Cloning and Characterization of a 72-kDa Inositol-polyphosphate 5-Phosphatase Localized to the Golgi Network*

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The inositol-polyphosphate 5-phosphatase enzyme family removes the 5-position phosphate from both inositol phosphate and phosphoinositide signaling molecules. We have cloned and characterized a novel 5-phosphatase, which demonstrates a restricted substrate specificity and tissue expression. The 3.9-kb cDNA predicts for a 72-kDa protein with an N-terminal proline rich domain, a central 5-phosphatase domain, and a C-terminal CAAX motif. The 3.9-kilobase mRNA showed a restricted expression but was abundant in testis and brain. Antibodies against the sequence detected a 72-kDa protein in the testis in the detergent-insoluble fraction. Indirect immunofluorescence of the Tera-1 cell line using anti-peptide antibodies to the 72-kDa 5-phosphatase demonstrated that the enzyme is predominantly located to the Golgi. Expression of green fluorescent protein-tagged 72-kDa 5-phosphatase in COS-7 cells revealed that the enzyme localized predominantly to the Golgi, mediated by the N-terminal proline-rich domain, but not the C-terminal CAAX motif. In vitro, the protein inserted into microsomal membranes on the cytoplasmic face of the membrane. Immunoprecipitated recombinant 72-kDa 5-phosphatase hydrolyzed phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,5-bisphosphate, forming phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3-phosphate, respectively. We propose that the novel 5-phosphatase hydrolyzes phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,5-bisphosphate on the cytoplasmic Golgi membrane and thereby may regulate Golgi-vesicular trafficking.

The inositol-polyphosphate 5-phosphatases (5-phosphatases)† are a large family of enzymes that remove the 5-position phosphate from the inositol ring of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2), phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3), and the inositol phosphates, ins, 1,4,5-trisphosphate (Ins(1,4,5)-P3) and ins 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P4). Nine distinct mammalian 5-phosphatases have been identified and characterized, while four 5-phosphatases have been described in Saccharomyces cerevisiae. All 5-phosphatases are defined by the presence of a conserved 300-amino acid domain, which contains two signature motifs, proposed to mediate substrate binding and catalysis (1, 2). Although all 5-phosphatase enzymes contain these signature motifs, there is considerable diversity in the substrate specificity of 5-phosphatase isoforms. The enzyme family has been subclassified on this basis into four types, I-IV. The type I enzymes, characterized by the 43-kDa 5-phosphatase (also called 5-phosphatase I), hydrolyze Ins(1,4,5)P3 and Ins(1,3,4,5)P4 but not any of the 5-position phosphoinositide substrates; the type II 5-phosphatases, including synaptojanin, 5-phosphatase II (originally designated the 75-kDa 5-phosphatase), and the protein product of the oculocerebrorenal syndrome (OCRL) gene, hydrolyze PtdIns(4,5)P2, PtdIns(3,4,5)P3, Ins(3,4,5)P4, and Ins(1,4,5)P2; type III enzymes, characterized by SHIP and SHIP-2, only hydrolyze PtdIns(3,4,5)P3 and Ins(1,3,4,5)P4, and finally the type IV enzymes, which have been partially purified, but not cloned, are specific for PtdIns(3,4,5)P3 only (1, 2).

The functional roles mediated by 5-phosphatase enzymes have been demonstrated by a number of recent studies in which gene-targeted deletion of specific 5-phosphatases has been achieved. Mice deficient in the type II 5-phosphatase synaptojanin exhibit neurological defects and die shortly after birth, providing evidence for a critical role for 5-phosphatase-mediated phosphoinositide regulation in synaptic vesicle recycling (3). Targeted disruption of the type III 5-phosphatase SHIP, which has a restricted expression to hematopoietic cells, results in dramatic hyperplasia of myeloid cells in the bone marrow, spleen, and lungs, resembling a myeloproliferative syndrome (4). Although mice deficient in the OCRL protein demonstrate no phenotype (5), humans with mutations in this gene, Lowe’s syndrome, demonstrate a variety of clinical features including growth and mental retardation, cataracts with severe visual impairment, renal Fanconi syndrome, and metabolic bone disease (6). Disruption in 5-phosphatase II results in testicular abnormalities. Mice deficient in both the OCRL protein and fluorescent protein; kb, kilobase(s); PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; BFA, brefeldin A; EST, expressed sequence tag; HPLC, high pressure liquid chromatography.

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The abbreviations used are: 5-phosphatase, polyphosphate 5-phosphatase; ins, inositol; PtdIns, phosphatidylinositol; GroPIns, glycerophosphorylinositol; OCRL, oculocerebrorenal syndrome; GFP, green fluorescent protein; kb, kilobase(s); PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; BFA, brefeldin A; EST, expressed sequence tag; HPLC, high pressure liquid chromatography.
5-phosphatase II die in utero (5). Collectively these studies suggest the 5-phosphatases have distinct functions that are nonredundant. This specificity may be mediated by the tissue and subcellular location of each 5-phosphatase or by specific interactions with a variety of other signaling proteins and pathways. Type II and III 5-phosphatases form complexes with other proteins via N-terminal SH2 domains and/or C-terminal proline-rich domains.

In this study, we report the cloning and characterization of a 72-kDa 5-phosphatase with a restricted tissue expression. The novel 5-phosphatase hydrolyzes PtdIns(3,4,5)P3 and PtdIns(3,5)P3 and localizes to the cytosolic face of the Golgi by the N-terminal proline-rich domain but not the C-terminal CAAX motif. We propose that the location of the 72-kDa 5-phosphatase to the Golgi may play a role in regulating vesicular trafficking through this compartment.

