Involvement of PITPnm, a Mammalian Homologue of Drosophila rdgB, in Phosphoinositide Synthesis on Golgi Membranes*

(Received for publication, November 16, 1998, and in revised form, March 11, 1999)

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Phosphatidylinositol transfer protein (PITP) is involved in phospholipase C-mediated signaling and membrane trafficking. We previously reported cloning and characterization of a gene encoding for membrane-bound PITP, named PITPnm, that is a mammalian homologue of the Drosophila retinal degeneration B (rdgB) gene (Aikawa, Y., Hara, H., and Watanabe, T. (1997) Biochem. Biophys. Res. Commun. 236, 559–564). Here we report the subcellular localization of PITPnm protein and provide evidence for its involvement in phosphatidylinositol 4-phosphate (PtdIns 4-P) synthesis. PITPnm is an integral membrane protein that largely localized in close association with membranes of Golgi vacuoles and the endoplasmic reticulum (ER). The amino terminus region of PITPnm was exposed to cytoplasmic side. Interaction with various phosphoinositides was observed in the amino terminus region spanning from 196 amino acids to 257 amino acids of PITPnm. At the amino terminus regions of 1–372 amino acids, PITPnm formed a complex with type III PtdIns 4-kinase. The transmembrane and carboxyl-terminal portions (residues 418–1242) functioned to retain the PITPnm in the Golgi vacuole. These results suggest that PITPnm plays a role in phosphoinositide synthesis on the Golgi vacuoles and possibly in the PtdIns signaling pathway in mammalian cells.

Phosphoinositides and their cleavage products play a critical role not only as second messengers in signal transduction at the cell surface but also as regulators that modulate the function of proteins involved in intracellular signal transduction (1–4). The mutation of proteins involved in the phosphoinositide signaling pathway leads to severe biological dysfunction. Recent studies have shown that targeted deletion of the murine phospholipase C (PLC)1 resulted in embryonic lethality (5) and that the vibrator mutation in mice caused neural degeneration (6). PITPa transfers either phosphatidylinositol (PtdIns) or phosphatidylcholine (PC) between membrane bilayers in vitro (7) and is an essential component of the PLC-mediated signal transduction pathway (8–10) as well as being important in membrane trafficking in vivo (11–13). In the epidermal growth factor-mediated signaling pathway, PITPa associates with epidermal growth factor receptor, PtdIns 4-kinase, and PLC-g (9). The Saccharomyces cerevisiae SEC 14 gene product is the major PITP of yeast and plays an essential role in maintaining the diacylglycerol pool that is required for Golgi secretory function (14) and cell viability (15). Rescue of SEC 14 defects in yeast with mammalian PITPs or two novel soybean Sec 14p homologues has been observed (16, 17).

Previously, we reported cloning and characterization of the first mammalian membrane-bound PITP, named PITPam. The PITPam protein contains a PITPa homology region (amino acids aa 1–257) and putative six-membrane spanning regions at the carboxyl terminus. The protein is abundantly expressed in brain, but also ubiquitously in other tissues, and is thought to be a counterpart of the Drosophila retinal degeneration B protein (rdgB) (18). Null mutation of this gene in Drosophila is responsible for the rdgB mutant whose phenotype is characterized by light-dependent retinal degeneration (19–21) and an olfaction defect (22). Expression of the mouse PITPam cDNA in rdgB mutant flies suppressed both the rdgB-dependent retinal degeneration and the abnormal electroretinogram (23), but the biochemical function and subcellular localization of the PITPam in mammalian cells still remains largely unknown.

To understand the role of the PITPam protein in signaling in mammalian cells, it is important to determine its subcellular localization and steps of the PtdIns signaling pathway in which the PITPam may be involved. In the present study, we demonstrate that PITPam interacts with type III PtdIns 4-kinase, localizes on Golgi membranes, and synergizes with PtdIns 4-kinase in the formation of PtdIns 4-P.

