A Phosphatidylinositol 3-Kinase and Phosphatidylinositol Transfer Protein Act Synergistically in Formation of Constitutive Transport Vesicles from the Trans-Golgi Network*

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The cytoplasmic domain of TGN38 (an integral membrane pro-

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tions and is stimulated by the activation of the p62pI

S-associated 25-kDa GTPase. Present evidence suggests that the essential function of the PI 3-kinase in exocytic vesicle forma-
tion from the TGN is the generation of a specific PI(3)P pool.
The supporting evidence comes from the demonstration that both catalytic activity and vesicle formation are equally inhibited
by wortmannin.

PITPs also have been demonstrated to function in vesicle
formation from the TGN in yeast and mammalian systems (3–5).
The yeast PITP (Sec14p) is required for the formation of yeast Golgi-derived secretory vesicles (4), and this essential Sec14p requirement can be bypassed by modulation of metabo-
lic flux through specific phospholipid biosynthetic pathways
(6–8). In mammalian membrane trafficking reactions both the
formation of TGN-derived transport vesicles of constitutive and
regulated secretory pathways and the regulated fusion of se-
cretory granules with the plasma membrane are stimulated by
PITP (5, 9). Whereas PITP cooperates with at least one other unidentified cytosolic factor to stimulate TGN-derived vesicle
production, the mechanism of PITP function in that reaction
remains unresolved (5). In the secretory granule fusion reac-
tion, PITP synergizes with phospholipid kinases to generate PI
4,5-bisphosphate (10). One of the mechanisms by which PITP
may stimulate phosphoinositide synthesis is by presenting PI
phosphatidylinositol transfer protein (PITP) is involved in driving vesicular traffic from
mammalian trans-Golgi network (TGN). We have tested the interaction between these cytosolic pro-
teins in an assay that measures the formation of consti-
tutive transport vesicles from the TGN in a hepatocyte
cell-free system. This reaction is dependent on a novel PI 3-kinase, and we now report that, under conditions of
limiting cytosol, purified PI 3-kinase and PITP function-
ally cooperate to drive exocytic vesicle formation. This
synergy was observed with both yeast and mammalian
PITPs, and it also extended to the formation of PI
3-phosphate. These collective findings indicate that the
PI 3-kinase and PITP synergize to form a pool of PI
3-phosphate that is essential for formation of exocytic
vesicles from the hepatocyte TGN.

Much effort has recently been focused on understanding the
molecular mechanisms that underlie the various vesicular traf-

ficking reactions that operate throughout the eukaryotic secre-
tory pathway. The p62pI

S is a cytosolic complex required for the
formation of polymeric IgA receptor (pIgA-R) containing exo-
cytic transport vesicle from the TGN of hepatocytes. The p62pI

S consists of a 62-kDa phosphoprotein and a 25-kDa GTPase and
regulates the activity of a novel PI-specific PI 3-kinase (1, 2).
In cytosol, the p62 molecule is phosphorylated and is not associ-
ated with the PI 3-kinase catalytic subunit. Upon receipt of
some unknown signal, p62 is dephosphorylated, the PI 3-
kinase regulatory p62pI

S and catalytic subunits assemble with
the cytoplasmic domain of TGN38 (an integral membrane pro-

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tion, PITP synergizes with phospholipid kinases to generate PI
4,5-bisphosphate (10). One of the mechanisms by which PITP
may stimulate phosphoinositide synthesis is by presenting PI
to PI kinases (11–13). This concept remains controversial (14).

In this paper, PITP is shown to be an essential component
required for the efficient, cell-free formation of pIgA-R contain-
ing exocytic vesicles from the hepatocyte TGN. PITP synergizes
with the p62pI

S-associated PI 3-kinase in the formation of PI(3)P,
and this synergy extends to formation of exocytic trans-
port vesicles from the TGN.

EXPERIMENTAL PROCEDURES

Materials

Unless otherwise indicated, all chemicals were obtained from Sigma or Boehringer Mannheim. Phosphatidylinositol was purchased from Avanti Polar Lipids, (Alabaster, AL). Production of specific antibodies against the pIgA-R and PITP has been described (15, 16).

