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A FUNCTIONAL ANALYSIS OF METAZOAN PHOSPHATIDYLINOSITOL TRANSFER PROTEINS

by

JAMES G. ALB, JR.

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham, in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT OF DISSERTATION
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Title A Functional Analysis of Metazoan Phosphatidylinositol Transfer Proteins

Genetic and biochemical approaches are shedding new insight on the distinct physiological functions of specific phospholipid metabolic pathways and the mechanisms by which phospholipids are mobilized between intracellular compartments. In particular, phosphatidylinositol transfer proteins have recently been revealed to play fascinating and unanticipated roles in the coordination of phospholipid metabolism with vesicle-trafficking and signal-transduction reactions.

We have shown that the mammalian phosphatidylinositol transfer proteins can functionally substitute for the yeast transfer protein in a manner that is similar, but not identical, to the native protein. Furthermore, analysis of phosphatidylinositol transfer protein mutants revealed that the PI and PC transfer activities of the protein could be uncoupled, suggesting a mechanism for regulation of the protein. Finally, we have generated a set of targeted mouse ES cells that have the transfer protein deleted. These targeted ES cells were then used to generate chimeric mice so this deletion mutation could be studied *in vivo*.

DEDICATION

This dissertation is dedicated to my high school Biology teachers (Highland High School, Highland, IN) Mr. Herb Schmidt and Mr. Larry Johnson. They not only taught me, each in their unique way, the essentials required to excel in class, but also to enjoy and be interested about science. Both of them opened a world to me that I found fascinating but complicated. They made me realize that science is exciting and should be studied with enthusiasm and dedication. Let this dissertation be one of their many students' way of saying that their sometimes thankless efforts were not wasted nor ever forgotten.

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There are too many people that deserve thanks to form a complete list. I hope those I have left out will realize that they have not been forgotten.

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My parents, James and Carole Alb, also need to be mentioned in this section as their support and encouragement over the years have truly made this body of work possible.

I have many friends that have influenced and supported me during my graduate career but not to the extent of my best friend, Robert Schwartz. He is truly one of a kind and they come no better.

Finally, I would like to give credit to all my fellow graduate students who have passed through the Bankaitis lab. Some have played greater roles in my development than others but I will not list them here; they know who they are.

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INTRODUCTION TO METAZOAN TRANSFER PROTEINS

The intracellular trafficking of lipids is a subject of which little is known compared to protein trafficking. Like protein trafficking, however, the intracellular lipid traffic pathways must also define a set of essential cellular activities. The membrane bilayer of all eukaryotic cells is set up in the endoplasmic reticulum, the major site of cellular lipid synthesis. Based on this, however, the reality remains that the lipid compositions of organellar membranes differ from each other. First, the lipid composition of specific organelles differ from each other and in some cases the presence of a lipid unique to a certain membrane is seen. Secondly, between the inner and outer leaflets of a membrane bilayer there exists an asymmetric lipid composition. This suggests that specific lipid transport and sorting must play a role in the formation of intracellular organelles as opposed to dedicated biosynthetic pathways within each organelle solely responsible for this asymmetry.

How are lipids transported and sorted within cells? Conventional vesicular trafficking of lipids occurs via the same route that proteins are trafficked--through vesicles making their way down the secretory/endocytic pathways (1-3). This is probably the major pathway for bulk lipid transport in the cell. Nonconventional vesicular lipid trafficking

involves vesicles acting as carriers of lipid that do not play a clear role in bulk protein transport through the secretory pathway (4, 5). This concept relies on the hypothesis that there is a class of carrier vesicles for protein traffic solely acting between the ER and *cis*-Golgi and from the *cis*-Golgi to the *medial*-Golgi, for example. Finally, nonvesicular lipid transport defines an expansive classification whose biochemical properties are not characteristic of vesicle formation or fusion. For example, collisional events between distinct organelles trigger lipid transport (6, 7).

Since diffusion of a phospholipid species from one membrane to another would presumably be much too slow a reaction to account for the rate at which these transport events occur, there may exist a mechanistic species or family of proteins to promote rapid transfer of lipids between membranes as monomers. The most likely candidate class of proteins that could promote this rapid transfer of lipids are the phospholipid transfer proteins (PL-TPs). These proteins have been studied for 30 years and have been operationally defined to catalyze such lipid transfer reactions.

Three general classes of PL-TPs have been identified and are classified by the ligand(s) they bind and transfer in an *in vitro* PL transfer assay. First, the monospecific lipid transfer proteins bind and transfer only one phospholipid species. These include the phosphatidylcholine-transfer protein (PC-TP) of mammalian liver (8-10) and small intestine

(11) and the phosphatidylglycerol-transfer protein (PG-TP) isolated from rat lung (12). Secondly, the nonspecific lipid transfer proteins have been identified and isolated from a variety of plant and mammalian species (13-15). This class of transfer proteins catalyzes the *in vitro* transfer of a variety of phospholipids, sterols, and glycolipids. The final class of lipid transfer proteins will transfer several phospholipid species between membranes with a preference for one of the phospholipid species. Examples include the bovine heart PC/sphingomyelin (SM) transfer protein (16), the liver surfactant PC/SM transfer protein (17, 18) and the mammalian and yeast phosphatidylinositol/PC transfer proteins (PI-TPs)(19-22).

In general, PL-TPs achieve efficient exchange transfer reactions in an energy independent manner *in vitro* (23); i.e. the transfer of a phospholipid species from one membrane (the "donor") to another (the "acceptor") then completing the cycle by returning to the original donor membrane bound to ligand it picked up at the acceptor membrane. This, in effect, simply exchanges one phospholipid species for another, even the same species, in a one to one ratio between two membranes. PL-TPs do not execute, however, efficient net transfer reactions *in vitro* which would result in effecting an actual change in the overall lipid composition of the two membrane species in the assay. This idea that PL-TPs seem to act as lipid exchange proteins, along with the fact that they perform in an energy independent manner, challenges the

thought that PL-TPs function to catalyze PL-transfer *in vivo*.

This study will specifically focus on the PI/PC-TPs (henceforth referred to as PI-TP), which have the ability to transfer both PI and PC between membrane bilayers *in vitro*. Like most PL-TPs, PI-TPs have a preferred ligand; in this case PI is favored over PC about 16-fold (24). Furthermore, PI-TPs as a class share a high level of primary sequence conservation (25), a fact that is unique among the PL-TPs. There is a divergence from this rule, however, within the known PI-TPs that break up their classification into two groups. Fungal PI-TPs are approximately 35 kD in molecular weight and are highly similar to each other at the level of primary sequence (26-29). Metazoan PI-TPs are also about 35 kD in molecular weight and share a high degree of primary sequence similarity to each other (25). When the two groups are compared to each other, however, they share no sequence similarity whatsoever (25, 26). Indeed, the fungal PI-TPs share approximately a 25% primary sequence homology with the mammalian retinaldehyde binding protein--a polypeptide that plays a vital role in mammalian vision (28). The amazing reality when comparing fungal and mammalian PI-TPs is that even with the lack of primary sequence homology between these two groups they function very similar in the *in vitro* transfer reaction; it is hard to distinguish between two proteins of different groups (i.e., fungal and mammalian PI-TPs) based solely on their *in vitro* transfer capabilities. Several

important exceptions to this include the recent finding that an isoform of a mammalian PI-TP, the rat PI-TPb isoform, has the ability to transfer the lipid sphingomyelin *in vitro* in addition to PI and PC (30). Furthermore, the mammalian PI-TP transfer activity is sensitive to N-ethylmaleimide (NEM) challenge whereas fungal PI-TP activity is not (31, 32).

Given the many details known about PI-TPs *in vitro*, the most important of which is the ability to bind and then release phospholipids, speculation about their *in vivo* activity has increased dramatically. For instance, does this ability to transfer phospholipids between two membranes *in vitro* also define its *in vivo* activity or could this PL transfer activity be an artifact? Assuming that the PL transfer activity is relevant *in vivo*, a means of regulating this activity must be imposed upon the protein species. Without regulation, the transfer protein would most likely be exchanging the same ligand between two membrane domains, accomplishing nothing.

The necessity for a detailed functional analysis of phospholipid transfer activity is crucial to understanding how and what the PI-TPs accomplish inside the cell; especially since there is not a structural understanding of how PI-TPs execute their PI and PC binding/transfer activities. Questions as to the fact of PI/PC binding domains within the protein and regulation of the transfer protein have remained unanswered until recently. These and other insights into the functional capabilities of the PI-TPs have lent themselves to

a better understanding of the possibilities that may exist defining *in vivo* function.

PI-TP Function in *Saccharomyces cerevisiae*. The first detailed analysis of PI-TP function *in vivo* was possible when it was discovered that the yeast *SEC14* gene product was in fact a PI-TP (26, 27). The *SEC14* gene product is an essential yeast gene whose function is essential for protein transport from the yeast Golgi complex. The finding that the Sec14p specifically localizes to the Golgi, a minor membrane complex in yeast, demonstrated an *in vivo* specificity for PI-TP targeting that was not apparent in the *in vitro* transfer reaction (26, 33).

Clues into the *in vivo* function of Sec14p were first obtained from genetic analysis of *sec14* mutant strains that could be bypassed for their normally essential requirement of *SEC14* (34-36). Mutations in the CDP-choline biosynthetic pathway for PC synthesis rendered the cells free from the Sec14p requirement. Mutations in the PE-methylation pathway for PC biosynthesis, however, did not render the cells free from their Sec14p requirement. Further analysis revealed that the primary *in vivo* consequence of Sec14p dysfunction is a specific CDP-choline pathway driven increase in Golgi PC content that is somehow toxic to cells (34, 35). Sec14p, at a very general level, then is required to maintain a reduced Golgi membrane PC level. Further experimentation then identified the fact that overproduction of Sec14p leads to a significant decrease in bulk cellular CDP-choline pathway

activity but not in methylation pathway activity. *In vitro* experiments then confirmed that Sec14p effects a specific inhibition of cholinephosphatase cytidylytransferase (CCT-ase), one of the three enzymes involved in the CDP-choline pathway reactions (37). Furthermore, the potency of the Sec14p mediated inhibition of CCTase appears to be a function of membrane PC content suggesting that Sec14p-PC is the CCT-ase inhibitor (as opposed to Sec14p-PI) (37). These collective data led to the development of the sensor model for Sec14p function (Fig 1). The protein is localized to the cytosolic surface of yeast Golgi membranes where it is hypothesized to execute a constitutive PL exchange reaction that generates a pool of PL bound Sec14p comprised of both Sec14p-PC and Sec14p-PI. SEC14p-PC acts as a negative effector of CCTase activity in the Golgi membranes. The PI binding activity of Sec14p is thought to constitute a competitive PL binding reaction that constantly regulates the amount of SEC14p-PC present in response to the PI/PC ratio present in Golgi membranes. This constitutive *in vivo* exchange reaction where SEC14p is "sensing" the PL content of yeast Golgi would manifest itself as an *in vitro* PL exchange molecule. The sensor model and the data that led to its formation highlights the potential for an indirect relevance of the *in vitro* PL transfer activity of a protein and its actual *in vivo* duty. Furthermore, the mechanism of function determined for one PL-TP should not be forced upon a homolog even in

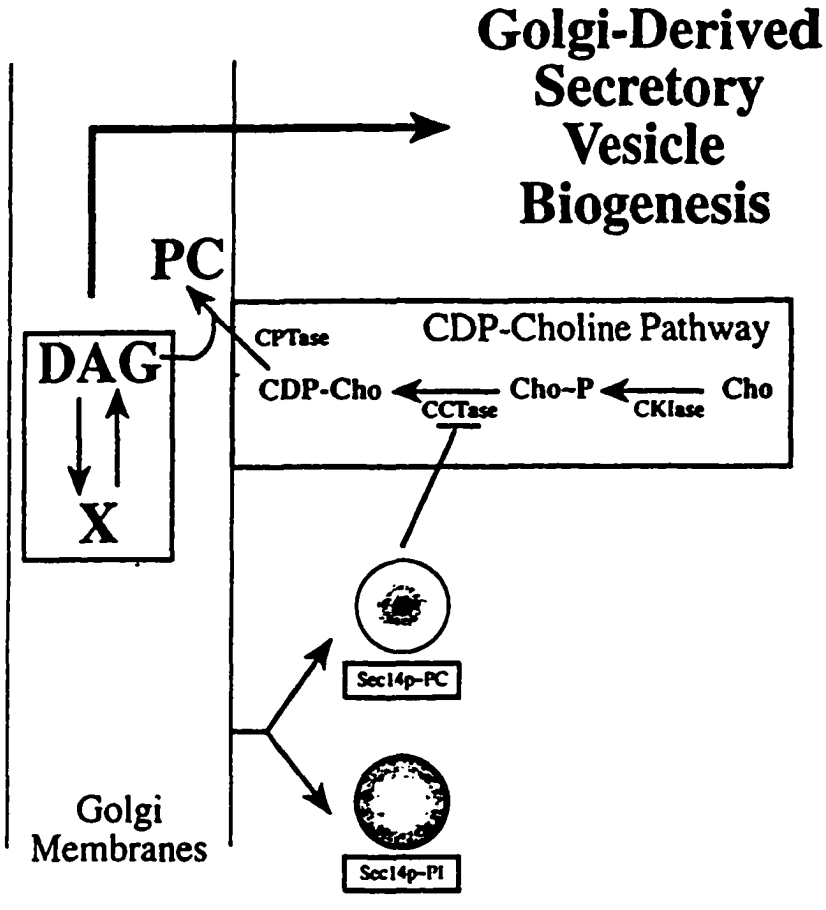

FIG. 1. The sensor model for Sec14p function in yeast. Sec14p senses the PC content of yeast Golgi membranes, rela-tive to PI levels, and the PC bound form of Sec14p (Sec14p PC) effects a negative feedback regulation on CDP-choline pathway activity in Golgi membranes. The purpose of this regulation is to spare a Golgi diacylglycerol (DAG) pool, or the pool of some other phospholipid precursor (X). Sec14p PI is a reservoir of Sec14p that does not repress CCTase. The Sec14p PC pool can change size in response to fluctuating PI and/or PC levels in Golgi membranes. Cho, choline; Cho-P, phosphorylcholine; CKIase, choline kinase; CPTase, cholinephosphotransferase.

instances where they exhibit similar PL substrate specificities *in vitro*.

Metazoan PI-TP Function. As mentioned previously, PI-TP function has been measured in all eukaryotes studied and moreover it has been detected in all mammalian tissues examined thus far (38-40). Immunoblot analysis generally reveals a single immunoreactive PI-TP species at about 36 kD in molecular mass. Initial cloning experiments identified a PI-TP isolated from rat brain as a 271 residue polypeptide with a predicted MW of 35 kD, henceforth referred to as rat PI-TPa (41). Conservation of all known mammalian PI-TP species is remarkable with the rat and human homologs of PI-TPa sharing at least a 99% primary sequence identity (41).

Recent work has identified at least one isoform of the rat PI-TPa within the species referred to as rat PI-TPb (30). The two isoforms share a 77% primary sequence identity and the rat PI-TPb has the ability to transfer both PI and PC in the *in vitro* transfer reaction (30). In addition, the rat PI-TPb isoform has the ability to transfer sphingomyelin *in vitro* due to what can be described as a relaxed phospholipid backbone specificity; it has the ability to accommodate both the ceramide backbone of sphingomyelin and the glycerol backbone of PI and PC (30). The *in vivo* relevance of this ability to transfer sphingomyelin is still unclear.

The physiological role of PI-TPs in higher eukaryotes is still very much a mystery. Clues to explain this lack of understanding have recently come to light with the inform-

ative work of several labs in which the mammalian PI-TP has surfaced in certain *in vitro* functional assays that reconstitute biochemical processes. These assays can be criticized in that they may not represent a true physiological role for the mammalian PI-TP; only other examples of an *in vitro* transfer reaction falsely coupled to a biologically functional assay. Nonetheless, these data suggest that mammalian PI-TP functions to present dedicated PI-kinases with substrate (PI) so that specific pools of phosphoinositides can be generated for use in several distinct biological reactions.

The first of these informative works was from the lab of Tom Martin looking at the mechanism of regulated exocytosis in neuroendocrine cells. Martin and co-workers have reconstituted Ca^{++} activated secretion in semi-intact cells in which the intracellular structure is directly accessible to biochemical measurements (42, 43). Furthermore, this method depletes the cells of cytosol, allowing fractionated cytosol to be assayed for necessary components utilized in stimulating the reaction of interest. In this case, PC12 cells were loaded with norepinephrine (NE), which is taken up into secretory granules, and were then mechanically permeabilized. NE release was measured by depolarizing the semi-intact cells and quantitating radioactivity in the supernatant.

Previous work had established the fact that release of NE required MgATP, Ca^{++}, and cytosol and occurred in two

sequential steps (44): *i*) a MgATP dependent **priming** event of secretory granules for fusion to the PC12 cell plasma membrane; and *ii*) a Ca^{++} dependent, MgATP independent **fusion** event prior to release. Three priming factors were identified from cytosol and named PEP1, PEP2, and PEP3 (Priming in Exocytosis Protein) (44-46). PEP3 was purified and identified as the rat PI-TPa while PEP1 purified as a 500-kD protein that co-purified with PI-4-P 5-kinase activity. These data and subsequent work demonstrated that priming at its simplest form requires phosphatidylinositol-4,5-biphosphate (PIP_2) biosynthesis, indicating a role for phos-phoinositide biosynthesis in Ca^{++} regulated secretion. Furthermore, it suggests that PI-TP's role in this biosynthetic process lies in presenting PI to PI-4-kinase for phosphorylation to PI-4-phosphate (PIP) and subsequent phosphorylation (by PI-4-phosphate 5-kinase) to PIP_2.

Recent work in the laboratory of Wieland Huttner suggests a role for PI-TP in the biogenesis of both constitutive and regulated secretory vesicles on the surface of the trans-Golgi network (TGN) membranes in neuroendocrine cells (47). Interestingly, this execution point is analogous to that of *SEC14* and differs from the execution point described by Martin whose work predicts the rat PI-TPa involved in preparing secretory granules for their consumption at the plasma membrane in a Ca^{++} triggered reaction (47). As in the work of Martin and co-workers, however, the key player in

Huttners' scenario seems to be the generation of PIP_2 as a requirement for vesicle biogenesis from the TGN.

Cytosol was fractionated from bovine adrenal medulla and found to contain two distinct fractions that restored biogenesis of both TGN-derived secretory granules and constitutive secretory vesicles in cell-free assay systems (47). The fractions were termed CAST 1 and CAST 2 (Cytosolic Activity Stimulating TGN vesicle formation). CAST 1 activity consisted of two proteins that were identified as being the bovine PI-TPa and PI-TPb isoforms. It was subsequently shown that Sec14p exhibited CAST 1 activity in this assay system (47), indicating that the bovine PI-TP's ability to function in this reconstituted system was most likely due to its phospholipid transfer capabilities, possibly presenting PI to dedicated PI-kinases for generation of PIP_2 as in the priming of secretory granules described previously.

It has been considered likely that PI-TP function has other roles in cells that are distinct from secretory pathway function, for instance, to replenish PI stores in the plasma membrane (PM) that have been depleted due to membrane signaling events (48). Hormones, growth factors, and neurotransmitters act through receptors on the PM that cause the rapid hydrolysis of PIP_2 whose regeneration requires PI (49). The work of Shamshad Cockroft and Justin Hsuan have entered this area due to their work of reconstituting phospholipase C-mediated inositol lipid signaling (50, 51). This reaction requires a plasma membrane receptor kinase, an activating

GTP-binding protein, and a PI specific phospholipase C (PI-PLC). They could elicit a strong stimulation of PI-PLCb2 mediated hydrolysis of inositol phospholipid with induction of long-term activated G-proteins and an essential cytosolic fraction. Maximal reconstitution of PI-PLC activity was due to a single cytosolic factor found to be the rat PI-TP (50). Furthermore, due to the linear relationship between rat PI-TP concentration and PI-PLC activity, it is suggested that the rat PI-TP fraction defines the rate limiting step in this reconstitution assay. As with the previously described systems, PI-TPs role in this assay is most likely to provide substrate (PI) so that a phosphoinositide pool, whose consumption is necessary for reconstituting PI-PLC activity, can be maintained.

Taken collectively, these data, which identify PI-TP as a factor required for secretory-vesicle/secretory-granule formation and receptor-mediated signaling, indicate interesting possibilities for the execution point of PI-TP *in vivo*. Can PI-TP function at both execution points (plasma membrane and TGN)? If PIP_2 generation is demonstrated to be required for secretory vesicle/granule formation, then an alternative possibility would be that the priming reaction represents an *in vitro* "repriming" step that is required for the regeneration of a PIP_2 domain at the TGN that was lost during the process of cell permeabilization (Fig. 2). This idea demonstrates the possible misinterpretations of the role and

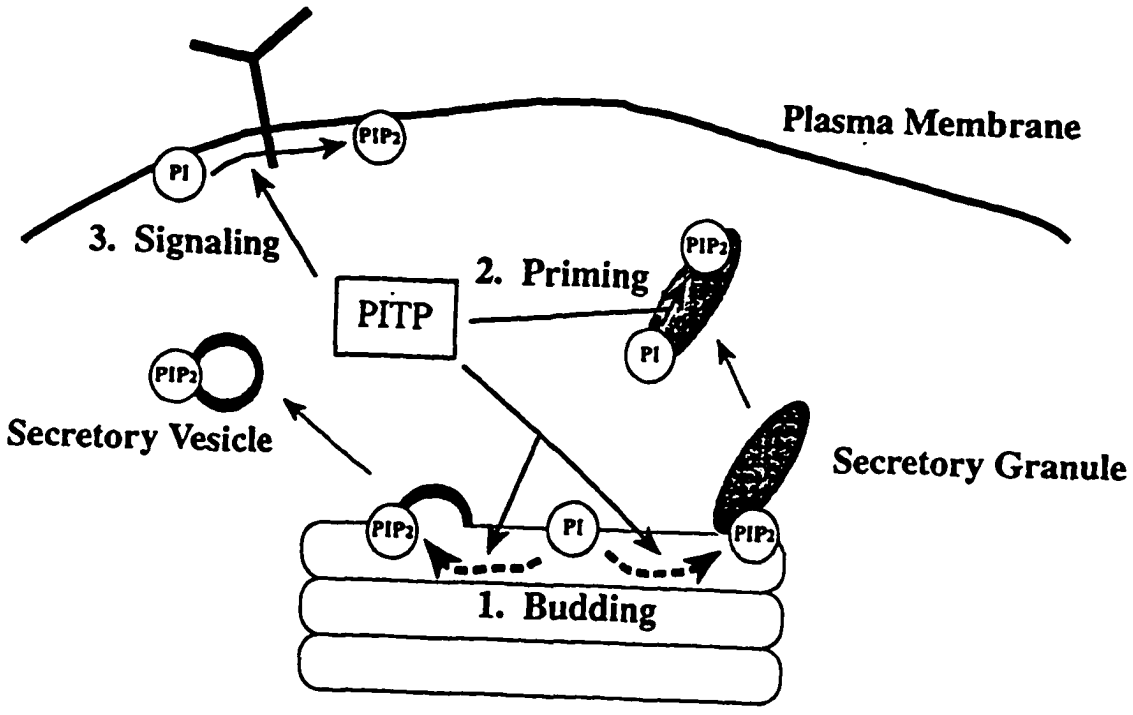

FIG. 2. PI-TP function in mammals. Biochemical data indicate a role for PI-TP in (*1*) the budding of both constitutive secretory vesicles and secretory granules from the TGN. It is not known if these reactions involve PIP_2 generation, but the possibilities raised that the budding reaction may build the requisite PIP_2 structure that is subsequently required to sustain fusion of secretory granules. Priming of secretory granules might then reflect a repriming required to generate a critical PIP_2 domain that was compromised during cell permeabilization. (*2*) The priming of pre-formed secretory granules for Ca^{++} regulated fusion to the plasma membrane. (*3*) PI-TP is also involved in the compartmentalized synthesis of PIP_2 that drives transmembrane receptor mediated signal transduction at the plasma membrane.

execution point(s) for PI-TP *in vivo* based on reconstitution assays.