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors—To construct an epitope tagged PITPam, an XhoI site was introduced into the 5′ and 3′ ends of the full-length mouse PITPam cDNA and ligated into the mammalian expression vector pcDNA 3.1 Myc-His (Invitrogen Corp., Carlsbad, CA). The XhoI–ClaI fragment (1.3 kilobases) was excised from full-length PITPam cDNA and ligated into the pcDNA Myc-His (designated Δ378–1242) and into pEGFP-N1 (CLONTECH Laboratories), respectively. The XhoI–BglII fragment (2.7 kilobases) was excised from full-length PITPam cDNA and ligated into the pcDNA 4xFLAG (designated Δ707–1242). A PITPa homology region of PITPam (aa 1–257) was amplified by the polymerase chain reaction (PCR) by using sense primers with an XhoI site at the 5′ end and antisense primers with a HindIII site at the 3′ end. The XhoI–HindIII fragment was gel-purified, digested with XhoI and HindIII, and then

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† The abbreviations used are: PLC, phospholipase C; PITP, phosphatidylinositol transfer protein; PtdIns, phosphatidylinositol; PC, phosphatidylcholine; PITPam, membrane-bound PITP; rdgB, retinal degeneration B; rdgA, retinal degeneration A; GFP, green fluorescent protein; GST, glutathione S-transferase; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; ER, endoplasmic reticulum; aa, amino acids(s); CDS, CDP-diacetylglucosamine synthase; norpA, no receptor potential A.
FIG. 1. Western blotting of PITPnm. A, a schematic illustration of a Myc-tagged construct of full-length PITPnm (wild type) and a deletion mutant of PITPnm, Δ(258–1242). TM, transmembrane domain. B, Western blotting with affinity-purified rabbit anti-PITPnm antibodies. The antibodies recognized one major band in the extracts prepared from wild type PITPnm-transfected COS-7 cells, X63 cells, and P19 cells. C, the cell extracts prepared from P19 cells were fractioned by differential solubilization as described under “Experimental Procedures.” P, pellet; S, supernatant, respectively. D and E, subcellular fractionation and differential solubilization of the full-length and PITPnm deletion mutant proteins, respectively. These blots were developed with anti-Myc antibody. The mobility of molecular mass markers is shown on the left.

ligated into the pcDNA 3.1 Myc-His (designated Δ(257–1242)). The full-length Myc-tagged construct was digested with PmaC1 and self-ligated (designated Δ(64–475)). The expression vector, pSRE, encoding for FLAG-tagged type III PtdIns 4-kinase cDNA has been described previously (24).

Cell Culture and Transfection—COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) with 10% fetal calf serum (Sanko Junyaku Co., Ltd., Tokyo, Japan). X63 and P19 cells were maintained in RPMI 1640 (Life Technologies, Inc.) and α-minimum essential medium (Life Technologies, Inc.) with 10% fetal calf serum. The expression vectors described above were transiently transfected into COS-7 cells using LipofectAMINE™ (Life Technologies, Inc.) according to the manufacturer’s instructions.

Bacterial Expression and Purification of Recombinant Proteins—The various truncated forms of GST-fusion proteins were expressed in Escherichia coli by standard procedures. The cDNAs encoding recombinant protein I (aa 1–372) and IV (aa 1–257) were amplified by PCR using sense primers with an XhoI site at the 5’ end and anti-sense primers with a BamHI site at the 3’ end. Each fragment was excised with BamHI and XhoI and ligated into the pGEX 5X-1 vector. XhoI-EcoRI and EcoRI-EcoRI fragments, which encode the recombinant proteins II (aa 1–196) and III (aa 196–372), respectively, were ligated into pGEX 2T and pGEX 1T. The mouse PITPα cDNA was cloned by PCR using mouse spleen cDNA libraries as template and the previously described primers (25). The positive clone was confirmed by nucleotide sequencing, digested with NdeI/BamHI, and ligated into the pGEX 2T vector.