EXPERIMENTAL PROCEDURES

Materials

Restriction and DNA-modifying enzymes were from New England Biolabs, Fermentas, or Promega. The mouse brain λZAP II cDNA library (Stratagene) was a gift from Dr. Ora Bernard (Walter and Eliza Hall Institute, Melbourne, Australia), Big Dye Terminator Cycle Sequencing kit was from PE Applied Systems (Foster City, CA). Synthetic peptides were from Chiron Mimotopes (Melbourne, Australia). All cell lines were from the American Type Culture Collection. Oligonucleotides were obtained from Geneworks (Adelaide, Australia). The pCR vector was a gift from Dr. Tony Tiganis (Monash University). The human multitissue Northern blot and pEGFP-C2 vector were obtained from CLONTECH. All other reagents were from Sigma unless otherwise stated. Antibodies specific for the trans-Golgi network protein p230 (72-kDa 5-phosphatase) were a kind donation from Dr. Paul Gleeson (Monash University, Australia). Monoclonal antibodies to β-COP were from Sigma; monoclonal antibodies to green fluorescent protein (GFP) were from Roche Molecular Biochemicals; and monoclonal antibodies to hemagglutinin tag were from Babco.

Methods

Isolation of Murine Full-length 5-Phosphatase cDNA—A mouse brain λZAP II cDNA library (1 × 109 recombinants) was screened using a cDNA (nucleotides 5–636) derived from the human EST clone U45974. The cDNA was labeled with [α-32P]dCTP and hybridized at 40 °C using standard techniques and conditions (9). The cDNAs from eight positive clones were identified, plaque-purified, and subcloned into the vector Bluescript II KS+. Of these, three clones of varying length (2.4, 3.3, and 3.9 kb) were completely sequenced on both strands using standard dideoxynucleotide sequencing procedures and an “oligo-walk” strategy (9). All three clones showed identical sequences in overlapping areas to the largest clone of 3.9 kb, which was designated M4. Sequence analysis was performed using DNASIS version 7.0 (Hitachi) software and ANGIS and BLAST-EST programs. The cDNA sequence is available at GenBankTAZAF236855.

Northern Blot Analysis—A membrane containing poly(A) RNA isolated from various adult mouse tissues and cell lines was probed with the 1.5-kb cDNA encoding the open reading frame of the M4 clone. The open reading frame was obtained by PCR amplification and subcloned in frame into the pCR-Blunt vector (Invitrogen). Isolated DNA fragments were sequenced on both strands using standard techniques and conditions (9). The resulting fusion plasmid (pEGFP-PRD) encodes amino acids 1–339 of the 72-kDa 5-phosphatase. The nucleotide sequence of all PCR-derived constructs was confirmed by dideoxy sequencing.

Transient Transfection of GFP- and HA-tagged 72-kDa 5-Phosphatase—The complete open reading frame of the 74-kDa encoding the 72-kDa 5-phosphatase was amplified by PCR and subcloned in frame into the HindIII site of pEGFP-C2 (pEGFP-M4) and into the XbaI site of pCGN (pCGN-M4), thereby adding N-terminal GFP and HA tags in frame, respectively, to the 72-kDa 5-phosphatase. Mutants in which the CAAX motif was either deleted or mutated were generated as follows. A cDNA fragment in which the nucleotides coding for the C-terminal CAAX motif were deleted from the open reading frame was amplified by PCR and subcloned into the HindIII site of pEGFP-C2 (pEGFP-ΔCAAX), and the XbaI site of pCGN (pCGN-ΔCAAX). A PCR strategy was also used to perform site-directed mutagenesis in which the cysteine residue (Cys545) of the 72-kDa 5-phosphatase within the CAAX motif was replaced with alanine. The resulting cDNA was subcloned into the HindIII site of pEGFP-C2 (pEGFP-C44A) to generate a recombinant GFP-tagged N-terminal mutant construct lacking both the 5-phosphatase domain and C-terminal CAAX motif was derived by EcoRI digestion of pEGFP-M4 followed by religation. The resulting fusion plasmid (pEGFP-PRD) encodes amino acids 1–339 of the 72-kDa 5-phosphatase. The nucleotide sequence of all PCR-derived constructs was confirmed by dideoxy sequencing.
tase in COS-7 Cells—The cDNAs encoding the open reading frames of the 72-kDa 5-phosphatase, CAAX-deleted or mutated constructs, generated as described above, were transiently transfected into COS-7 cells using LipofectAMINE reagent according to the manufacturer’s protocol (Life Technologies, Inc.). Forty-eight hours after transfection, cells were washed twice, resuspended in lysis buffer, briefly sonicated and then centrifuged at 100,000 g. The resulting HA-tagged proteins was localized using anti-HA monoclonal antibody and detected using fluorescein isothiocyanate-conjugated anti-mouse IgG. Co-localization was performed using specific Golgi markers, β-COP or p230, and detected using secondary tetramethylrhodamine isothiocyanate-conjugated anti-mouse IgG antibody or rhodamine-conjugated anti–rabbit IgG antibody, respectively. Transfected cells were visualized by confocal microscopy.