Methods

Subcellular Fractionation Procedures—Stacked Golgi fractions (SGF) were isolated from rat liver according to Taylor et al. (17). Briefly, livers were removed, finely minced, and resuspended at 6 g/10 ml 0.5 mM sucrose in 100 mM KPO4, pH 6.8, 5 mM MgCl2, and 1 μg/ml each of a mixture of proteolytic inhibitors: chymostatin, leupeptin, antipain, and pepstatin. All sucrose solutions contained the same buffer and proteo-
lytic inhibitors. The homogenate was centrifuged (1500 × g for 10 min) to pellet unbroken cells, cell debris, and nuclei. This pellet contained at least 50% of the cell protein. The resulting supernatant (PNS) was loaded in the middle of a sucrose step gradient in an SW28 tube; steps
of 1.3 and 0.86 M sucrose were overlaid with the PNS supernatant (0.5 M) followed by a 0.25 M layer and centrifuged for 1 h at 100,000 × g (Beckman Instruments, Palo Alto, CA). The 0.5 M sucrose soluble fraction was collected and used for the preparation of cytosol. The SII fraction (0.5/0.86 M interface) was adjusted to 1.15 M sucrose with 2 M sucrose and ultracentrifuged. The SII-14 inserted into an acetic acid resin (Qiagen), and eluted with a linear gradient of imidazole (0–40 mg/ml (Amicon, Beverly MA). Protein assays (DC Protein Assay, Bio-Rad) were carried out on all fractions. Aliquots of these fractions were frozen in liquid nitrogen and stored at −70 °C.

**Cell-free Assay of pIgA-R Containing Exocytic Vesicle Formation from the TGN**—The cell-free assay of budding from an immobilized SGF was carried out as described (22). Each assay contains 2.5 mg of magnetic core and shell beads with approximately 50 μg of SGF immobilized. The immobilized fraction is characterized in Ref. 23. For the budding reaction the immobilized fraction was incubated in 2.5 ml containing 0.70 mg/ml cytosol, 25 mM HEPES, pH 6.7, 25 mM KCl, 1.5 mM magnesium acetate, 1.0 mM ATP, an ATP regeneration system (8.0 mM creatine phosphate, 0.043 mg/ml creatine phosphokinase), and 5 mg/ml bovine serum albumin were present. The ATP regenerating system and cytosol were omitted, the background budding is ~5%. There is no detectable PTP on the SGF, therefore, in antibody inhibition studies we used 20 μg protein A which is ~60% efficient. When the ATP regenerating system and cytosol are omitted, the amount of budding from the cell-free system is ~70% efficient. When the ATP regenerating system and cytosol are omitted, the amount of budding from the cell-free system is ~70% efficient. When the ATP regenerating system and cytosol are omitted, the amount of budding from the cell-free system is ~70% efficient. When the ATP regenerating system and cytosol are omitted, the amount of budding from the cell-free system is ~70% efficient. When the ATP regenerating system and cytosol are omitted, the amount of budding from the cell-free system is ~70% efficient. The presence of at least a 100:1 molar ratio of phospholipid:Sec14p with phosphatidylcholine (PC) because these two lipids are not synthesized in E. coli Lysate—Hexahistidine-tagged rat PITPα was generated and cloned to the rat PITPα structural gene in the pQE31 vector (Qiagen). E. coli expressing the His6-tagged PITPα were harvested, resuspended in ice cold lysis buffer, and disrupted as above. The homogenate was serially centrifuged at 5,000 × g, 12,000 × g, and 100,000 × g, and the 100,000 × g supernatant was used in the PI 3-kinase and transfer assays. The bacterial high speed supernatant (1 mg) contained one unit of PI transfer activity. **Phosphatidylinositol Transfer Assays**—PI transfer assays have been described previously (20). Briefly, rat liver microsomes were employed as [3H]PI donors in the transfer reaction, and unlabeled PC liposomes served as acceptor vesicles (21). Prior to the start of the reaction, 1 ml of donor microsomes was mixed with 0.5 ml of EDTA, and 5 ml Tris-HCl, pH 7.4) were incubated with either purified Sec14p or lysates containing rat PITPα lyses at 37 °C. After 30 min the reactions were centrifuged at 10,000 × g for 10 min to pellet the donor microsomes, and 1 ml of the supernatant was collected for scintillation counting. Under these conditions, the PI transfer reaction is linear for 60 min in preparations of yeast membranes or rat liver microsomes. The assay is capable of sustaining 20% transfer or less. One unit of activity is defined as the amount of transfer protein that catalyzes the transfer of 1% radiolabeled phospholipid in 1 min (21).