Immunofluorescence Analysis— transiently transfected COS-7 cells grown on glass coverslips were fixed with 4% paraformaldehyde in PBS for 30 min. Fixed coverslips were permeabilized, and nonspecific reactive sites were blocked for 1 h at room temperature in PBS containing 0.3% Triton X-100, 1% bovine serum albumin, and 0.2% skim milk. Cells were then incubated with affinity-purified polyclonal anti-PITPnm antibodies (18) or anti-c-Myc (9E10) antibody (Santa Cruz Biochemistry, Inc., Santa Cruz, CA) overnight at 4 °C. A monoclonal antibody to the Golgi marker protein (Sigma) was used to identify the Golgi vacuole. Antibodies were detected by Cy3TM- or Cy2TM-labeled goat anti-rabbit and anti-mouse IgG (H+L) (Amersham Pharmacia Biotech, Buckinghamshire, UK). The coverslips were observed under confocal laser microscopy (Leica Laser Technik, Heidelberg, Germany).

Subcellular Fractionation—For subcellular fractionation, cells were swollen in homogenizing buffer (20 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 1 mM phenylmethylsulfonyl fluoride) and disrupted by Dounce homogenization. Nuclei and unbroken cells were removed by centrifugation at 800 × g for 15 min at 4 °C. The supernatants were centrifuged at 100,000 × g for 1 h and separated into membrane and cytosol fractions. For alkaline extraction of the membrane fraction, the second membrane pellet obtained above was resuspended in 0.1 M Na2CO3 (pH 11) and incubated on ice for 10 min as described previously (26). The samples were repelleted by centrifugation at 100,000 × g for 1 h.

Phosphoinositide Binding Assays—The enzyme-linked immunosorbent assay (27) was performed with the following modification. The various phosphoinositides (100 µg/well) dissolved in chloroform/methanol (1:9) were coated onto 96-well multiplates and dried at 60 °C. The lipid-coated plates were blocked with 5% skim milk (w/v) in PBS for 1 h at room temperature and various amounts of recombinant proteins were added to each well and incubated at 4 °C overnight. After washing the plates with PBS, antibody against GST was added to the wells and incubated at room temperature for 1 h. The plates were then washed and incubated with peroxidase-conjugated anti-rabbit immunoglobulins (Zymed Laboratories Inc.). The interactions were visualized with the TMB Microwell Peroxidase Substrate System (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The lipid-protein co-sedimentation assay was performed as described previously (28–30). Briefly, protein samples were added to tubes containing lipid vesicles (100 µM total lipid concentration) and PBS in a final volume of 100 µl. Vesicles were made to contain increasing molar percentages of PtdIns (Sigma): (0% (100% PC), 1% (99% PC), 2.5% (97.5% PC), 5% (95% PC), 10% (90% PC)) or the indicated concentrations of PtdIns 4-P or PtdIns 4,5-P2 (Sigma). After incubation for 30 min, the lipid vesicles were precipitated by centrifugation at 100,000 × g for 30 min. The distribution of the recombinant proteins between the supernatant and pellet fractions was determined by SDS-polyacrylamide gel electrophoresis, and the gels were stained with Coomassie Brilliant Blue. Densitometric analysis was performed on the stained gel.

PtdIns 4-Kinase Activity—PtdIns 4-kinase activity was assayed as
described previously (24) with the following modification. Briefly, the reaction mixture (100 μl/assay) contained 50 mM Tris-HCl (pH 7.4), 1 mM EGTA, 20 mM MgCl₂, 0.1% Triton X-100, and 5 mg/ml sonicated PtdIns. The reaction was started by adding 50 μM ATP and 10 μCi of \([g-32P]\)ATP (Amersham Pharmacia Biotech) at 37 °C and stopped by addition of 160 μl of 1 N HCl. The lipid was extracted with 320 μl of 1:1 (v/v) chloroform:methanol. The organic layer was analyzed by thin layer chromatography (TLC) on a silica gel plate (Merck, Darmstadt, Germany). The TLC plate was stained with iodine to identify the lipid spots, and the radioactivity was quantified by scintillation counting after excision of the spots corresponding to PtdIns 4-P. A similar protocol was employed for assaying PtdIns 4-kinase activity, with the exception that the assay buffer did not include exogenous PtdIns or Triton X-100.

