Human Type 2 Phosphatidic Acid Phosphohydrolases

SUBSTRATE SPECIFICITY OF THE TYPE 2a, 2b, AND 2c ENZYMES AND CELL SURFACE ACTIVITY OF THE 2a ISOFORM

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Phosphatidic acid (PA), lysophosphatidic acid, cere- mide 1-phosphate (C1P), and sphingosine 1-phosphate (SIP) are lipid mediators generated by phospholipases, sphingomyelinas, and lipid kinases. The major pathway for degradation of these lipids is dephosphorylation catalyzed by members of two classes (1 and 2) of phosphohydrolase activities (PAPs). cDNAs encoding two type 2 PAPs, PAP-2a and -2b, have been expressed by transient transfection and shown to catalyze hydrolysis of PA, C1P, and SIP (Kai, M., Wada, I., Imai, S., Sakane, F., and Kanoh, H. (1997) J. Biol. Chem. 272, 24572-24578). We report the cloning and expression of a third type 2 PAP enzyme (288 amino acids, predicted molecular mass of 32.6 kDa), PAP-2c, which exhibits 54 and 43% sequence homology to PAPs 2a and 2b. Expression of HA epitope-tagged PAP-2a, -2b, and -2c in HEK293 cells produced immunoreactive proteins and increased membrane-associated PAP activity. SF9 insect cells contain very low endogenous PAP activity. Recombinant expression of the three PAP enzymes using baculovirus vectors produces dramatic increases in membrane-associated Mg$^{2+}$-independent, N-ethylmaleimide-insensitive PAP activity. Expression of PAP-2a but not PAP-2b or -2c resulted in high levels of cell surface PAP activity in intact insect cells. Kinetic analysis of PAP-2a, -2b, and -2c activity against PA, lysophosphatidic acid, C1P, and SIP presented in mixed micelles of Triton X-100 revealed differences in substrate specificity and susceptibility to inhibition by sphingosine, Zn$^{2+}$, and propranolol.

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The nucleotide sequence(s) in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AF017116, AF017786, AF047769 (human PAP-2a, -2b, and -2c, respectively).

The abbreviations used are: PAP, phosphatidic acid phosphohydrolase; PLD, phospholipase D; PC, phosphatidylcholine; PA, phosphatidic acid; LPA, lysophosphatidic acid; C1P, ceramide 1-phosphate; SIP, sphingosine 1-phosphate; NEM, N-ethylmaleimide; DG, diacylglycerol; HA, hemagglutinin; EST, expressed sequence tag; hPAP, human PAP.

catalyzed hydrolysis of phosphatidylcholine (PC). PA generated in this manner appears to act on a variety of cell-specific target proteins that include typtical protein kinase C isoforms, the Raf protein kinases, the Ras GTPase-activating protein, several proteins involved in cytoskeletal organization, and the neutrophil respiratory burst oxidase. In this case, PAP-catalyzed hydrolysis of PA serves to terminate the signaling functions of PA and concurrently generates diacylglycerol for activation of conventional Ca$^{2+}$ and phospholipid-dependent protein kinase C enzymes. DG kinase generates PA by ATP-dependent phosphorylation of DG. Presumably the subcellular localization, catalytic, and regulatory properties of these different lipid-metabolizing enzymes will dictate the source, fate, and signaling functions of PA and DG (2, 3).

PAP activity is widely expressed, and two classes of these enzymes can be distinguished in mammalian cells on the basis of their subcellular distribution and catalytic properties (4). Type 1 activity (PAP-1) is associated with the cytosol and endoplasmic reticulum and appears to redistribute from the soluble to membrane compartment upon treatment of hepatocytes with glucagon and glucocorticoids (4, 5). PAP-1 activity is sensitive to inhibition by sulfhydryl reagents (most notably N-ethylmaleimide (NEM)) and displays an absolute requirement for Mg$^{2+}$. PAP-2 activity has been localized to the plasma membrane and, in contrast to PAP-1, is independent of Mg$^{2+}$ and insensitive to inhibition by NEM (4–8). PAP-2 activity is presumably directed toward the inner leaflet of the plasma membrane, although several reports also describe a cell surface enzyme with PAP-2-like properties that may serve to terminate the receptor-directed signaling functions of lysy-PA (LPA) and related compounds (9, 10). Sphingosine 1-phosphate (SIP) and ceramide 1-phosphate (C1P) phosphatase activities have also been described in mammalian cells, and purified preparations of rat liver PAP-2 also catalyze the hydrolysis of these lipid phosphomonoesters (11). C1P, SIP, and their hydrolysis products exhibit a number of interesting biological activities and may function as intra- and possibly extracellular signaling molecules (12). The inference drawn from these studies is that PAP-1 most likely functions in lipid synthesis, while PAP-2 may have an important role to play in modulating the signaling functions of PA and LPA as well as lipid phosphomonoesters derived from sphingomyelin.

