Identification and cDNA Cloning of 35-kDa Phosphatidic Acid Phosphatase (Type 2) Bound to Plasma Membranes

POLYMERASE CHAIN REACTION AMPLIFICATION OF MOUSE H$_2$O$_2$-INDUCIBLE hic53 CLONE YIELDED THE cDNA ENCODING PHOSPHATIDIC ACID PHOSPHATASE*

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We previously described the purification of an 83-kDa phosphatidic acid phosphatase (PAP) from the porcine thymus membranes (Kanoh, H., Imai, S.-i., Yamada, K. and Sakane, F. (1992) J. Biol. Chem. 267, 25309–25314). However, we found that a minor 35-kDa protein could account for the PAP activity when the purified enzyme preparation was further analyzed. We thus determined the N-terminal sequence of the 35-kDa candidate protein and prepared antipeptide antibody against the determined sequence, MFDKTRLPYVALDVL. The antibody almost completely precipitated the purified enzyme activity. Furthermore, the antibody precipitated from the radiiodinated enzyme preparation a single 35-kDa protein, which was converted to a 29-kDa form when treated with N-glycanase. We also found that the immunoprecipitable PAP activity was exclusively associated with the plasma membranes of porcine thymocytes. These results indicated that the 35-kDa glycosylated protein represents the plasma membrane-bound (type 2) PAP.

We surprisingly noted that the N-terminal sequence of the porcine PAP was almost completely conserved in the internal sequence encoded by a mouse partial cDNA clone, hic53, reported as an H$_2$O$_2$-inducible gene (Egawa, K., Yoshiwara, M., Shibanauma, M., and Nose, K. (1995) FEBS Lett. 372, 74–77). We thus amplified from the mouse kidney RNA the hic53 clone by polymerase chain reaction, and obtained a cDNA encoding a novel protein of 283 amino acid residues with a calculated M, of 31,894. Methionine reported as an internal residue was found to serve as an initiator, and the C-terminal 64 residues were lacking in hic53. The protein contains several putative membrane-spanning domains and two N-glycosylation sites. When transfected into 293 cells, the cDNA gave more than 10-fold increase of the membrane-bound PAP activity, which could be precipitated by the antipeptide antibody. In [35S]methionine-labeled cells, the translational product was confirmed to be a 35-kDa protein, which became 30 kDa in cells treated with tunicamycin, an inhibitor of N-glycosylation. We thus succeeded in identifying the porcine type 2 PAP and subsequently in determining the primary structure of a mouse homolog of the PAP.

Phosphatidic acid phosphatase (PAP)1; EC 3.1.3.4) supplies diacylglycerol in the classical pathway of glycerolipid biosynthesis by dephosphorylating phosphatidic acid. The metabolic importance of PAP was demonstrated in particular for triacylglycerol synthesis, as evidenced by the translocation of soluble enzyme into the microsomal membranes in cells cultured with fatty acids (1, 2). More recently, PAP has attracted considerable interest in cellular signal transduction mediated by phospholipase D (3). In this case, phosphatidic acid cleaved from the major membrane phospholipid, phosphatidylcholine, is converted by PAP to diacylglycerol, which serves as a lipid second messenger by activating protein kinase C (4). It is thus likely that PAP acts in a coordinate manner with phospholipase D in signal-stimulated cells. Phosphatidic acid and its metabolic derivative, lysophosphatidic acid, are also known to be potent mitogens and activators when exogenously added to different cells (reviewed in Ref. 5). These observations indicate the crucial role of PAP in the regulation of the relative concentrations of diacylglycerol and phosphatidic acid, both of which serve as signal mediators and as intermediates of glycerolipid synthesis.

So far the existence of at least two PAP isoforms has been proposed in rat liver (6, 7). The first isoform, designated PAP1, is associated with the cytosol and microsomes, and appears to be responsible for glycerolipid biosynthesis. The second isoform (PAP2) is bound to the plasma membranes and has been considered to be involved in cellular signal transduction. The two PAP isoforms can be distinguished from each other by distinct enzymologic properties with respect to dependence of Mg$^{2+}$ and sensitivity to thiol-reactive reagents such as N-ethylmaleimide (6, 7). Although measured in crude enzyme preparation, the two PAP isoforms appeared to undergo different activity alterations in several liver diseases (8). The decrease of the PAP2 activity in transformed fibroblasts suggested its importance in regulating the balance of diacylglycerol and phosphatidic acid (9).