Co-immunoprecipitation Analysis—Transfected COS-7 cells were harvested and lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 0.5% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA) with protease inhibitors, 10 μg/ml leupeptin, and aprotinin. Lysates were incubated for 1 h at 4 °C with gentle rocking and centrifuged at 10,000 × g for 20 min at 4 °C. Lysates were precleared by incubation with 0.1% albumin-coated protein G-Sepharose beads. The supernatants were divided and incubated for 2 h with a monoclonal anti-FLAG-M2 antibody (Eastman Kodak Co.), anti-c-Myc antibody, or control anti-mouse IgG (Zymed Laboratories Inc.) antibodies bound to protein G-Sepharose beads. The beads were washed three times with the lysis buffer.

Electron Microscopy—Cells were collected and washed twice with 0.1 M cacodylate buffer (pH 7.4). The cell pellets were fixed for 1 h at 4 °C with 2% paraformaldehyde in a buffer containing 4% sucrose. After dehydration in a graded series of ethanol solutions, the samples were embedded in LR Gold resin (London Resin Company Ltd., Berkshire, UK) at −20 °C and sectioned. The sections were mounted on nickel grids, blocked with PBS containing 1% bovine serum albumin for 40 min, and then incubated for 4 h at 37 °C with an appropriate dilution of affinity-purified anti-PITPnm primary antibody. Sections were subsequently incubated for 1 h with goat anti-rabbit IgG conjugated with 10 nm gold particles (British BioCell International, Cardiff, UK) diluted 1:100. After staining with uranyl acetate and lead citrate, the sections were observed in a JEM-1200 EX electron microscope (JEOL, Tokyo, Japan) operated at 100 kV.

RESULTS

**PITPnm Is an Integral Membrane Protein**—The PITPnm protein contains a PITPα homology domain at the amino terminus (aa 1–257), an acidic domain (aa 341–362), and six putative membrane-spanning domains (wild type in Fig. 1A). To confirm that PITPnm is an intrinsic membrane protein, the full-length cDNA coding for mouse PITPnm was inserted into the pcDNA Myc-His expression vector and transiently trans-
Membrane-bound Phosphatidylinositol Transfer Protein in Golgi

PITP

Is Localized to the Golgi Vacuoles, Rough ER, and Vesicles—To further discern the intracellular localization of the PITP
protein and recombinant products were subjected to high pH (0.1 M Na2CO3, pH 11.3) or high salt (1 M NaCl) extraction. Under these conditions both the endogenous and transfected PITP
were found exclusively in the pellet fractions (Fig. 1, C and D). In contrast, the deletion mutant, the Δ-(258–1242) (Fig. 1A), which lacks the acidic and whole membrane domains of PITP
, was detected as a 33-kDa protein present in the cytosolic fraction under the same experimental conditions (Fig. 1E). These results indicate that PITP
is an integral membrane protein with its carboxyl terminus anchored in the lipid bilayer.

PITP
Is Localized to the Golgi Vacuoles, Rough ER, and Vesicles—To further discern the intracellular localization of the PITP
, immunofluorescence studies were performed using confocal laser microscopy. COS-7 cells transiently expressing Myc-tagged full-length PITP
CDNA were stained with anti-PITP
antibodies and/or with anti-c-Myc antibodies. The anti-PITP
antibody did not stain nontransfected cells (Fig. 2A). Immunoreactive transfected cells accounted for approximately 15–20% of the total cells, and the anti-PITP
and anti-Myc antibodies yielded identical staining patterns (Fig. 2, B and C). Both antibodies revealed reticular staining pattern near the nucleus and in the cytoplasm, which is a characteristic of ER localization. In addition, of note, a clear juxtanuclear labeling pattern reminiscent of the Golgi complex was observed. To confirm this possibility, P19 embryonal carcinoma cells were doubly stained with anti-PITP
antibody and anti-Golgi 58 K protein antibody. In this staining, complete colocalization was observed (Fig. 2F). The immunoreactive products were densely aggregated in the form of caps in juxtaposition to the nuclei, which is the labeling pattern of the Golgi vacuoles (Fig. 2, D and E), suggesting that the majority of the endogenous PITP
may be present in Golgi vacuoles. In contrast, the Δ-(258–1242) mutant was detected not only in Golgi vacuoles (Fig. 1A), but also diffusely throughout the cytoplasm (data not shown). This is consistent with the results of subfractionation of the lysates from COS-7 cells transfected with the Δ-(258–1242) of PITP
(Fig. 1E).