The tight association of PAP with membranes and the low abundance of the proteins has hampered purification attempts. PAP-1 has not yet been isolated. Several groups have prepared highly enriched preparations of PAP-2 from a number of tissue sources (6–8). Rat liver PAP-2 was identified as a glycosylated protein of about 50 kDa that could be converted to a 28-kDa protein by N-glycanase treatment (6). A mouse cDNA (mPAP-2a) encoding a protein that is highly similar to a heat-inducible gene product (Hic53) has recently been isolated. When ex-
pressed in HEK 293 cells, this protein localizes to the plasma membrane and is accompanied by a Mg2+-independent PAP activity (13). A human cDNA encoding a second mammalian PAP-2 enzyme, hPAP-2b (the human homolog of a previously described rat intestinal epithelial cell endoplasmic reticulum-resident protein, Drf42), has been cloned (14, 15). Transient expression of PAP-2a and -2b in HEK-293 cells produced increases in membrane-associated PA and C1P phosphatase activities. Hydrophobicity analysis suggests that PAP-2a and -2b are integral membrane proteins with six hydrophobic membrane-spanning regions (13–16). Comparison of the sequence of mPAP-2a with other phosphatases defines a protein motif comprised of three regions of conserved sequence. PAP-2 homologs have been identified in yeast and Drosophila (16–20).

We sought to identify further human PAP homologs, and a search of expressed sequence tag data bases identified a number of candidate sequences. Full-length cDNAs encoding three of these (PAP-2a, PAP-2b, and a novel PAP-2 isoform, PAP-2c) have been cloned. These enzymes have been expressed as HA epitope-tagged proteins by transient transfection of HEK-293 cells and insect cells using baculovirus vectors. These systems have been used to investigate the cell surface activity of the three PAP enzymes, to examine their specificity for lipid phosphomonoester substrates presented as components of non-ionic detergent micelles and susceptibilities to inhibition by a number of agents that have been widely used as modulators of PAP-2 activity in intact and broken cell systems.

**Experimental Procedures**

**Isolation and Analysis of hPAP-2a, -2b, and -2c cDNAs—** We conducted blast searches of the GenBank™ data base of expressed sequence tags (ESTs) using regions of sequence conserved among mouse PAP-2a and previously reported human homologs. The complete cDNA was constructed from overlapping ESTs, and a complete cDNA was amplified from reverse-transcribed HL-60 cell cDNA by polymerase chain reaction using primers with the sequences 5′-GCTCTAGAACCATGTTGACAAGACGCGG-3′ (forward) and 5′-CAGCCCGGGTCGCAGCCTGCTG-3′ (reverse) using standard methodology (21). The cDNA was ligated into pGEM-7ZF. One of the ESTs identified (accession number U77929) appeared to correspond to a complete open reading frame, encoding hPAP-2b. This EST was obtained from the IMAGE Consortium, and the hPAP-2b cDNA was sequenced by a combination of manual sequencing using Sequenase 2.0 and automated sequencing performed in the center for analysis of macromolecules at Stony Brook. An open reading frame containing the complete cDNA sequence of hPAP-2b was constructed from overlapping ESTs, and a complete cDNA was amplified from reverse-transcribed HeLa cDNA using primers with the sequences 5′-GGTCTAGAACCATGTTGACAAGACGCGG-3′ (forward) and 5′-GGGGATCCATCTGACCAGAAGGTGGC-3′ (reverse) using standard methodologies (21). The cDNA was ligated into pGEM-ZF+. One of the ESTs identified (accession number U77931) was a complete open reading frame encoding hPAP-2c. This EST was obtained from the IMAGE Consortium, and the hPAP-2c cDNA was sequenced by a combination of manual sequencing using Sequenase 2.0 and automated sequencing performed in the center for analysis of macromolecules at Stony Brook.

**Transient Expression of hPAP-2a, -2b, and -2c in HEK-293 Cells—** The PAP-2a, -2b, and -2c cDNAs were subcloned into pcDNA, which is a cytomegalovirus-based vector for expression of proteins with an NH2-terminal HA epitope tag. HEK-293 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. 35-mm diameter lysine-coated dishes of 50% confluent cells were transfected with 1 μg of pcDNA hPAP-2a, pcDNA hPAP-2b, or pcDNA hPAP-2c using lipofectamine in Opti-MEM (Life Technologies, Inc.). The transfection medium was removed after 24 h and replaced with complete Dulbecco's modified Eagle's medium. The cells were harvested 24 h later by washing in phosphate-buffered saline followed by scraping into ice-cold lysis buffer containing 20 mM Tris, pH 7.5, 5 mM EDTA, 0.1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride. The lysate was disrupted by sonication on ice with a probe-type sonicator, and the material was used in assays within 24–48 h as described below. In some cases, the lysate obtained was centrifuged into total membrane and cytosolic fractions by centrifugation at 300,000 × g at 4 °C. The membrane fraction was resuspended in ice-cold lysis buffer by vortexing.

