization. As M-RdgB1/Nir2 is a tightly associated membrane protein, and a potential phosphorylation of Thr_{59} is required for M-RdgB1/Nir2 localization to lipid droplets, it is likely that M-RdgB1/Nir2 incorporates onto the lipid

droplet during the biogenesis of the droplet itself. How such an event sorts the potentially phosphorylated M-RdgB1/Nir2 from the unmodified form is unknown. Furthermore, as the high-resolution structure of PITPα reveals, Thr_{59} is not readily accessible for phosphorylation in the soluble PITP::phospholipid complex, although the PITP-domain protein fold might be different in the context of M-RdgB1/Nir2.

It is also unclear what role M-RdgB1/Nir2 may play on lipid droplet surfaces. M-RdgB1/Nir2 may mobilize DAG from the lipid droplet reservoir into the TGN. The imported DAG could contribute to the DAG pool that functions in PKD recruitment. Why the TGN would require such a DAG supply line is unclear; given that mammalian Golgi membranes are veritable DAG producing machines as a consequence of housing the enzyme that catalyzes SM biosynthesis. Production of one mole SM via the action of SM synthase consumes one mole PtdCho and one mole ceramide while generating one mole DAG as additional product (see Luberto and Hannun, 1998). On this basis, PITPβ is ideally positioned to regulated Golgi DAG homeostasis (Bankaitis, 2002). The available data do not support this proposal, however. Rather, the data identify M-RdgB1/Nir2 as a major regulator of TGN DAG homeostasis and suggest the CDP-choline pathway is involved in regulating DAG homeostasis (Litvak *et al.*, 2005).

M-RdgB/Nir proteins exhibit other domains that potentially regulate important cellular processes. For example, cytokinesis requires coordinated regulation of cellular cytoskeletal networks and proteins that engage the plasma membrane. Regulation of this process involves the action of the small GTPase Rho, and M-RdgB1/Nir2 function appears to interface with that of Rho. Microinjection of M-RdgB1/Nir2 antibodies into cells blocks completion of cytokinesis by inciting cleavage furrow regression. The result is production of multinucleate cells (Litvak *et al.*, 2002b). In this regard, while M-RdgB1/Nir2 normally localizes to the Golgi, endogenous M-RdgB1/Nir2 also localizes with RhoA to the cleavage furrow and the midbody. Co-immunoprecipitation experiments confirm that M-RdgB1/Nir2-RhoA association is significantly enhanced during mitosis in HeLa cells (Litvak *et al.*, 2002b). In this regard, the interaction between M-RdgB1/Nir2 and RhoA is mediated by a Rho inhibitory domain (Rid) in M-RdgB1/Nir2 that is essential for cytokinesis (Tian *et al.*, 2002; Litvak *et al.*, 2002b). Again, this cell division function for M-RdgB1/Nir2 resembles that executed by $Sec14p^{SP}$ in completion of cytokinesis in fission yeast and, potentially, by Patellins in cell plate formation in higher plants (Nakase *et al.*, 2000; Petermann *et al.*, 2004; see above). The precise role of the M-RdgB1/Nir2 PITP-domain in regulating cytokinesis remains to be resolved.

CONCLUDING REMARKS

In this review, we emphasize the point that PITPs and PITP-like proteins sit at the nexus of signaling pathways that control interesting and important cellular and physiological functions. PITPs execute roles in both uni- and multicellular eukaryotes, and these organisms span the gamut of the eukaryotic kingdom. The few functional studies that have been done on these remarkably under-investigated proteins demonstrate there exists a specific coupling of the functions of individual PITPs to defined cellular activities associated with membrane trafficking and/or membrane biogenesis. The studies of the function of specific PITPs in higher organisms in particular demonstrate PITP insufficiencies result in intestinal malabsorbtion disorders, glucose homeostatic and neurodegenerative diseases in mammals. PITPs are also critical for maintenance of mycelial growth programs in dimorphic yeast; *i.e.*, growth programs that are critical factors in the pathogenesis of infectious yeast agents. Thus, from multiple biomedical standpoints, PITP studies will impact our understanding of the molecular basis of disease.

It is similarly clear that PITPs regulate interesting developmental pathways for polarized membrane biogenesis in higher plants in ways that may influence the symbiotic program that permits nitrogen fixation, and are also likely to regulate plant stress responses. Whether the plant paradigm for PITP-mediated regulation of polarized exocytosis translates to the function of PITPs, particularly Sec14p-like PITPs, in mammalian cells remains to be seen. In any event, as hunger presents the major health problem facing the human race, studies of how PITPs function in plants are likely to assume increasing basic and applied importance over the coming years.

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