By immunoelectron microscopy (Fig. 3), the immunoreactive protein was restricted to the cytoplasmic side of the membrane of Golgi vacuoles (Fig. 3A, arrows) and rough ER (Fig. 3, B and C, arrows). To determine the orientation of the amino-terminal end of the PITP
in Golgi vacuoles, a rabbit antibody that specifically recognized the amino-terminal region (aa 260–372) was used for staining. These results indicate that the amino-terminal portion of the PITP
protein is exposed to cytoplasmic surface but not to the lumen. In addition, the labeled PITP
was also found in vesicles; however the nucleus and mitochondria were not labeled (data not shown). No specific gold labeling was observed in samples stained with rabbit preimmune serum or stained only with gold-conjugated secondary antibody. In summary, the morphological data confirm that PITP
is localized to the membrane of the Golgi vacuoles, ER, and vesicles and that the amino-terminal region of PITP
is exposed to cytoplasmic surface.

Binding of Phosphoinositides to the PITPα Domain of PITP
—To test whether the amino-terminal region of PITP
, which is homologous to soluble PITPα, is able to bind phosphoinositides, the lipid-protein interaction was examined using an enzyme-linked immunosorbent assay and a lipid-protein co-sedimentation assay. Expression vectors encoding four different truncated GST fusion proteins (I-IV) of PITP
and the GST fusion protein of PITPα were constructed (Fig. 4A). These proteins were expressed in E. coli and purified (Fig. 4B). In vitro lipid-binding activities of these recombinant proteins were determined using PtdIns, PtdIns 4-P, and PtdIns 4,5-P2 (Fig. 4C). As a positive control, binding activity of the mouse PITPα was also measured. Recombinant proteins I (aa 1–378), III (aa 196–372), and IV (aa 1–257) bound avidly to PtdIns, PtdIns 4-P, and PtdIns 4,5-P2 (Fig. 4C). Binding activities reached a plateau at 15 μg/well protein, while protein II (aa 1–195) and control GST protein alone did not bind to the lipids. These data indicated that the residues 196–257 of PITP
comprise a binding site for PtdIns, PtdIns 4-P, and PtdIns 4,5-P2 and that binding affinity is similar to that of soluble PITPα. The binding activity of these recombinant proteins to phosphoinositides was also independently confirmed using a lipid-protein co-sedimentation assay (Fig. 4D). Densitometric analysis of recombinant proteins in lipid-bound (pellet) and lipid-unbound (supernatant) fractions was performed on the stained gel. Specific binding of the PITPα and recombinant protein I to PtdIns, PtdIns 4-P, and PtdIns 4,5-P2 was observed with increasing molar percentage of phosphoinositides relative to PC as a control, supporting the previous report that PITPα has binding affinity for PtdIns 4,5-P2 micelles (31). The recombinant protein III and IV also bound to these vesicles (data not shown). In contrast, protein II and the control GST protein alone showed little or no binding activity. These results are

![Fig. 3. Immunoelectron micrographs of COS-7 cells transfected with full-length PITP
-Myc. A, a visualization of the Golgi vacuoles (GV) in COS-7 cells transfected with PITP
-Myc. Localization of PITP
-Myc was detected using gold particle-labeled anti-PITP
antibodies. Only the cytoplasmic side of the membrane of GV was labeled (arrow). B and C, gold particles are present in the cytoplasmic side of the membranes of rough ER, but not in the mitochondria (M) (arrow). Bar = 100 nm.](image-url)
consistent with that of the enzyme-linked immunosorbent assay.