Despite the apparent importance of PAP in lipid metabolism and in signal transduction, little is known about the molecular properties of this enzyme. The purification of PAP with the enzymologic properties characteristic to the type 2 isozyme has

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) D84376.

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1 The abbreviations used are: PAP, phosphatidic acid phosphatase; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; HPLC, high performance liquid chromatography; CAPS, 3-(cylohexylamino)propanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride.
been reported by several groups but with somewhat variable results. Previously, we highly purified the membrane-bound PAP from porcine thymus (10). We interpreted the data as the purification of an 83-kDa enzyme, since this protein always appeared in parallel to the PAP activity fractionated in a variety of chromatographic procedures. A PAP of the same molecular mass (83 kDa) has also been obtained from rat liver by a similar purification protocol (11). However, the PAP2 recently purified from rat liver plasma membranes exhibited the molecular mass of 51–53 kDa (12). The PAP2 of rat liver membranes, on the other hand, was identified as a 31-kDa protein on SDS gels very recently (13). Subsequent to the purification of the 83-kDa enzyme, we cloned a cDNA encoding this protein. However, the cDNA failed to give PAP enzyme activity in repeated cDNA expression experiments. These results led us to reinvestigate the enzyme purification from the porcine thymus membranes. In an attempt to further identify the enzyme, we found that a minor 35-kDa protein but not the predominant 83-kDa protein present in the final enzyme preparation could account for the purified PAP activity. Although the 35-kDa protein could not be purified further, we confirmed using antipeptide antibody raised against the N-terminal amino acid sequence that the 35-kDa protein represents the PAP isof orm bound to the plasma membranes of the porcine thymus.

During our attempts to clone the cDNA encoding PAP based on the knowledge of the N-terminal sequence and another internal sequence obtained for the 35-kDa enzyme, we unexpectedly noted that the N-terminal (but not the internal) sequence was highly conserved as the internal sequence encoded by a H2O2-inducible mouse cDNA clone, hic35 (14). The analysis of the hic35 clone done by PCR amplification from the mRNA of mouse kidney demonstrated that the clone indeed encodes the 35-kDa PAP, although a considerable portion of the C-terminal sequence of the PAP is different from that described for the hic35 clone.

**EXPERIMENTAL PROCEDURES**

**Materials**—The sources of most of the chemicals have been described (10, 15). [%30P-labeled phosphatidic acid was prepared from [γ-30P]ATP (ICN Biochemicals) and 1,2-di(oleoyl)glycerol (Sigma) using diacylglycerol kinase from Escherichia coli (Calbiochem) (16). Na125I and [35S]methionine were obtained from DuPont NEN. Peptide N-glycosidase F (N-glycanase) and endoglycosidase H were obtained from New England BioLabs and Seikagaku (Tokyo, Japan), respectively. Keyhole limpet hemocyanin, tunicamycin, and heat-killed Staphylococcus aureus (strain Cowan) were the products of Sigma. Sephadex G-25, protein A-Sepharose, and BrCN-activated Sepharose were obtained from Pharmacia Biotech Inc. Total RNA from mouse kidney and 293 cells was kindly donated by Dr. H. Kimura (Research Center for Molecular Genetics, Hokkaido University). 293 cells were maintained in Dulbecco’s modified Eagle’s minimum essential medium supplemented with 10% fetal calf serum (Life Technologies, Inc.). DNA-modifying enzymes and restriction enzymes were purchased from Takara Shuzo Co. Oligonucleotides were synthesized using an Applied Biosystems 380B DNA synthesizer. All other chemicals were of the highest quality commercially available.

**Assay of PAP Activity**—The PAP activity was determined exactly as described previously (10) by measuring [%32P]Pi radioactivity liberated during a 5-min incubation at 37 °C. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 pmol of [%32P]Pi/min under the described conditions.