**Bacterial Expression of PAP-2a, -2b, and -2c—** The PAP-2a, -2b, and -2c cDNAs were subcloned into pFastBac (Life Technologies, Inc.), and recombinant baculoviruses were generated by sequential transformation of DH10Bac cells, isolation of recombinant bacmid vectors, and transfection of SF9 cells using Cellfectin reagent. Recombinant baculoviruses were selected and propagated using standard procedures to generate monolayers of exponentially growing insect cells cultured in complete Grace's medium containing antibiotics, antimycotics, 10% fetal bovine serum and supplemented with lactic acid and Yeastolate (generally 20 × 106 cells in a 225-cm2 flask) were infected with recombinant baculoviruses for expression of the PAP enzymes or of a control protein (PLD2) at a multiplicity of 10. The cells were cultured for 48 h at 27 °C. For assays using intact insect cells, the monolayer of cells was carefully washed with unsupplemented Grace's medium, and the cells were dislodged by gentle pipetting and transferred to a 50-ml conical centrifuge tube. The intact cells were sedimented by centrifugation at 100 × g for 10 min at room temperature and resuspended in unsupplemented Grace's medium. The cells were kept at 27 °C and used within 6 h of isolation. Where indicated, for determinations of total lactate dehydrogenase or PAP activity, these cells were disrupted by ultrasound on ice (see below), and the sonicated material was kept on ice before use.

For studies using SF9 cell membranes or detergent-extracted membrane proteins, the monolayers of infected cells were washed gently with phosphate-buffered saline and lysed by the addition of 4 ml of ice-cold lysis buffer and scraping. The cell suspension was transferred to a 15-ml conical tube, and the cells were disrupted by sonication (Vertis Systems Sonifier), 10 s-10 s-pulses on ice. The disrupted cells were centrifuged at 20,000 × g at 4 °C for 20 min. The cytosolic fraction was removed, and the membrane fraction was resuspended in ice-cold lysis buffer. Detergent extracts were prepared from the membranes by the addition of Triton X-100 and β-octylglucopyranoside to final concentrations of 1% followed by incubation at 4 °C with constant rocking for 1 h. The solubilized material was centrifuged at 20,000 × g at 4 °C for 30 min, and the supernatant was removed.

**Preparation of Substrates—** [32P]PA, C1P, and LPA were prepared by phosphorylation of DG, C8 ceramide, or monooleoylglycerol (Avanti Polar Lipids) using Escherichia coli DG kinase (Calbiochem) and [γ-32P]ATP (ICN) (11). The reactions were terminated by extraction with acidified CHCl3 and MeOH, and the dried organic phases obtained were resuspended in 0.4 ml of 20:9:1 CHCl3/MeOH/H2O (solvent A) and neutralized by the addition of a small volume of 20% NH4OH in MeOH. This material was applied to an Econosil NH2 5U high pressure liquid chromatography column (250 × 4.2 mm) (Alltech Associates). The column was washed with 20 ml of solvent A and then eluted with a 40-ml linear gradient of 0–1 M ammonium acetate in solvent A, 0.5-ml fractions were collected, and associated radioactivity was determined by liquid scintillation counting. 32P-Labeled products were pooled and extracted from the eluant by the addition of 3 ml HCl and CHCl3 to give two phases. [32P]PA and [32P]C1P prepared in this way were dried and resuspended in a small volume of solvent A. The lipids were stored at −20 °C until use. [32P]SIP was prepared by acid hydrolysis of [32P]C1P and purified by thin layer chromatography on silica gel plates (11) or by phosphorylation of sphingosine using Swiss 373 cells as a source of sphingosine kinase (23). Radiolabeled material was identified by autoradiography, the silica was scraped from the plate, and lipids were eluted by the addition of acidified CHCl3/MeOH. The eluted material was stored at −20 °C. Dipalmitoyl PA was obtained from Avanti Polar Lipids. Unlabeled C8 C1P and SIP were obtained from Biomol Inc. Sphingosine was from Avanti Polar lipids.

**PAP Assays—** The assay procedures used were adapted from those described by others (6, 7). In brief, assays were performed in medium containing 20 mM Tris, pH 7.5, 1 mM EDTA, and 2 mM DETA, and in some cases the MgCl2 concentration of the assay medium was varied by the addition of MgCl2, as indicated. C1P and SIP were stored as 1 mg/ml solutions in 1 ml Triton X-100. Labeled lipids were dried in vacuo and resuspended in 6.4 ml Triton X-100. Unlabeled PA was mixed with the radiolabeled substrate before drying and resuspension, while C1P and SIP were added to appropriate dried radiolabeled substrates from stock solutions in Triton X-100. In some experiments, dodecyl β-D-maltoside was substituted for Triton X-100. The substrates were dispersed by bath sonication and vortexing. Unless otherwise noted, the assay volume was 100 μl, and each assay contained final.
concentrations of 3.2 mM Triton X-100 and 100 μM 32P-labeled lipid substrate. For investigations of the dependence of enzyme activity on the surface concentration of substrate, the substrate concentration was 100 μM, and the detergent concentration was varied as indicated. Transfected cell fractions, SF9 cell fractions, or detergent-extracted membrane proteins (generally 0.1–5 mg of protein) were added directly to these incubations. In some cases, the source of PAP activity was incubated with 5 mM NEM at 37 °C before addition to the incubations. Assays were performed at 37 °C and were terminated by the addition of ice-cold 10 mg/ml bovine serum albumin and 10% trichloroacetic acid. The samples were centrifuged for 5 min in a microcentrifuge, and [32P]PO4 released into the supernatant was quantitated by liquid scintillation counting. This assay was validated by demonstrating that the water-soluble radioactivity released from the substrates was [32P]PO4 by quantitative extraction with ammonium molybdate.