Finally, other higher eukaryotic PI-TPs have surfaced recently, most notably is the *Drosophila* RdgBp (retinal degeneration B protein) work done by David Hyde and colleagues (51, 52). This is a novel member of the PI-TP family in that it is not soluble but rather membrane bound and genetic defects in this protein lead to retinal degeneration in flys. *RdgB* is a 1,054 residue polypeptide with the first 280 amino acids sharing a 42% primary sequence identity to the rat PI-TPa (53). Not surprisingly, this domain has been shown to transfer PI *in vitro*. The novelty of this protein lies in several areas that include the fact that it is a high molecular weight protein of 160 kDa and the primary sequence indicates that it may be membrane bound with six potential membrane spanning domains (54). In reality, biochemical fractionation corroborates this idea as alkaline Na_2CO_3 treatment of membranes indicates *RdgB* as an integral membrane protein, localizing to the Subrhabdomeric Cisternea (SRC) located within the rhab-domeres. *RdgB* also exhibits two potential Ca^{++} binding do-mains between the PI-TP and membrane spanning regions (55). The interesting fact that *RdgB* is not a soluble protein leaves one to question the validity of a simple phospholipid transfer model imposed upon this protein as its true role within the *Drosophila* visual cascade. In fact, recent evidence suggests that RdgBp is

required for rhodopsin matur-ation in a manner that is not strictly dependent on phos-pholipid transfer activity (55).

The above described reconstitution assays, along with the *Drosophila* RdgB data, implicate PI-TP as a cofactor in several diverse cellular reactions and suggest a functional role for the PI transfer activity of metazoan PI-TP. Furthermore, the assays in which the rat PI-TPa plays a crucial role in reconstituting suggest a more conventional model by which rat PI-TPa utilizes its *in vitro* transfer activity of PI, the preferred ligand *in vitro*, as opposed to Sec14p whose defined role of downregulating the CDP-choline pathway is Sec14p-PC specific. This raises a potential problem, however, due to the fact that the reconstitution assays could only be effective if rat PI-TPa was to effect a net transfer reaction of PI, a reaction very poorly achieved *in vitro*. Given this fact and assuming an *in vivo* relevance of the reconstitution assays, a form of regulation must be imposed upon the rat PI-TPa so that a net transfer of PI could be achieved *in vivo*. For instance, an uncoupling of the dual specificity of the protein for its two ligands, PI and PC, would suggest the possibility of regulating the protein so that a vectorial transfer of PI could be achieved. This ability to affect a vectorial transfer of one ligand, say PI, would also support the ideas proposed in the reconstitution experiments by showing that a critical component, rat PI-TPa, could effectively achieve what is being proposed *in vivo*.

At a more fundamental level, the reconstitution assays impose a critical, if not absolutely essential, role for mammalian PI-TP in several cellular processes. While these assays mimic cellular activities, they may not predict a true representation of cellular events (or the order of) *in vivo*. A mammalian model in which PI-TPa is compromised, which would allow one to determine the affect on intact cells and an entire animal, is necessary to study the true role(s) of PI-TP *in vivo*.

PHOSPHOLIPID TRANSFER ACTIVITY IS RELEVANT TO BUT NOT SUFFICIENT FOR THE ESSENTIAL FUNCTION OF THE YEAST *SEC14* GENE PRODUCT

by

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ABSTRACT

To investigate several key aspects of phosphatidylinositol transfer protein (PI-TP) function in eukaryotic cells, rat PI-TP was expressed in yeast strains carrying lesions in *SEC14*, the structural gene for yeast PI-TP (Sec14p), whose activity is essential for Golgi secretory function *in vivo*. Rat PI-TP expression effected a specific complementation of *sec14*^{ts} growth and secretory defects. Complementation of *sec14* mutations was not absolute as rat PI-TP expression failed to rescue *sec14* null mutations. This partial complementation of *sec14* lesions by rat PI-TP correlated with inability of the mammalian protein to stably associate with yeast Golgi membranes and was not a result of rat PI-TP stabilizing the endogenous *sec14*^{ts} gene product. These collective data demonstrate that while *in vitro* PI-TP activity of Sec14p clearly reflects some functional *in vivo* property of Sec14p, the PI-TP activity is not the sole essential activity of Sec14p. Those data further identify an efficient Golgi targeting capability as a likely essential feature of SEC14p function *in vivo*. Finally, the data suggest that stable association of Sec14p with yeast Golgi membranes is not a simple function of its lipid-binding properties, indicate that the amino-terminal 129 Sec14p residues are sufficient to direct a catalytically inactive form of rat PI-TP to the Golgi and provide the first evidence to indicate that a mammalian PI-TP can stimulate Golgi secretory function *in vivo*.

INTRODUCTION

All eukaryotic cells contain a set of cytosolic proteins that have the capacity to act as diffusible carriers for the energy-independent transport of phospholipid monomers between membrane bilayers *in vitro* (1, 2). These phospholipid transfer proteins (PL-TPs) have been the subject of considerable analysis from the standpoint of the mechanisms by which these proteins catalyze the cell-free phospholipid transfer reaction. Although there has been much speculation concerning the possible involvement of PL-TPs in various aspects of intracellular trafficking of lipids in cells, very little information has been forthcoming with respect to the question of what precise cellular functions PL-TPs fulfill *in vivo*. The primary reasons for this lack of progress have been reviewed elsewhere (3). Nevertheless, the conservation of antigenic and biochemical properties of PI-TPs from mammals to *Drosophila* has been suggested to be indicative of some important, but as yet entirely undefined, cellular function for the PI-TPs in higher eukaryotes (4).

The yeast *Saccharromyces cerevisiae* exhibits a PI-TP whose molecular mass and catalytic properties are very similar to those of the mammalian PI-TP (5), although the mammalian and yeast proteins share no primary sequence homology (4, 6). The first opportunity for a detailed *in vivo* analysis of PL-TP function was identified by the finding that the *S. cerevisiae SEC14* gene product (SEC14p), whose function is essential for protein transport from the yeast Golgi complex

(6, 7), is the yeast PI-TP (8). Subsequent work has established that SEC14p exhibits a specific association with yeast Golgi membranes *in vivo* and that this association is stable as evidenced by copurifica-tion of SEC14p with yeast Golgi membranes (9). Finally, it has been shown that defects in one specific avenue for phosphatidylcholine (PC) biosynthesis bypass the normally essential cellular requirement for SEC14p fuction (9). On the basis of these data, SEC14p has been hypothesized to function in the maintenance of an appropriate phospholipid compostition in yeast Golgi membranes, a parameter that has been proposed to by a critical determinant of Golgi secretory competence in yeast (3, 9).

While the Sec14p studies have provided the first insights into the cellular function of a PI-TP, a number of general issues remain unresolved. First, it has not yet been unambiguously established that the PI-TP activity of Sec14p directly reflects either some essential functional feature of this protein or the sole essential function of Sec14p. The finding that the *sec14*^{ts} gene product elaborates thermolabile PI-TP activity *in vitro* (8), while consistent with the notion that phospholipid transfer activity is somehow important to SEC14p function, nonetheless does not constitute proof of this because Sec14p^{ts} may itself exhibit some general (but reversible) denaturation at restrictive temperatures (7). Second, there is presently no information regarding what the biochemical basis is for the stable and specific association of Sec14p with Golgi membranes *in vivo*. The specific

localization of Sec14p to Golgi membranes, a minor membrane system in yeast, indicates an *in vivo* specificity of PI-TP membrane targeting that is not at all apparent in the *in vitro* transfer reaction (9). Finally, there are no data that directly bear on the question of whether mammalian and yeast PI-TPs share any functional relatedness in cells.

In this chapter, the characterization of yeast strains that express the heterologous rat PI-TPa in the face of dysfunction of the endogenous Sec14p is described. The expression of rat PI-TPa results in a dramatic and specific complementation of *sec14^{ts}* associated growth and secretory defect, and demonstrate that unlike Sec14p, the rat PI-TP does not exhibit an obvious affinity for yeast Golgi membranes. These results make four important points which provide new insights with regard to the three issues identified above. First, the complementation data provide the first direct evidence to indicate that the *in vitro* PI-TP activity of Sec14p reflects some functional property of this protein *in vivo*. Second, the findings indicate that provision of PI-TP activity to a yeast cell is not sufficient to account for all of the *in vivo* functions of the Sec14p. These data reveal at least one other essential activity of the Sec14p, likely a Golgi targeting function, which is distinct from the PI-TP activity. Third, the data indicate that the stable association of Sec14p with yeast Golgi membranes is not a simple function of the specific phospholipid binding properties of Sec14p and that the amino terminal 129 Sec14p residues are sufficient to

specify stable association of a heterologous protein to the yeast Golgi. Finally, the data provide the first evidence to indicate that the mammalian PI-TP can also stimulate Golgi secretory function in an *in vivo* system.

MATERIALS AND METHODS

Yeast Strains, Media, and Transformation. *S. cerevisiae* strains CTY182 (*MAT*a *ura3-52 Dhis3-200 lys2-801*^{am}); CTY1-1A (*MAT*a *ura3-52 Dhis3-200 lys2-801*^{am} *sec14-1*^{ts}); CTY393 (*MAT*a *ura3-52 lys 2-801 Dhis3-200 cki::HIS3*) and CTY230 (*MAT*a *ura3-52 Dhis3-200 cki sec14-129::HIS3*) have been described Bankaitis *et al.*, and Cleves *et al.* (3, 6, 9). Strain CTY303 is isogenic to strain CTY230 and congenic to strain CTY160. The *sec14DP::hisG* allele represents an eviction of essential *SEC14* promoter sequences and 95% of *SEC14* coding sequences, and their replacement with the *Escherichia coli*. *hisG* structural gene by the method of Alani *et al* (10). Yeast were transformed with the appropriate plasmids by the lithium acetate method to uracil prototrophy (11). Complex yeast pep-tone dextrose (YPD) and yeast minimal media have been des-cribed by Sherman *et al* (12).

Rat PI-TP Expression Constructs. To construct the *SrPI-1* gene, the pUC9(SEC14) derivative plasmid pRE26 (6) was digested with HindIII, the ends rendered flush with DNA polymerase I and ligated to SphI linkers to yield pRE246. pRE247 was generated from pRE246 by inserting coding sequence for the *c-myc* epitope EQKLISEEDL between *SEC14* codons 2 and 3 using standard site-directed mutagenesis as described by

Kunkel (13). The epitope tag was designed such that the codons specifying KL introduced a unique Hind III site into pRE247 and the precise nucleotide sequence of the *c-myc* epitope tag is as follows: 5'GAACAAAAGCTTATTTCCGAAGATTTA-3'. PRE247 was digested with *Hind*III and *Bam*HI and the rat PI-TP cDNA-derived fragment was inserted as an ~0.8 kb *Hind*III-*Bam*HI restriction fragment from pRE252 to generate the *SrPI-1* plasmid pRE248. This rat PI-TP cDNA-derived fragment was generated by PCR (14) using the full-length rat PI-TP cDNA clone PI-12 as template (4), the oligonucleotides 5'-CCAAG-CTTGTTAACAGCGACATGGTGCTGGTCCAAGG-3' and 5'-AAGGGATCCAGCGCT-AGTCATCTGCTG-3' as 5' and 3'-synthetic primers in the PCR reaction, subsequent digestion of the PCR product with *Hind*III-*Bam*HI, and subcloning of the digestion product into the corresponding half-sites of pTZ19R (15) to yield pRE253. The hallmark *Hind*III and *Hpa*I sites for the 5' oligonucleotide and the *BamHI* site of the 3' oligonucleotide are underlined. Nucleotide sequence analysis of the *SrPI-1* structural gene revealed that PCR introduced a single missence mutation relative to the rat PI-TP structural gene sequence. Codon 171 (AAT) of the rat PI-TP gene was changed to GAT in *SrPI-1*, resulting in an Asn to Asp substitution at the corresponding position (not shown). The *SrPI-1* construct was mobilized as a 2.5 kb *Eco*RI-*Sph*I restriction fragment from pRE248 and subcloned into the corresponding half-sites of the yeast multicopy vector pSEY18[17] to generate pSEY18(*SrPI-1*). To generate the isogenic *SrPI-1myc* construct, a 0.4kb *Eco*RI-*Hind*III

restriction fragment carrying the transcriptional and translational control elements of the *mcy*-tagged *SEC14* was generated by PCR using pRE247 as template, 5'-GGGAATTCACGCGTGAATATCTTCCTC-3' and 5'-CCAAGCTTTAAATCTTCTTCGGAAATAAGTTTTTGTTCAACCATTGTGTTTTAGGGCG-3' as forward and reverse synthetic primers, respectively, and appropriate restriction endonuclease digestion of the PCR product. The *EcoRI* and *Hind*III site of each corresponding oligonecleotide is underlined. This fragment was exchanged for the *Eco*RI-*Hind*III fragment of pRE248 to generate the *SrPI-1myc* plasmid pRE251. pSEY18(*SrPI-1myc*) was generated by mobilizing *SrPI-1myc* as a 2.5kb *Eco*RI-*Sph*I restriction fragment from pRE251 into the corresponding half-sites of pSEY18 (16). To generate yeast centromeric plasmids bearing each of the SrPI constructs, the *SrPI-1* and *SrPI-1myc* genes were mobilized as 2.5kb *Eco*RI-*Sph*I restriction fragments and subcloned into the corresponding half-sites of the yeast centromeric vector pSEYc68 (16) to yield pSEYc68(*SrPI-1*) and pSEYc68(*SrPI-1myc*).

The *SEC14*-rat PI-TP *SrPI-129myc* gene fusion was constructed by digesting plasmid pRE247 with *Hpa*I and *Bam*HI, and exchanging into the cleaved plasmid an ~800 bp *Hpa*I-*BamHI* restriction fragment derived from pRE248 which carries the entire rat PI-TP cDNA to yield pRE249. The resultant gene fusion encodes a chimera that contains the amino-terminal 129 SEC14p residues fused to the initiator Met of rat PI-TP. The *SrPI-129myc* gene fusion was mobilized as a *Eco*RI-*Sph*I restriction fragment and subcloned into the corresponding

halfsites of the yeast centromeric plasmid pSEYc68 and the yeast episomal plasmid pSEY18 to yield pSEYc68(*SrPI-129myc*) and pSEY18(*SrPI-129myc*), respectively.

Preparation of Cytosol and Phospholipid Transfer Assays. Yeast spheroplasts were prepared from cells grown in the appropriately supplemented minimal media as described by Bankaitis *et al* and Cleves *et al* (6, 9), washed in 1.2 M sorbitol, 10mM Tris-HCL pH 7.4 and lysed by resuspension in 0.6 M sorbitol, 10mM Tris-HCL pH 7.4, 0.5 mM PMSF and vortexing in the presence of glass beads for 5min (as 30 s bursts with cooling on ice between bursts). Lysate was serially clarified by centrifugation at 5000, 20,0000 and 100,000xg for 5, 20, and 80 min, respectively, and the final soluble fraction was defined as unfractionated cytosol. PI and PC transfer assays, unless otherwise stated, were performed at a standard temperature of 37°C as described by Aitken *et al* (17). Radiolabeled PI microsomes were prepared by *in vitro* enzymatic incorporation of [1,2-^{3}H(N)]inositol into PI as described by Paulus and Kennedy (18).

Ion Exchange Chromatography, Chemical Inhibition and Rat PI-TP Immunodepletion Experiments. DEAE-Sephacel chromatography employed a 26 x 2.5 cm column equilibrated in CB (5 mM sodium phosphate pH 7.4, 8 mM 2-mercaptoethanol and 3 mM NaN_3) running at a flow rate of 30ml/h. Column elution employed a 500 ml linear 0.0-0.3 M NaCl gradient in CB. One hundred microliters of each fraction were assayed for PI and PC transfer activity and relative protein

was determined by A_{280}. Quantitatative ELISA measurements were performed as described by Cleves *et al* (9). Anti-myc antibodies, an equal mixture of monoclonal antibodies Myc1-9E10.2 and CT14-G4.3 and rabbit polyclonal anti-rat PI-TP antibodies were used at 1.02 and 0.86 ug/ml, respectively for ELISAs.

Chemical inhibition of transfer activity involved addition of NEM (Sigma Chemical Co., St. Louis, MO)to cytosol to a final concentration of 2.5 mM immediately prior to initiation of the transfer reaction by addition of radiolabeled phospholipid.

For immunodepletion experiments, 22.2 mg anti-myc antibodies (Myc1-9E10.2 and CT14G4.3) plus 13.6 mg of affinity-purified anit-rat PI-TP and 11.5 mg anti-SEC14p antibodies were coupled to cyanogen bromide-activated Sepharose 4B (per ml swelled beads) as per manufacturer's instructions. Cytosol (1ml, 10 mg total protein) was incubated with the appropriately coupled beads at 4°C overnight with gentle, but constant, agitation. The beads were removed by centrifugation and PI transfer activity of the treated cytosol was determined as above. Protein was determined using the bicinhoninic acid (BCA) assay (Pierce Biochemical).

Biochemical Fractionation. Yeast strains were grown to logarithmic phase (0.8 OD_{600} units/ml) in appropriately supplemented minimal medium with glucose (2%) as carbon source, shifted to 37°C for 30 min and cycloheximide was added to the culture at 0.1 mg/ml. Following a 15 min incu-

bation at 37°C, cells were harvested and osmotic lysates were prepared and subsequently fractionated to yield the indicated supernatant, pellet and purified Golgi fractions as previously described by Cleves *et al* (9). Biochemical markers were followed by quantitative ELISA, as previously reported by Cleves *et al* (9), using affinity-purified polyclonal mouse anti-KEX2p serum (4ug/ml), polyclonal rabbit anit-SEC14p serum (6ug/ml) and polyclonal rabbit anti-rat PI-TP serum (4ug/ml). Secondary antibodies conjugated to horseradish peroxidase were used at 400 ng/ml for development of ELISAs.

Production of Anit-Rat PI-TP Antibodies in Rabbits. Plasmid pRE245, which contains the 0.8 kb rat PI-TP open reading frame was employed as template in a PCR reaction using oligonucleotides 5'-TATGGATCCGACATCGTCGATCGAAGC-CAA-3' and 5'-AAGAAGCTTAGCGCTAGTCATCTGCTG-3' as forward and reverse primers, respectively. The respective *BamHI* and *HindIII* restriction sites engineered into the oligonucleotides are underlined. The 386 bp PCR product was digested with *BamHI* and *HindIII* and subcloned into the *E. coli TrpE* expression vector pATH3 (from A. Tzagaloff). The resultant plasmid, pRE254, encodes a 467 residue fusion protein representing the 128 carboxy-terminal residues of the rat PI-TP. *E. coli* strain RRI (19) bearing pRE254 produced nearly 10 mg of fusion protein per liter culture after an expression period of 8 h in tryptophan-free medium supplemented with indoleacrylic acid (20ug/ml). The hybrid protein was found to reside in the supernatant of a 0.2% SDS, 50 mMTris-HCL pH 7.5

and 50 mM EDTA wash of the pellet fraction of total cell lysate. Purified protein (500 ug) was resolved by SDS-PAGE, the hybrid protein was excised from the gel, emulsified in an equal volume of Freund's complete adjuvant and injected into a New Zealand White rabbit via a subcutaneous route. A primary injection and two boosts were administered over a 2 month period.

Immunofluorescence. Immunofluorescence experiments were performed as previously described by Cleves *et al* with the following modifications (9). Cells were grown in the appropriately supplemented minimal media to mid-logarithmic growth phase, shifted to 37°C for 30 min, cyclohexamide was added to the culture at 0.1 mg/ml and following a 15 min incubation at 37°C, the cells were fixed *in situ* with a 20 min exposure to formaldehyde (4% final concentration). The cells were subsequently pelleted in a clinical centrifuge and resuspended in 50 mM potassium phosphate buffer (pH 6.7), adjusted to a final concentration of 1 mM $MgCl_2$ and 4% formaldehyde, and allowed to fix overnight at 4°C. The formaldehyde-fixed spheroplasts were deposited onto coverslips by 5 min centrifugation at 1000xg in a Cytospin centrifuge (Shandon Inc., PA). Cells were then dehydrated on the coverslips by 6 min incubation in ice-cold methanol, permeabilized by a 30-60 s rinse in cold acetone, treated with 0.1% Tween 20, 1% BSA phosphate buffered saline (blocking buffer) and incubated with mouse anit-KEX2p and rabbit anti-rat PI-TP antibodies in blocking buffer at concentrations of 44 and 49 ug/ml, respec-

tively. Spheroplasts were subsequently washed in blocking buffer and incubated with sheep anit-mouse antibodies (15ug-/ml) for 2 h to decorate further the bound anti-KEX2p antibodies. Finally, after extensive washing with blocking buffer, FITC-conjugated Donkey anti-sheep and Texas red-conjugated donkey anti-rabbit antibodies for 2 h at a concentration of 30 ug/ml each. After further washing in blocking buffer, the respective staining profiles were visualized with a Leitz Axioplan microscope equipped for epifluorescence with UV filters and an HBO 100 W mercury lamp.