**RESULTS AND DISCUSSION**

PITP Plays an Essential Role in Budding of Exocytic Vesicles from the Hepatocyte TGN—PITP is a cytosolic factor that plays an essential role in secretory vesicle formation from the yeast Golgi complex and constitutive and regulated secretory granules in neuroendocrine cells (4–6). We have used two independent approaches to examine whether PITP is required for cell-free formation of pIgR-containing vesicles budding from the rat hepatocyte TGN. First, the cell-free assay was challenged with a polyclonal antibody that recognizes both rat PITPα and PITPβ isoforms (16). A concentration-dependent inhibition of vesicle formation was observed (Fig. 1). The ~65% budding efficiency of the control assay was reduced to ~40, 25, and 10%...
Preimmune serum the budding efficiency remained at was specific as in the presence of an equivalent amount of concentrations ranging from 0.25 to 1.25 mg/ml (Fig. 2).

Cytosol titration experiments demonstrated that for- mation of pIgA-R containing vesicles was linear at cytosol. Cytosol was capable of stimulating vesicle formation when the assay was carried out in the presence of limiting concentrations of yeast Sec14p or p62.

As a second approach to test for a role for PITP in formation of exocytic vesicles from the TGN, we examined whether PITP was capable of stimulating vesicle formation when the assay was carried out in the presence of limiting concentrations of cytosol. Cytosol titration experiments demonstrated that formation of pIgA-R containing vesicles was linear at cytosol concentrations ranging from 0.25 to 1.25 mg/ml (Fig. 2A). At higher cytosol concentrations, pIgA-R vesicle formation plateaued at an efficiency of approximately 70%. For subsequent experiments, cytosol was limited to 0.25 mg/ml, a concentration that provides an ~12% efficiency of pIgA-R vesicle formation, and increasing concentrations of PITP were added. Yeast PITP (Sec14p) was employed because the recombinant protein was quite stable and could be readily purified to homogeneity. Previous studies had demonstrated the interchangability of Sec14p and mammalian PITP in phosphoinositide-dependent systems reconstituted from mammalian cells (5, 10, 12). At the highest concentration of PITP, vesicle formation was restored to an efficiency of approximately 40% (Fig. 2B). Similarly, supplementation of the budding assay with p62 restored vesicle budding to an efficiency of ~40%. Neither component alone was sufficient to restore the assay to the full budding efficiency of ~70%. Strikingly, co-addition of PITP and p62 elicited a dramatic stimulation in vesicle formation relative to that achieved by addition of either component alone. The co-addition of both components at concentrations that individually restored vesicle budding activity to efficiencies of ~25% effected a cooperative restoration of vesicle formation to maximal efficiency (~70%).

In summary, antibodies against PTPα specifically inactivated the vesicle formation reaction (Fig. 1). When the cell-free assay is carried out in limiting amounts of cytosol, introduction of either yeast Sec14p or p62-associated PI 3-kinase to the assay stimulated vesicle formation (Fig. 2B). Importantly, PITP and p62-associated PI 3-kinase synergized to drive pIgA-R vesicle formation to the full efficiency achieved with an optimal amount of cytosol (Fig. 2B). These data indicate that PITP and p62-associated PI 3-kinase functionally cooperate in the formation of exocytic vesicles from the TGN.

Synergy between PTPα and the p62-associated PI 3-Kinase Extends to Synthesis of PI(3)P—The similar wortmannin sensitivities of p62-associated PI 3-kinase activity and vesicle formation from the TGN suggested that PI(3)P formation underlies the p62-associated PI 3-kinase requirement for vesicle formation. If PI 3-kinase activity and PI(3)P production are prerequisites for exocytic vesicle formation, the functional synergy between the p62-associated PI 3-kinase and PITP should extend to PI(3)P production. Moreover, the production of PI(3)P should be stimulated by GTP because activation of the p62-associated small GTPase results in activation of PI 3-kinase activity (2).

To establish that the mammalian PTPα exhibits the capability to stimulate p62-associated PI 3-kinase activity, recombinant PTPα (in the form of a bacterial high speed supernatant) was added to the immunopurified p62-associated PI 3-kinase. As shown in Fig. 3A, a bacterial high speed supernatant from E. coli not expressing PTPα sustained only a low level of PI 3-kinase activity. By contrast, addition of bacterial high speed supernatant that contained PTPα supported a

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**Fig. 1.** PITP is essential for exocytic vesicle formation. The cell-free assay was carried out as described under “Methods” in the presence of increasing concentrations of an antiserum (5, 10, and 15 μg) that recognizes rat PTPα and PITPβ. Controls assays were as follows: Control/ATP, the absence of cytosol and ATP; Control/+ATP, the presence of cytosol and ATP; and Preimmune 15 μg, in the presence of preimmune serum. The budded fraction was resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, immuno- blotted with antibodies against the pIgA-R, detected with125I-protein A, visualized by autoradiography, and quantitated with a Phospho-Imager. The budding efficiency of formation of pIgA-R containing vesicles (amount of the 116-kDa form of the pIgA-R in budded fraction/total amount 116-kDa form in immobilized SGF) was calculated and is plotted for each condition.