For assays of PAP activity using intact SF9 cells, [32P]dipalmitoyl-PA (approximately 10,000 dpm) was resuspended in unsupplemented Grace's medium containing 2 mg/ml bovine serum albumin to a final concentration of 20 mM by bath sonication. Assays were initiated by adding 1 ml of this substrate preparation to 1 ml of cells (generally 400 × 10^3 cells) and incubation at 37 °C with constant shaking. Aliquots of the suspension were removed at various times for determination of PAP activity by measurement of [32P]PO4 release as described above. In some cases, samples were removed for determination of lactate dehydrogenase activity.

**RESULTS**

**Sequence Comparison of PAP-2a, -2b, and -2c**—Fig. 1 shows an alignment of the deduced amino acid sequences of hPAP-2a, -2b, and -2c. PAP-2a comprises 289 amino acids corresponding to a protein of 32,788 kDa. PAP-2b comprises 311 amino acids corresponding to a protein of 35,120 kDa. PAP-2c comprises 288 amino acids corresponding to a protein of 32,577 kDa. PAP-2c is 54% identical to PAP-2a and 43% identical to PAP-2b. These values increase to 68 and 54%, respectively, when conservative amino acid substitutions are accounted for. It is notable that in comparison with PAP-2a and -2c, PAP-2b contains an extended N terminus with an enrichment of basic amino acid residues. The C-terminal 40 amino acids of the three proteins are also highly divergent. All three proteins contain a single consensus site for N-linked glycosylation (residue 140 in PAP-2c).
Hydropathy analysis of all three sequences suggests that PAP-2a, -2b, and -2c are integral membrane proteins with six membrane-spanning regions of 17–25 hydrophobic amino acid residues. These sequences are underlined in Fig. 1 and denoted as regions I–VI. Mutagenesis of rat PAP-2b supports this putative transmembrane topology (15).

Expression of PAP-2a, -2b, and -2c in HEK293 Cells—We expressed PAP-2a, -2b, and -2c as HA epitope-tagged proteins by transient transfection of HEK293 cells using a cytomegalovirus promoter-based vector. Cells were harvested 48 h post-transfection. PAP activity was determined in lysates of control and transfected cells using PA as substrate. The cells expressing PAP-2a, -2b, and -2c exhibited 7-, 15-, and 7-fold increases in membrane-associated PAP activity, respectively (Fig. 2A).

Total protein from the transfected cells was separated by SDS-polyacrylamide gel electrophoresis on a 10% gel and analyzed by Western blotting using the HA epitope-specific 12CA-5 monoclonal antibody. In comparison with samples from untransfected cells, major immunoreactive proteins with estimated molecular masses of 33 and 37 kDa, 34 kDa, and 34 and 33 kDa were detected in cells expressing PAP-2a, -2b, and -2c, respectively (Fig. 2B). The immunoreactive material of higher molecular weight observed in the case of PAP-2a and -2c presumably represents the glycosylated forms of the proteins. We found that immunoreactive species of lower mobility could be partially converted to the faster migrating species upon treatment of membrane extracts with N-glycanase prior to SDS-polyacrylamide gel electrophoresis on a 10% gel and analyzed by Western blotting as described under “Experimental Procedures.” Molar weights of prestained markers are shown. The data shown are representative of three separate experiments.

Expression of PAP-2a, -2b, and -2c in Insect Cells Using Baculovirus Vectors—Recombinant baculovirus-infected Sf9 insect cells have proved to be an effective system for studying the regulation of integral membrane signaling proteins including adenylyl cyclase isoforms and G-protein-coupled receptors (27, 28). In comparison with the transient transfection studies described above, this approach also provides a simple and consistent means to produce recombinant protein that is necessary for detailed investigations of the catalytic properties of these enzymes. Recombinant baculoviruses for expression of PAP-2a, -2b, and -2c were prepared and monolayer cultures of insect cells infected with these viruses and a virus expressing a control protein (murine PLD2) as described under “Experimental Procedures.” Cells were harvested 48 h post-transfection and fractionated into cytosolic and membrane fractions. PAP activity in these fractions was determined using PA as substrate. In comparison with control cells, cells expressing PAP-2a, -2b, and -2c exhibited dramatic (1140-, 540-, and 460-fold) increases in membrane-associated PAP activity (Table I). Cytosolic PAP activity was unaltered in cells expressing PAPs-2a, -2b, and -2c. PAP activity could be effectively extracted from the SI9 cell membranes by a combination of 1% Triton X-100 and 1% β-octylglucopyranoside. This procedure routinely solubilized 70–80% of PAP-2a, -2b, and -2c activity and approximately 30% of total membrane protein. Type 2 PAP activities are characteristically independent of Mg2+ and insensitive to inhibition by NEM. Our standard assay medium contains 2 mM EDTA and no added Mg2+.