Identification of PtdIns 4-Kinase-associating Regions of PITPnm—PITPnm binds PtdIns, which is a substrate for PtdIns 4-kinase (Fig. 4, C and D), and the amino-terminal residues (aa 1–257) of PITPnm are more than 46% identical to those of mouse PITPα. Therefore it was important to investigate whether PITPnm interacts with PtdIns 4-kinase. Although multiple PtdIns 4-kinases exist in mammalian cells (32), it has been reported that type III PtdIns 4-kinase is localized on the Golgi membrane (24). COS-7 cells were transfected with pcDNA 3 vector containing Myc-tagged PITPnm and a pSRE vector containing FLAG-tagged type III PtdIns 4-kinase cDNAs. The cells were then solubilized with 0.5% Nonidet P-40, and the cell lysates were immunoprecipitated with either anti-FLAG or anti-c-Myc antibodies. The immunoprecipitates were then immunoblotted with anti-c-Myc or anti-FLAG antibodies. As shown in Fig. 5B, full-length PITPnm-

![Image](image_url)
Myc protein and PtdIns 4-kinase-FLAG protein were immunoprecipitated by anti-FLAG and anti-c-Myc antibodies, respectively, indicating that the full-length PITPnm protein associated with PtdIns 4-kinase. Furthermore, both PITPnm and PtdIns 4-kinase appeared to accumulate mainly in Golgi vacuoles (Fig. 2, G–I). These results strongly suggest that PITPnm associates with type III PtdIns 4-kinase on the Golgi membranes in vivo. To define the region of PITPnm that mediates the association with PtdIns 4-kinase, we constructed four deletion mutants of Myc-tagged PITPnm (Fig. 5A). These deletion mutant cDNAs were co-transfected into COS-7 cells together with PtdIns 4-kinase cDNA. The expression of these mutants was confirmed by Western blot analysis (Fig. 5B). Two PITPnm deletion mutants, Δ(258–1242) and Δ(65–474), did not immunoprecipitate any bands detectable with the anti-FLAG antibody (Fig. 5C, b and d), while two other PITPnm deletion mutants, Δ(708–1242) and Δ(379–1242), did so (Fig. 5C, c and e). Because the Δ(379–1242) mutant had the same molecular size as that of immunoglobulin heavy chain, a GFP-tagged mutant protein was used instead of Myc-tagged protein. Taken together, these results indicate that both the PITPα homology region and acidic domain in the PITPnm are required for forming a complex with type III PtdIns 4-kinase in vitro.

The localization of these PITPnm deletion mutants was also investigated (Table I). The truncated proteins lacking the membrane-spanning region did not localize to the Golgi vacuoles, but the Δ(708–1242) was partially localized there. These results indicate that the transmembrane and carboxyl-terminal luminal domain are required for the Golgi vacuole localization. In fact, the complete transmembrane domain (aa 405–1242)-GFP tagged protein completely localized on the Golgi (data not shown).