**Purification of PAP**—PAP was purified exactly as described previously (10) from the KCl-washed thymus membranes suspended in buffer A, containing 25 mM Tris-HCl (pH 7.4), 5 mM MgCl2, 1 mM EDTA, 20 mM glycerol, 1 mM PMSF, 10 μg/ml each of leupeptin and pepstatin, and 0.02% NaN3. After being solubilized from the membranes with 1% each of octylglucoside and Triton X-100, the enzyme was finally purified by the Affi-Gel blue column chromatography, reproducibly resulting in the purification of PAP more than 2000-fold from the membranes, with the specific enzyme activity ranging from 15 to 18 units/mg of protein as described (10). This final enzyme preparation was further analyzed in the present study.

**Native PAGE of PAP Preparation**—Native PAGE was performed by the method of Davis (17). The final PAP preparation (10 μg of protein) was loaded onto a 7.5% polyacrylamide disc gel. The gel and electrode buffer contained 0.1% Triton X-100. The electrophoresis was performed at 35 °C for 6 h at 4 mA per gel column (0.5 × 6 cm). After electrophoresis the gel was cut into 0.2-cm pieces, crushed, and agitated at 4 °C for 16 h in 100 μl of buffer A containing 0.2% Triton X-100. The aliquots (5 μl) of gel extracts were subjected to the PAP activity assay, and the remaining extracts were further analyzed by SDS-12.5% PAGE (18) under reducing conditions.

**Determination of Partial Amino Acid Sequences of the 35-kDa PAP**—In the case of determination of the N-terminal sequence, the purified PAP preparation (35 μg of protein) was subjected to chloroform/methanol precipitation (19) and subjected to SDS-12.5% PAGE. After electrophoresis, the gel was incubated for 20 min in the transfer buffer, containing 30 mM CAPS (pH 11.0) and 0.05% SDS. Electroblotting to an Immobilon-P® membrane (Millipore Corp.) was carried out at 400 mA for 6 h at 4 °C using a Bio-Rad apparatus. The membrane was stained with Coomassie Brilliant Blue, and the 35-kDa band excised was directly applied to a protein sequencer (Applied Biosystems model 477A) equipped with an on-line phenylthiocyanatodervative analyzer (model 120A). The in situ gel digestion of the 35-kDa protein was done by the method of Helman et al. (20). The SDS-gel pieces containing 35-kDa enzyme (<20 μg) from approximately 200 μg of the final enzyme preparation were treated with 200 ng of lysyl endopeptidase (Wako, J apan) for 20 h at 37 °C. The buffer used was 0.1 M Tris-HCl (pH 8.8) containing 0.02% Tween-20. The generated peptides were extracted from the gels with 0.1% trifluoroacetic acid, 60% acetonitrile. The peptides were then separated by a reversed-phase HPLC using a Brownlee Aquapore column (100 × 2.1-mm; Applied Biosystems), and the collected peptides were subjected to sequence analysis.

**Preparation and Affinity Purification of Antibody—**A peptide, MFQDKTRLYIVALVDV (C), corresponding to the N-terminal sequence determined for the 35-kDa candidate PAP with an additional cysteine residue attached to the C terminus, was synthesized by a peptide synthesizer (model 431A, Applied Biosystems). The peptide was purified by a reversed-phase HPLC using an Inertisil C18 column (GL Science, J apan). Two rabbits were immunized with the peptide (150 μg each) coupled to keyhole limpet hemocyanin (21) in complete Freund’s adjuvant (Nakarai, J apan). Booster injections were repeated every 2 weeks thereafter using the same amount of the conjugated peptide emulsified in incomplete Freund’s adjuvant. The serum was collected 10 days after each booster injection, and IgG was obtained by a protein A-Sepharose column chromatography (21). For the affinity purification of the antibody, the antigen peptide (6 μg) conjugated to bovine serum albumin (Sigma) was coupled to 3 ml of BrCN-activated Sepharose according to the manufacturer’s instructions. Immunogen was then applied to the peptide column and affinity-purified by elution at pH 2.5.