To confirm the synthesis of recombinant GFP- or HA-tagged proteins, transfected cells were washed briefly with wash buffer (40 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM benzamidine, 12 mM β-mercaptoethanol) before being scraped from the plate and transferred to a microcentrifuge tube. An aliquot was removed at this time and represents the total cell lysate sample. The remaining cells were brieﬂy sonicated and then centrifuged at 100,000 × g for 1 h at 4 °C. The supernatant (cytosol) was collected, and the pellet was resuspended in 500 μl of wash buffer plus 1% Triton X-100, incubated for 2 h at 4 °C with gentle agitation, and centrifuged at 100,000 × g for 1 h at 4 °C to obtain the Triton-insoluble fraction. The Triton-soluble fraction was resuspended in 500 μl of the Triton-wash buffer.

Brefeldin A studies were performed as follows. Tera-1 or COS-7 cells 48 h after transfection were incubated with Dulbecco’s modiﬁed Eagle’s medium plus 10% fetal calf serum containing 148 h after transfection were incubated with Dulbecco's modiﬁed Eagle’s medium plus 10% fetal calf serum containing 1% of [3H]inositol-labeled yeast lysates, which were extracted following hypotonic stress with 1 M NaCL for 10 min as described (19).

**RESULTS**

**Isolation and Characterization of a Full-length Mouse 5-Phosphatase Clone (M4)—**A 1.6-kb human cDNA EST clone U45974 encoding the predicted partial coding sequence of a novel human 5-phosphatase was used to screen a mouse brain ZAP II cDNA library, resulting in the isolation of eight positive clones. The largest clone (M4) contained a 3.9-kb insert as determined by restriction digestion and Southern blot analysis. Sequence analysis demonstrated that the 3.9-kb mouse brain cDNA (M4) predicts for a 1944-bp open reading frame encoding a 72-kDa polypeptide, with the initiator methionine occurring at nucleotide 595 and the termination codon at 2538 (Fig. 1a). The 5′-untranslated region contains a number of in-frame stop codons prior to the proposed initiator methionine. In addition, the region prior to the initiator methionine bears no similarity to other cDNA sequences in various data bases. M4 contains a 3′-untranslated region with a poly(A) tail commencing at nucleotide 3869. The amino acid sequence predicts for an N-terminal proline-rich domain with 13% prolines over 221 amino acids containing five PXXP motifs, which may form complexes with SH3 domain-containing proteins. The C terminus predicts for a CAAX motif (CTVS), which in other proteins, is a signal for post-translational modification by farnesylation and thereby membrane attachment. Hydrophobicity analysis reveals that the M4 protein product contains no significant hydrophobic domains and does not predict for a transmembrane domain (not shown).

M4 demonstrates significant sequence identity with the human EST clone U45974, which was used to screen the library. The amino acid sequence bears 84% identity over 175 amino acids with the two 5-phosphatase signal sequences motifs. In addition, the C terminus of the protein predicted by human EST U45974 is 50 amino acids longer than that encoded by the mouse 3.9-kb CDNA (M4) and unlike the mouse clone does not predict for a C-terminal CAAX motif. However, we have resequenced clone U45974 and identified a sequence error that results in a frameshift. When corrected, the translated protein predicted by the EST ends 147 nucleotides shorter and predicts...
for a C-terminal CAAX motif. We therefore propose that the 3.9-kb cDNA M4 represents the mouse homologue of the partial human EST U45974.

The protein encoded by M4 cDNA demonstrates significant sequence identity with other 5-phosphatases. Analysis of data bases indicates that the 3.9-kb cDNA bears significant cDNA and amino acid sequence identity with a partial rat cDNA recently reported to represent a novel 5-phosphatase designated pharbin (for “phosphatase that induces arborization”) (21). Pharbin represents a rat cDNA comprising 2.7 kb but lacks the 5'-prime end of the cDNA and initiator methionine found in the mouse 3.9-kb cDNA M4 reported here (Fig. 1a).

The pharbin sequence bears 94% nucleotide sequence identity over 2044 base pairs and 88% amino acid identity over 647 amino acids with the M4 protein. We propose that pharbin represents a partial rat homologue of the novel 72-kDa 5-phosphatase predicted by M4. Recently Majerus and colleagues (22) have identified a novel human 70-kDa 5-phosphatase that demonstrates 76% amino acid identity over 649 amino acids with the 72-kDa 5-phosphatase reported here. The greatest sequence diversity appears to be in the N-terminal proline rich domain.

In Vitro Translation of the 72-kDa 5-Phosphatase—To establish that the 3.9-kb cDNA could produce a protein product of the predicted size, the full-length cDNA and a construct commencing immediately prior to the proposed initiator methionine were cloned into the expression vector pSVTf and translated in vitro to determine the size of the translated polypeptide. Each construct was used to direct mRNA synthesis and translated in a wheat germ expression system in the presence of [35S]methionine. The resulting labeled proteins were analyzed by SDS-PAGE and fluorography. Translation of the full-length cDNA (not shown) and the construct commencing immediately prior to the putative initiator methionine both gave rise to a prominent polypeptide species of 72 kDa, consistent with the size predicted by the open reading frame of the 3.9-kb cDNA (Fig. 1b).

**RNA Analysis of Human and Mouse 72-kDa 5-Phosphatase**
Expression—The full-length 3.9-kb cDNA M4 and the human 1.6-kb EST U45974 were used to probe membranes containing mRNA isolated from mouse or human tissues and cell lines (Fig. 2, a and b). To compare the level of RNA in each sample, the membranes were subsequently hybridized to an actin probe. The mouse M4 cDNA detected the restricted expression of a single mRNA species of approximately 3.9 kb in mouse testis and GC-2 cells (spermatocyte) with low levels in brain and in the cell lines C6 and NT, which are derived from rat brain glioma and neuroteratocarcinoma cells, respectively (Fig. 2a). The human EST U45974 demonstrated a similar pattern of expression in human tissues with most prominent expression of a 3.9-kb mRNA species in testis with low level expression in ovary and prostate (Fig. 2b).