RESULTS

Expression of the Rat PI-TP Structural Gene in Yeast. Initial attempts to express the rat PI-TP structural gene involved placing the corresponding cDNA under control of an inducible yeast Gal promoter. However, such constructs failed to support synthesis of the rat protein in yeast. This may be attributable to the rat PI-TP cDNA exhibiting a 200 nucleotide untranslated leader sequence (4). Thus, two SEC14-rat PI-TP hybrid genes (SrPI) were constructed that place expression of the rat PI-TP under the transcriptional and translation initiation controls of *SEC14* (Fig. 1). *SrPI-1* encodes the rat PI-TP with a six residue amino-terminal extension, whereas the *SrPI-1myc* gene product (SrPI-1myc) differs from SrPI-1 only in that it also contains a myc epitope tag at its amino terminus (20). To determine whether the hybrid *SrPI* genes directed the synthesis of polypeptides with the expected antigenic properties in yeast, lysates

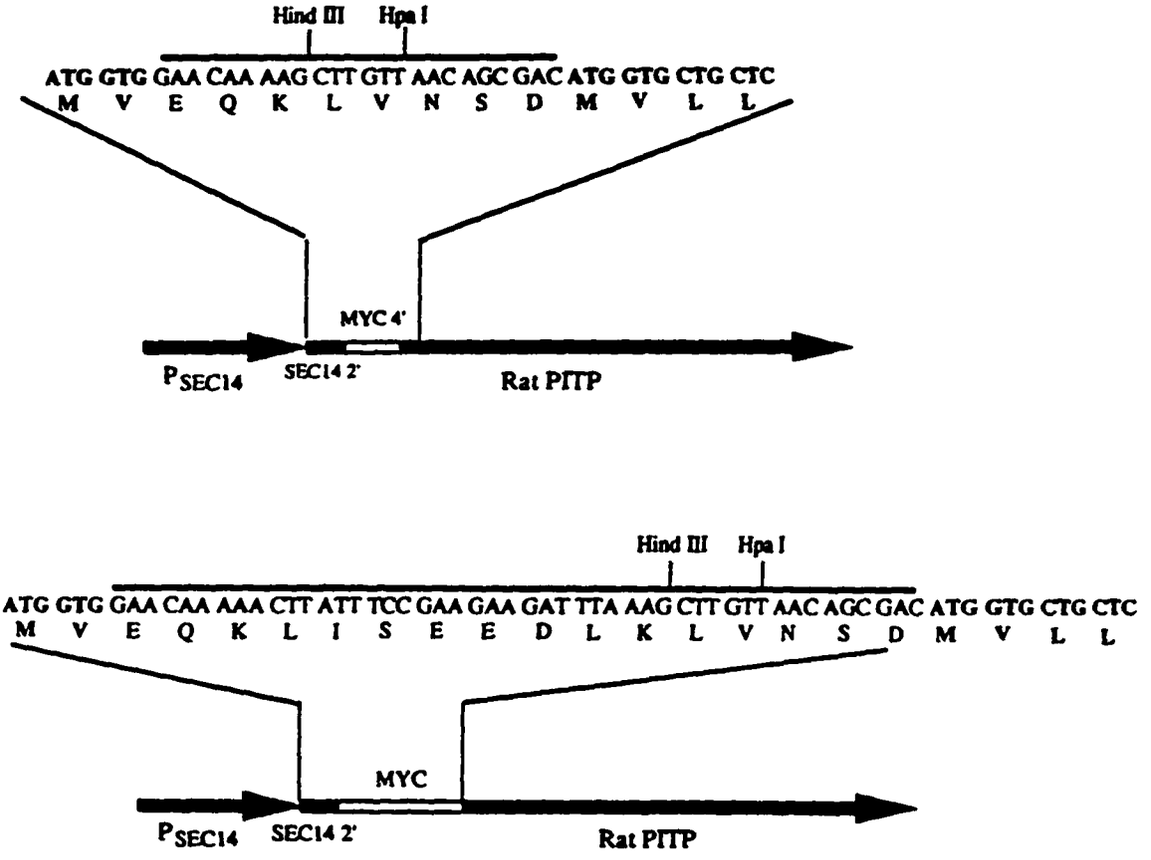


FIG. 1. Expression of SrPI-1 and SrPI-1myc in yeast. Two chimeras were generated that result in an in-frame fusion of the entire rat PI-TP cDNA coding sequence (in bold) to the first two *SEC14* codons (SEC14 2'; also in bold). In the case of the *SrPI-1* construct (top), an eight codon linker (MYC 4'; in plain type) was introduced between the *SEC14* and rat PI-TP codons during the construction. This linker contains single *Hpa I* and *Hind III* restriction sites as indicated. In the otherwise identical *SrPI-1myc* construct (bottom), this linker was expanded to 16 codons that encode the c-*myc*-derived EQKL-ISEEDL epitope (MYC; in plain type). For both *SrPI* constructs, transcriptional expression in yeast is under *SEC14* promoter control and translation initiation is also under *SEC14* control. Translation termination is provided by the rat PI-TP cDNA termination codon and transcriptional termination signals are presumed to be provided by dedicated *SEC14* sequences. These *SrPI* genetic constructs were subcloned into the yeast *URA3* multicopy plasmid vector pSEY18 to yield pSEY18(*SrPI-1*) and pSEY18(*SrPI-1myc*).

prepared from the *sec14-1^{ts}* yeast strains CTY1-1A, CTY1-1A/pSEY18(*SrPI-1*), and CTY1-1A/pSEY18(*SrPI-1myc*) were displayed by SDS-PAGE and immunoblotted with antibodies directed against the yeast vocuolar carboxypeptidase Y (CPY) and either affinity-purified antibodies directed against the rat PI-TP or a monoclonal antibody directed against the myc epitope. For all three lysates, a signal derived from the 61kD CPY antigen was apparent at approximately equal intensities and indicated a proper normalization of protein load for each lane (Fig. 2). Probing of these lysates with rat PI-TP antibody revealed new immunoreactive signals in lysates prepared from CTY1-1A/pSEY18(*SrPI-1*), and CTY1-1A/pSEY-18(*SrPI-1myc*), but not CTY1-1A. These rat PI-TP immunoreactive species exhibited apparent M_rs of ~34 and 36 kDa, respectively. These values were in good agreement with the expected M_rs for the SrPIs as authentic rat PI-TP exhibits an M_r of 35 kDa by SDS-PAGE (4). Immunoblotting of these lysates with the myc-directed monoclonal antibody CT14-G4.3 revealed an immunoreactive signal only in the CTY1-1A/pSEY-18(*SrPI-1myc*) lysate and this signal comigrated with the rat PI-TP immunoreactive species detected in that same lysate (Fig. 2). It is noted that the intracellular steady-state levels of the SrPI-1myc species were reproducibly 4 to 6 fold reduced relative to those of SrPI-1 (Fig. 2). These data demonstrate that by the criteria of molecular mass and an-tigenic properties, the *SrPI-1* and *SrPI-1myc* genes direct synthesis in yeast of the appropriate polypeptide species.

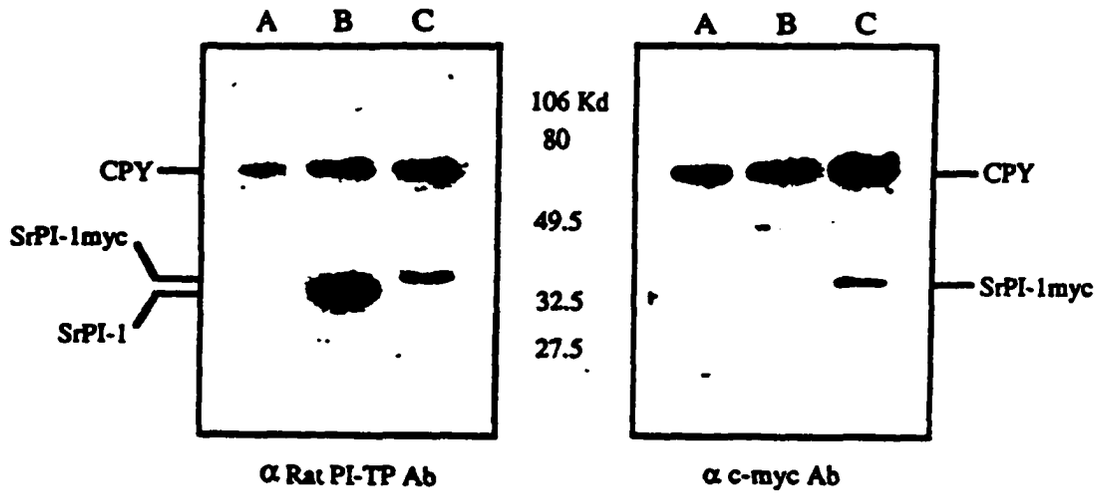
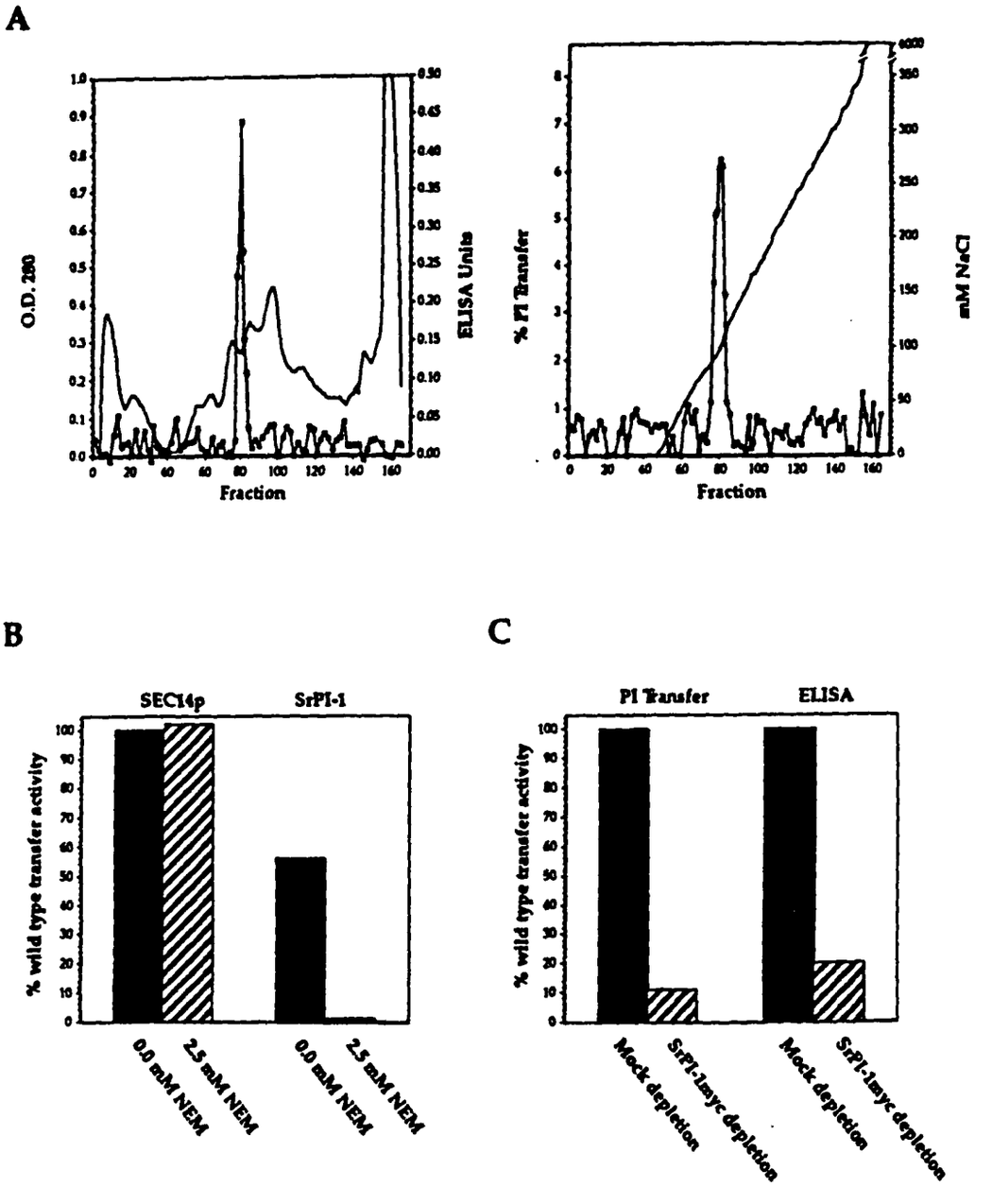


FIG. 2. Identification of *SrPI* gene products. Immunoblots of cell-free lysates prepared from the *sec14-1*^{ts} yeast strain CTY1-1A (lanes A), strain CTY1-1A carrying pSEY18(*SrPI-1*) (lanes B), or CTY1-1A carrying pSEY18(*SrPI-1myc*) (lanes C) were probed with antibodies to the yeast vacuolar proteinase CPY and either affinity-purified rabbit polyclonal antibodies directed against the rat PI-TP or the monoclonal antibody CT14-G4.3 directed against the c-*myc* epitope, as indicated. Immunoblots were developed using the enhanced chemiluminescence (ECL) system (Amersham).

SrPIs are Active PI-TPs in Yeast. To determine whether SrPI-1 and SrPI-1myc were active PI-TPs in yeast, we took advantage of strain CTY230 which is totally lacking in endogenous PI and PC transfer activity due to disruption of Sec14p (9). This strain survives the normally lethal consequences associated with the *sec14* disruption allele because it carries a suppressor *cki* allele that effects a complete bypass of the cellular requirement for Sec14p (9). Cytosol was prepared from strains CTY230/pSEY18(*SrPI-1*) and CTY230/pSEY18(*SrPI-1myc*), applied to a DEAE-Sephacel column and bound proteins were subsequently eluted with a linear NaCl gradient(see Materials and Methods). Fractions were then analyzed for PI-TP activity and myc-immunoreactive antigen. Representative data for CTY230/pSEY18(*SrPI-1myc*) are shown in Fig. 3*A*. A robust PI transfer activity was measured in CTY230/pSEY18(*SrPI-1myc*) cytosol prepared for the assay that eluted in a single sharp peak at ~90 mM NaCl. This peak coincided with the SrPI-1myc peak as determined by quantitative ELISA using either myc-directed (Fig. 3*A*) or rat PI-TP directed antibody probes (not shown). Moreover, PC transfer activity was also measured in a single peak that coeluted with the SrPI-1myc peak and essentially identical elution profiles were also obtained for PI-TP activity and rat PI-TP immunoreactivity from CTY230/pSEY18(*SrPI-1*) lysates fractionated in this manner (not shown). These data indicate that SrPI-1myc and SrPI-1 syn-thesis resulted in the elaboration of a novel PI-TP activity in yeast which eluted from an

FIG. 3. Biochemical characterization of SrPI-1myc PI-TP activity in yeast. (*A*) Cytosol prepared from a pSEY18-(*SrPI-1myc*) derivative of the SEC14p-deficient yeast strain CTY230 was applied to a DEAE-Sephacel column and washed with a linear 0-0.3 M NaCl gradient. Collected fractions (5ml) were assayed for PI-TP activity (open squares), SrPI-1myc antigen (ELISA units; solid squares), NaCl and total protein. PI-TP activity is given as percent radiolabeled PI transferred after correction for background (0.5-2% of total transfer). (*B*) Unfractionated cytosol prepared from the wild-type strain CTY182 or CTY230/pSEY18(*SrPI-1*) was assayed for SEC14p and SrPI-1 associated PI-TP activity, respectively, in the presence (solid bars) or absence (hatched bars) of NEM as indicated. PI transfer is expressed relative to wild-type cytosol (set at 100%). (*C*) Unfractionated cytosol prepared from CTY230/pSEY18(*SrPI-1myc*) was challenged with Sepharose 4B beads coupled either to anti-SEC14p immunoglobulin (mock depletion; hatched bars) of a mixture of anti-myc and anti-rat PI-TP immunoglobulin (SrPI-1myc depletion; solid bars), and the unbound fraction assayed for PI-TP activity and SrPI-1myc antigen, respectively. PI-TP activity is expressed relative to the activity of mock-depleted cytosol which is set at 100%. SrPI-1myc antigen quantitated by ELISA and is expressed relative to the amount of antigen present in mock-depleted cytosol which is set at 100%.



ion exchange column at low salt. The ion exchange elution profiles of SrPI-1 and SrPI-1myc associated PI-TP activities are consistent with those described for authentic rat PI-TP (4).

To determine whether SrPI-1 and SrPI-1myc are themselves active PI-TPs with biochemical properties of the rat PI-TP, we tested whether the novel PI-TP activities in SrPI-1 and SrPI-1myc producing strains were sensitive to challenge with the sulfhydryl alkylating agent *N*-ethylmaleimide (NEM) and whether these activities were sensitive to specific immunodepletion with SrPI-directed antibodies. As shown by the data in Fig. 3*B*, cytosol prepared from CTY230/pSEY18(*SrPI-1*) exhibited ~55% of the PI-TP activity of cytosol prepared from the wild-type strain CTY182. Only the SrPI-1 associated PI-TP activity was sensitive to NEM challenge, a characteristic of mammalian, but not yeast, PI-TPs (21). A similar NEM sensitivity was also recorded for the PI-TP activity of CTY230/pSEY18 (*SrPI-1myc*) which generally yielded a specific PI-TP activity that was ~15% of the activity measured for wild-type CTY182 cytosol (not shown). Finally, incubation of cytosol with beads coupled to a mixture of myc-directed and rat PI-TP directed antibodies led to an 8-fold reduction in the SrPI-1myc content of cytosol and a proportional (10-fold) reduction in cytosolic PI-TP activity (Fig. 3*C*). By contrast, mock challenge of CTY230/pSEY18(*SrPI-1myc*) cytosol with Sepharose 4B beads coupled to polyclonal SEC14p-directed antibodies neither depleted cytosol of SrPI-1myc antigen nor

reduced the PI-TP activity of cytosol. Taken together, the chemical inhibition and immunodepletion data unambiguously identify SrPIs as active PI-TPs that exhibit the biochemical characteristics of rat PI-TP.

SrPI Expression Results in a Specific Complementation of *sec14^{ts}* Growth and Secretory Defects. The threshold levels of Sec14p required for wild-type growth and secretory efficiency of yeast are 7- to 10-fold lower than the Sec14p levels of wild-type strains (22). Those measurements suggested to us that at least pSEY18(*SrPI-1*)-driven expression of PI-TP activity should complement *sec14*-associated growth and secretory defects if PI-TP activity reflected the essential functional property of Sec14p. To test this hypothesis, multicopy and centromeric yeast plasmids bearing either *SrPI-1* or *SrPI-1myc* were introduced into the *sec14-1^{ts}* strain CTY1-1A and the growth properties of the various transformants were tested. Phenotypic analysis clearly demonstrate that introduction of either the *SrPI-1* or *SrPI-1myc* genes on multicopy plasmids effected a dramatic complementation of the growth defect suffered by *sec14-1^{ts}* strains at 37°C (Fig. 4). Indeed, growth was restored to essentially wild-type rates. Analyses of the secretion efficiency of these strains similarly revealed a significant complementation of the *sec14-1^{ts}* secretory defect. The secretory efficiency of the parental *sec14-1^{ts}* strain was improved from 0.22 to ~0.70 for the SrPI-producing derivatives on a

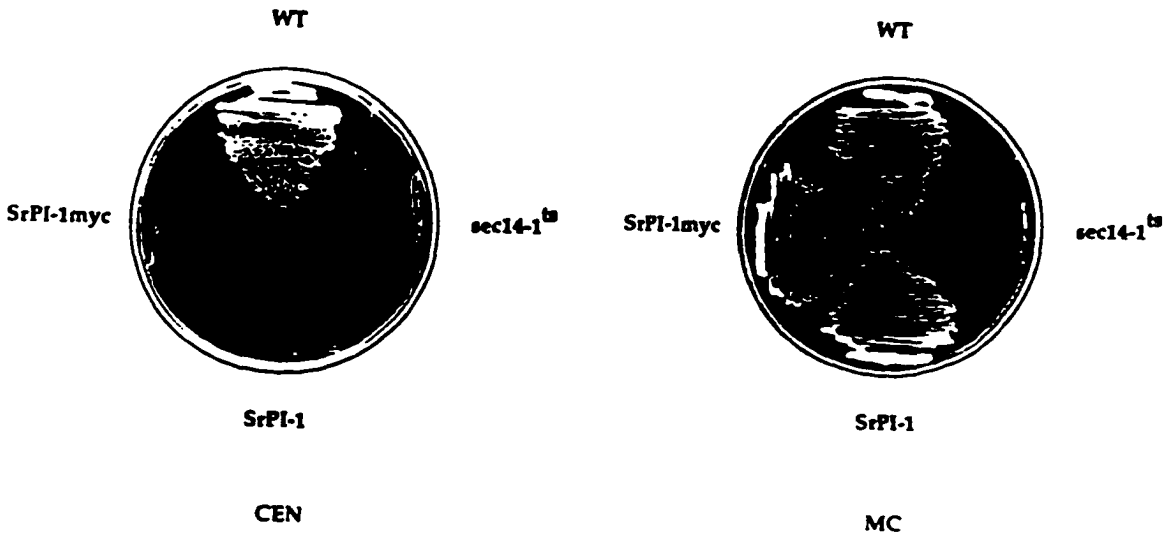


FIG. 4. SrPI expression complements *sec14^{ts}* associated growth and secretory defects. The wild-type yeast strain CTY182 (WT), the isogenic *sec14^{ts}* strain CTY1-1A and CTY1-1A carrying either *SrPI-1* or *SrPI-1myc* expression plasmids, as indicated, were streaked for isolation on YPD solid medium and incubated at 37°C for 48h. MC indicated that the corresponding *SrPI* constructs were carried by CTY1-1A in a multicopy configuration as pSEY18(*SrPI-1*) and pSEY18(*SrPI-1myc*), respectively. CEN denotes that the corresponding *SrPI* constructs were carried by CTY1-1A on the low copy yeast *URA3* centromeric vector pSEYc68 as pSEYc68(*SrPI-1*) and pSEYc68-(*SrPI-1myc*), respectively.

scale where wild-type secretory efficiencies approach 1.0 (See Fig. 5A). Moreover, the observed complementation was specific for *sec14-1^{ts}* as ex-pression of neither *SrPI-1* or *SrPI-1myc* from multicopy plas-mids effected any detectable suppression of yeast growth de-fects associated with *ts* alleles in any of the other 22 SEC genes tested (not shown). We also note that presentation of the *SrPI-1* or *SrPI-1myc* genes on centromeric vectors, configurations expected to reduce substantially the intracell-ular levels of the SrPIs (~10-fold) relative to those sustained by expression of *SrPI* genes from multicopy configurations, precluded complementation of *sec14-1^{ts}* (Fig. 4). This was a satisfying result as such reduction in *SrPI* gene dosage was expected to reduce *SrPI*-encoded PI-TP levels below those provided by threshold levels of Sec14p. Finally, meiotic segregation analyses and plasmid shuffle experiments revealed that *SrPI* gene expression from multicopy plasmids failed to rescue the lethality associated with *sec14* disruption mutations (not shown). Thus, the data indicate that *SrPI* expression was able to substitute partially for Sec14p function in yeast.