**Labeling of the PAP Preparation**—The purified PAP (6 μg of protein) was treated with 100 μl of Na252I and 1ODO-BEADES (Pierce). After iodination, bovine serum albumin (1 mg/ml) was added as a carrier to the reaction mixture, and the labeled proteins were obtained from a Sephadex G-25 column equilibrated with buffer A containing 1% Triton X-100. The labeled proteins were subjected to immunoprecipitation followed by enzymatic treatments as described below.

**Immunoprecipitation of PAP**—The final PAP preparation or crude detergent extracts of the membranes (10) containing approximately 1 milliunit of PAP activity were incubated at 4 °C with varying amounts of the antipeptide antibody. The incubation was done at 100 μl of buffer A containing bovine serum albumin (1 mg/ml) and 0.1-1.0% Triton X-100. The different concentrations of Triton X-100 did not affect the immunoprecipitation. The mixture was subsequently added with 10 μl of 10% (w/v) suspension of S. aureus in buffer A containing 0.1% Triton X-100, and was further incubated for 1 h. The mixture was then centrifuged at 13,000 rpm for 30 min at 4 °C and the resultant supernatant was collected. After being washed twice with 500 μl of buffer A containing 0.1% Triton X-100, finally suspended in 80 μl of the same buffer. The aliquots of both supernatant and suspended cells were assayed for the PAP activity. In these experiments, the preimmune IgG or the antibody preincubated with the antigen peptide served as control.

**Subfractionation of Porcine Thymus Membranes**—The thymus membranes were subfractionated following the method of Owens et al. (22). In brief, the fresh thymus (10 g) was minced and homogenized using a Dounce homogenizer (10 strokes with a type B pestle) with 40 ml of the

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2 S-I. Imai and H. Kanoh, unpublished results.
lysis buffer containing 10 mM Hapes (pH 7.2), 0.15 mM NaCl, 1 mM PMSF, and 10 μg/ml each of leupeptin and pepstatin. The homogenate was centrifuged first at 4000 × g for 15 min, and the postmitochondrial membranes were subsequently obtained by further centrifuging at 100,000 × g for 1 h. The membrane pellet was treated with a hypotonic wash and suspended in 2 ml of the lysis buffer containing 65% (w/v) sucrose. The membrane suspension was layered on top of 75% sucrose and a sucrose gradient (45–25%) was further overlaid in a centrifuge tube. After centrifugation at 32,000 rpm for 17 h in a Hitachi RPS40T rotor, 31 fractions (~0.4 ml) were collected from the bottom of the tube.

After assaying the marker enzyme activities and protein contents, the fractionated membranes were recovered by centrifugation, and the membrane-associated PAP was solubilized by buffer A containing 1% each of octylglucoside and Triton X-100 (10).

Enzymatic Treatments of PAP—To obtain a 125I-labeled PAP preparation (0.6 μg of protein) was immunoprecipitated with 7 μg of the antibody and 20 μl of 10% S. aureus suspension. The immunoprecipitates were suspended in 20 μl of Laemmli sample buffer (18) and boiled for 2 min. The boiled suspensions were subsequently diluted 10-fold with 50 mM sodium phosphate buffer (pH 7.5) containing 1% Triton X-100 and with 100 mM citrate buffer (pH 5.5) containing 1% Triton X-100 for the treatments with N-glycanase (10 units) and endoglucosidase H (1 milliunit), respectively. The mixture was then added to protease inhibitors (1 mM PMSF and 10 μg/ml of pepstatin) and incubated for 16 at 37°C. The reaction was stopped by boiling in the SDS-sample buffer. The radiolabeled protein separated by an SDS-12.5% PAGE were visualized by a BAS2000 image analyzer (Fuji).