In Situ Hybridization of Mouse Tissues—To further determine the cellular location of the novel 72-kDa 5-phosphatase, we explored the expression pattern of this 5-phosphatase in adult testis and brain using in situ hybridization (Fig. 3, a–f). The 5-phosphatase demonstrated a restricted cellular expression in testis. In situ hybridization using antisense probes revealed that the M4 RNA was present in pachytene and diploctene spermatocytes; however, the more mature elongating spermatids were negative. In addition, spermatogonia demonstrated only faint staining, while no definite hybridization was observed in Sertoli cells. Corresponding sense riboprobes did not hybridize to any cell type.

In situ hybridization using antisense M4 riboprobes revealed positive neurons throughout the entire brain (Fig. 3, d–f). M4 positive neurons were found in the cerebral cortex, hippocampus, diencephalon, brainstem, and cerebellum. The intensity of hybridization within individual neurons varied, and this could reflect the level of M4 mRNA expressed. It was also noted that not all neurons within a given part of the brain were intensely stained. For instance, within the substantia nigra, approximately 50% of neurons were positive. This is in contrast to the hippocampus or dentate gyrus, where virtually all of the neurons hybridized to M4 riboprobe (Fig. 3d). Neurons displaying intense staining were found at several sites including the hippocampus, dentate gyrus, dorsal thalamus, nucleus accumbens (Fig. 3e), bed nucleus of the stria terminalis, cerebellum (Fig. 3f), and the brainstem. There was no evidence that neuronal cells in the brain expressed M4.

Tissue Distribution of the 72-kDa 5-Phosphatase—To examine the tissue and subcellular localization of the 72-kDa 5-phosphatase, Western blot analysis of Triton-soluble and -insoluble fractions of a variety of mouse tissues and cell lines were undertaken using anti-peptide antibodies specific to the 5-phosphatase. Affinity-purified antibodies to a C-terminal peptide in the 5-phosphatase recognized a 72-kDa polypeptide in the Triton-insoluble fraction, most prominently in fractions derived from mouse adult testis and brain, but not heart, thymus, liver, spleen, lymph node, or kidney (Fig. 4a). A number of cross-reacting low molecular weight polypeptides were occasionally detected in the soluble fraction of adult liver and spleen (not shown). We cannot determine if these species represent proteolytic fragments of the 72-kDa 5-phosphatase, which were present despite the use of multiple protease inhibitors, or cross-reacting species. No reactive 72-kDa 5-phosphatase was observed in several cell lines analyzed, including monkey kidney COS-7, glioma (C6), neuroteratocarcinoma (NT), erythroleukemia (K562), spermatogonia (GC-1), and Sertoli (TM4) (results not shown). However, cell lysates derived from spermatocytes (GC-2) (not shown) and teratocarcinoma (Tera-1) (Fig. 4b) demonstrated the presence of a reactive 72-
kDa polypeptide species. Expression of the 72-kDa polypeptide was detected in the Triton-soluble fraction, in addition to the insoluble fraction in Tera-1 cells. Collectively, these studies demonstrate that the 5-phosphatase demonstrates a relatively restricted tissue and cellular expression distribution, most prominent in testis, located in the detergent-insoluble fraction.

**Fig. 3.** 72-kDa 5-phosphatase expression in testis and brain. *a, b, and c* paraffin-embedded testis sections from 14-week-old mice hybridized to the M4 riboprobes and counterstained with hematoxylin. *a* and *b*, testis hybridized with antisense M4 probe; *c*, testis hybridized to M4 sense probe (*a* and *c*, 230-fold magnification; *b*, 460-fold magnification). *Sp*, spermatogonia; *Sc*, spermatocytes; *Se*, Sertoli cells; *L*, leydig cells; *Rs*, round spermatids; *Es*, elongating spermatids. *d, e, and f*, paraffin sections of the brain from 14-week-old mice hybridized to M4 antisense (*left panel*) or sense riboprobes (*right panel*). *d*, M4 mRNA expression in hippocampal subfield CA4, in dentate gyrus (DG), and in the dorsal thalamus (DT). *HF*, hippocampal fissure; *ML*, molecular layer. *e*, M4-positive neurons in nucleus accumbens (Acb) and in the bed nucleus of the striatal terminalis (BSTL). These structures surround the anterior commissure (ac); *f*, M4-positive neurons within the cerebellum and brainstem. In the cerebellum, M4-positive neurons were present within the granule cell layer (GCL) and Purkinje cell layer (PCL). Positive cells were occasionally identified within the molecular layer (ML). Large numbers of M4-positive neurons were also present in the dorsal brainstem (DBS). Sections were counterstained with hematoxylin. Scale bar, 500 μm for all photomicrographs.