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**Fig. 2.** PITP and p62-associated PI 3-kinase are synergistic in restoration of vesicle formation at limiting concentrations of cytosol. The cell-free assay was carried out as described under “Methods” in the presence of increasing amounts of cytosol (0–2.5 mg/ml). The efficiency of formation of pIgA-R containing vesicles is plotted versus the concentration of cytosol in each assay (A). Cell-free assays were carried out in the presence of limiting amounts of cytosol (0.25 mg/ml) with the addition of increasing concentrations of purified Sec14p (0–100 μg) (B, diamonds) or the immunopurified p62-associated PI 3-kinase (0–60 μg) (squares). To test for additive effects of using the two components in combination, increasing concentrations of Sec14p were added in the presence of 20 μg of p62-associated PI 3-kinase (B, circles).
3–5-fold concentration-dependent stimulation of the p62$^{plic}$-associated PI 3-kinase activity (Fig. 3A). This stimulation was amplified further by activation of the 25-kDa GTPase bound to p62 (Fig. 3B). Whereas p62$^{plic}$-associated PI 3-kinase was individually stimulated 3- and 5-fold by a fixed concentration of GTPyS (100 nM) and PITPα, respectively, the simultaneous addition of both reagents resulted in ~30-fold stimulation (Fig. 3B). These data revealed a powerful synergy between the GTPase bound to p62 and PITPα in stimulation of the p62$^{plic}$-associated PI 3-kinase activity.

**p62$^{plic}$-associated PI 3-Kinase Can Utilize PITP-bound Phospholipid as Substrate—**There are presently two general views for how PITP might stimulate p62$^{plic}$-associated PI 3-kinase activity. The "substrate presentation" model posits that PITP acts as a co-factor that presents PI to PI kinases and thereby stimulates the initial rate of the headgroup phosphorylation reaction (11–13). The "lipid transfer" model proposes that PITP merely sustains PI kinase activity by effecting transfer of PI down a chemical gradient that is itself created by depletion of PI by metabolic enzymes such as PI kinases and PI phospholipases (14).

To examine the possibility that PITP presents substrate to the p62$^{plic}$-associated PI 3-kinase, PI 3-kinase assays were performed in a membrane-free system using purified Sec14p loaded with either PC (Sec14p-PC) or PI (Sec14p-PI). These experiments allowed the interaction between a lipid kinase and PITP to be examined in a purified system. The source of PI in the kinase assays was limited to that which was stoichiometrically bound to Sec14p (20 μg/ml), and this concentration of PI was an order of magnitude lower than that present in the standard assay (i.e. 200 μg/ml) (Fig. 4A). Under these assay conditions, the p62$^{plic}$-associated PI 3-kinase alone had minimal PI 3-kinase activity. Introduction of Sec14p-PC in the assay reduced that activity to base-line levels. By contrast, Sec14p-PI stimulated the p62$^{plic}$-associated activity 5-fold. These data suggest that the p62$^{plic}$-associated PI 3-kinase is capable of directly phosphorylating PI directly bound to PITP and that this presentation of PI enhances kinase activity.

To further characterize these interactions, additional PI was added to a parallel set of kinase assays to bring the total PI concentration (Sec14p-bound and free) to the levels present in the standard assay (200 μg/ml). A stimulation of PI(3)P formation was observed under all assay conditions (Fig. 4B). Addition of either Sec14p-PC or Sec14p-PI to the assay increased PI 3-kinase activity 6–7-fold. This stimulatory effect of Sec14p-PC is attributed to its ability to rapidly exchange bound ligand for the excess PI present in the reaction mixture, effectively converting Sec14p-PC to Sec14p-PI during the course of the reaction.

Activation of the GTPase bound to p62 provided an additional enhancement of PI 3-kinase activity as measured by PI(3)P production (Fig. 4C). Sec14p-PI and GTPyS individually stimulated this activity some 4-fold, and co-addition of both Sec14p-PI and GTPyS effected a synergistic 10–12-fold stimulation of the PI 3-kinase activity.