Amplification of Mouse cDNA Encoding PAP—Since we found that the N-terminal sequence of the 35-kDa PAP is highly conserved as the internal sequence encoded by the mouse hPAP clone (Ref. 14; see “Results”), we designed four primers for PCR amplification of the hPAP clone. The published hPAP sequence: 5'-GGACGGACGACATGGAGAGAGC-3' (nucleotides 68–91 in Gen) at the 3'-amplimer. The 5'-amplimer. The reaction mixture (20 μl) contained the total RNA (20 μg), PP4 primer (20 pmol), and 200 units of Superscript II (Life Technologies, Inc.) and was incubated at 50°C for 1 h. The synthesized first strand DNA was recovered by ethanol precipitation, and one-tenth of the resulting DNA preparation was used as a template for the subsequent PCR amplification. The amplification mixture (100 μl) contained Expand High Fidelity PCR System (Boehringer Mannheim) and 50 pmol each of the PP1 and PP3 primers. Amplification reactions were done on a GeneAmp PCR system 2400 (Perkin-Elmer Corp.) at 94°C for 20 s, 60°C for 30 s, and 65°C for 2 min for 40 cycles. A 1212-base pair fragment amplified (designated mPAP) was gel-purified, treated with T4 polynucleotide kinase, and subcloned into pbLUEscript II SK+ (Stratagene) at the Hind III site. Purified plasmid DNA was linearized with the dideoxynucleotide sequencing chain termination method (23) using a Sequenase 2.0 kit (U.S. Biochemical Corp.). Sequencing of multiple clones gave the same result. Sequences were analyzed and aligned with related sequences as described before (24).

Expression of mPAP cDNA in 293 Cells—In order to obtain a cDNA with a shorter 5'-noncoding sequence suitable for its expression, mPAP was amplified from the mouse kidney RNA by PCR as described above except for the use of PP2 instead of PP1 as the 5'-amplimer. The resultant mPAP (961 base pairs) containing the entire open reading frame was subcloned in pbLUEscript in correct orientation, digested with KpnI-XbaI, and subcloned into the expression plasmid, pREP9 (Invitrogen) at the KpnI-NheI site. The mPAP clone (2 μg/35-mm dish) was then transfected into 293 cells by calcium phosphate precipitation (11). After 3 days, the cells were suspended in 0.5 ml of lysis buffer containing 5 mM Hapes-NaOH (pH 7.2), 5 μg/ml each of leupeptin and pepstatin, and 0.2 mM PMSF. The cells were homogenized by passing 5 times through a 30-gauge needle and were separated to soluble and membrane fractions by centrifugation at 250,000 × g for 30 min in a TLA100 rotor. Both soluble and membrane fractions were assayed for the PAP activities. In some experiments, the membranes were suspended in buffer A, solubilized by adding 1% each of Triton X-100 and octylglucoside, and subjected to immunoprecipitation with the antipeptide antibody as described above. In some experiments, 293 cells after 2.5 days of mPAP transfection were labeled for 12 h with [35S]methionine (50 μCi/ml) in a methionine-poor medium containing 10% of the normal concentration. The cells were then solubilized by sonication in buffer A containing 1% each of Triton X-100 and octylglucoside, and the labeled PAP immunoprecipitated by the antipeptide antibody was analyzed by SDS-PAGE.

Identification of 35-kDa PAP from Porcine Thymus Membranes—We previously purified the membrane-bound PAP and described an apparent purification of an 83-kDa enzyme from porcine thymus (10). The 83-kDa protein was eluted in different column chromatographies always in parallel to the PAP activity. We estimated that the 83-kDa protein accounted for 85–90% of the purified protein when examined with Coomassie Blue-stained SDS-PAGE gels (10). In our successive experiments, the purification protocol described reproducibly resulted in a similar PAP preparation having specific enzyme activity ranging from 14 to 18 units/mg of protein. However, we noted in repeated experiments that all of the several minor proteins in the final PAP preparation behaved very similarly to the predominant 83-kDa protein when the enzyme was further fractionated by a variety of procedures including gel filtration and affinity chromatographies using several immobilized lectins (not shown). Furthermore, the cDNA cloned for the 83-kDa protein failed to express PAP activity in the cDNA transfection experiments using COS-7 cells.2 We thus felt it necessary to reconfirm the identity of the porcine thymus PAP. We first applied the purified PAP to a nondenaturing PAGE and examined the proteins extracted from the native gel slices by assaying the PAP activity and by silver-staining the proteins separated by the subsequent SDS-PAGE (not shown). When the intensities of the stained proteins and the enzyme activities recovered from the native gel pieces were compared, we noted that the PAP activity was best accounted for by a minor broad band at 35 kDa but not by others, including the predominant 83-kDa protein (not shown).