**Fig. 4.** Tissue and subcellular distribution of the 72-kDa 5-phosphatase. *a*, the Triton-soluble (S) or -insoluble (I) fraction from the indicated mouse tissues was isolated as described under “Experimental Procedures,” and 100 μg of each subcellular fraction was analyzed by 10% SDS-PAGE under reducing conditions followed by immunoblotting with affinity-purified anti-peptide antibody to the C terminus of the 72-kDa 5-phosphatase. Molecular mass markers are shown on the left. *b*, the total cell lysate, Triton-soluble (S) fractions, or insoluble (I) fractions (100 μg) from the Tera-1 (teratocarcinoma) cell line were analyzed by immunoblots using anti-peptide antibodies to the 72-kDa 5-phosphatase.
Inositol-polypolyphosphate 5-Phosphatase Activity—Numerous attempts were made to express the novel 72-kDa 5-phosphatase as a recombinant protein in Escherichia coli, S. cerevisiae, and S2 Drosophila cells using a variety of expression vectors. When expressed in E. coli or S. cerevisiae, the recombinant protein was detected only in the detergent-insoluble fraction and was resistant to extraction. Therefore, the open reading frame of the 72-kDa 5-phosphatase was cloned in frame into the mammalian expression vector pCGN, which contains an N-terminal HA tag, and transiently expressed in COS-7 cells. The recombinant 5-phosphatase was immunoprecipitated using antibodies to the HA tag, and immunoprecipitates were examined for 5-phosphatase enzyme activity. In addition, in parallel immunoprecipitations, the presence and level of recombinant HA-72-kDa 5-phosphatase protein was determined by immunoblot analysis using antibodies to the HA tag. Immunoprecipitated 5-phosphatase showed minimal activity toward the substrates Ins1,4(5)P2, Ins1,3,4,5(6)P4 or PtdIns4(5)P2. The latter analysis was undertaken in the presence or absence of detergent, since it has recently been reported that the human 70-kDa and SHIP PtdIns(4,5)P2 5-phosphatase activity is sensitive to specific detergents (22).

The recombinant 5-phosphatase demonstrated significant enzyme activity converting PtdIns(3,4,5)P3 to PtdIns(3,4)P2 (Fig. 5, a–b). The labeled products of this reaction were excised from the TLC, deacylated, and analyzed by HPLC with comparison with known standards. The GroPtdIns(3,4)P2 product migrated in a position immediately prior to a GroPtdIns(4,5)P2 standard, confirming that the enzyme is a PtdIns(3,4,5)P3 5-phosphatase (Fig. 5c). Although it has been reported that pharbin shows Ins1,4(5)P2, Ins1,3,4,5P4, and PtdIns4(5)P2 5-phosphatase activity (21) (PtdIns(4,5)P3 and PtdIns(3,5)P2 were not tested), we demonstrated that levels of immunoprecipitated protein that did not hydrolyze Ins1,4(5)P2, Ins1,3,4,5P4, or PtdIns4(5)P2 demonstrated significant PtdIns(3,4,5)P3 5-phosphatase activity. These studies suggest that a major substrate for the 72-kDa 5-phosphatase is PtdIns(3,4,5)P3.

A novel lipid PtdIns(3,5)P2 has been recently identified in yeast, fibroblasts, COS-7 cells, platelets, and T cells (23–26). It has been proposed that this phosphoinositide is a signaling molecule induced in response to stress. We generated PtdIns(132P)3(5)P2 by phosphorylating PtdIns 5-P using highly purified recombinant phosphatidylinositol 3-kinase (16). Recombinant immunoprecipitated 72-kDa 5-phosphatase hydrolyzed PtdIns(3,5)P2, forming PtdIns 3-P (Fig. 6a). Products of the 5-phosphatase reaction were analyzed by HPLC chromatography of deacylated lipids with comparison with the migration of [3H]inositol-labeled yeast, which had been stressed with 1.1 M NaCl for 10 min (Fig. 6b) (19). The labeled deacylated lipid generated by phosphatidylinositol 3-kinase phosphorylation of PtdIns 5-P migrated in a position consistent with GroPtdIns(3,5)P2 and following 5-phosphatase treatment, PtdIns 3-P was the major product (Fig. 6c). In control studies, analysis of the products of PtdIns(3,5)P2 5-phosphatase assays of vector-transfected cell immunoprecipitates showed predominantly GroPtdIns(3,5)P2 with little GroPtdIns 3-P generated, confirming the results observed by TLC analysis (not shown).

The 72-kDa 5-Phosphatase Is Associated with the Golgi in Tera-1 Cells—We investigated the intracellular location of the 72-kDa 5-phosphatase. Indirect immunofluorescence of Tera-1 cells, using affinity-purified C-terminal peptide antibodies was performed. This antibody stained an area adjacent to the nucleus that resembled the Golgi complex (Fig. 7a). Preimmune serum faintly stained the cytoplasm but not the Golgi (Fig. 7b). Double immunostaining using affinity-purified anti-peptide antibody and antibodies specific for the Golgi protein β-COP demonstrated that the 72-kDa 5-phosphatase co-localized with the Golgi marker. No plasma membrane staining was observed; however, we cannot exclude the possibility that plasma membrane association was lost as a result of the incubation with Triton X-100 required for indirect immunofluorescence. To exclude the possibility that the apparent co-localization was due to plasma membrane-derived 72-kDa 5-phosphatase trafficking through the endosomal recycling compartment, we tested for sensitivity to brefeldin A. This fungal metabolite causes the disassembly of the cis and medial Golgi and promotes the retrograde transport of Golgi components to the endoplasmic reticulum (27, 28). Brefeldin A treatment resulted in the release of β-COP, a component of the Golgi-derived transport vesicles, into the cytoplasm. Similarly, following brefeldin A treatment (30 min), the 72-kDa 5-phosphatase was found in vesicles co-localizing with β-COP marker and relocated to the Golgi following 16-h recovery (Fig. 7, c and d). Collectively, these results are consistent with the contention that the 72-kDa 5-phosphatase is localized to the Golgi network.