Activity of the extracted PAP-2a, -2b, and -2c was unchanged by the addition of MgCl2 to the assays to give a Mg2+ concentration of 5 mM. Similarly, preincubation of the extracted material with 5 mM NEM produced very modest decreases in PAP-2a, -2b, and -2c activity (Table I). Baculovirus-infected SI9 cells are therefore an excellent model system for investigating the catalytic properties of recombinantly expressed PAP-2 enzymes.

Cell Surface Activity of PAP-2a, -2b, and -2c in Baculovirus-infected Insect Cells—Cell surface PAP activity has been detected in many different cell types, but the identity of the enzyme responsible has not been determined. To investigate the cell-surface expression of PAPs-2a, -2b, and -2c, we measured PAP activity of intact baculovirus-infected insect cells expressed using [32P]dipalmitoyl-PA presented in 1 mg/ml bovine serum albumin. Fig. 3 shows time courses of PAP activity determined as 32P release measured using intact cells or equiv-
Monolayer cultures of Sf9 cells were infected with recombinant baculoviruses for expression of PAP-2a, -2b, and -2c and a control protein (murine PLD2). The cells were harvested, washed, and resuspended in unsupplemented Grace’s medium as described under “Experimental Procedures.” PAP activity was determined using PA as substrate in samples of intact cells (●) or cells that had been disrupted by sonication (▲). Lactate dehydrogenase release from the cells was determined as described under “Experimental Procedures” and expressed as a percentage of activity in cells that had been disrupted by sonication (▲). The data shown are means ± S.D. of triplicate determinations and are representative of three separate experiments.

### TABLE I

| Substrate | Control | PAP-2a | PAP-2b | PAP-2c |
|-----------|---------|--------|--------|--------|
| Membrane  | 0.05 ± 0.001 | 57 ± 3 | 27 ± 2 | 23 ± 2 |
| Cytosol   | 0.2 ± 0.03  | 0.2 ± 0.03 | 0.2 ± 0.04 | 0.2 ± 0.04 |
| Detergent Extract | 0.25 ± 0.04 | 225 ± 22 | 167 ± 21 | 123 ± 12 |
| Detergent Extract + Mg<sup>2+</sup> | 0.3 ± 0.05 | 220 ± 23 | 158 ± 30 | 128 ± 11 |
| Detergent Extract + NEM | 0.2 ± 0.04 | 191 ± 9 | 147 ± 11 | 115 ± 14 |

**Fig. 3. PAP activity in intact insect cells.** Monolayer cultures of Sf9 cells were infected with recombinant baculoviruses for expression of PAP-2a (A), -2b (B), and -2c (C). The cells were harvested, washed, and resuspended in unsupplemented Grace’s medium as described under “Experimental Procedures.” PAP activity was determined using PA as substrate in samples of intact cells (●) or cells that had been disrupted by sonication (▲). Lactate dehydrogenase release from the cells was determined as described under “Experimental Procedures” and expressed as a percentage of activity in cells that had been disrupted by sonication (▲). The data shown are means ± S.D. of triplicate determinations and are representative of three separate experiments.
was increased, and the data obtained are shown in Fig. 4. For all three enzymes, activity increased with increasing substrate concentration in an apparently saturable manner, suggesting that a surface dilution kinetic model was appropriate for analysis of these enzymes. The data were analyzed by weighted nonlinear regression fit to the equation

\[ V = \frac{V_{\text{max}} A}{K_a + A} \]

where \( V \) is the initial rate and \( A \) is the substrate concentration in \( \text{mol} \% \) to calculate \( V_{\text{max}} \) and \( K_a \). Values \( R \) for this analysis ranged from 0.84 to 0.97. The results obtained are presented in Table III.

### Table II

| Substrate hydrolysis | Control | PAP-2a | PAP-2b | PAP-2c |
|----------------------|---------|--------|--------|--------|
| PA CIP               | PA CIP  | PA CIP | PA CIP |
| Triton X-100         | 0.5 ± 0.01 | 0.1 ± 0.02 | 320 ± 12 | 170 ± 14 | 220 ± 28 | 160 ± 24 | 90 ± 8.1 | 120 ± 17 |
| Dodecyl-β-D-maltoside| 0.6 ± 0.02 | 0.1 ± 0.01 | 413 ± 32 | 189 ± 21 | 244 ± 22 | 174 ± 21 | 128 ± 12 | 147 ± 24 |

### DISCUSSION

PAP-2c is a third member of the family of PAP-2 isoenzymes. This enzyme shares the putative transmembrane topology of the other PAP-2 isoenzymes, having six regions of predominantly hydrophobic amino acids linked by extramembrane regions. This proposed transmembrane structure is similar to that of the membrane-spanning portions of membrane-bound adenylylcyclases and transport proteins of the P-glycoprotein superfamily, which consist of a short N terminus and two transmembrane regions consisting of six hydrophobic spans that link globular cytoplasmic domains (28). Comparison of the sequences of hPAP-2a and hPAP-2b with previously recognized homologs identifies three regions of conserved sequence (denoted as A, B, and C in Fig. 1), which contain invariant amino acids that define a signature sequence motif shared among several proven or putative lipid phosphatases, the mammalian glucose 6-phosphatases, and some bacterial nonspecific acid phosphatases. This group also contains yeast and Dro sophila PAP-2 homologs (13–20). These conserved regions lie predominantly within the hydrophilic regions of the proteins, and homologous sequences are also found in a soluble globular proteins that include bacterial acid phosphatase, mammalian glucose 6-phosphatase, and a fungal vanadium-dependent chloroperoxidase (29). The structure of this latter enzyme has been recently determined (30). The pentacoordinate vanadate cofactor resembles the transition state structure of phosphate. This suggests a two-step reaction in which a charge relay system involving conserved His and Asp residues in region C establishes a histidine-phosphate bond and the catalytic histidine residue in region B acts as an acid to cleave this intermediate, releasing the dephosphorylated substrate. This residue then acts as a base to facilitate nucleophilic attack on the phospho-histidine intermediate by a water molecule. Catalytically inactive PAP-2 mutants would be valuable tools for in vivo studies.