**A Role for PITP in PI 3-Kinase Activity—**The collective data reported herein demonstrate that the p62$^{plic}$-associated PI 3-kinase is stimulated both by Sec14p and mammalian PITPα. More dramatically, activation of the small GTPase bound to p62 in the presence of PITPα supported a synergistic activation of the p62$^{plic}$-associated PI 3-kinase of up to 30-fold. In addition to activating the generation of PI(3)P, the cooperation of PITPα and the p62$^{plic}$-associated PI 3-kinase are essential for pIgA-R vesicle formation from the TGN. We propose that a specific pool of PI(3)P is generated "on demand" at the site at which it will be utilized. In this regard, we emphasize that the p62$^{plic}$-
associated PI 3-kinase assembles with the cytosolic domain of an integral membrane protein of the TGN (TGN38) (2). This organization suggests that trans-Golgi proteins (perhaps even cargo proteins) provide positional cues for assembly of this specific PI 3-kinase.

There are multiple steps in formation of vesicles from the TGN, and the cell-free assay more than likely measures all of these steps. These include sorting of molecules, tubule formation, and vesicle budding (22, 25, 26). We propose that PI(3)P is a key regulatory molecule in the vesicle formation and that its generation may provide signaling molecule required for activation/integration of any one of these steps.

Recently others have shown a similar PI 3-kinase activity associated with TGN46, the human orthologue of TGN38 (27). Those studies failed to reveal either a GTPase or a PTP-mediated activation of the associated PI 3-kinase activity. Unfortunately, the relevant experiments performed in that study are difficult to evaluate because the specific activities for PI transfer of the PTP, and the concentrations of GTPyS employed are not provided, so it is difficult to relate these two bodies of work in a meaningful manner.

Finally, our data speak to how PTPs might cooperate with PI 3-kinase in exocytic vesicle formation from the TGN. The demonstration that p62associated PI 3-kinase activity is stimulated when the sole source of PI in the kinase assay was PTP-bound lipid strongly suggests that p62associated PI 3-kinase is at least capable of utilizing the PI presented by PTP as an effective (and perhaps even optimal) substrate for phosphorylation (Fig. 4A). These findings are consistent with the notion that the PTP-bound PI pool represents the physiologically relevant substrate for the p62associated PI 3-kinase. Moreover, the finding that PC-bound PTP was inactive in the stimulation of PI 3-kinase argues against a role for the PC-bound PTP effecting some allosteric stimulation of the kinase. An important caveat to these membrane-free PI 3-kinase experiments is that Sec14p was the source of PTP. Our choice of Sec14p for these studies was driven by its stability and our ability to rapidly purify it to homogeneity. The mammalian PTP was unstable after purification, which made these experiments impossible. It is important to note that present evidence indicates that the in vitro function of Sec14p is directed at maintaining a pool of Golgi membrane diacylglycerol in a manner that employs converging functions for the PC- and PI-bound forms of Sec14p (8). There is no evidence that Sec14p synergizes with a yeast PI 3-kinase in a physiologically relevant way. Nonetheless, both mammalian PTPs and

![FIG. 4. Sec14p and an activated GTPase synergistically enhance the rate of PI(3)P production by p62-associated PI 3-kinase. PI 3-kinase assays were carried out for increasing amounts of time (0–20 min) using the immunopurified p62-associated PI 3-kinase (50 ng) (no membrane was present in the assay). The substrate was PI (squares), PI bound to Sec14p (diamonds), or PC bound to Sec14p (circles). The total lipid concentration of the assay was 20 μg/ml, the amount that is stoichiometrically bound to Sec14p (A). PI 3-kinase assays were carried out as in A with the addition of PI to adjust its final concentration to 200 μg/ml. The symbols are the same as in A. To test whether activation of the small GTPase bound to p62 influences the PI 3-kinase activity, assays were carried out using 200 μg/ml PI in the presence or absence of GTPγS (100 nM) under the following conditions: p62-associated PI 3-kinase (filled squares); p62-associated PI 3-kinase and GTPγS (half-filled squares); p62-associated PI 3-kinase and Sec14p (closed diamonds); and p62-associated PI 3-kinase and Sec14p and GTPγS (half-filled diamonds) (C). Note that the PhosphorImager units change increase from A (150,000 maximum), B (200,000 maximum), to C (600,000 maximum).]
Sec14p behaved similarly in the cell-free assay of pIgA-R vesicle formation from the TGN with regard to synergy with p62\textsuperscript{cplx}-associated PI 3-kinase activity. The synergy observed between the p62\textsuperscript{cplx}-associated PI 3-kinase activity and both mammalian PITP\textsubscript{a} and Sec14p indicates that a functional interaction between these factors is critical for PI(3)P generation. Further, a spatially and temporally regulated burst of PI(3)P synthesis is a prerequisite for exocytic vesicle formation from the TGN.

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