Despite our efforts we could not separate the 35-kDa candidate protein from other proteins by a variety of chromatographies. We therefore electroblotted the proteins onto Immobilon-P membranes and directly applied the membrane pieces...
containing the 35-kDa band to a protein sequencer. We obtained the N-terminal sequence of MFDKTRLPYVALDVLXVLLAGLPFA. We also obtained an internal sequence of EEDSHTTLH from a fragment of 35-kDa protein digested in gel with lysylendopeptidase. At this stage of the investigation, we could exclude the possibility that the 35-kDa protein was a proteolytic product of the 83-kDa protein, since the amino acid sequencededuced for the 83-kDa protein did not contain any of the determined sequences.  

In order to investigate whether the 35-kDa protein represented PAP, we next synthesized a peptide corresponding to the first 15 N-terminal residues (MFDKTRLPYVALDVL) and obtained antipeptide antibody. As shown in Fig. 1A, the affinity-purified antipeptide antibody could precipitate more than 95% of the purified PAP activity. In this case, the precipitated activity was almost quantitatively recovered by directly assaying the suspended immune complex. In this experiment we confirmed that no precipitation of the enzyme activity occurred when tested with preimmune IgG, the antibody preincubated with the antigen peptide, or with IgG passed through the peptide column used for the affinity purification of the antibody (not shown). The almost complete immunoprecipitation strongly suggested that the purified PAP activity was exclusively accounted for by the 35-kDa protein. On the other hand, the antipeptide antibody precipitated only 60% of crude PAP activity detergent-extracted from the thymus membranes (Fig. 1B). The reason for this partial immunoprecipitation remains unknown and needs to be studied further. However, the results may suggest the presence in the membranes of a PAP isoform(s) immunologically distinct from the 35-kDa enzyme.

The antipeptide antibody failed to react with the denatured enzyme, thus being inapplicable to Western blotting analysis.

The putative N-glycosylation sites are circled. The amino acid sequences determined for the porcine 35-kDa PAP are double underlined, where identical amino acids are in boldface type. The putative N-glycosylation sites are circled.

Fig. 2. Immunoprecipitation of 125I-labeled PAP preparation with antipeptide antibody. The 125I-labeled enzyme preparation (lane 1; 0.6 μg of protein) was treated with 7 μg each of preimmune IgG (lane 2), the antibody preincubated with the antigen peptide (lane 3), and the antibody (lane 4). The immune complex was precipitated with S. aureus, and the immunoprecipitated proteins were subjected to SDS-12.5% PAGE followed by detection with Fuji BAS 2000. In some experiments the immunoprecipitates were treated before SDS-PAGE with N-glycanase (lane 5) or endoglucosidase H (lane 6). Molecular mass standards indicated in kDa are bovine serum albumin, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, and soybean trypsin inhibitor.

Fig. 3. Subfractionation of porcine thymus membranes by a sucrose density gradient centrifugation. Porcine thymus membranes were subfractionated as described under “Experimental Procedures,” and 31 fractions (-0.4 ml) were collected from the bottom of the tube as indicated in the figure. A, the membrane subfractions were assayed for protein (Ç—Ç), and the activities of esterase (E—E) and alkaline phosphodiesterase (●—●). B, the membrane subfractions were recovered by recentrifugation and solubilized with 1% each of Triton X-100 and octylglucoside. The detergent extracts were assayed for total (Ç—Ç) and the immunoprecipitable PAP activities (●—●) using the antipeptide antibody as described under “Experimental Procedures.”

Fig. 4. Nucleotide sequence and deduced amino acid sequence of mPAP encoding mouse PAP. The mPAP cDNA was obtained by PCR amplification of mouse kidney mRNA using amplimers (PP1–PP3, indicated by arrows) synthesized according to the DNA sequence of the hic53 clone (14). Nucleotides and amino acids are numbered at the right. An in-frame stop codon in the 5′-untranslated sequence is boxed. The amino acid sequences determined for the porcine 35-kDa PAP are double underlined, where identical amino acids are in boldface type. The putative N-glycosylation sites are circled.
In order to confirm that the antibody solely reacted with the 35-kDa protein, the 125I-labeled PAP preparation was treated with the antibody, and the precipitated proteins were analyzed by SDS-PAGE (Fig. 2). In this experiment, the immune complex was treated prior to SDS-PAGE with N-glycanase and endoglucosidase H to see whether the PAP protein was glycosylated. The results shown in Fig. 2 clearly demonstrated that the antibody precipitated a single 35-kDa protein, which migrated as a 29-kDa band when treated with N-glycanase. In contrast, the 35-kDa protein was resistant to endoglucosidase H, suggesting that the N-linked sugars are complex type (27).