SrPI-Mediated Complementation of *sec14-1^{ts}* Does not Involve Stabilization of the *sec14-1^{ts}* Gene Product. The ability of *SrPI*-encoded PI-TP activity to complement *sec14-1^{ts}* but not *sec14* null mutations clearly indicated some contribution of the *sec14-1^{ts}* gene product to the complementation mechanism. One trivial mechanism for complementation could involve some general SrPI-mediated

stabilization of Sec14p^{ts} and a resultant resuscitation of Sec14p^{ts}-associated PI-TP activity at normally restrictive temperatures. To test this hypothesis, cytosol was prepared from the *sec14-1^{ts}* strain CTY1-1A/pSEY18(*SrPI-1myc*) that had been cultured for at least 20 generations at 37°C, a restrictive temperature for Sec14p^{ts}, and either challenged with NEM (2.5 mM) to inhibit any SrPI-associated PI-TP activity, or left unchallenged. PI-TP activity of challenged and unchallenged cytosol was then measured at 30°C and 37°C (permissive and restrictive conditions for Sec14p^{ts}, respectively). The results shown in Fig. 5*B* demonstrate that all PI-TP activity in either assay incubations was completely NEM-inhibitable. These data indicated that SrPI-1myc was the sole source of the PI-TP activity measured in the transfer assays run at both temperatures. These results make it unlikely that SrPI mediated complementation of *sec14-1^{ts}* simply occurs through some general stabilizing or otherwise amplifying effect on Sec14p^{ts} activity and suggest that both Sec14p^{ts} and SrPIs contribute independently to the mechanism of complementation.

SrPI Association with Yeast Golgi Membranes is Not a Simple Function of Their Phospholipid Binding Properties. Sec14p localizes as a peripheral yeast Golgi membrane protein that can be recovered in stable association with highly enriched yeast Golgi membranes (9). To determine whether SrPIs exhibit an affinity for yeast Golgi membranes we compared their intracellular dis-tribution with that of Sec14p and an integral membrane pro-tein of yeast

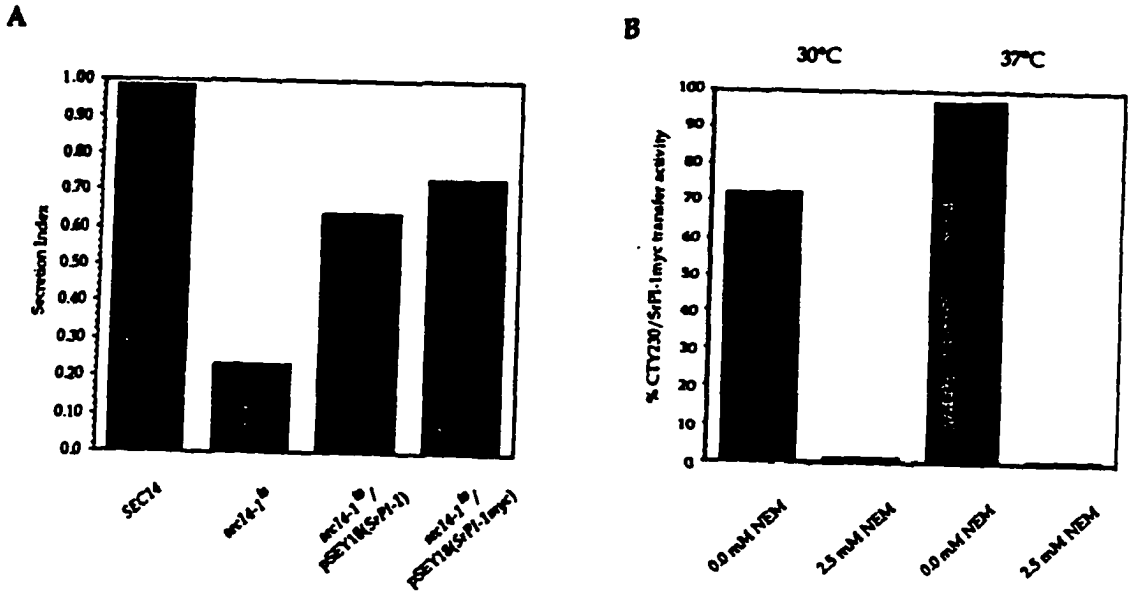
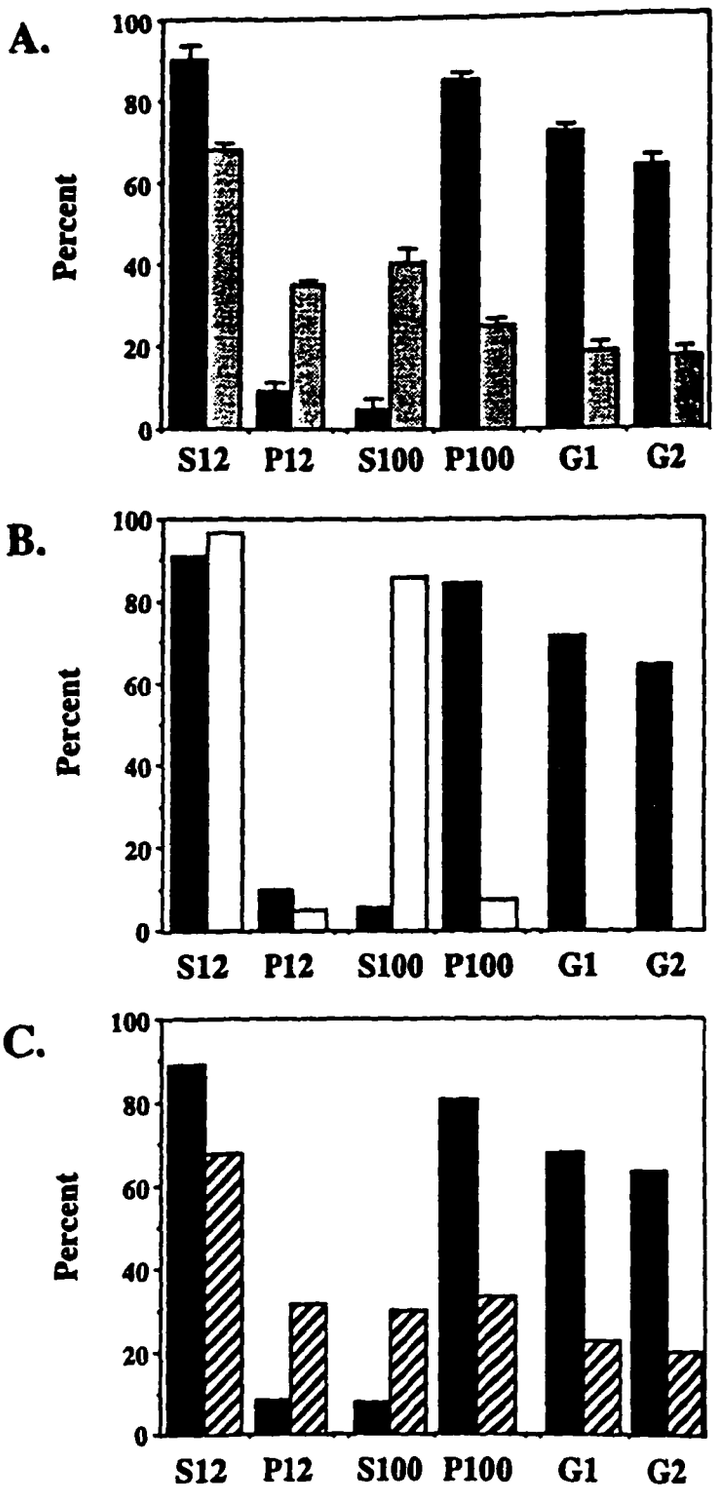


FIG. 5. SrPI expression does not detectably rescue Sec14p^{ts} associated PI-TP activity. (*A*) Isogenic yeast strain CTY182 (*SEC14*), CTY1-1A (*sec14^{ts}*), CTY1-1A/pSEY18-(*SrPI-1*) and CTY1-1A/pSEY18(*SrPI-1myc*) were cultured to early logarithmic growth phase at 30°C with shaking, shifted to YPD medium for 2 h under the same incubation conditions and finally shifted to YP medium (0.1% glucose) for 2 h at 37°C. The cells were subsequently harvested, and total and latent invertase activities were determined for each as described previously (13). From these values, the secretion indices, which reflect the efficiency of invertase secretion by these strains, were calculated and are displayed for each strain. Wild-type secretory efficiency yields a theoretical maximum secretion state of 1. (*B*) Unfractionated cytosol was prepared for the *sec14-1^{ts}* yeast strain CTY1-1A/pSEY18 (*SrPI-1myc*), which was grown to steady-state at 37°C and either treated with NEM (2.5 mM final concentration) or not treated, as indicated, and assayed for PI-TP activity at 30°C and 37°C. PI-TP specific activities are expressed relative to the PI-TP specific activity measured at 37°C for the SEC14p-deficient strain. The reference PI-TP specific activity is set at 100%.

Golgi (i.e. Kex2p). Similar results were obtained for both SrPI-1 and SrPI-1myc, and the latter data are presented here. Fractionation experiments indicated that SrPI-1myc fractionated very predominantly to cytosolic frac-tions prepared from yeast harboring a functional Sec14p (i.e. 90% of total cellular SrPI-1myc recovered in the 100,000 g supernatant fraction; not shown). These results raised the concern that endogenous Sec14p interfered with Golgi membrane binding by SrPI-1myc. As a result, we repeated these exper-iments in a SrPI-1myc-producing strain without the *SEC14* gene so as to eliminated any potential interference of SrPI-1myc binding to Golgi membranes by functional Sec14p. The dele-tion of *SEC14* in the SrPI-1myc-producing strain was made possible by the presence of a *cki* allele, which effects a bypass of the normally essential cellular requirement for Sec14p by specifically reducing the efficiency of PC synthesis via the CDP-choline pathway (9). These experiments utilized the congenic *cki* strains CTY393 and CTY303/pSEY18-(*SrPI-1myc*) cultured under the conditions described in Material and Methods. The representative data (Fig. 6*A*) show that a significant fraction of total Sec14p (60%) was associated with intracellular membranes prepared from the *cki* strains CTY393 (i.e. recovered from the P12 and P100 fractions). Of the 28% of total Sec14p measured in the P100 fraction, 75% was recovered in the G1 fraction and sub-sequently the G2 fraction, upon further resolution of the P100. G1 and G2 represent highly enriched Golgi fractions (defined as KEX2p

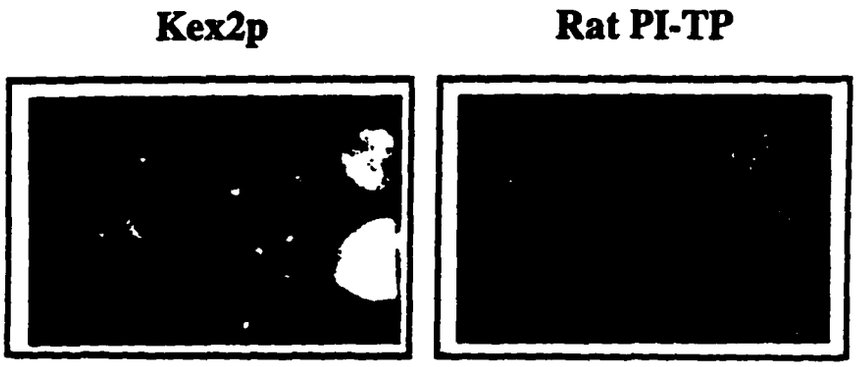
FIG. 6. Localization to yeast Golgi membranes is not dependent on PI-TP activity. Subcellular fractions were prepared from osmotic lysates derived from (*A*) strain CTY393 (*SEC14, cki*), (*B*) strain CTY303, its isogenic *sec14DP::hisG*, cki derivative, carrying pSEY18(*SrPI-1myc*) and (*C*) strain CTY303 carrying pSEY18(*SrPI-129myc*), by a sequential series of differential and equilibrium centrifugation as previously described (6). Fractions were evaluated for the integral Golgi membrane protein KEX2p (solid bars) and either Sec14p^{ts} (stippled bars), the catalytically active PI-TP SrPI-1myc (open bars) or SrPI-129myc that exhibits no catalytic PI-TP activity (cross-hatched bars), by quantitative ELISA. Differential fractions of a clarified 1000xg supernatant fraction of total osmotic lysate centrifuged at 12,000xg (S12 and P12) and the supernatant and pellet fractions of the S12 centrifuged at 100,000xg (S100 and P100). The G1 fractions were obtained by pooling the peak KEX2p fractions of a P100 membrane population resolved by equilibrium centrifugation in a 30-60% self-forming sorbitol gradient. The G2 fractions were obtained by pooling the peak KEX2p fractions of the G1 fraction resolved by equilibrium centrifugation on a 40-65% self-forming sorbitol gradient. Values for each of the markers are expressed as a percentage of the total measured in the original clarified osmotic lysate (set at 100%).



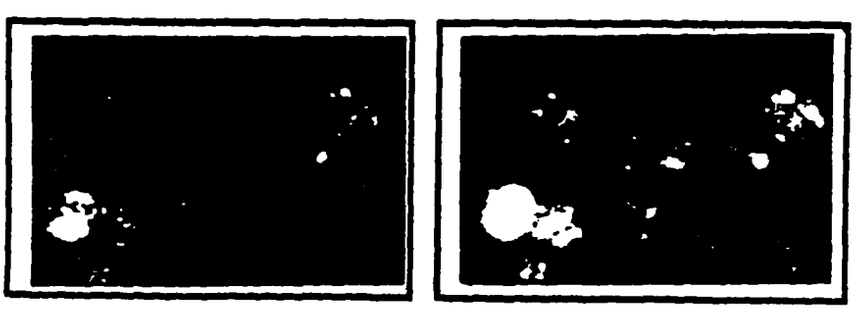
containing fractions), with G2 representing the most highly enriched Golgi fraction (9). These Sec14p and Kex2p fractionation profiles are in good agreement with those previously reported and indicate that the *cki* lesion had no effect on the Golgi targeting/binding properties of the Sec14p (9). The SrPI-1myc expressed in the *cki* yeast strain CTY303/pSEY18 (*SrPI-1myc*), however, fractionated as a cytosolic species. Only some 15% of total SrPI-1myc was found to be membrane-associated and the highly enriched Golgi fractions G1 and G2 exhibited only trace quantities of SrPI-1myc (Fig. 6*B*). This fractionation profile was observed regardless of whether cells were cultured at 30°C or 37°C (not shown) and these data indicated that in contrast to Sec14p, SrPIs do not exhibit a stable association with yeast Golgi membranes. We have also employed Δ*sec14, bsd2-1* mutant strains of yeast for these fractionation experiments with the same results recorded for the D*sec14, cki* yeast strain (not shown). The *bsd2-1* mutation effects bypass of Sec14p without detectably compromising cellular PC biosynthetic capability, thereby eliminating the possibility that SrPI-1myc localization is somehow sensitive to rates of PC synthesis via the CDP-choline pathway (9). Immunofluorescence studies provided further support for this conclusion as the SrPI-1myc staining profile for the *cki* strain employed for these fractionation experiments was of a diffuse cytosolic nature with no detectable colocalization of SrPI-1myc and Kex2p (i.e. yeast Golgi membranes) in any of the cells analyzed (Fig. 7*A*). We

FIG. 7. Immunofluorescence localization of SrPI-1myc and SrPI-129myc. Strain CTY303 expressing either (*A*) SrPI-1myc from pSEY18(*SrPI-1myc*) or (*B*) SrPI-129myc from pSEY18(*SrPI-129myc*) was prepared for immunofluorescence as described in Materials and Methods. The respective SrPI species were each stained with a primary rabbit anti-rat PI-TP polyclonal antiserum and secondary Texas red-conjugated donkey anti-rabbit antibodies. The yeast Golgi integral membrane protein Kex2p antiserum, a secondary polyclonal sheep anti-mouse antiserum, and a FITC-con-jugated tertiary donkey anti-sheep antiserum. The primary antibodies employed to yield the recorded staining patterns are indicated above the corresponding panel.

A. pSEY18 (*SrPI-1myc*)



B. pSEY18 (*SrPI-129myc*)



note, however, that these experiments cannot formally exclude some minor association of SrPI-1myc with Golgi membranes *in vivo*.

To investigate further the uncoupling of Golgi localization from Sec14p phospholipid binding/transfer activities we determined whether Sec14p primary sequence could direct a heterologous protein to yeast Golgi. To this end, we employed a Sec14-rat PI-TP gene fusion (designated *SrPI-129-mcy*), which encodes a chimera in which the amino-terminal 129 Sec14p residues have been fused to the rat PI-TP at its initiator Met residue (see Materials and Methods). The *SrPI-129myc* polypeptide is stable when expressed in yeast cells, is catalytically inactive as a PI-TP *in vitro* and is consequently unable to complement *sec14-1*^{ts} growth and secretory defects when expressed from multicopy configurations in such mutant strains (not shown). The representative data in Fig. 6*C* show that the SrPI-129myc produced in the CTY303/pSEY18 (*SrPI-129myc*) strain fractionated in a manner indistinguishable from that exhibited by authentic Sec14p and in a manner markedly dissimilar to that exhibited by SrPI-1myc (compare Fig. 6*A*, *B*, and *C*), Indeed, 20% of the total cellular SrPI-129myc was recovered in the most enriched Golgi fraction (G2), indicating a stable association of SrPI-129myc with yeast Golgi membranes in this Δ*sec14, cki* strain. As in the case of SrPI-1myc, the fractionation profile of SrPI-129myc was not affected by whether the experiments were performed in Δ*sec14, cki* or Δ*sec14, bsd2-1* genetic backgrounds or whether

the cells were cultured at 25°C or 37°C (not shown). Double label immunofluorescence experiments provided independent conformation of the fractionation data as the SrPI-129myc immunostaining profile exhibited both punctate and diffuse cytosolic components (Fig. 7*B*). The punctate aspect of SrPI-129myc staining coincided significantly with that recorded for the Golgi membrane protein Kex2p (Figure 7B). These data show that the amino-terminal 129 SEC14p residues are sufficient for Golgi targeting. One possible complication to the evidence for such a conclusion is that the heterologous reporter employed is a PI-TP (albeit a catalytically inactive one) that may nevertheless contribute significantly to the observed localization. We consider this possibility to be unlikely for two reasons. First, PI-TP activity is clearly insufficient to confer detectable yeast Golgi localization to a heterologous polypeptide *in vivo* (see above). Second, expression of the amino-terminal 129 SEC14p residues (i.e., the *sec14-129::HIS3* gene product) (6) as the sole SEC14p species in yeast indicates this truncated SEC14p to represent a stable polypeptide *in vivo* that exhibits no detectable PI-TP activity (9). Nevertheless, this truncated SEC14p colocalizes with KEX2p *in vivo* (not shown). Taken together, these data indicate: (*i*) that Golgi targeting can be effectively uncoupled from the phospholipid binding/transfer activity of Sec14p and (*ii*) that the amino-terminal 129 Sec14p residues are sufficient and necessary to direct a heterologous polypeptide to yeast Golgi.

DISCUSSION

Although the rat PI-TP and the yeast Sec14p share very similar catalytic properties from the standpoint of substrate specificity and substrate preference in the *in vitro* phospholipid transfer reaction (5), these polypeptides share no detectable primary sequence homology (4, 6). This conservation of biochemical function, in the absence of any structural similarity at the primary sequence level, provided an opportunity to address the question of whether the sole essential function of Sec14p *in vivo* was in some fashion represented by its PI-TP activity *in vitro*. If this were strictly the case, one might predict that expression of a catalytically active rat PI-TP in yeast should complement the growth and secretory defects associated with *sec14* defects. The data indicate that the two genetic constructions (i. e., *SrPI-1* and *SrPI-1myc*) that place the rat PI-TP structural gene under the transcriptional and translational control of the yeast *SEC14* gene drove expression in yeast of a novel PI-TP activity that exhibited the immunological and biochemical properties of rat PI-TP (Fig. 1, 2, and 3*A-C*). Expression of the *SrPI-1* and *SrPI-1myc* genes in yeast from multicopy plasmids yielded intracellular levels of PI-TP ac-tivity that exceeded those provided by the threshold levels of Sec14p required to sustain yeast cell growth (Fig. 3*B*, see above) and expression of such heterologous PI-TP levels was clearly sufficient to effect a dramatic complementation of both the yeast growth and Golgi secretory defects associated with

dysfunction of the *sec14-1^{ts}* gene product at the normally restrictive temperature of 37°C (Fig. 4 and 5*A*). Moreover, this complementation was specific as multicopy plasmid-directed expression of these same *SrPI* constructs failed to result in any phenotypic complementation of *ts* defects in 22 other SEC genes tested whose products are not known to be PI-TPs. Finally, reduction of *SrPI*-driven rat PI-TP levels in yeast using a centromeric plasmid that should reduce rat PI-TP levels below the threshold of PI-TP levels provided by Sec14p resulted in failure to complement the growth defects of *sec14-1^{ts}* strains (Fig. 4). The collective data indicate that expression of a mammalian PI-TP was able to relieve the growth and Golgi secretory defects suffered by yeast strains experiencing dysfunction of their endogenous Sec14p. As rat PI-TP and Sec14p share similar *in vitro* catalytic properties, but no primary sequence homology, these findings provide the first direct evidence that the PI-TP activity of Sec14p reflects some fundamental functional fea-ture of this poly-peptide. Indeed, this finding represents the first direct demonstration that the *in vitro* phospholipid transfer activ-ity is somehow relevant to the function of that protein *in vivo*.

One striking feature of the rat PI-TP mediated comple-mentation of *sec14* defects, however, was the inability of *SrPI-1* or *SrPI-1myc* expression to complement the lethality associated with *sec14* null mutations. These data clearly demonstrate that rat PI-TP cannot fully substitute for Sec14p

function in yeast and indicate that Sec14p^{ts} contributed in some fashion to the rat PI-TP mediated complementation of the defective gene. Since expression of rat PI-TP in *sec14-1^{ts}* yeast strains failed to detectably stabilize, or otherwise amplify, Sec14p^{ts} activity at nonpermissive temperatures (Fig. 5*B*), it is concluded that rat PI-TP and Sec14p^{ts} contribute independently to the rescue of efficient growth and Golgi secretory function in *sec14-1^{ts}* strains expressing the heterologous PI-TP activity. In this regard, we note that Sec14p^{ts} is not a completely nonfunctional species as its overexpression (a condition generated by providing yeast with multiple copies of the *sec14-1^{ts}* gene) results in phenotypic suppression of the growth and secretory defects of *sec14* mutants (not shown). This data is interpreted to indicate that while neither Sec14p^{ts} nor rat PI-TP can alone provide the threshold level of PI-TP activity at Golgi membranes required to support Golgi secretory function at 37°C, the combined activities of these two PI-TP species do.