Like PAP-2a and PAP-2b, PAP-2c is exclusively membrane-bound when expressed in mammalian or insect cells. As with many integral membrane proteins, purification of the type 2 PAP enzymes has been difficult. We made baculoviruses for expression of PAP-2a, -2b, and -2c and found that baculovirus-infected insect cells were an excellent model system for generating recombinant PAP enzymes. These cells exhibited extremely low levels of endogenous PAP activity. Infection with appropriate viruses resulted in dramatic increases in PAP activity. Membranes or detergent-extracted membrane proteins from these cells provided an abundant source of activity for kinetic studies. The Sf9 cell system should be effective for investigating the regulation of the type 2 PAP enzymes and may also provide an appropriate source for purification of recombinant proteins.

We used Triton X-100 mixed micelles to investigate the substrate specificity of PAP-2a, -2b, and -2c. The activity of all three enzymes was dependent on the surface concentration of substrates, suggesting that a surface dilution kinetic model was appropriate for analyzing the kinetic behavior of the enzymes in this model system. According to this model, which was originally developed for studies of phospholipase \( \Lambda_e \), enzyme activity depends on both bulk and surface concentrations of substrate, because enzyme binding to the micelle interface precedes substrate binding and catalysis (31, 32). Since the PAP-2 enzymes are clearly integral membrane proteins, their association with detergent micelles would be expected to be effectively irreversible, so this type of analysis can be simplified to measurements of the dependence of enzyme activity on the surface concentration of substrate. The major limitation of this approach is that substrate or enzyme molecules can exchange between detergent micelles, and for highly active enzymes this rate of substrate exchange can be limiting (33). It is also possible that the detergent employed for these measurements may have selective affects on enzyme activity, although our finding that the enzymes displayed similar profiles of activity with
substrates presented in Triton X-100 or dodecyl β-D-maltoside micelles argues against this possibility. The data from these experiments were used to calculate $V_{\text{max}}$ and $K_m$ values for the different substrates. Although the extremely high levels of expression obtained with the insect cell system rendered interference from endogenous activities insignificant, because the enzyme preparations used were not pure, comparison of the absolute $V_{\text{max}}$ values of the enzymes is not meaningful. The data obtained do, however, provide a quantitative comparison of the relative activities of the individual PAP enzymes for the different substrates tested. Our results indicate that PAP-2a, -2b, and -2c can hydrolyze PA, LPA, C1P, and S1P and that there are differences in the selectivity of the enzymes for these four substrates. PAP-2a displayed comparable $V_{\text{max}}$ values for all four substrates, with highest activity displayed against LPA and PA. PAP-2b showed a similarly higher relative $V_{\text{max}}$ activity with LPA as substrate, while PAP-2c displayed significantly higher activity against sphingosine phosphate. $K_m$ values for the substrates varied more widely. Specificity constants ($V_{\text{max}}/K_m$) provide a means to quantitate the specificity of the three enzymes for the different substrates. These values are given in Table III, and they indicate a relative rank order of substrate selectivity of LPA > PA > S1P > C1P for PAP-2a, LPA ~ PA > C1P > S1P for PAP-2b, and PA > C1P > LPA > S1P for
PAP-2c. The $K_m$ values obtained with PA as substrate are comparable with those reported for a yeast PAP (32) but somewhat higher than previously determined for a liver form of the enzyme (11). Our results are in broad agreement with studies that used transiently transfected HEK293 cells as a source of enzyme activity to demonstrate that PAP-2a and -2b could hydrolyze PA, C1P, and S1P, although we did not observe the selectivity of PAP-2b for S1P reported by these investigators (13, 14).

The diversity of PAP-2 enzymes revealed by cDNA cloning was somewhat unexpected, since purification studies suggested that this enzyme behaved as a single activity from a number of different mammalian tissues (4–8). PAP-2 activity has been studied extensively in rat liver, where a highly purified enzyme preparation has been reported to hydrolyze PA, C1P, and S1P with comparable avidity. The finding that each substrate inhibited the hydrolysis of the other in a competitive manner further supports the contention that this PAP-2 enzyme hydrolyzes all three substrates (11). The relationship of the rat liver PAP-2 enzyme to the cloned isoenzymes is not known, and this issue clearly requires further investigation. The S. cerevisiae genome contains four open reading frames encoding proteins with extensive homologies to the type 2 PAPs. Recent work has identified a member of one class as a PA and diacylglycerol pyrophosphate phosphatase and the members of the remaining class as S1P phosphatases (18–20). PA and C1P did not inhibit the hydrolysis of S1P by these yeast enzymes (19), suggesting that they are highly specific for S1P. In comparison with mammalian PAP-2a, -2b, and -2c, the yeast S1P phosphatases have several unique structural features including an extended C terminus and several nonconservative substitutions of amino acid residues around the presumed active site. We have identified a cDNA encoding a fourth human type 2 PAP enzyme, which has a number of provocative homologies to the yeast S1Pases. This may prove to be a mammalian S1P-selective PAP-2 isoenzyme.