Intracellular Location of the 72-kDa 5-Phosphatase in Transfected COS-7 Cells—To confirm the intracellular location of the 72-kDa 5-phosphatase and to investigate the role of the C-terminal CAAX motif in membrane association, recombinant 72-kDa 5-phosphatase was transiently expressed in COS-7 cells as an N-terminal fusion protein with GFP (Fig. 8) or with HA (not shown). Recombinant GFP-5-phosphatase localized predominantly to an area adjacent to the nucleus, which colocalized with antibodies to the trans-Golgi protein p230 (Fig. 8b). In high expressing cells, we noted faint cytoplasmic staining; however, in contrast to the reported localized plasma membrane expression of pharbin and other 5-phosphatases with C-terminal CAAX motifs, no plasma membrane staining was observed. Similar intracellular localization was observed when the 72-kDa 5-phosphatase was expressed as an N-terminal HA-tagged protein (results not shown). Cells expressing GFP alone demonstrated predominantly nuclear staining (Fig. 8a). Brefeldin A treatment resulted in relocation of recombinant GFP-5-phosphatase to the cytosol, which relocated to the Golgi upon recovery (Fig. 8, c and d).

To confirm that the GFP-tagged 5-phosphatase expressed was intact, lysates from cells transfected with GFP or HA-tagged 5-phosphatase were harvested, and cytosolic, Triton-soluble membrane, and Triton-insoluble membrane subcellular fractions were examined by immunoblot analysis using antibodies specific to the GFP or the HA tag (results not shown). A 101-kDa GFP fusion protein was detected predominantly in the Triton-insoluble fraction (Fig. 9e), consistent with the location of the native protein, as shown by subcellular fractionation of mouse tissues and cell lines. Little proteolysis of the GFP or HA-tagged recombinant 5-phosphatase was observed.

In contrast to our studies, a recent report has proposed that recombinant Mec-tagged pharbin localizes to the plasma membrane (21). In addition, other 5-phosphatases such as the 43-kDa 5-phosphatase, which contains a C-terminal CAAX motif, localize to the plasma membrane (29). We therefore compared the intracellular location of recombinant GFP-43-kDa 5-phosphatase under identical conditions to GFP-72-kDa 5-phosphatase (Fig. 8, e and f). Analysis of cells expressing recombinant GFP-5-phosphatases was performed in the absence of detergent, and multiple sections throughout the cell were analyzed by confocal microscopy. Although faint plasma membrane staining was occasionally observed in cells expressing GFP-72-kDa 5-phosphatase, the predominant staining localized to the Golgi. In contrast, the 43-kDa 5-phosphatase was prominently expressed at the plasma membrane. Collectively, these studies demonstrate that the localization of the 72-kDa 5-phosphatase is on the Golgi.
FIG. 5. The 72-kDa 5-phosphatase is a PtdIns(3,4,5)P₃ 5-phosphatase. HA-tagged 72-kDa 5-phosphatase or empty vector was transiently transfected in COS-7 cells. 72 h after transfection, cell lysates were prepared and immunoprecipitated using antibodies to the HA tag and captured on Protein A-Sepharose and washed extensively. Immunoprecipitates were examined by PtdIns(3,4,5)P₃ 5-phosphatase assays (a) or immunoblot analysis (b) using antibodies to the HA tag to demonstrate immunoprecipitation of the HA-tagged 72-kDa 5-phosphatase. a, PtdIns(3,4,5)P₃ 5-phosphatase assays were performed on immunoprecipitates from empty vector-transfected cells (lanes 1 and 2) or HA-72-kDa 5-phosphatase-transfected cells (lanes 3 and 4) as described under "Experimental Procedures." The migration of PtdIns(3,4,5)P₃ and PtdIns(3,4,5)P₄, as shown on the right, b, immunoblot analysis of empty vector-transfected (lane 1) or HA-72-kDa 5-phosphatase-transfected cells in parallel immunoprecipitation reactions used for PtdIns(4,5)P₂ (lane 2) or PtdIns(3,4,5)P₃ 5-phosphatase assays (lane 3), respectively. Protein A-Sepharose-captured immunoprecipitates were washed extensively and assessed for the presence of the HA-tagged 72-kDa 5-phosphatase by immunoblot analysis using antibodies to the HA tag. The migration of molecular mass markers is shown on the left. c, the labeled PtdIns(3,4,5)P₃ (closed circles) and PtdIns(3,4)P₂ (open circles) from 5-phosphatase reactions were excised from the TLC, deacylated, and analyzed by HPLC chromatography with comparison with known standards, which are indicated by arrows at the top.