There are several general possibilities as to why rat PI-TP failed to substitute completely for Sec14p *in vivo*. Perhaps rat PI-TP operates in yeast by an *in vivo* mechanism that is unrelated to that employed by Sec14p; their shared PI-TP activities notwithstanding. For example, one protein may genuinely function as a PI-TP *in vivo*, whereas the *in vitro* PI-TP activity of the other might indirectly reflect a specific phospholipid binding activity that is utilized in some other fashion *in vivo*. Alternatively, rat PI-TP may

function in a mechanisticly similar fashion to Sec14p, yet be rendered an incomplete surrogate by its inability to execute some other essential activity of Sec14p; perhaps a Golgi targeting capability that is a distinguishing property of Sec14p (9). Although these two general hypothesis are not mu-tually exclusive, the available data lead us to favor the latter possibility as rat PI-TP clearly exhibited a reduced affinity for yeast Golgi membranes relative to Sec14p (Fig. 6*A* and *B*). The demonstration that rat PI-TP was significantly less effective at associating with yeast Golgi membranes than is the endogenous Sec14p suggests that Sec14p localization to yeast Golgi membranes is not a simple function of some unique Golgi phospholipid domain recruiting and/or retaining Sec14p by virtue of its specific phospholipid binding properties. If this particular targeting mechanism were operative in cells, we would have expected rat PI-TP to localize in a manner similar to Sec14p since these PI-TPs share very similar phospholipid binding properties. Rather, some other, perhaps receptor-mediated, mechanism contributing to the localization of Sec14p to Golgi membranes is implied. This idea receives considerable experimental support from the demonstration that the amino-terminal 129 Sec14p residues efficiently and stably targeted a catalytically inactive rat PI-TP to yeast Golgi membranes *in vivo*, and that the truncated *sec14-129::HIS3* gene product (consisting of the fisrt 129 SEC14p amino acids) itself targeted to Golgi membranes *in vivo* (Fig. 6*C* and 7*B*). These data describe a clear

uncoupling of Sec14p Golgi localization from PI-TP catalytic activity.

Finally, our characterization of rat PI-TP expressing yeast strains has provided the first direct evidence to indicate that mammalian and yeast PI-TPs can play functionally similar roles in an *in vivo* system. This is an intriguing finding in light of the recent observation that mammalian PI-TP associates with the Golgi complex in rodent fibroblasts (23). Although the present data do not permit the extrapolation of what the functional roles of mammalian PI-TPs are in their cognate cells, these findings nevertheless provide a first step toward addressing that fundamental question. In this regard, the dramatic phenotype associated with *SrPI* mediated complementation of the *sec14* lesion provides for the first time a facile *in vivo* assay with which a comprehensive structure-function analysis of the function of a mammalian PI-TP can be undertaken.

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MUTANT RAT PHOSPHATIDYLINOSITOL/PHOSPHATIDYLCHOLINE TRANSFER PROTEINS SPECIFICALLY DEFECTIVE IN PHOSPHATIDYLINOSITOL TRANSFER: IMPLICATIONS FOR THE REGULATION OF PHOSPHOLIPID TRANSFER ACTIVITY

by

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ABSTRACT

The mammalian phosphatidylinositol/phosphatidylcholine transfer proteins (PI-TPs) catalyze exchange of PI or PC between bilayers *in vitro*. We find that Ser-25, Thr-59, Pro-78, and Glu-248 make up a set of rat PI-TP residues, substitution of which effected a dramatic reduction in the relative specific activity for PI transfer activity without significant effect on PC transfer activity. Thr-59 was of particular interest as it is a conserved residue in a highly conserved consensus protein kinase C phosphorylation motif in metazoan PI-TPs. Replacement of Thr-59 with Ser, Gln, Ile, Asn, Asp, or Glu effectively abolished PI transfer capability but was essentially silent with repect to PC transfer activity. These findings identify rat PI-TP residues that likely cooperate to form a PI head-group binding/recognition site or that lie adjacent to such a site. Finally, the selective sensitivity of the PI transfer activity of rat PI-TP to alteration of Thr-59 suggests a mechanism for *in vivo* regulation of rat PI-TP activity.

INTRODUCTION

All eukaryotic cells harbor cytosolic phospholipid transfer proteins (PL-TPs) that can transport lipid monomers between membrane bilayers *in vitro* (1-3). The phosphatidylinositol transfer proteins (PI-TP's) represent an interesting class of PL-TPs in that PI-TPs can utilize either PI or phosphatidylcholine (PC) as transfer substrates and they also define two highly conserved protein families. The

mammalian PI-TPs are 35 kD proteins that share a very high degree of primary sequence identity (4-6). Yet another PI-TP homolog is represented by the *Drosophila* RdgB protein, an integral membrane protein with an N-terminal rat PI-TP like domain (42% identity with the mammalian PI-TP) that catalyzes PI transfer *in vitro* when expressed as a soluble polypeptide (7). The fungal Sec14 proteins are also 35kD in molecular mass and are highly homologous to each other (8-11) but do not resemble metazoan PI-TPs (5, 8). While the evidence suggests that Sec14p functions in *Saccharomyces cerevisiae* as a PL sensor that controls the PC content of yeast Golgi membranes by regulating the activity of the CDP-choline pathway for PC biosynthesis (12-14), the *in vivo* function of mammalian PI-TPs is unresolved. It is widely assumed that the PI and PC binding/transfer activities of metazoan PI-TPs are somehow relevant to *in vivo* function. However, as there exists no understanding of how PI-TP executes or regulates its PL binding/transfer activities, the necessity for a functional analysis of the PL transfer activities of metazoan PI-TP is emphasized.

Herein, the uncoupling of the PI and PC transfer activities of both the rat and *Drosophila* (RdgBp) PI-TP is described and the genetic and biochemical data that identify at least one consensus protein kinase C (PKC) phosphorylation site in rat PI-TP (Thr-59) as a structural element required for efficient PI transfer are reported. Finally, the data

suggest a means by which PI-TP activity could be regulated in mammalian cells.

MATERIALS AND METHODS

Yeast Strains, Plasmids, Media, and Transformation. *Saccharomyces cerevisiae* CTY182 (*Mata ura3-52 Dhis3-200 lys2-801*); CTY1-1A (*Mata ura3-52 Dhis3-200 lys2-801 sec14-1^{ts}*); CTY303 (*Mata ura3-52 Dhis3-200 cki sec14D-P::hisG*) and the YEp (*URA3, SrPI-1*) plasmid, pCTY161, and basic yeast methods have been described (8, 15-17).

Random Mutagenesis of the SrPI-1 Expression Construct. Random chemical mutagenesis employed hydroxylamine (18). Aliquots of mutagenized DNA were transformed into *Escherichia coli*. MC1066. Ampicillin-resistant transformants were selected and replica plated onto M9 minimal medium lacking uracil to assess the frequency of noncomplementation of the *pyrF::*Tn5 uracil auxotrophy. Base M9 minimal medium has been described (19). Suitably mutagenized DNA aliquots (3-5% Ura-plasmids) were used to transform yeast strain CTY1-1A to Ura+ at 25°C, and transformants that grew at 25°C but failed to grow at 37°C were rescreened for the temperature-sensitive phenotype. Those temperatures-sensitive mutants whose growth defect at 37°C was not uracil-remedial were kept for analysis.

Phospholipid Transfer Assays. Yeast cytosol was prepared, and PI and PC transfer assays were performed as described (15, 20, 21). Quantitative ELISAs were performed by using a direct sandwich assay with polyclonal rabbit anti-

rat PI-TP serum directed against the C-terminal 128 residues of rat PI-TP (12, 13). Secondary mouse anti-rabbit antibodies conjugated to horseradish peroxidase were used for development of signal in the presence of *o*-phenylenediamine. After quenching, A_{450} was measured on an EL311sx automated microplate reader.

Expression of the soluble RdgB-PITP domain in *E. coli* was achieved by inoculating 1 l of SuperBroth with the appropriate strain to be used in transfer assays with a 1/100 dilution of an overnight culture. The sample was grown to an OD_{600} of 0.3 at 37°C then IPTG was added to a final concentration of 1 mM and the cultures allowed to incubate for an additional 3 h. Cells were harvested by centrifugation, washed in cold lysis buffer (50 mM $NaPO_4$ [pH 7.1], 300 mM NaCl, 1 mM NaN_3, 0.2 mM PMSF, and 5 mM EDTA), and pellets were resuspended in 10 ml of cold lysis buffer containing 1/2 volume of 0.1 mm glass beads (Bio-Spec Products). Samples were vigorously vortexed seven times in 1 min bursts with cooling on ice between bursts. The lysates were clarified by serial centrifugation for 5 min at 2,000 x g, 20 min at 20,000 x g, and finally 60 min at 100,000 x g to yield the final cytosol fraction. Total protein concentration of the cytosol fraction was determined using the BCA assay (Pierce Biochemicals).

Nucleotide Sequence Analysis. Sequencing of the SrPI-1 mutants was performed by the chain-termination method

(22) by using double-stranded plasmid DNA as template and the Sequenase version 2.0 sequencing kit (Amersham).

Site Directed Mutagenesis. To mutagenize codon 59 of rat PI-TP, pCTY161 was digested with Hpa I and Bam HI to liberate the 0.8 kb rat PI-TP coding sequence. This fragment was cloned into the SK Bluescript plasmid (Stratagene) to yield pRE547. Single-stranded DNA was prepared and mutagenized as described (23) by using synthetic primers to effect the T59 substitutions. Mutants were confirmed by nucleotide sequence analysis and the mutagenized Hpa I-Bam HI cassettes were cloned into the respective half-sites of pCTY161.

The vector pTVh1 (23), which contains the RdgB-PITP domain, was digested with Xho I and Hind III to liberate the 1.2 Kb restriction fragment and subcloned into the pBluescript SK vector (Stratagene) to yield pSKPT. Single-stranded DNA was subsequently prepared and mutagenized (21) using the mutagenic primer 5'-GGTAATGGTCAATACGCAAAGAAAATCTA-TCACGT-GGG- 3' to affect the T59A substitution (mutagenized codon is underlined). The other mutations were generated in an identical fashion by substituting the underlined positions to affect the T59E, T59I, and T59D mutations. After sequence analysis determined each mutation was correct and unique, the mutagenized PI-TP was cloned into the T7 RNA polymerase/-promoter vector pT7-5 (Tabor, Harvard Medical School) via PCR techniques that introduced unique 5' Eco RI and 3' Hind III sites. The subsequent clones were confirmed correct by

sequence analysis and transformed into the *E. coli* strain BL21(DE3) (Novagen).

RESULTS

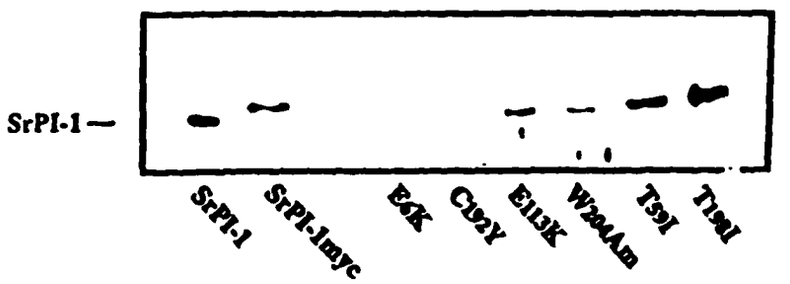
Isolation and Characterization of Mutant Rat PI-TPs That Fail to Rescue *sec14-1^{ts}*. Expression of rat PI-TP in yeast effects a phenotypic rescue of *sec14-1^{ts}* mutants at 37°C, and the rat PI-TP engineered for such expression is referred to as the SrPI-1 protein (15). From 6000 mutagenized SrPI-1 expression plasmids, we identified 31 that genuinely failed to rescue growth of *sec14-1^{ts}* yeast at 37°C. Nucleotide sequence analysis of these 31 mutant plasmids indicated that 21 of the corresponding mutations mapped within the SrPI-1 structural gene (Fig. 1A). Immunoblot analyses identified three mutant categories: (*i*) seven mutants that either failed to express detectable levels of SrPI-1 antigen or expressed greatly reduced levels of SrPI-1 antigen, (*ii*) three mutants that expressed reduced levels of full-length SrPI-1 (10-40% of wild-type levels), and (*iii*) six mutants that expressed substantially wild-type (>40%) levels of full-length SrPI-1 (Figure 1). In this collection, we noted five missense mutations that exerted either wholesale (E6K, P12S, or C192Y) or considerable (E113K or H85Y) destabilizing effects on SrPI-1. These five mutations likely identify residues that either play important roles in the rat PI-TP folding pathway or are required for the maintenance of stable rat PI-TP tertiary structure. The *rpi57* allele represented a peculiar case of significant translational

FIG. 1. Primary sequence of SrPI-1 mutants that fail to rescue yeast *sec14-1^{ts}* growth defects at 37°C. (*A*) The 21 mutations that render SrPI-1 incapable of rescuing yeast *sec14-1^{ts}* growth defects at 37°C identify 16 unique *rpi* (rat PI-TP) alleles. Each *rpi* mutation is identified by the corresponding base change involved, by the codon affected, and by the corresponding amino acid substitution or termination mutation introduced at the affected codon. The *rpi* mutations are also compiled as a function of the relative steady-state levels of their respective products compared to the steady-state levels of their respective products compared to the steady-state levels of the wild-type SrPI-1. These values were estimated by immunoblot analysis with the anti-rat PI-TP serum and by quantitative ELISA. (*B*) Immunoblot analysis of the relative steady-state levels of representative *rpi* gene products. Clarified extracts were prepared from yeast strain CTY303 carrying the appropriate YEp (*rpi, URA3*) plasmids that were cultured in uracil-deficient medium to midlogarithmic growth phase at 25°C. Lysates (10 *ug* protein) were individually resolved by SDS/PAGE. Immunoblots were developed by using the enhanced chemiluminescence (ECL) system (Amersham). SrPI-1 is indicated at left, and the corresponding mutational alterations are indicated at bottom. Am designates an amber mutation.

A

Mutant *rpi* allele	*rpi* Mutation	Relative Protein Level
rpi-11	R146 (CGA) --> Nonsense (TGA)	< 10%
rpi-31 *rpi-35*	R74 (CGA) --> Nonsense (TGA)	
rpi-36 *rpi-54*	E6 (GAA) --> K (AAA)	
rpi-43 *rpi-48*	E66 (CAG) --> Nonsense (TAG)	
rpi-53	E188 (CAG) --> Nonsense (TAG)	
rpi-59	P12 (CCT) --> S (TCT)	
rpi-65	C192 (TGT) --> Y (TAT)	
rpi-17	E113 (GAA) --> K (AAA)	10% - 40%
rpi-57	W204 (TGG) --> Nonsense (TAG)	
rpi-58	H48 (CAT) --> Y (TAT)	
rpi-13	P78 (CCA) --> L (CTA)	> 40%
rpi-15	T198 (ACA) --> I (ATA)	
rpi-22 *rpi-34*	T91 (ACA) --> I (ATA)	
rpi-38 *rpi-66*	H66 (CAC) --> Q (CAG)	
rpi-46	E244 (GAA) --> K (AAA)	
rpi-52	S23 (TCT) --> F (TTT)	

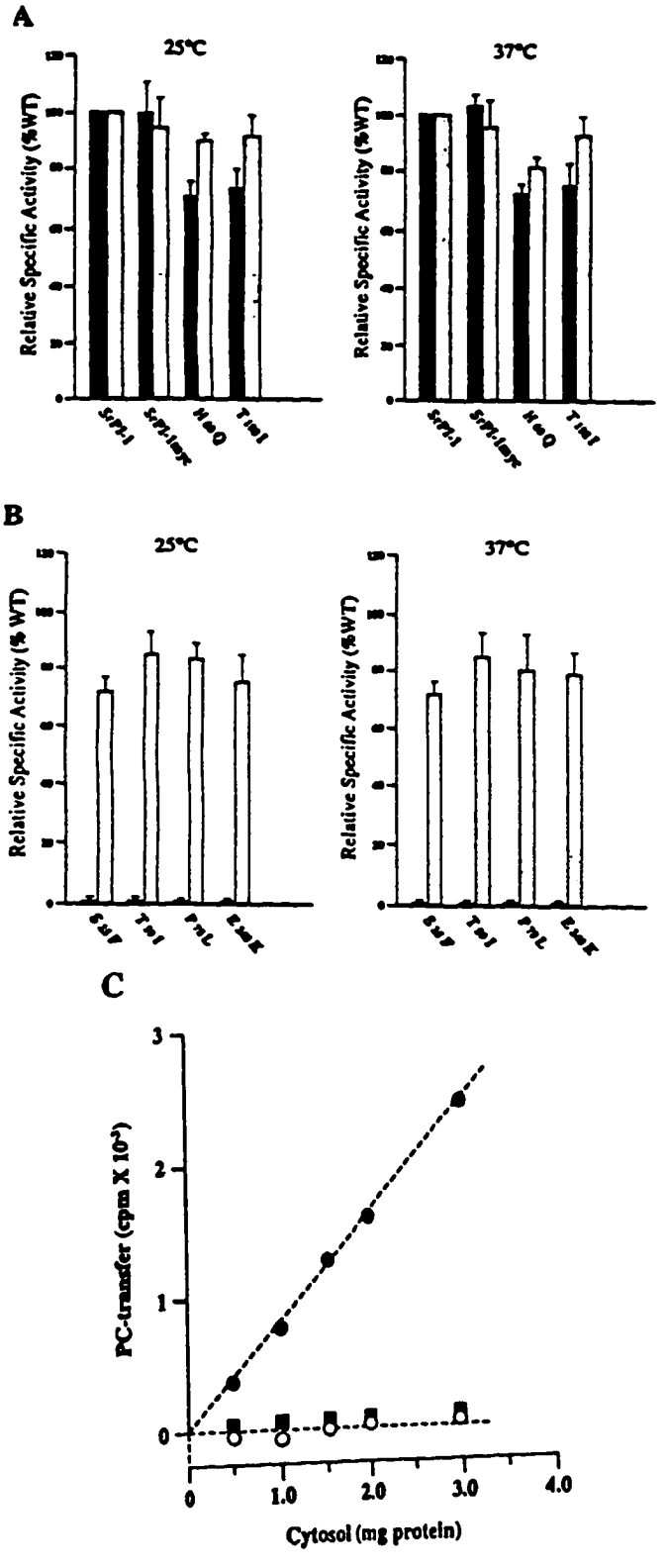
B



read-through across a termination codon. Finally, the six remaining dys-functional SrPI-1 (i.e., S25F, T59I, H60Q, P78L, and E248K) were expressed at steady-state levels approaching those of SrPI-1 and represented the most interesting category of mutants, henceforth referred to as SrPI-1*.

SrPI-1 Mutants Exhibit Defects in PL Transfer Activity. Analysis of the PL transfer properties of the six SrPI-1* identified two mutant classes. Class I SrPI-1* (H60Q and T198I) exhibited relative specific activities for PI and PC transfer at 25°C and 37°C that were at least 70% of the parental SrPI-1 specific activities measured at those temperatures (Fig. 2*A*). It is not obvious why these mutants failed to complement *sec14-1^{ts}* defects. While both mutations reduced the expression of bulk PI and PC transfer activities in yeast relative to those sustained by SrPI-1 (i.e., H60Q and T198I PI-TP cytosols exhibited 63 +/- 3.8% and 56.4 +/- 4.1% of the bulk PI transfer activity for SrPI-1 cytosol at 37°C and 65.2 +/- 3.1% and 63.3 +/- 6.1% of the bulk PC transfer activity measured for SrPI-1 cytosol at 37°C), the bulk PI and PC transfer activities measured for H60Q and T198I cytosols nevertheless exceeded those measured for SrPI-1myc cytosol (Fig. 2*A*). Yet, SrPI-1myc expression phenotypically rescues *sec14-1^{ts}*. These results suggest that rescue of *sec14-1^{ts}* growth and secretory defects by SrPI-1 expression may not solely rely on the ability of SrPI-1 to effect PI or PC transfer in yeast.

FIG. 2. SrPI-1* mutations define two biochemical classes of mutants that are distinguished by their effects on the PL transfer properties of rat PI-TP. (*A*) Cytosolic fractions prepared from derivatives of strain CTY303, a strain lacking endogenous PI/PC transfer activity, carrying isogenic YEp plasmids driving individual expression of the wild-type SrPI-1, SrPI-1myc, and the indicated SrPI-1*. The corresponding cytosols (1 mg of cytosolic protein) were assayed for PI (solid bars) and PC (stippled bars) transfers at 25°C and 37°C. The specific activities for PI and PC transfers were determined for each SrPI-1 species by dividing the percent radiolabeled PL transferred during the experiment by the amount of SrPI-1 antigen detected in the cytosol preparations by specific ELISA. The percent radiolabeled PL transferred was corrected by subtraction of background (determined by mock transfer reactions using buffer as input sample). Cytosol (1 mg) from strain CTY303 exhibited PI and PC transfer efficiencies that were indistinguishable from mock transfer reactions. Relative SrPI-1 specific activities were calculated by comparing the specific activities of SrPI-1myc and SrPI-1* cytosols to that measured for SrPI-1 cytosol (set to 100%). Transfer activities were linear with respect to protein and up to levels of 25% and 15% transfer of PI and PC, respectively. Determinations represent the average obtained from at least three experiments. (*B*) Relative PI (solid bars) and PC (stippled bars) transfer activities of the indicated SrPI-1* were determined as described in panel A. Determinations represent the average obtained from at least three experiments. (*C*) The indicated amount of cytosol prepared from *E. coli* (solid squares), yeast strain CTY303 (open circles), and a T59I SrPI* expressing derivative of CTY303 (solid circles) were assayed for PC transfer at 37°C. The transfer values were corrected for background as determined by mock transfer reactions using buffer as input sample. Only the T59I strain exhibited measurable PI transfer activity.