**Involvement of PITPnm in PtdIns 4-kinase activity**—To examine further the involvement of PITPnm in phosphoinositides synthesis, we determined PtdIns 4-kinase activity in the PITPnm immunoprecipitates (Fig. 6). A cDNA from the full-length PITPnm and other PITPnm deletion mutants were transfected into COS-7 cells. Cell lysates from each transfected cell were immunoprecipitated with anti-Myc and subjected to a PtdIns 4-kinase assay (Fig. 6A). PtdIns 4-kinase activity was detected in anti-Myc immunoprecipitates of the full-length PITPnm, the Δ(708–1242) and the Δ(379–1242) in the presence of 0.3% Triton X-100, indicating that these proteins could interact with endogenous PtdIns 4-kinase in COS-7 cells. The cDNAs encoding the full-length PITPnm or other deletion mutants were co-transfected into COS-7 cells with type III PtdIns 4-kinase cDNA. Anti-Myc immunoprecipitates were subjected to the PtdIns 4-kinase assay in the presence of exogenous PtdIns and Triton X-100. The PtdIns 4-kinase activity was
detected in anti-Myc immunoprecipitates of full-length PITPnm, Δ-(379–1242) and Δ-(708–1242) (Fig. 6B). The production of PtdIns 4-P in anti-Myc co-immunoprecipitates with type III PtdIns 4-kinase (Fig. 6B) was at least 100-fold higher than that in anti-Myc immunoprecipitates alone (Fig. 6A). These results further support the notion that PITPnm can interact with the type III PtdIns 4-kinase. The interaction between the PITPnm and type III PtdIns 4-kinase was not affected even in the presence of 0.5% Triton X-100 (data not shown). In contrast, in anti-Myc immunoprecipitates of two other PITPnm deletion mutants, the Δ-(258–1242) and Δ-(65–474), PtdIns 4-kinase activity was undetectable (Fig. 6B). The endogenous PtdIns 4-kinase activity in the PITPnm immunoprecipitates in the absence of exogenous PtdIns and 0.3% Triton X was also determined. As shown in Fig. 6C, only the immunoprecipitates from the full-length PITPnm but none of the PITPnm deletion mutants enhanced the production of PtdIns 4-P, indicating that PtdIns 4-kinase could utilize endogenous PtdIns, which appeared to be held within the full-length PITPnm protein and produced PtdIns 4-P. These data indicate that PITPnm-dependent PtdIns 4-P synthesis requires not only the interaction with PtdIns 4-kinase but also the proper intracellular localization of the PITPnm on Golgi membrane. These results provide evidence that the PITPnm is involved in the biosynthesis of PtdIns 4-P and possibly also in other polyphosphate inositides on the membrane of the Golgi.

**DISCUSSION**

The present study demonstrates that PITPnm protein, a mammalian homologue of *Drosophila* RdgB protein, associates with the type III PtdIns 4-kinase in the Golgi membrane through the region (1–257 aa) homologous to PITPa and the adjacent acidic region (341–362 aa) (Fig. 5C). The PtdIns 4-kinase activity was detected in the immunoprecipitates containing full-length PITPnm molecules, and production of PtdIns 4-P was observed without addition of exogenous PtdIns. It has been showed that type III PtdIns 4-kinase activity of this molecule was markedly stimulated in the presence of Triton X-100 (24). Amounts of PtdIns 4-P in PITPnm immunoprecipitates were also markedly increased in the presence of Triton X-100. Biochemical analysis on the interaction between phosphoinositides (PtdIns, PtdIns 4-P, and PtdIns 4,5-P2) and various PITPnm truncated proteins indicated that the region consisting of the residues 196–257 in PITPnm, which is also conserved in soluble PITPa, is obligatory for binding phosphoinositides. Taken together, these data indicate that PITPnm forms a ternary complex with PtdIns and PtdIns 4-kinase in *vivo*.

PITPnm protein was mainly localized in the Golgi (Fig. 2). Retention of PITPnm in the Golgi membrane depends on both six putative transmembrane and carboxyl-terminal luminal domains of the molecule, but the transmembrane domain does have a major effect on its localization in Golgi. The experiments with deletion mutants indicated that PITPnm-dependent PtdIns 4-P production (Fig. 6) required not only its interaction

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### Table I

**Localization of PITPnm and its various deletion mutant molecules on Golgi vacuole and their interaction with type III PtdIns 4-kinase**

| Interaction with type III PtdIns 4-kinase | Localization on Golgi vacuoles |
|----------------------------------------|-------------------------------|
| Wild type                              | +                             |
| Δ-(258–1242)                           | −                             |
| Δ-(379–1242)                           | +                             |
| Δ-(65–474)                             | −                             |
| Δ-(708–1242)                           | +                             |

*Note: Δ-(708–1242)-Myc-tagged proteins were partially localized on the Golgi vacuoles.*