Fractionation studies using rat hepatocytes indicate that PAP-2 activity is enriched in the plasma membrane, and recombinantly expressed mouse PAP-2a is also localized to this compartment in HEK 293 cells (4, 13). Several cells have been reported to express a cell surface PAP activity (9, 10). We measured PAP activity in intact Sf9 cells expressing PAPs-2a, -2b, and -2c. The results obtained clearly indicate that when expressed in these cells, the active site of PAP-2a has effective access to extracellular substrates. The simplest explanation for these results is that this is an ectoenzyme that is oriented in the plasma membrane with its active site facing the extracellular space. Although we cannot rule out the possibility that a highly active “flippase” delivers PA substrate to this enzyme at an intracellular site, we consider this possibility unlikely because hydrolysis of PA by PAP-2a-expressing insect cells was rapid and proceeded without significant accumulation of radio-labeled substrate by the cells. PA, LPA, and S1P are receptor-active compounds, and it is probable that one function of PAP-2a is to terminate the signaling functions of these lipid agonists. Studies using fluorescent PA analogs reveal that dephosphorylation of PA to form DG precedes DG uptake into the cell. Cell surface PAP-2a may also provide a mechanism for formation of DG from extracellular substrates (34). Unfortunately, immunocytochemical analysis reveals that the HA-tagged PAP-2a fails to exit the endoplasmic reticulum when expressed in HEK293 cells, so we have therefore not yet been able to examine cell surface PAP activity in mammalian cells expressing this enzyme.3

As discussed above, the PAP-2 enzymes appear to be glycoproteins that share the same predicted transmembrane topology. Our results suggest that, in comparison with PAP-2a, PAP-2b and -2c are localized to different intracellular membrane compartments. Clearly, localization studies, preferably using PAP-2 isoenzyme-selective antibodies to study endogenous enzymes, are needed to further define the subcellular distribution of the PAP-2 isoenzymes. Rat PAP-2b is localized to the endoplasmic reticulum in cultured rat intestinal epithelial cells (15). A portion of type 1 PAP activity in rat liver co-localized with endoplasmic reticulum markers during cell fractionation studies (4, 35). Despite this apparent co-localization, PAP-2b is clearly a type 2 PAP enzyme, and presumably the endoplasmic reticulum-localized PAP-1 activity reported in

\[ K_m \]

3 V. A. Sciorra and A. J. Morris, unpublished observations.
rat liver represents the product of a separate gene. These findings are particularly interesting in light of the growing body of information that points to a role for PLD-generated PA in controlling vesicular transport between the endoplasmic reticulum and Golgi apparatus as well as the cis and trans compartments of the Golgi. PA has been proposed to play an essential role in recruitment of cytosolic coatomer complexes to the surfaces of the respective membrane compartments involved in these transport processes, which in turn initiates formation of coated transport vesicles (36–38). Such a mechanism would require tight control of PA levels, and localization of PA activity to this membrane compartment may therefore play an important role in this process.

Clearly, establishment of the roles played by the PAP-2 enzymes in cellular lipid metabolism remains an important priority. Our results suggest roles for the PAP-2 enzymes in the metabolism of both phospholipid- and sphingolipid-derived signaling molecules. Further work is required to establish the physiological role of these enzymes in metabolism of these bioactive lipid substrates. In this regard, it is noteworthy that PA has been proposed to play an essential role in recruitment of cytosolic coatomer complexes to the plasma membrane (39). These findings raise the possibility that the PAP-2 enzymes play selective roles in signaling processes controlled by these two PLDs, and future work will be directed toward investigating this possibility.

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Note Added in Proof—While this paper was in press, Hooks and co-workers (Hooks, S. B., Ragan, S. P., and Lynch, K. R. (1998) FEBS Lett. 427, 189–192) also reported the cloning and expression of a human PAP-2c cDNA.