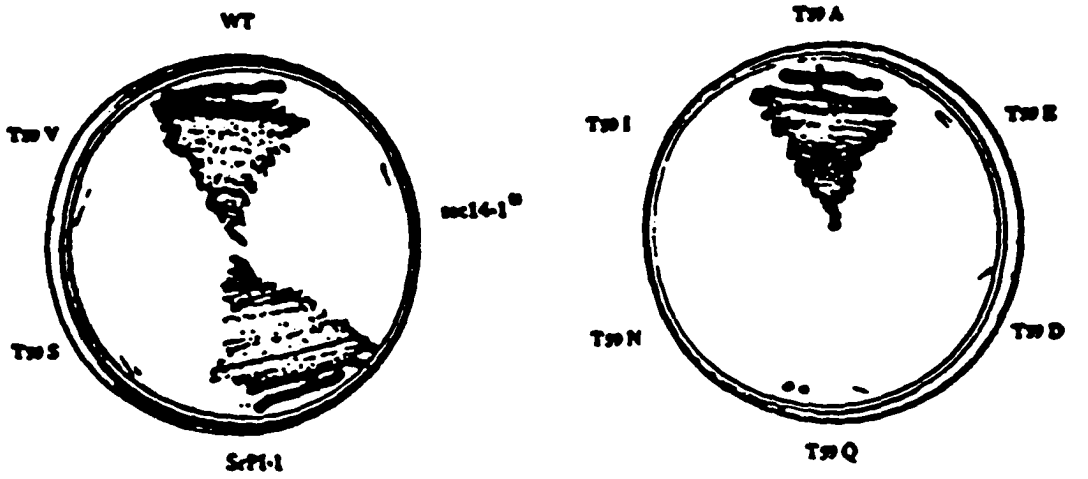
The class II mutants (S25F, T59I, P78L, and E248K) exhibited no detectable PI transfer activity at either 25°C or 37°C (<0.3% SrPI-1 specific activity; Fig. 2*B*). In contrast, the relative specific PC transfer activities measured for these SrPI-1* at both temperatures always exceeded 70% of that measured for SrPI-1 (Fig. 2*B*). That the PC transfer activities measured in these mutant cytosols were derived from the SrPI-1* was indicated by the demonstration that isogenic yeast cytosol devoid of SrPI-1* failed to exhibit PC transfer activity in excess of background or of that measured for *E. coli* cytosol (Fig. 2*C*). The magnitude of the PI transfer defects recorded for the class II SrPI-1*, in the face of substantially wild-type PC transfer capability, demonstrated that (*i*) class II mutations did not result in global SrPI-1 folding defects and (*ii*) PI transfer defect was not merely attributable to enhanced proteolysis of SrPI-1* relative to SrPI-1 in cell free lysates. Finally, the remarkable defects in the specific activity of PI transfer measured for the class II SrPI-1* were not the result of modest reductions in steady-state SrPI-1* levels, thereby diminishing SrPI-1* associated PI transfer activity to levels below the sensitivity of the assay. Titration experiments demonstrated the (*i*) PI transfer activity was linear between 100 *ug* and 2.5 mg of wild-type SrPI-1 cytosol per assay (i.e., the PI transfer assay recorded activities that were at least 10-fold reduced relative to standard assays) and (*ii*) addition of 3 mg of class II SrPI-1* cytosol to the PI

transfer assay still failed to generate detectable activity (not shown). Finally, it is noted that two of the four class II SrPI-1* mutations (T59I and E248K) involved residues positioned within or adjacent to consensus PKC phosphorylation sites in SrPI-1 (Fig. 1*A*).

PI Transfer Activity of Rat PI-TP is Exquisitely Sensitive to the Replacement of Thr-59. The specific PI transfer defect associated with T59I indicated a sensitivity of the PI transfer activity to the nature of the amino acid side chain at position 59 of rat PI-TP. Interestingly, Thr-59 is an invariant residue among both the mammalian PI-TPs and *Drosophila* RdgBp, and it resides in a consensus PKC motif that is itself highly conserved among these PI-TPs (4-7). Thus, we surveyed PI transfer activity as a function of a range of missense mutations at residue 59. The T59V, T59S, T59D, T59E, T59Q and T59N mutants uniformly behaved as class II SrPI-1* with regard to (*i*) their inabilities to phenotypically rescue *sec14-1^{ts}* growth defects at 37°C (Fig. 3*A*), (*ii*) the similarities of their steady-state protein levels to that of SrPI-1 as determined by immunoblot analysis (not shown), and (*iii*) their specific PI transfer defects in the face of near wild-type PC transfer capability at both temperatures (Fig. 3*B*). The class II SrPI-1* phenotype of the T59S mutant emphasized the sensitivity of SrPI-1 catalyzed PI transfer to the identity of the amino acid at position 59 as T59S repre-sents a conservative substitution. Moreover, the finding that the T59D and T59E mutations

FIG. 3. PI transfer activity of SrPI-1 is selectively attenuated by mutations involving Thr-59 of SrPI-1. (*A*) Biological activity of SrPI-1 in yeast is sensitive to alterations at Thr-59. The wild-type strain CTY182 (WT), the isogenic *sec14-1*^{ts} strain CTY1-1A, and CTY1-1A derivative strains expressing the indicated Thr-59 SrPI-1* were streaked for isolation on YPD solid medium and incubated at 37°C for 48 h. (*B*) Relative specific activities for PI and PC transfer associated with SrPI-1, SrPI-1myc, and the indicated Thr-59 SrPI-1* were determined. The relative specific activities measured at the indicated temperatures are identified. Determination represents the averages from at least three experiments.

A



B

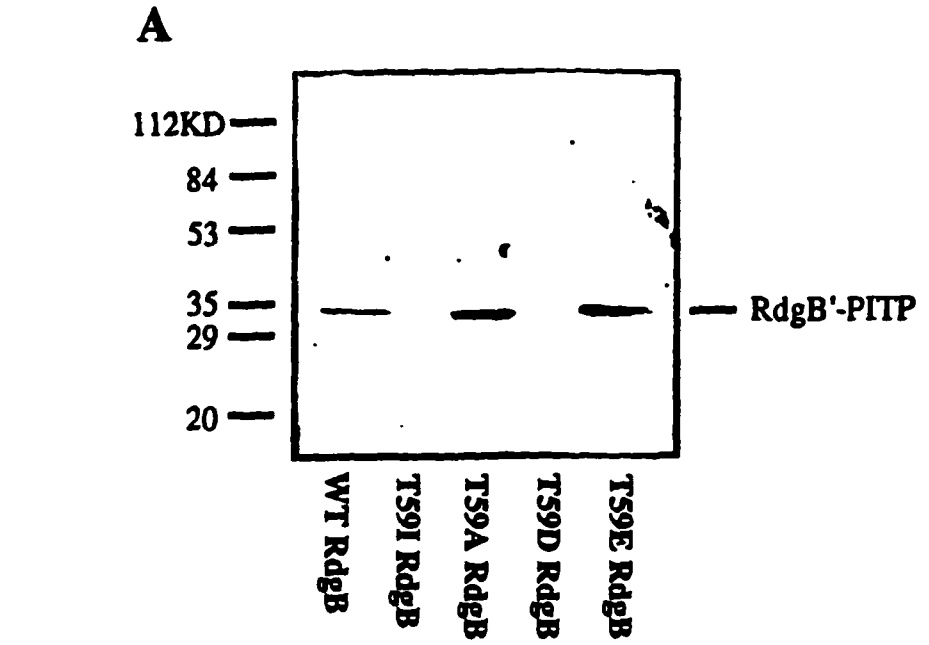
	25°C		37°C	
Allele	PI-Transfer	PC-Transfer	PI-Transfer	PC-Transfer
SrPI-1	100	100	100	100
SrPI-1myc	99 ± 11	95 ± 11	104 ± 4	96 ± 9
T59V	<0.3 ± 0	87 ± 8	<0.3 ± 0	92 ± 6
T59E	<0.3 ± 0	77 ± 3	<0.3 ± 0	78 ± 3
T59D	<0.3 ± 0	92 ± 4	<0.3 ± 0	95 ± 6
T59S	<0.3 ± 0	82 ± 3	<0.3 ± 0	85 ± 3
T59N	0.5 ± 0	101 ± 3	<0.3 ± 0	104 ± 7
T59Q	0.3 ± 0	90 ± 5	<0.3 ± 0	95 ± 6
T59A	90 ± 4	77 ± 3	55 ± 4	82 ± 1

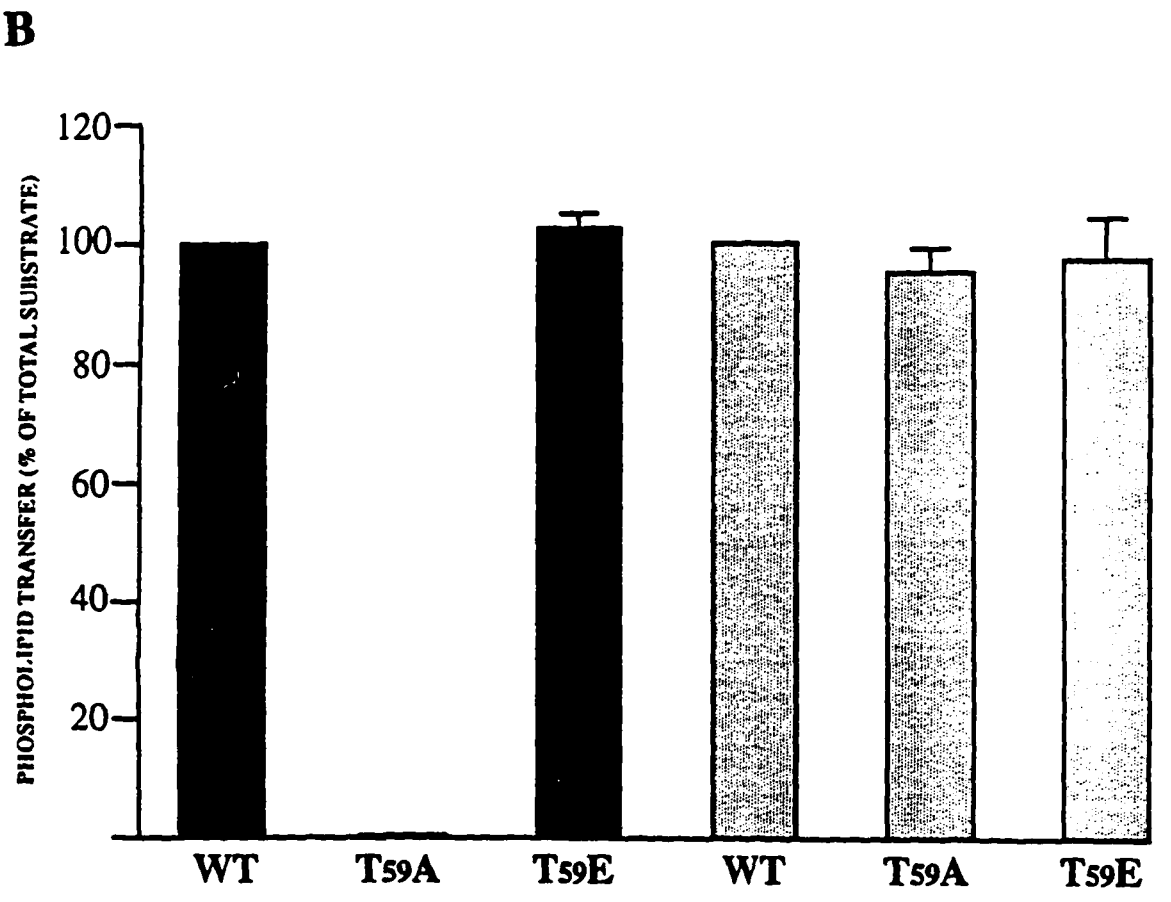
selectively inactivated PI transfer activity suggests that phosphorylation of Thr-59 will also specifically inactivate the PI transfer activity of rat PI-TP.

One exceptional case was encountered. The T59A SrPI-1* supported phenotypic rescue of *sec14-1^{ts}* (Fig. 3*A*), and the T59A SrPI-1* cytosol exhibited PI and PC transfer activities *in vitro* (Fig. 3*B*). The specific PI transfer activity of this SrPI-1* was only 50 +/- 4% of that recorded for SrPI-1, however, while the specific activity for PC transfer was considerably less affected (Fig. 3*B*). Thus, while Thr-59 was not obligatorily required for SrPI-1 to effect significant PI transfer, the T59A mutation nevertheless effected a specific attenuation of PI transfer activity.

RdgB PI-TP Domain PL Transfer Activity Resembles, But Does Not Mimic, That of the Mammalian PI-TPa Gene. It had been previously established that the PI-TP domain of the *Drosophila* RdgBp exhibited PI transfer activity (7), however, its ability to transfer the ligands PC or sphingomyelin (SM, as can the mammalian PI-TPb isoform) had not been established. To determine this, RdgB-PITP was expressed in *E. coli* (see Materials and Methods) and its ability to transfer PI, PC and SM was assessed. Furthermore, the comparison between the rat PI-TP and RdgB-PITP was extended to include some of the missense mutations introduced at Thr-59 (a residue conserved in RdgBp). As seen in Fig. 4A, the wild-type, T59A, and T59E RdgB-PITP proteins were expressed in *E. coli* and steady state levels were approximately

FIG. 4. Biochemical analysis of mutant RdgB-PITP constructs in *E. coli*. (*A*) Immunoblot of the relative steady-state levels of the wild-type and four mutant RdgB-PITP gene products. Extracts were prepared from the *E. coli* strain BL21(DE3) by resuspension in sample buffer, boiling of the sample, and clarification by microcentrifugation. Prior to resolution by SDS/PAGE, protein concentrations were determined by the DotBlot protein assay (Research Products International) so that 10 ug of total protein of each sample was loaded. Immunoblots were hybridized with a rabbit polyclonal antibody raised against the RdgB-PI-TP domain and developed using the ECL detection kit (Amersham). (*B*) Cytosolic fractions prepared from *E. coli* expressing either WT RdgB-PITP, T59A, or T59E proteins. The cytosol from each sample (1 mg of total cytosolic protein) was assayed for PI (solid bars) and PC (stippled bars) transfers at 37°C. Specific activities were determined essentially as described in Fig. 2. Relative activities were calculated by comparing the specific activities of the mutant proteins to that measured for WT cytosol (set at 100%). Determinations represent the average obtained from at least three experiments.





equal. Note that two other Thr-59 mutants that were made, T59I and T59D, were not stable when expressed in this system.

Phospholipid transfer assays established the fact that these proteins were active in *E. coli* as determined by the wild-type protein's ability to transfer both PI (5.3 +/- 0.1%) and PC (6.4 +/- 1.2%, Fig. 4*B*). SM transfer activity was not detected in RdgB-PITP, therefore establishing that this protein more closely resembles the mammalian PI-TPa isoform than the b isoform. Interestingly, the T59E and T59A mutations in RdgB-PITP had opposite consequences regarding PI transfer activities than what would be expected based on the SrPI-1* Thr-59 transfer data presented previously. Whereas the SrPI T59E mutant abolished PI transfer activity and the T59A mutant exhibited a 50% reduction in PI transfer, the RdgB-PITP T59E mutant transferred PI at WT levels (5.7 +/- 0.1%) and the T59A mutant had lost the ability to transfer PI altogether (compare Fig's. 3*B* and 4*B*). PC transfer activity was not effected in either of the RdgB-PITP mutants (Fig. 4*B*), resembling the results seen in the rat PI-TP Thr-59 mutants. Thus, while the T59 residue represents a crucial amino acid in RdgB-PITP biochemical function, there is a difference when comparing it to the rat PI-TP in what can be substituted at position Thr-59 to maintain full bio-chemical activity.

DISCUSSION

Recent studies have implicated rat PI-TP as a cofactor in the priming of regulated exocytosis in semi-intact PC12

cells and in the stimulation of PI-specific phospholipase C activity in permeabilized HL60 cells (24, 25). These findings have largely been interpreted in the context of rat PI-TP effecting vectorial transfer of PI to signaling membranes. The discussion of the above results will be restricted to (*i*) rat PI-TP residues that are required for PI transfer and (*ii*) what the data imply for regulation of rat PI-TP activity *per se*. While we interpret our results in the context of a PL transfer function for rat PI-TP, the concepts described also apply to regulation of differential PL binding by rat PI-TP (22).

Two corollaries are implied by a vectorial PI transfer reaction. First, it predicts a critical role for vectorial PI transfer in rat PI-TP function *in vivo* and predicts that vectorial PI transfer must be sustained in the direction of the signaling membrane. If vectorial PI transfer is balanced by countertransfer of PC (25), there should exist a mechanism to uncouple the PI and PC transfer activities of rat PI-TP at the signaling membrane. The findings that the S25F, T59I, P78L, and E248K mutations manifested specific PI transfer defects *in vitro*, in the face of unadulterated PC transfer capabilities, demonstrate that the PI and PC transfer activities can be uncoupled in the context of rat PI-TP itself (Fig. 2*B*), as well as in the *Drosophila* RdgB-PITP domain based on the biochemical data obtained from the Thr-59 mutants (Fig. 4*B*). The specificity of these mutations further suggests that (*i*) Ser-25, Pro-78, Thr-59, and Glu-248

cooperate to form a PI head group recognition/binding site in rat PI-TP or (*ii*) these residues lie adjacent to such a site.

Second, as efficient rat PI-TP mediated PL transfer *in vitro* is executed via PL exchange reactions that exhibit neither membrane specificity nor vectoriality (3), models invoking rat PI-TP driven vectorial PI transfer to specific signaling membranes in mammalian cells suggests the *in vivo* superimposition of some regulatory action upon rat PI-TP. While such regulation can potentially be achieved by a coupling of PL transfer to modification of the transferred PI monomer (26, 27), our finding that the class II T59I and E248K SrPI-1* involve consensus PKC phosphorylation motifs in rat PI-TP suggests the additional possibility that at least one consensus PKC phosphorylation site serves as a regulatory site on rat PI-TP. In particular, the genetic data demonstrating the sensitivity of PI transfer activity to mutations involving Thr-59 identify this residue as a potential site on rat PI-TP through which the PL transfer activities of rat PI-TP can be selectively regulated (Fig. 3).

The close proximity between the residue present at position 59 and PI transfer function is underscored by two striking allele-specific effects on PL transfer activity: First, it is noted that H60Q elicited only modest effects on the specific activities of PI and PC transfer. In contrast, all Thr-59 mutations tested, including T59Q, selectively attenuated PI transfer activity (Fig. 2*A*, *B*, and 3*B*). Second, it was found that, while both T59I and T198I mutations

involve consensus PKC phosphorylation sites, only the former elicited selective PI transfer defects (Fig. 2*A* and *B*).

The sensitivity of PI transfer to Thr-59 predicts that phosphorylation of this residue by mammalian PKC will effectively and selectively downregulate the PI transfer activity of PI-TP. A model for how a PKC/protein phosphatase (PPase) cycle might operate in the context of vectorial PI transfer by rat PI-TP *in vivo* is depicted in Fig. 5. The demonstration that phosphorylation of rodent PI-TP is stimulated by PKC agonists *in vivo* (28), when coupled with our finding that the T59D and T59E mutants were specifically defective in PI transfer (Fig. 3), supports the basic tenets of the proposed PKC/PPase cycle. This model is also appealing from a physiological perspective. As PKC is recruited to membranes engaged in inositol PL-driven signaling events and activated there (29), the kinase is properly poised to effect downregulation of the PI transfer activity of rat PI-TP at membranes expected to function as efficient PI acceptors, but inefficient PI donors, in vectorial PI transfer reactions. This cycle also identifies a strategy for reconstitution of an efficient vec-torial PI transfer reaction with purified components. Finally, the fact that the RdgB-PITP site directed Thr-59 mutants had altered PI transfer capabilities, in the face of normal PC transfer activities, lends support to the fact that the PI and PC transfer activities can be uncoupled in metazoan PI-TPs. The results obtained from expressing the RdgB-PITP do-main of the *Drosophila* RdgBp

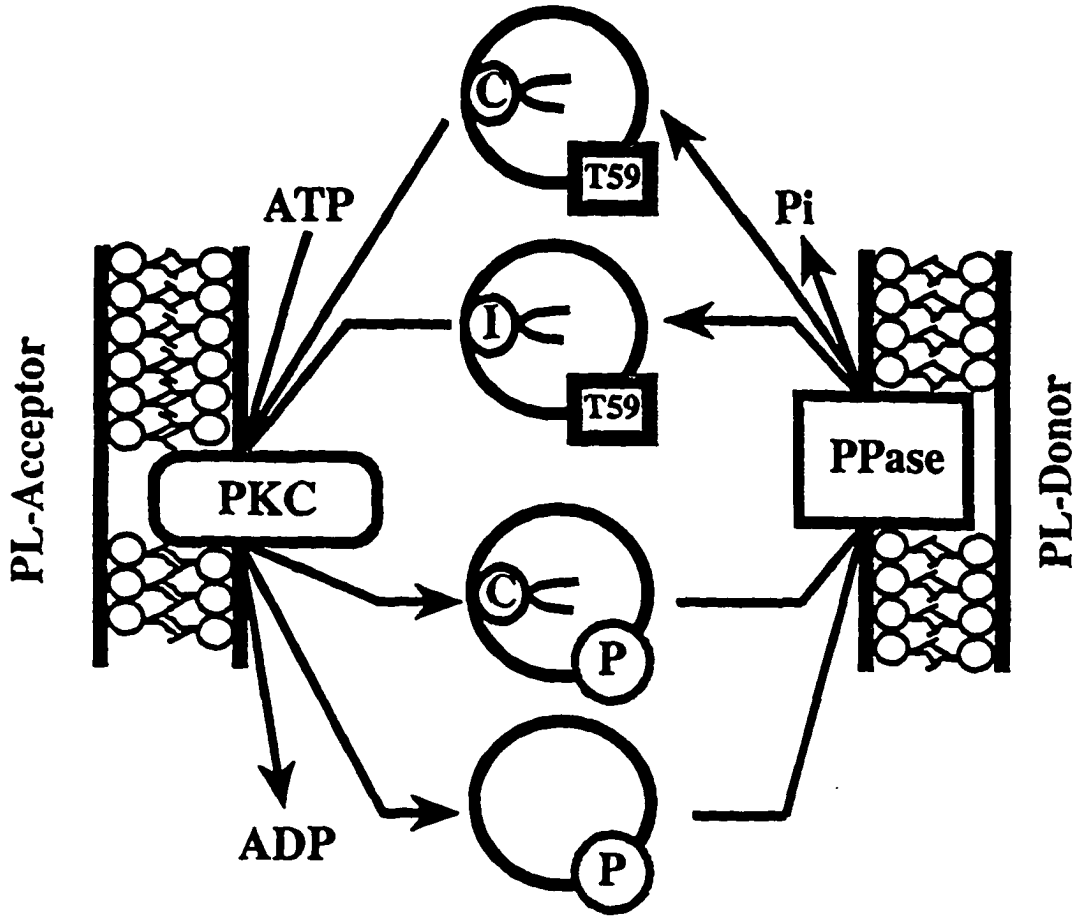


FIG. 5. PKC/PPase cycle for vectorial transfer of PI from a donor to an acceptor membrane in mammals. Upon discharge of PI (I) or PC (C) into the acceptor membrane (upper legs of cycle), a resident PKC phosphorylates rat PI-TP on Thr-59 and renders it unable to reload with PI. Rat PI-TP then disengages from the acceptor membrane either as a PL-free protein or as a PC-bound species (lower legs of cycle). Upon reengagement of PI-TP with the PL-donating membrane, a resident PPase dephosphorylates PI-TP and permits reloading with PI for the next round of transfer.

established this gene as a ho-molog of the mammalian PI-TPa isoform (as opposed to the PI-TPb isoform) but still indicated that it may have a similar, but not identical role, *in vivo* (based on the different con-sequences of specific mutations at position Thr-59 of each protein). In fact, indications are that *RdgB* is required in the *Drosophila* visual cycle for proper trafficking of rho-dopsin to the rhabdomere and that mutations in the PI-TP domain of RdgBp results in retinal degeneration phenotypes in mutant flies (30).