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**Fig. 6.** Analysis of PtdIns 4-kinase activity in the PITPnm co-immunoprecipitates. COS-7 cells were transfected with the PITPnm deletion mutants and used for immunoprecipitation with anti-Myc antibody. The resultant immunocomplexes were subjected to a lipid kinase assay in the presence of exogenous PtdIns and 0.3% of Triton X-100 (A). The PtdIns 4-P products were extracted, separated by thin layer chromatography, and visualized by autoradiography. The full-length PITPnm and other deletion mutants were co-transfected into COS-7 cells with type III PtdIns 4-kinase and immunoprecipitated with anti-Myc. Each immunocomplex was also subjected to PtdIns 4-kinase assay in the presence of Triton X-100. A, assay of PtdIns 4-P synthesis against endogenous PtdIns present in the anti-Myc co-immunoprecipitate. The results from duplicate assays are presented, and the variation was below 10%. Background counts ranging from 80 to 100 cpm at time 0 were subtracted.
with PtdIns 4-kinase but also its localization in Golgi membrane. These results suggest that PITPnm may play a role in production of phosphoinositides in Golgi through the interaction with PtdIns 4-kinase.

Although both the PITPs homologous region and adjacent acidic region of PITPnm are required to form complex with type III PtdIns 4-kinase, by contrast, the PITPn homologous region (aa 1–257) alone did not interact with the type III PtdIns 4-kinase (Fig. 5C). Two deletion mutants, the Δ(708–1242) and Δ(379–1242), which lack putative transmembrane domains, could also interact with type III PtdIns 4-kinase. However, endogenous PtdIns 4-kinase activity was not detected in the immunoprecipitates of these deletion mutants, unless excessive exogenous PtdIns and detergent were present (Fig. 6), implying that these deletion mutants may be unable to bind endogenous PtdIns that is localized in Golgi membrane. Thus, the acidic region and/or transmembrane domain in PITPnm, both of which are not conserved in PITPn, may play a role in stable binding of endogenous PtdIns. The phosphoinositide pool in cells appears to be compartmentalized in subcellular organelles, and the biological function of each organelle requires the specific PtdIns signaling molecules (33). It is therefore possible that endogenous PtdIns 4-P synthesis by PITPnm strongly depends on its localization in Golgi, and that the PtdIns pool in Golgi may be maintained by PITPnm together with PtdIns 4-kinase.

Expression of mouse PITPnm, a mammalian homolog of rdgB, in rdgB mutants flies rescued abnormal electronretrography and light-enhanced retinal degeneration (23). By contrast, expression of a mouse soluble PITPn failed to restore the rdgB mutant phenotypes (34). This finding might be due to the inability of soluble PITPn to localize in subrhabdomeric cisternal (SRC) membranes, where RdgB protein localizes in flies (35, 36), so that soluble PITPn did not supply the PtdIns to the PtdIns 4-kinase. Thus, PITPnm, similar to the Drosophila RdgB, may be photoreceptor cell-specific protein whose function cannot be compensated for by the ubiquitously expressed soluble PITPn. CDP-diaacylglycerol synthase (CDS) is an enzyme required to convert phosphatidic acid into CDP-diaacylglycerol, the precursor of PtdIns (37, 38). Null mutants of CDS are defective in signaling but only in response to light activation, suggesting that phosphoinositide pools required for signaling in photoreceptors might be distinct from the general pool (37). This could be rationalized by taking into account that the photoreceptor is highly specialized and a high signaling demand is imposed on these cells (39). Thus, although PITPnm has functional similarity with the soluble PITPn, that is both supply PtdIns 4-kinase with PtdIns, the physiological function of PITPnm in vivo is different from that of PITPn, because of its additional functional acidic and transmembrane domains, and its different subcellular localization and tissue-specific distribution.

In Drosophila photoreceptors, there is a large amount of G-protein-mediated PLCs (40), which hydrolyze PtdIns 4,5-P2 into inositol triphosphate and diacylglycerol after illumination (40, 41). Light causes retinal degeneration in the rdgB mutant flies (19–21), suggesting that activation of a G protein precedes the steps in the transduction cascade that lead to photoreceptor degeneration in the rdgB mutant. Activation of PLC also precedes the steps that lead to degeneration, because mutation of the norpA gene, which encodes light-activated PLC (42), blocked the light-enhanced degeneration in rdgB photoreceptors in the double mutant norpA/rdgB (20). Interestingly, null mutants of the photoreceptor-specific genes CDS and retinal degeneration A (rdgA) that are involved in PtdIns 4,5-P2 regeneration pathway had a phenotype similar to the rdgB mutant.
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