FIG. 6. The 72-kDa 5-phosphatase hydrolyzes PtdIns(3,5)P₂. a, PtdIns(3,5)P₂ 5-phosphatase assays were performed on buffer alone (lane 1) or immunoprecipitates from vector-transfected cells (lane 2), or HA-72-kDa 5-phosphatase-transfected cells (lane 3) and analyzed by TLC. The migration of PtdIns3P and PtdIns(3,5)P₂ is shown on the right. b, [³H]inositol-labeled yeast was stimulated with 1.1 M NaCl for 10 min, and labeled lipids were extracted, deacylated, and analyzed by HPLC chromatography as described under "Experimental Procedures." c, PtdIns(3,5)P₂ 5-phosphatase assays were performed on immunoprecipitates from 5-phosphatase-transfected cells. At the end of the reaction assays, lipids were deacylated and analyzed by HPLC chromatography. The migration of deacylated lipid products was compared with deacylated [³H]inositol-labeled yeast lipids shown in b, analyzed under the same conditions.
Recent studies have shown that the enzymes that modify prenylated CAAX motifs are located in the endoplasmic reticulum (30–32). In addition, the CAAX motif alone is sufficient to target Ras to the Golgi and endomembranes (33). To investigate the role of the C-terminal CAAX motif in Golgi membrane localization, recombinant mutated 72-kDa 5-phosphatase that lacked the C-terminal CAAX motif, GFP-CAAX, or contained a point mutation, GFP-72C644A-5-phosphatase, was transiently expressed in COS-7 cells. Both mutant 5-phosphatase recombinant proteins localized to the Golgi (Fig. 9a–c). Mutant 5-phosphatases expressed with an HA tag also demonstrated Golgi localization (results not shown). Since the C-terminal CAAX motif appears to play little role in Golgi-membrane localization, we investigated whether the 72-kDa 5-phosphatase N-terminal domain, which contains a number of proline-rich motifs, might mediate Golgi localization (Fig. 9d). The N-terminal proline-rich domain was expressed as a C-terminal fusion protein with GFP in COS-7 cells and demonstrated a Golgi localization comparable to that of the intact recombinant protein. Therefore, the N-terminal proline-rich domain, but not the C-terminal CAAX motif, mediates the intracellular location of the 72-kDa 5-phosphatase. In control studies, immunoblot analysis using GFP antibodies demonstrated all mutant and truncated recombinant 5-phosphatase proteins were expressed predominantly in the Triton-insoluble fraction and migrated at the predicted molecular weight with little proteolysis (Fig. 9e).

The 72-kDa 5-Phosphatase Inserts into the Cytoplasmic Face of Membranes—Many proteins insert into the cytoplasmic face of the Golgi. All known resident Golgi proteins are peripheral or integral membrane proteins. The 72-kDa 5-phosphatase lacks a signal sequence and therefore is expected to undergo post-translational insertion into membranes. The membrane orientation of the 72-kDa 5-phosphatase was determined by a protease protection assay. 72-kDa 5-phosphatase mRNA was translated into [35S]methionine-labeled protein in the presence of canine pancreatic microsomes. After translation, the microsomes were washed to remove proteins that were not incorporated into microsomes and collected by centrifugation. The microsomes contained the 72-kDa 5-phosphatase, indicating that the protein was associated with the microsomal membrane (Fig. 10). Recombinant 72-kDa 5-phosphatase lacking the C-terminal CAAX motif, inserted into membranes comparable with the wild type protein. Proteinase K treatment of microsomes containing the labeled recombinant 72-kDa 5-phosphatase, either intact or lacking the CAAX motif, resulted in digestion of the 5-phosphatase, indicating that the 72-kDa 5-phosphatase was exposed on the surface of the microsome. In contrast, in control studies the secretory protein β-lactamase was translocated into the lumen and was resistant to proteinase treatment, demonstrating that the microsomes were functional and intact. These studies are consistent with the contention that the 72-kDa 5-phosphatase localizes to the cytoplasmic face of the Golgi membrane.

**DISCUSSION**

This study has described the isolation and characterization of a new member of the inositol-polyphosphate 5-phosphatase family.
family. The 72-kDa 5-phosphatase demonstrates a restricted tissue and cellular distribution based on Northern and immunoblot analysis, with the highest apparent expression levels observed in the round spermatids of the testis. We propose that the enzyme is located on the cytoplasmic surface of the Golgi membrane. As the 72-kDa 5-phosphatase hydrolyzes PtdIns(3,4,5)P3 and PtdIns(3,5)P2, the enzyme may regulate phosphoinositide-mediated vesicular trafficking from the Golgi.

To date, nine mammalian and four yeast 5-phosphatases have been cloned and characterized, with numerous spliced isoforms described. The requirement for so many 5-phosphatases may be to regulate phosphoinositides within specific intracellular membranes and compartments, in addition to the inner wall of the plasma membrane. The intracellular locations of several mammalian 5-phosphatases have been reported. 5-Phosphatase I is predominantly located at the plasma membrane via carboxyl prenylation of the C-terminal CAAX motif (29). 5-Phosphatase II is located in the cytosol and mitochondria and is also prenylated via a C-terminal CAAX motif (34–36). Synaptotagmin 2A associates with the PDZ domain of a novel outer mitochondrial protein OMP25, via a unique C-terminal motif (37). OCRL is detected in the lysosomes (38), and two studies have detected the protein in the Golgi of epithelial cells (39), where the enzyme may function to regulate PtdIns(4, 5)P2 levels and thereby trafficking events. SHIP and SHIP-2 associate with tyrosine kinase receptors, most probably at the plasma membrane (40, 41). The recently described 107-kDa proline-rich inositol-polyphosphate 5-phosphatase localizes to membrane ruffles (42). Although the partial rat Myc-tagged recombinant 70-kDa pharbin has been reported to localize to the plasma membrane (21), we have compelling evidence that the intact native 72-kDa 5-phosphatase is predominantly expressed on the Golgi. Indirect immunofluorescence using specific anti-peptide antibodies in Tera-1 cells, where the native enzyme is highly expressed, co-localize with Golgi-specific markers. Second, recombinant GFP or HA-