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GENERATION OF MOUSE EMBRYONIC STEM CELLS DEFICIENT IN PITPa PROTEIN AND TARGETED CHIMERIC MICE

by

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Format adapted for dissertation

ABSTRACT

The rat phosphatidylinositol transfer protein (PI-TP) has the ability to catalyze the transfer of phosphatidylinositol (PI) or phosphatidylcholine (PC) between membranes *in vitro*. Recently, several reconstitution assays have identified the rat PI-TP to be an essential cytosolic factor in regulated and constitutive secretion in PC12 cells and receptor-mediated signaling in HL60 cells. These assays, however, may not represent the true *in vivo* role of mammalian PI-TP. To investigate the physiological role of a mammalian PI-TP, the mouse PI-TPa gene was cloned from a lambda phage library and used to generate a PI-TPa knockout cassette that was targeted in embryonic stem (ES) cells. Chimeric mice were generated but failed to pass the mutation to their germ line, probably due to technical rather than physiological reasons. Furthermore, PI-TPa heterozygous knockout cell lines were treated with high concentrations of G418 to produce homozygous knockout ES cell lines that were viable. The viability of these cells indicates that the roles assigned to mammalian PI-TP based on the reconstituted systems may not represent the true *in vivo* role(s) of the protein.

INTRODUCTION

Phosphatidylinositol/phosphatidylcholine transfer proteins (PI-TPs) represent a class of phospholipid transfer proteins (PL-TPs) that can utilize either phosphatidylinositol (PI) or phosphatidylcholine (PC) as substrates in an *in vitro* phospholipid transfer reaction (1). Furthermore,

PI-TPs are found in all eukaryotic cells (1-3) and, because of such, it is not surprising that they share a high degree of primary sequence identity(4-6). The exception being the fungal PI-TPs which share no identity to the metazoan PI-TPs but share a high degree of primary sequence identity among themselves (7-10). The fungal PI-TPs do, however, share a degree of functional identity to the higher eukaryotic PI-TPs as (*i*) they are almost indistinguishable when measuring phospholipid transfer *in vitro*, one exception being that the mammalian PI-TPs are sensitive to N-ethylmaleimide (NEM) while fungal PI-TPs are not (11); (*ii*) the rat PI-TP has been previously shown to complement yeast PI-TP (Sec14p) defects (12); and (*iii*) Sec14p can functionally substitute for mammalian PI-TP in reconstitution assays measuring regulated exocytosis and transmembrane signaling(13, 14).

Mammalian PI-TPs have been implicated in various roles including (*i*) the priming of secretory granules in regulated exocytosis in PC12 cells (13), (*ii*) the biogenesis of Golgi-derived vesicles in both regulated and constitutive exocytosis in neuroendocrine cells (14), and (*iii*) receptor-mediated transmembrane signaling events which require replenishment of phosphoinositide pools (15). All of these assays share the fact that they reconstitute various cellular events that require cytosolic factors. In all three cases, mammalian PI-TPa was isolated as an essential cytosolic factor required to reconstitute the given assay, thereby defin-

ing mammalian PI-TPa as an essential gene in several cellular events.

To investigate the role of mammalian PI-TPa in an organism as opposed to reconstituting cellular events, data are presented that describes the cloning of the mouse PI-TPa gene and subsequent generation of a knockout cassette targeted to R1 embryonic stem cells along with the generation of chimeric mice. Furthermore, the double-knockout of the targeted PI-TPa knockout cassette in embryonic stem (ES) cells is reported to be success-ful indicating that, at least in mouse ES cells, the PI-TPa gene is non-essential; an unexpected result given the essential nature of the gene in the above described reconstitution assays.

MATERIALS AND METHODS

Screening Mouse CDNA and Genomic Libraries. A mouse brain cDNA lambda phage library was obtained from Stratagene and propagated according to their instructions. Approximately 50,000 plaques were screened using random primed ^{32}P labeled probe generated from the open reading frame of the rat PI-TP cDNA (pRE245). Plaques were screened under stringent aqueous conditions as follows: (*i*) filters were baked at 80°C for 2 h then incubated in Hybridization solution (3X SSC, 5X Denhardts, 0.5% SDS, 1 mg denatured salmon sperm DNA) for 3 h at 62°C; (*ii*) after addition of labeled probe, filters were incubated overnight in the hybridization solution; and (*iii*) filters were washed in 2X

SSPE, 0.5% SDS at room temperature followed by two washes in 0.2X SSPE, 0.5% SDS at 65°C.

Positive plaques were isolated and rescreened and the insert DNA were cloned into the Hind III/Bam HI cohesive sites of the vector pTZ19u (16). PCR techniques were then employed to clone the open reading frame of the mouse PI-TPa cDNA utilizing the 5' primer CCAAGCTTGTTAACAGCGACATGGTGC-TGCTCAAGG which contained Hind III and Hpa I restriction enzyme sites (underlined) and the 3' primer AAGGGATCCAGCG-CTAGTCATCTGCTG containing a Bam HI restriction site. The polymerase chain reaction (PCR) product was then digested with Hind III and Bam HI and cloned into the respective sites in pTZ19u resulting in plasmid pRE263.

A mouse 129SVJ genomic lambda phage library was obtained from Stratagene. Approximately 100,000 plaques were screened as described above with the exception being that pRE263, the mouse PI-TPa open reading frame, was used to generate probe. Positive plaques were isolated, rescreened, and the insert mouse genomic DNA were isolated as unique Sst I restriction fragments and cloned into Sst I digested pTZ-18u, resulting in plasmids pRE264 (12Kb), pRE559 (3.0Kb), and pRE546 (1.3Kb).

Southern Hybridizations. Preparation of ES cell DNA was performed as follows. ES cells were grown to confluency on a p100 plate, trypsonized (25 mM) to detach cells from plate, spun down to pellet the cells, and finally washed once with PBS. Cells were resuspended in 0.3 mls lysis buffer

(0.5% SDS, 50 mM NaCl, 25 mM EDTA) in which proteinase K was added to a final concentration of 10 ug/ml. The cell suspension was then incubated overnight at 55°C. Finally, one half volume of a saturated NaCl solution was added and the mixture was vortexed vigorously and centrifuged for 20 min at 10,000 rpm and 2 volumes of 100% ethanol was added to the supernatant. The DNA ball was then extracted with a pasteur pipette and placed in 70% ETOH until needed.

Genomic ES cell DNA (5 ug) was digested overnight with the appropriate restriction enzyme was loaded per well of a 0.8% agarose 15 cm gel (1X TAE buffer) and the gel was run at 35 volts for 48 hours to ensure good separation of the DNA throughout the length of the gel. The DNA was then transferred to Hybond-N nylon membrane (Amersham).

Hybridization conditions for the membrane were as follows: (*i*) filters were baked at 80°C for 2 h then incubated in pre-hybridization solution (50% Formamide, 3XSSC, 10X Denhardt's, 20 ug/ml salmon Sperm DNA, and 2% SDS) at 42°C overnight; (*ii*)Pre-hybridization buffer was then replaced with Hybridization buffer (50% Formamide, 3XSSC, 1X Denhardt's, 20 ug/ml Salmon Sperm DNA, 5% Dextran Sulfate, and 2% SDS) containing the labeled probe and allowed to incubate at 42°C overnight; (*iii*) the filters were finally washed in 1X SSC, 0.1% SDS at 65°C, then 0.1X SSC, 0.1% SDS at 65°C.

Western Analysis. Protein was prepared from ES cells as follows: confluent p100 dishes of each cell line were trypsonized (25 mM) to detach cells from the plate, spun down

to pellet the cells, and washed once in PBS. The cell pellet was then resuspended in 0.3 mls of SDS/PAGE sample buffer, boiled for 5 min and microfuged for 1 min to pellet the cell debris. The supernatant was subsequently recovered and the protein concentration of each sample was determined by the DotBlot Protein Assay (Research Products International). 150 ug of each sample was then loaded onto a 10% SDS-PAGE gel and run under standard conditions. After transferring the protein to nitrocellulose, the membrane was blocked in a mixture of 5% milk protein, 10% BSA overnight. Hybridization to the polyclonal chicken-anti-mouse PI-TPa antibody was then allowed to proceed for 24 h. Secondary antibody consisted of rabbit-anti-chicken and allowed to incubate for 6 h. The Western was finally developed using the ECL kit from Amersham.

ES Cell Chromosome Preps. 50 ul of a 2 ug/ml stock of Vinblastine sulfate (Sigma) was added to a confluent p100 plate of ES cells and allowed to incubate for 4 h. Cells were then trypsinized, spun down, and finally resuspended in 10 mls of a hypotonic solution (3% sodium citrate, 2.24% potassium chloride) and incubated for 30 min at 37°C. Cells were then spun to pellet and resuspended in 3.5 ml of Carnoy's fixative (one part glacial acetic acid, three parts methanol). The cells were pelleted and washed in fixative two more times before finally being resuspended in a final volume of 0.5 ml of the fixative. Cells were then dropped from a pasteur pipette at eye level onto a glass slide and

allowed to dry. The slides were then stained with Giemsa (10%) for 10 min before rinsing with water and viewing under a microscope.

Construction of the Targeting Vector. The mouse PI-TPa genomic clone pRE264 (see above) contained approximately 12 kb of genomic sequence containing five exons of the PI-TPa gene and was used as the backbone to build the targeting vector. To this end, PCR was utilized to move the Kpn I site from the right of exon A to the left of it generating a 2 kb PCR product with Hind III-Kpn I cohesive ends (see Fig. 2*A*). This was cloned into the cohesive ends of pTZ19u generating pRE553. To increase the frequency of gene targeting, the herpes simplex virus TK cassette was cloned from vector pPNT (17) as a Hind III-Sal I fragment into the cohesive sites of vector pRE553 to generate vector pRE554. Exons A, B, and C (see Fig. 2*A*) were interrupted by a mutant *neo* cassette generated by PCR from the plasmid pNTK (17). In this cassette, unique Kpn I sites were introduced by way of oligonucleotides at the 5' and 3' ends to facilitate cloning into the Kpn I site at the end of vector pRE554, thus generating vector pRE555. In effect, this replaced almost 3 kb of genomic DNA containing three exons of the PI-TPa with the 2 kb *neo* cassette. Finally, the 7 kb of genomic DNA flanked by Kpn I and Sst I sites remaining from pRE264 were cloned into the respective sites of pRE555 generating the targeting vector pRE557 (see Fig. 1*A*).

Isolation of Targeted ES Cell Clones. The ES cell line R1 (129/SV-derived) was cultured on a feeder cell layer and electroporated, using 5 x 10^7 cells and 50 ug of the linearized targeting vector DNA as described (18, 19). Colonies doubly resistant to G418 (0.5 mg/ml) and ganciclovir (5 uM) were selected and expanded on feeder layers in 24-well plates. Homologous recombinants were identified by the Southern blot analysis described previously. ES cells that were identified as those heterozygous for the PI-TPa sequence were cultured in the presence of a higher dose of G418 (2.0 mg/ml) (20). After 10 days of cultivation, colonies were isolated and expanded, and DNA were extracted and examined for lack of the intact PI-TPa gene using both Southern blotting and Western analysis as described above.

Generation of Chimeric Mice. Chimeras were produced by microinjecting the targeted agouti ES cells into 3.5-day-old C57/black blastocysts (8-15 ES cells per blastocyst) and transplanting the embryos into the uteri of albino CD-1 pseudopregnant females. These surrogate mothers were allowed to carry to term the implanted embryos and chimeric offspring were determined by any amount of agouti coat color seen on the offspring.

RESULTS

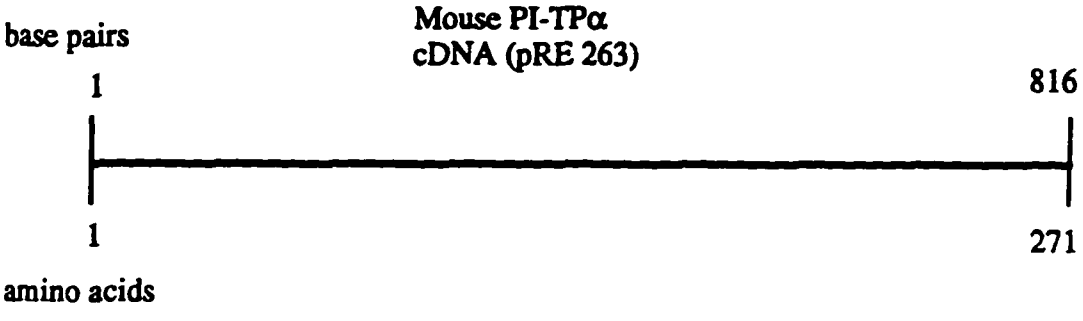
Cloning and Characterization of Mouse PI-TPa Gene. Using the rat PI-TPa open reading frame (ORF) to generate ^{32}P labeled random primed probe, a lambda phage mouse brain cDNA library (Stratagene) was screened under stringent

conditions (see Materials and Methods) in an attempt to clone the mouse PI-TPa gene. Five plaques were isolated and, upon restriction enzyme analysis, determined to have identical restriction enzyme digestion patterns to that of the rat PI-TPa cDNA clone (pRE245). The clone containing the largest mouse cDNA insert, named pRE263, was sequenced and a potential ORF of 816 bp was identified that differed from the rat PI-TPa ORF at only 16 nucleotide positions. The predicted amino acid sequence of this ORF (271 amino acids) differed from that of the rat PI-TPa primary sequence at only one position, 167, a 99.6% identity between the two sequences. Based on these data, pRE263 was identified to be the mouse PI-TPa cDNA clone.

In an attempt to obtain genomic mouse PI-TPa clone(s) so they may be used to generate a knockout targeting construct, pRE263 was used to generate random primed probe to screen a lambda phage mouse genomic library (Stratagene). Three clones were obtained, pRE264 (12 kb), pRE559 (3.0 kb), and pRE546 (1.3 kb), and were determined not to be overlapping by restriction enzyme digestion analysis. Upon sequence analysis, pRE546 contained one exon identical to bases 301-375 of the mouse PI-TPa ORF or amino acids 101-125 (Fig. 1). PRE264 was determined to contain five contiguous exons spanning bases 376-767 of the mouse PI-TPa ORF, or amino acids 126-257 (Fig. 1). Finally, pRE559 contained one exon determined to be upstream of the two previous clones, mapping to bases 52-198 or amino acids 18-66 (Fig. 1).

FIG. 1. Mouse PI-TPa genomic clones. PRE559, 546 and 264 were cloned independently from a lambda phage mouse genomic library (Stratagene) using the mouse PI-TPa cDNA clone pRE263 to generate probe. Exons contained within each clone are represented by black boxes with pRE559 containing one exon of 48 amino acids, pRE546 containing one exon of 24 amino acids, and pRE264 with 5 contiguous exons of 27, 7, 17, 37, and 40 amino acids (from left to right).

A



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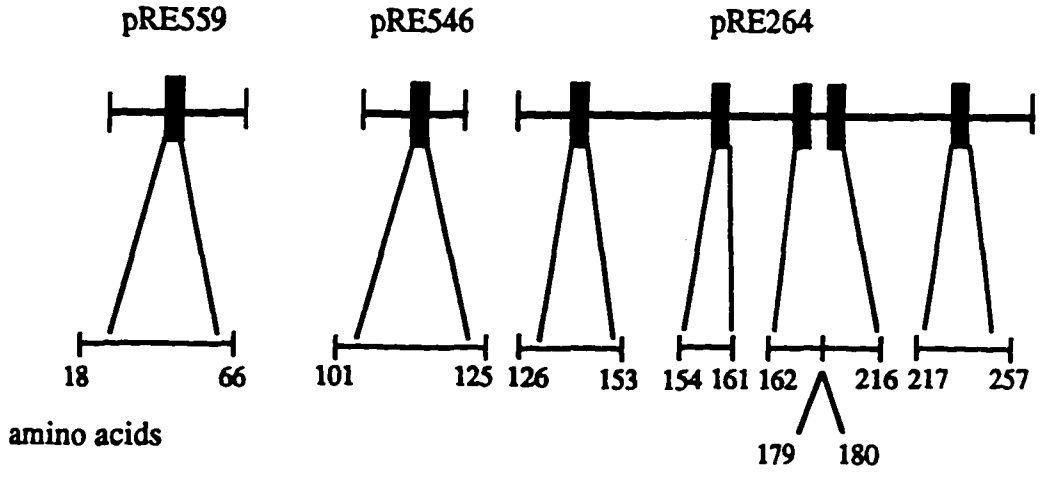


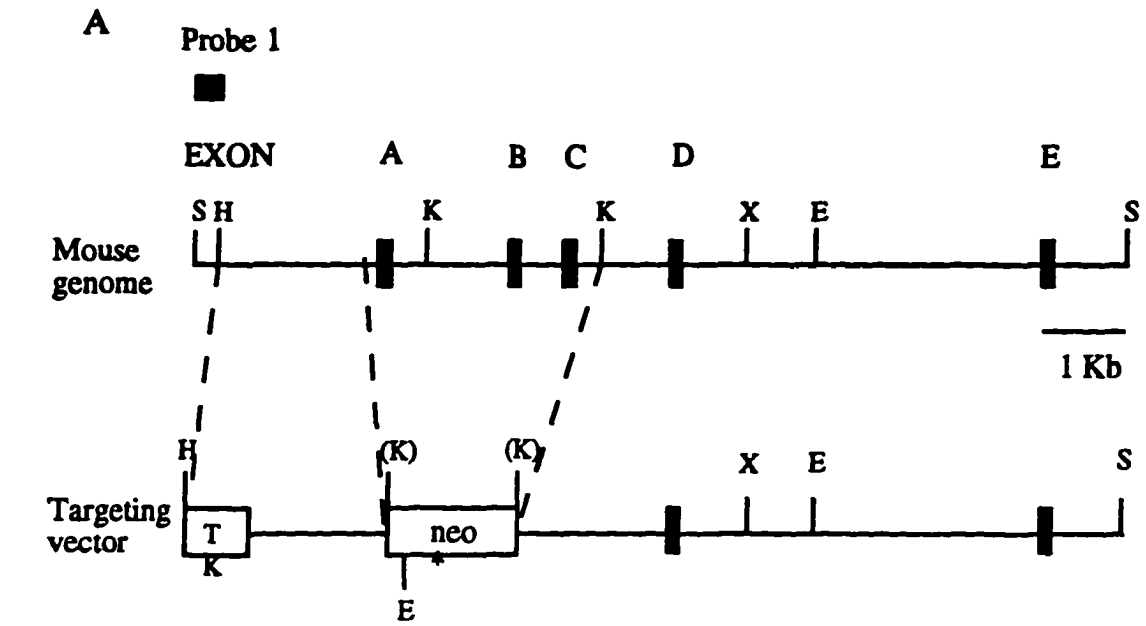
Generation of Targeting Construct. PRE264 (see above) was used to generate a knockout targeting construct based on several criteria: (*i*) its size (12 kB) should be sufficient to ensure homologous recombination in the mouse genome at an acceptable frequency, about 3-7%; (*ii*) in the event that the targeted knockout gene produced a truncated protein, the exons within this clone contained amino acid residues that have previously been shown to be important both functionally and biochemically to this protein (see Chapter 2), thereby making it unlikely to be a **functional** truncated protein; and (*iii*) the size of the clone being large, it would not be necessary to "build" a targeting construct from two or more cloned fragments from the phage library.

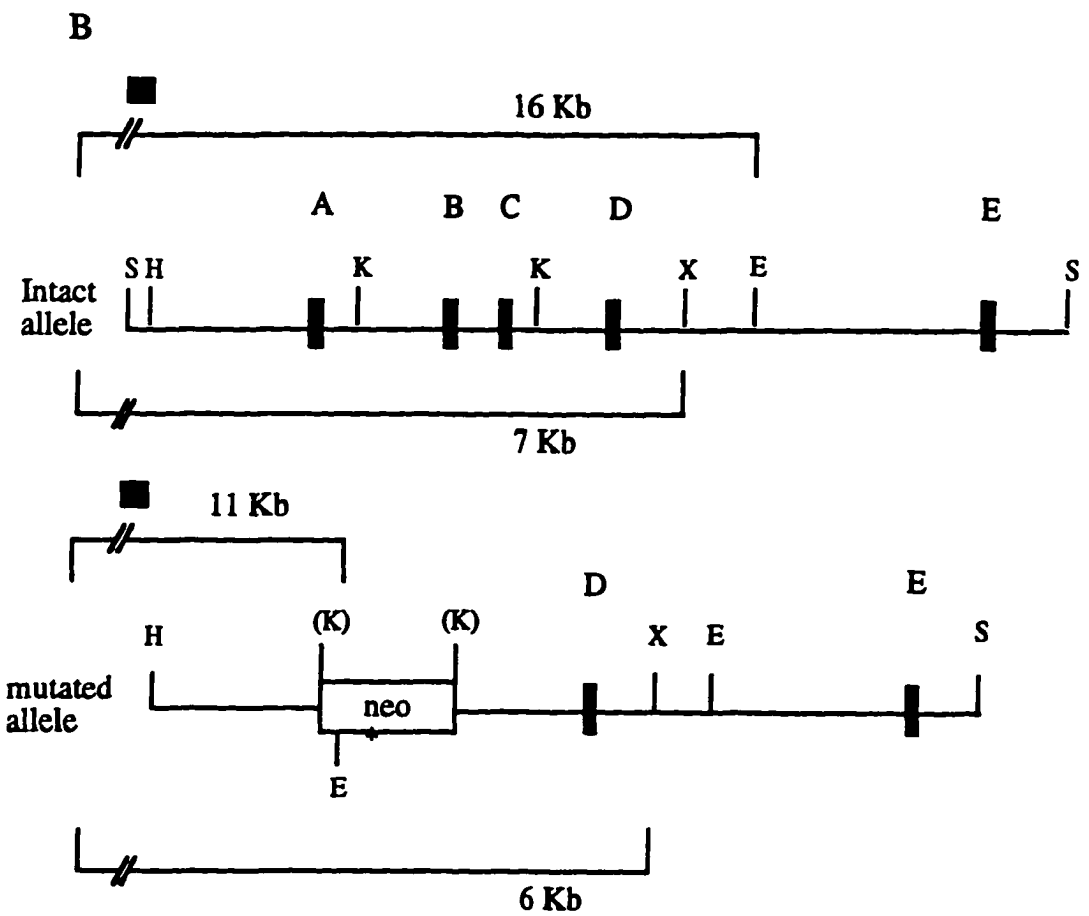
A positive/negative selection strategy was employed in the construction of the targeting cassette. The region of the clone (pRE264) containing exons A, B, and C (amino acid residues 162-257 of the mouse PI-TPa cDNA) were replaced by a mutated *neo* cassette (Fig. 2*A*) which confers resistance to the drug G418 in targeted mammalian cells. The use of a mutated *neo* cassette will be discussed in a later section. The resulting plasmid was then used to generate the targeting construct with the final addition of the herpes simplex virus thymidine kinase (TK) cassette at the 5' end of the construct (Fig. 2A). The addition of the TK cassette results in a negative selection for the drug ganciclovir (i.e., expression of the TK gene in mammalian cells confers a **sensitivity** to ganciclovir).