REFERENCES
1. Brindley, D. N., and Waggoner, D. W. (1996) Chem. Phys. Lipids. 80, 45–57
2. Exton, J. H. (1997) J. Biol. Chem. 272, 15579–15582
3. Morris, A. J., Engbercht, J. E., and Frohman, M. A. (1996) Trends. Pharmacol. Sci. 20, 182–185
4. Jamal, Z., Martin, A., Gomez-Munoz, A., and Brindley, D. N. (1996) J. Biol. Chem. 271, 2988–2996
5. Gomez-Munoz, A., Hamza, E. H., and Brindley, D. N. (1992) Biochim. Biophys. Acta 1127, 49–56
6. Waggoner, D. W., Martin, A., Dewald, J., Gomez-Munoz, A., and Brindley, D. N. (1995) J. Biol. Chem. 270, 19422–19429
7. Kanoh, H., Imai, S-I., Yamada, K., and Sakane, F. (1992) J. Biol. Chem. 267, 25309–25314
8. Fleming, I. N. Yeaman, S. J. (1995) Biochem. J. 308, 983–989
9. Perry, D. K., Stevens, V. L., Wielanski, T. S., Lambeth, J. D. (1993) J. Biol. Chem. 268, 25302–25310
10. Xie, M., and Low, M. G. (1994) Arch. Biochem. Biophys. 312, 254–259
11. Waggoner, D. W., Gomez-Munoz, A., Dewald, J., and Brindley, D. N. (1996) J. Biol. Chem. 271, 16506–16509
12. Spiegel, S., Foster, D., and Kolesnick, R. (1996) Curr. Opin. Cell Biol. 8, 169–167
13. Kai, M., Wada, I., Imai, S., Sakane, F., and Kanoh, H. (1996) J. Biol. Chem. 271, 18931–18938
14. Kai, M., Wada, I., Imai, S-I., Sakane, F., and Kanoh, H. (1997) J. Biol. Chem. 272, 24572–24578
15. Barilia, D., Plateroti, M., Nobili, F., Muda, A. O., Xie, Y., Morimoto, T., and Perozzi, G. (1996) J. Biol. Chem. 271, 29928–29936
16. Stukey, J., and Carman, G. M. (1997) Protein Sci. 6, 469–472
17. Zhang, X., Zhang, J., Purcell, K. J., Cheng, Y., and Howard, R. (1997) Nature 385, 64–67
18. Mao, C., Wadleigh, M., Jenkins, G. M., Hannun, Y. A., and Obeid, L. M. (1997) J. Biol. Chem. 272, 28699–28694
19. Toke, D. A., Bennett, W. L., Dillen, D. A., Wu, W.-L., Chen, X., Ostrand, D. B., Oshio, J., Creemst, A., Veelker, D. R., Fischl, A. S., and Carman, G. M. (1998) J. Biol. Chem. 273, 3278–3284
20. Mandala, S. M., Thornton, R., Tu, Z., Kurtz, M. B., Nickels, J., Broach, J., Menzeleeve, R., and Spiegel, S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 150–155
21. Frohman, M. A. (1994) PCR Methods and Applications, pp. s40–s57, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
22. O’Reilly, D. R., Miller, L. R., and Lucklow, V. A. (1992) in Baculovirus Expression Vectors: A Laboratory Manual, pp. 107–180, W. H. Freeman and Co., New York
23. Olivera, A., Rosenthal, J., and Spiegel, S. (1994) Anal. Chem. 223, 306–312
24. Hammond, S. M., Jenco, J. M., Nakashima, S., Cadwallader, K., Gu, Q., Cook, S., S., Nnazwa, Y., Prestwich, G. D., Frohman, M. A., and Morris, A. J. (1997) J. Biol. Chem. 272, 3860–3869
25. Lindau, M., and Gomperts, B. D. (1991) Biochim. Biophys. Acta 1071, 429–471
26. Martin, J. B., and Dry, D. M. (1949) J. Biol. Chem. 176, 956–967
27. Butkerait, P., Zheng, Y., Hallak, H., Graham, T. E., Miller, H. A., Burris, K. D., Molinoff, P. B., and Manning, D. R. (1995) J. Biol. Chem. 270, 16891–16899
28. Tassirig, R., Tang, W. J., and Gilman, A. G. (1997) Methods Enzymol. 238, 95–108
29. Neuwald, A. F. (1997) Protein Sci. 6, 1764–1767
30. Meserschmidt, A., and Wever, R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 392–396
31. Carman, G. M., Deems, R. A., and Dennis, E. A. (1995) J. Biol. Chem. 270, 18711–18714
32. Lin, Y. P., and Carman, G. M. (1990) J. Biol. Chem. 265, 166–170
33. Jain, M. K., Gelb, M. H., Rogers, J., and Berg, O. G. (1995) Methods Enzymol. 249, 567–614
34. Pagano, R. E., and Longmuir, K. J. (1985) J. Biol. Chem. 260, 1909–1916
35. Gomez-Munoz, A., Waggoner, D. W., O’Brien, L., and Brindley, D. N. (1916) J. Biol. Chem. 270, 26318–26325
36. Bi, K., Roth, M. G., and Ktistakis, N. T. (1998) Curr. Biol. 7, 301–307
37. Ktistakis, N. T., Brown, H. A., Waters, M. G., Sternweis, P. C., and Roth, M. G. (1996) J. Cell Biol. 134, 295–306
38. Chen, Y-G., Siddhanta, A., Austin, C. D., Hammond, S. M., Sung, T-C., Frohman, M. A., Morris, A. J., and Shields, D. (1997) J. Cell Biol. 138, 495–504
39. Colley, W., Sung, T-C., Roll, R., Jenco, J., Hammond, S. M., Autscheller, Y., Bar-sagi, D., Morris, A. J., and Frohman, M. A. (1997) Curr. Biol. 7, 191–201