**FIG. 8. Expression of GFP-72-kDa 5-phosphatase to the Golgi.** GFP vector (a), or GFP-72 kDa 5-phosphatase (b, c, d, and e) were transiently transfected into COS-7 cells and visualized by confocal microscopy. Cells were counterstained using an antibody against the trans-Golgi protein p230 (anti-TGN). Areas of co-localization appear as yellow in the merged images (merged). c and d, COS-7 cells transiently expressing GFP-72-kDa 5-phosphatase were exposed to 1μg/ml BFA (c) for 30 min, after which time the BFA was removed, and the cells were allowed to recover for an additional 16h (d). Both BFA-treated and recovered cells were labeled with antibodies to the specific Golgi marker, p230 (anti-TGN). The merged images appear as yellow (merged). GFP-72-kDa (e) or GFP-43-kDa 5-phosphatase (f) was transiently transfected into COS-7 cells using identical conditions and visualized using confocal microscopy. The indicated bar in d represents 20 μM.
tagged 72-kDa 5-phosphatase showed Golgi localization. Third, brefeldin A treatment of Tera-2 cells, which express the native enzyme, or COS-7 cells, expressing recombinant 72-kDa 5-phosphatase, disrupts the prominent perinuclear localization, consistent with a Golgi localization. Fourth, we have shown little plasma membrane expression under conditions in which the 43-kDa 5-phosphatase, another CAAX motif-containing 5-phosphatase, is highly expressed at the plasma membrane. It is possible that the 72-kDa 5-phosphatase traffics via the Golgi to the plasma membrane, and plasma membrane expression may vary between cell lines or under various cellular conditions yet to be shown.

**FIG. 9. The N-terminal proline-rich domain mediates Golgi localization.** COS-7 cells were transiently transfected with the indicated GFP-72-kDa 5-phosphatase constructs and visualized by confocal microscopy. The cells were counterstained with antibodies to the specific Golgi marker β-COP or p230 (anti-TGN) as indicated, and in the merged images colocalized areas appear yellow. a, GFP-72-kDa 5-phosphatase; b, GFP-ΔCAAX; c, GFP-C644A; d, GFP-proline-rich domain (PRD). The recombinant proteins produced are shown below each image. Bar, 20 μM. e, immunoblot analysis of recombinant GFP-72-kDa and mutant GFP-72-kDa 5-phosphatase as indicated expressed in COS-7 cells. Transfected cells were lysed, and subcellular fractions from the cytosol (C), Triton-soluble (S), or Triton-insoluble (I) compartments (100 μg) were analyzed by immunoblot using antibodies to GFP. The migration of molecular mass markers is shown on the right.
Recent studies have suggested that the CAAX motif alone may target proteins such as Ras to the Golgi and endoplasmic reticulum, where enzymes that modify the prenylated CAAX motif are located (33). A second signal is required for plasma membrane expression, which comprises either palmitoylation sites or a polybasic domain. However, we have shown that the C-terminal CAAX motif of 72-kDa 5-phosphatase appears to play little role in mediating Golgi localization or in attachment to the membrane. Similarly, recombinant Myc-tagged pharbin localization in 10T1/2 fibroblasts to the plasma membrane is not mediated by the C-terminal CAAX. However, overexpression of pharbin, but not CAAX-deleted pharbin, resulted in elongation of transfected fibroblasts. The prominent localization of the 72-kDa enzyme in the round spermatid and the potential role the 5-phosphatase plays in mediating the radical transformation of the haploid round spermatid into the elongating spermatozoan will be important to establish and is the subject of ongoing studies.

The 72-kDa 5-phosphatase demonstrates an unusual structure for a 5-phosphatase, since it contains an N-terminal proline-rich domain. The average proline content of proteins in eukaryotes is 4–5%, while the N-terminal proline-rich motif of 72-kDa 5-phosphatase demonstrates 13% prolines over 220 amino acids. This is the second recently described 5-phosphatase to contain an N-terminal proline-rich motif. The 107-kDa inositol-polyphosphate 5-phosphatase contains both an N-terminal and C-terminal proline-rich domain and requires both the N- and C-terminal proline-rich domains for association with membrane ruffles (42). The 72-kDa 5-phosphatase N-terminal proline-rich domain serves to localize the enzyme to the cytoplasmic surface of Golgi membranes. It may be targeted to this compartment as a result of hydrophobic interactions with the Golgi membrane or more likely as a result of specific protein-protein interactions with other Golgi proteins located on the cytoplasmic surface. The presence of several PXPF motifs within this domain suggests this localization may be mediated by binding to SH3 domain-containing proteins.

PtdIns(3,5)P$_2$ represents a newly described phosphoinositide. To date, there have been no reports of its metabolism by mammalian 5-phosphatase enzymes. The definitive signaling role for this lipid is still being determined. However, in yeast it has been proposed to play a role in Golgi to vacuole mediated trafficking, perhaps in response to acute osmotic adaptation (23, 43). Much recent evidence suggests phosphatidylinositol 3-kinases drive vesicular traffic from yeast and mammalian trans-Golgi network (44, 45). A specific interaction of the Golgi coamter protein α-COP with PtdIns(3,4,5)P$_3$ has been shown in in vitro assays (46). Both the D3 and D5 phosphates are essential for the α-COP-PtdIns(3,4,5)P$_3$ interaction. In addition, PtdIns(3,4,5)P$_3$ regulates ARNO (ADP-ribosylation factor nucleotide binding site opener) (47, 48), GRP-1 (general receptor for phosphoinositides) (49), and centrinin a (50), which may directly influence the ADP-ribosylation factor GTPases. The latter are critical enzymes in the formation of coated vesicles. We have shown that the 72-kDa 5-phosphatase hydrolyzes PtdIns(3,4,5)P$_3$ and PtdIns(3,5)P$_3$ and is located on the Golgi. Collectively these studies suggest a potential role for the enzyme in regulating vesicular trafficking.

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