Targeted ES Cell Lines. The resulting targeting construct, pRE557, was electroporated into ES cells (strain R1/129SV) and cells showing increased resistance to both ganciclovir and G418 were selected (165 colonies). The DNA of resistant clones were digested with restriction enzymes and hybridized with probe 1 (Fig. 2*A*). Homologous recombinants were characterized by appearance of an 11 kb Eco RI fragment and a 6 kb Xba I fragment (Fig. 2*B*). The PI-TPa sequence is flanked by both Eco RI and Xba I sites about 16 kb and 7 kb apart, respectively. The *neo* cassette contains an Eco RI site, thus, as a result of the targeted integration of the *neo* gene, a new Eco RI site is generated 5 kb away from the endogenous Eco RI site to produce a novel 11 kb Eco RI fragment (Fig. 2*B* and *C*). The *neo* cassette does not contain a Xba I fragment; however, since the *neo* cassette is approximately 1 kb smaller than the DNA it replaces in the wild type (WT) allele (Fig. 2*B* and *C*), a novel 6 kb Xba I fragment is gen-erated in the targeted allele. Four targeted cell lines were recovered (JGA6, JGA11, JGA25, and JGA152) out of a total of 102 cell lines screened, a 4% targeting frequency.

To obtain homozygosed cell lines for future *in vitro* experimentation, targeted cell lines JGA11, 25, and 152 were screened for increased resistance to high concentrations of G418 (see Materials and Methods). The utilization of the mutant *neo* cassette was essential for this line of investigation, as a wild-type *neo* cassette targeted to only one

FIG. 2. Targeted disruption of the mouse PI-TPa gene. (*A*) The targeting vector. The upper line labeled mouse genome shows part of the gene with appropriate restriction enzyme sites. S, Sst I; H, Hind III; K, Kpn I; X, Xba I; and E, Eco RI. The direction of the mouse PI-TPa gene transcription is from right to left. In the targeting vector line, *neo** indicates the mutated *neo* cassette to confer resistance to the drug G418 and TK represents the herpes simplex virus TK cassette which confers sensitivity to the drug ganciclovir. Both cassettes are driven by the PGK promoter. (*B*) Configurations of normal and mutated alleles. The expected restriction fragment lengths are shown, together with position of probe 1. Ligation of *neo** cassette to the mouse gene fragment yielded a new Eco RI restriction site (11 kb vs. 16 kb) along with shifting the size of the Xba I fragment (7 kb vs. 6 kb). To prepare probe 1, the 0.3 kb Sst I/Hind III fragment from the mouse genome fragment (pRE 264) was gel purified. (*C*) Southern blot analysis of DNAs from wild-type (WT) and targeted ES cell line (JGA152). Genomic DNAs prepared from WT and targeted ES cell clones were digested with Eco RI and Xba I and hybridized to probe 1. A representative Southern is shown comparing the Eco RI digested WT DNA to both Eco RI and Xba I digested targeted DNA. (*D*) Southern blot analysis of DNAs from a targeted cell line (JGA152) and a homozygously targeted cell line (JGA152-28). Notice the loss of the upper 16 kb Eco RI fragment in the cell line JGA152-28 indicating a properly targeted double-knockout cell line.





allele will confer resistance to very high concentrations of G418 (at least 2 mg/ml). The mutant cassette, however, is less efficient at conferring resistance at high G418 concentrations and must rely on the cell's ability to lose the chro-mosome not containing the targeted *neo* gene and duplicate the chromosome that does contain the gene (20). This results in the cell having two copies of the mutated *neo* gene, which will confer resistance to the high G418 concentrations (2 mg/ml). More importantly, it results in the transformation of the targeted heterozygous cell line into a homozygously targeted cell line (at a frequency of up to 50%) (20). As seen in Fig. 2*D*, the WT 16 kb Eco RI fragment disappeared and only the 11 kb fragment is seen, indicating that both alleles had been properly targeted creating a homozygous mouse PI-TPa knockout. Strains JGA11, 25, and 152 were all treated in this fashion to produce homozygous knockout ES cell lines JGA11-11, JGA25-5, and JGA152-28, respectively, at a properly targeted frequency of approximately 10% for all three cell lines.

Heterozygous and homozygous targeted cell lines were all finally confirmed to be properly targeted by Western analysis of endogenous mouse PI-TPa protein. Polyclonal chicken antibodies raised specifically against a C-terminal mouse PI-TPa peptide were utilized and revealed that protein samples derived from PI-TPa$^{+/-}$ ES cells contain at most one half the amount of PI-TPa protein detected in the WT cell line (Fig. 3). Furthermore, the PI-TPa$^{-/-}$ samples contained

no detectable mouse PI-TPa immunoreactive species (Fig. 3). This Western data and the previously described Southern analysis indicate proper targeting of the knockout gene in the cell lines indicated.

Generation of Chimeric Mice. Prior to injection of targeted ES cells into recipient blastocysts, each cell line was analyzed for chromosome composition in order to determine if any cell line was mutated, such as a chromosome trisomy. Mice have 40 chromosomes (20 pairs) and can easily be checked for chromosome anuploidy. Chromosome preps were prepared (see Materials and Methods) and photographed so that they could be counted. Fig. 4 demonstrates one such prep on strain JGA152. Approximately 80% of the chromosome spreads checked for this strain contained the expected number of chromosomes (40) for a WT cell line. Since any percentage above 70% is considered acceptable (there are many handling problems that cause loss of one or more chromosomes in any given spread), this strain was determined to be acceptable for injection into blastocysts. The other two strains to be injected, JGA11 and JGA25, were analyzed in the same manner and similar results were obtained (data not shown).

ES cell strains JGA11, 25, and 152 were microinjected into C57/black blastocysts and a total of eight chimeric mice were generated. As seen in Fig 5, two chimeric mice were obtained from cell line JGA152, three chimeric mice were obtained from cell line JGA11, and three chimeric mice from cell line JGA25. Unfortunately, none of the 8 chimeric mice

FIG. 3. Immunodetection of the PI-TPa protein. Cell extracts were prepared from the three ES cell lines indicated by resuspending cells in SDS/PAGE sample buffer, boiling for 5 min and finally spinning the sample and collecting the supernatant. DotBlot protein assays (Research Products International) were performed so that 150 ug of total cell lysate could be loaded per lane of a 10% SDS/PAGE gel. The Western blot was hybridized with chicken polyclonal antibody raised against a C-terminal peptide specific for the mouse PI-TPa protein. Signal was detected using the enhanced chemiluminesence system (ECL) from Amersham.

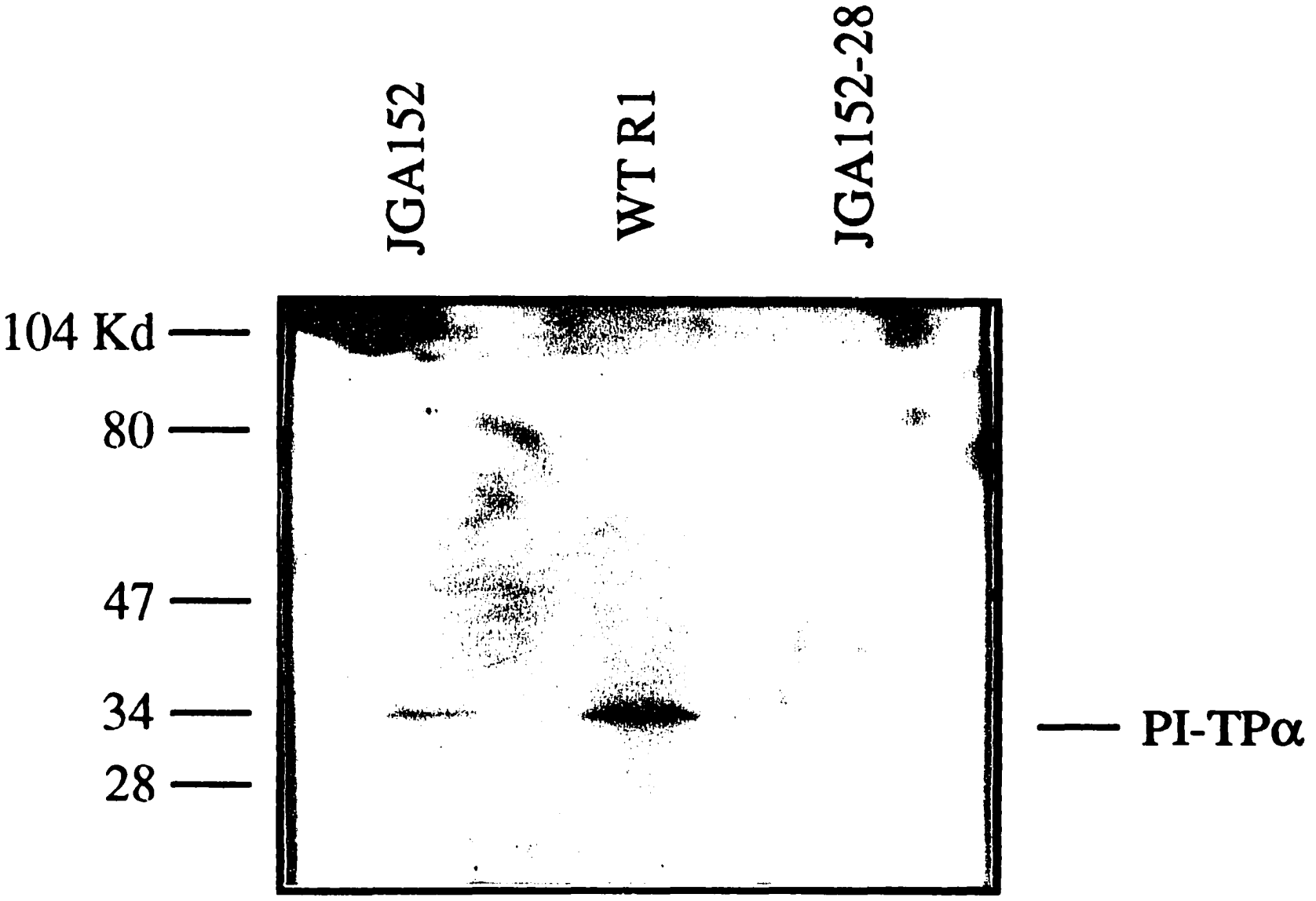


FIG. 4. Chromosome spreads from ES targeted cell line JGA152. ES cells were prepared as described in the Materials and Methods then dropped onto cold microscope slides to rupture the cells and spread the chromosomes. Slides were air dried, stained with Giemsa (10% stock), then examined under a light microscope. WT cell lines are defined as containing 40 chromosomes.

have passed the knockout gene to their germ line and, therefore, not to their offspring. From numerous matings for each mouse, only WT offspring have been recovered from some 85 F1 generation mice (Fig. 5). There are several possible reasons for this result that will be discussed in the ensuing section.

DISCUSSION

Recent studies of regulated and constitutive exocytosis in semi-intact PC12 cells (13, 14) and in the stimulation of PI-specific phospholipase C activity in permeabilized HL60 cells (15) have indicated a crucial role for mammalian PI-TP. In fact, PI-TP was isolated as one of only several cytosolic factors needed to reconstitute the system in both the regulated and constitutive exocytosis assays and the single cytosolic factor from bovine brain cytosol that was necessary for maximal reconstitution of PI-PLC activity. Furthermore, PI-PLC activation was found to be linear with respect to PI-TP concentration (15), suggest-ing that PI-TP represents the rate-determining step in the reconstituted reaction. The common factor in all these ass-ays is that PI-TP is a required component for maximal reconstitution. Interestingly, when Sec14p is introduced into the systems in place of mammalian PI-TP, it serves as an effec-tive surrogate (13, 21). This has been taken to indicate that simply by providing a means to generate phosphoinositide pools (i.e., by transferring PI to where it is needed by the transfer protein) the assays can be reconstituted. Since Sec14p and

FIG 5. Chimeric mice and their offspring obtained from targeted cell lines. Three targeted agouti cell lines (JGA152, JGA11, and JGA25) were microinjected into 3.5 day old C57/black blastocysts, implanted into pseudopregnant females (albino CD-1), and allowed to develop. Chimeric offspring were defined as having an agouti coat color (to varying degrees indicated as a % above) mixed into the black coat color derived from the host blastocyst. Chimeric mice are identified by the cell line used to generate the mouse, i.e., JGA152.1 was the first chimeric mouse obtained from the targeted cell line JGA152. When chimeric mice matured, they were mated to C57/black mice to generate F1 offspring. Germline transmission of the knockout gene would be indicated in those F1 offspring that *i*) had a 100% agouti coat color and *ii*) from tail DNA preps used in Southern hybridizations as only 1/2 off all agouti mice (generated from a heterozygously targeted cell line) would have the knockout gene. Southern hybridizations were not necessary because all offspring from the above chimeras were black, indicating that the knockout gene derived from the targeted agouti ES cells was not transmitted to the germ line even in the very highly chimeric mice (JGA11.2 and JGA11.3).

Chimeric Mouse Strain	# of offspring	Offspring coat color
JGA152.1 40% female	10	Black
JGA152.2 40% male	6	Black
JGA11.1 70% female	8	Black
JGA11.2 95% male	14	Black
JGA11.3 95% male	28	Black
JGA25.1 40% male	10	Black
JGA25.2 50% male	9	Black
JGA25.3 45% female	0	Black

rat PI-TPa (and b isoform) have this interchangeability both *in vitro* and *in vivo*, one is led to believe that a unification of their roles, and therefore a greater functional understanding of the PI-TP role(s), is forthcoming. One paradox to this proposal, however, is the sensor model for Sec14p function which proposes that the PC-bound form of Sec14p represses the CDP-choline pathway (see Introduction) (22). This fact is not consistent with a role for Sec14p in PIP_2 biosynthesis, nor does any of the mammalian reconstitution assays suggest a role for the mammalian PI-TP that is "Sec14p-like".

To address the issue of the functional role of PI-TP in a mammalian system, as opposed to reconstituting a cellular system, we cloned the mouse PI-TPa gene so that a knockout targeting construct could be generated. With the knockout cassette in hand, a series of ES cells were generated that were first heterozygous PI-TPa minus cells, then, after manipulation, homozygous PI-TPa minus cells were generated. Finally, a series of chimeric mice were generated from the heterozygous PI-TPa minus ES cells that, as of yet, have failed to pass the mutation to their germ line.

A somewhat surprising result was the fact that we could obtain a homozygous PI-TPa knockout ES cell line given the fact it is a required cofactor in all of the reconstitution assays. Furthermore, past results claim that when HepG2 cells are stably transfected with anitsense PI-TP cDNA they suffer a dramatic loss of viability (23), a phenotype not

seen in PI-TPa deficient ES cells. The conflict between these data and the fact that PI-TPa can be deleted from mouse ES cells without an immediately observable phenotype illustrates the danger of assigning an *in vivo* role to mammalian PI-TP based on *in vitro* results. There are several possible explanations for these conflicting results, however. First, it is possible that in the PI-TPa$^{-/-}$ ES cells there is upregulation of the PI-TPb isoform that compensates for the loss of the a gene. We have obtained preliminary results based on Western analysis that this is not the case. This data, however, does not rule out the possibility that steady-state levels of PI-TPb are sufficient to compensate for loss of the PI-TPa. There is also the possibility that there is a third PI-TP isoform in mammalian cells that has not been identified that can compensate for the loss of the PI-TPa gene. Since it has only been in recent years that the PI-TPb isoform was identified and purified from cytosolic fractions, there may still be undiscovered isoforms. This isoform, however, would have to be structurally distinct from the PI-TPa and b genes since antibodies that recognize these species do not cross react with other protein species in mammalian cells.

Finally, the fact that we have been unsuccessful in generating F1 agouti mice (PI-TPa$^{+/-}$) does not lend itself to interpretation regarding the necessity for the PI-TPa gene. As seen in Fig. 5, only two of the chimeric mice generated are considered "highly chimeric" (>80%). This is not a sufficient number of highly chimeric mice to base an analysis

of germ line transmission on. In fact, since none of the chimeric mice have had F1 offspring that even carried the agouti coat color (which is used as an indicator that the germ line of the chimeric mouse is derived from the agouti ES cells independently of the segregation of the mutant allele); (Fig. 5) it is reasonable to assume that this lack of germ line transmission is a technical problem as opposed to a mutant phenotype.

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SUMMARY

In this dissertations, we have investigated several key aspects of PI-TP function in eukaryotic cells. One of these included the use of the yeast *Saccharomyces cerevisiae* as a surrogate system in which the rat PI-TP could be expressed and therefore studied on both a structural and functional level. It was found that, when the rat PI-TP was expressed in yeast strains carrying lesions in *SEC14* (the structural gene for the yeast PI-TP, Sec14p), a specific complementation of *sec14*^{ts} growth and secretory defects was observed. This was the first evidence that a mammalian PI-TP could stimulate Golgi secretory function *in vivo*. Furthermore, these experiments provided evidence that the transfer activity of Sec14p reflects a fundamental functional feature of the protein. This was demonstrated because, in the face of the lack of primary sequence identity between the two protein species (mammalian and yeast PI-TPs), their similar *in vitro* catalytic properties were relevant to *in vivo* function, as witnessed by the ability of the rat PI-TP to complement Sec14p lesions.

The use of this yeast surrogate system was then utilized in a structure/function analysis of the rat PI-TP. Since in the face of Sec14p defects the expression of the heterologous rat PI-TP is mandatory for cell viability, it

was possible to randomly mutagenize the rat PI-TP and screen for those species that have lost the ability to complement the *sec14^{ts}* lesion. This line of investigation proved to be successful as two classes of mutant rat PI-TPs were isolated that had lost the ability to complement the Sec14p defect. The Class I mutants (H60Q and T198I) were biochemically nearly identical to the WT rat PI-TP as they had not lost the ability to transfer the phospholipid ligands, PI and PC, *in vitro*. These results indicated that the transfer activity of Sec14p does not represent the sole *in vivo* role of this protein as *sec14^{ts}* yeast strains expressing these biochemically active proteins were not viable at the *sec14^{ts}* non-permissive temperature (37°C). The Class II mutants (S25F, T59I, P78L, and E248K) represented the most interesting class of mutants as they had altered biochemical activities. While PC transfer activity was measured at WT levels, PI transfer activity was abolished in all four mutants. These data were the first to establish that the PI and PC transfer activities of the mammalian PI-TPs could be uncoupled.

We then generated a series of site-directed mutants at position Thr-59 of both the rat PI-TPa and *Drosophila RdgB* (an insoluble PI-TP required in the fly visual cycle) genes. This residue was deemed important based on the fact it represents a potential protein kinase C phosphorylation site and is highly conserved among all known metazoan PI-TPs. It was found that the ability to catalyze the transfer of PI was very sensitive to the residue at this position in both the

mammalian and *Drosophila* polypeptides. Furthermore, it highlighted the difference between the two metazoan proteins, as different residues at Thr-59 had opposite biochemical consequences.

This ability to uncouple the transfer of the two ligands lended itself to a mechanism by which mammalian PI-TPs may be regulated. The idea of regulated phospholipid transfer is necessary in the context of recent studies in which mammalian PI-TPs were isolated as required cytosolic factors in regulated and constitutive secretion in PC12 cells, as well as in receptor mediated signaling in HL60 cells. These assays share a common feature in that they all predict the maintenance of specific phosphoinositide pools (provided by the PI-TP) that are required in each assay system. This imposes a vectorial transfer of phospholipid (specifically PI) between two membrane species, a reaction that is poorly achieved *in vitro*. The fact that the Class II rat PI-TP mutants (two of which were mutated in consensus protein kinase C motifs) had lost the ability to transfer PI, but not PC, lended itself to just such a means by which the protein could be regulated so that a net transfer of a ligand could be achieved between membranes *in vivo*. The PKC/protein phosphatase cycle for vectorial transfer of PI from a donor to an accep-tor membrane proposes that a resident PKC phosphorylates PI-TP after discharge of ligand (PI) at an acceptor membrane rendering it unable to reload with PI. It subsequently dis-charges from the membrane in an unbound or PC

bound form where it engages the donor membrane and a resident protein phosphatase dephosphorylates the PI-TP so that it may re-load with PI. The cycle is completed when the PI loaded PI-TP re-engages the acceptor membrane, the ligand is discharged and the cycle starts again. This would accomplish a net transfer of PI from one membrane to another thereby making it possible to generate a localized phosphoinositide pool.

Finally, we report the generation of embryonic stem (ES) cells that have a targeted disruption (both heterozygous and homozygous) of the PI-TPa gene. The heterozygous disrupted cell lines were used to generate a series of chimeric mice. These mice, however, have not passed the disruption to their germ line resulting in the lack of F1 PI-TPa$^{+/-}$ mice. This lack of success is attributed to technical rather than physiological reasons. An interesting result was the success in generating the homozygous PI-TPa knockout cell lines. Given the requirement of the PI-TP in several reconstituted cellular events, it was thought that the generation of a PI-TPa$^{-/-}$ cell line would not be possible. This result indicates that attributing an *in vivo* role to PI-TP based on *in vitro* transfer assays and reconstituted events could lead to misunderstanding the true role(s) PI-TP play within cells. These cell lines, however, will be useful as they can be used to study *in vivo* consequences attributable to loss of the PI-TPa gene.

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