The antibody showed no cross-reactivity with the predominant 83-kDa protein. Taken together with the immunoprecipitation data given in Fig. 1, these results show that the purified PAP activity is exclusively due to the 35-kDa glycosylated protein, although it remains to be seen whether the native PAP functions as a monomer or a homooligomer. It is interesting to note that the 51–53-kDa PAP from rat liver plasma membranes was also converted to a 28-kDa form when treated with N-glycanase (12), suggesting that the rat and porcine PAPs share a similar structure.

As reported previously (10), the properties of the purified PAP, such as independence of Mg2+, activation by Triton X-100, and insensitivity to thioreactive reagents, fulfilled the criteria proposed as the characteristics of the type 2, plasma membrane-bound PAP isoform (6, 7). The results of the endogluconsidase H treatment of the 35-kDa enzyme (Fig. 2) also suggested the post-Golgi localization of this protein (27). We therefore tried to confirm that the presently identified 35-kDa PAP was indeed localized in the plasma membranes. For this purpose, we subfractionated the postmitochondrial membranes from porcine thymus by a sucrose density centrifugation. As shown in Fig. 3A, the activity of esterase, the marker for the endoplasmic reticulum, formed a peak with protein near the bottom of the tube. On the other hand, the activity of the plasma membrane marker, alkaline phosphodiesterase I, gradually increased from the middle of the tube, reaching a plateau at the top of the gradient. The two major membrane subfractions were thus clearly separated from each other. We extracted the membrane-bound PAP and found that the total as well as the immunoprecipitable PAP activities closely followed that of the plasma membrane marker enzyme (Fig. 3B). In agreement with the results given in Fig. 1, the antibody precipitated only from 50 to 65% of total PAP activity solubilized from the subfractionated membranes. We detected very little PAP activity in the fractions of the endoplasmic reticulum. The findings confirmed that the immunoreactive and most of the extracted enzyme activities are due to plasma membrane-bound PAP isoforms. It is known that the endoplasmic reticulum contains the type 1 PAP isoform (6, 7). It seems therefore likely that the present methods of the enzyme assay and solubilization are not suitable for the PAP1 activity associated with the endoplasmic reticulum.

Amplification and Expression of the Mouse cDNA Encoding 35-kDa PAP—The reevaluation of enzyme purification using the anti-peptide antibody showed that the minor 35-kDa protein, the 125I-labeled PAP preparation was treated with the antibody, and the precipitated proteins were analyzed by SDS-PAGE (Fig. 2). In this experiment, the immune complex was treated prior to SDS-PAGE with N-glycanase and endogluconsidase H to see whether the PAP protein was glycosylated. The results shown in Fig. 2 clearly demonstrated that the antibody precipitated a single 35-kDa protein, which migrated as a 29-kDa band when treated with N-glycanase. In contrast, the 35-kDa protein was resistant to endogluconsidase H, suggesting that the N-linked sugars are complex type (27). The antibody showed no cross-reactivity with endogluconsidase H, indicating that the N-linked sugars are complex type (27). The antibody showed no cross-reactivity with endogluconsidase H, suggesting that the N-linked sugars are complex type (27). The antibody showed no cross-reactivity with endogluconsidase H, suggesting that the N-linked sugars are complex type (27). The antibody showed no cross-reactivity with endogluconsidase H, suggesting that the N-linked sugars are complex type (27).
the 35-kDa enzyme. Despite these discrepancies we amplified
the mouse hic53 clone using amplimers synthesized according
to the published cDNA sequence (14). In the reverse tran-
scriptase-PCR amplification we used total RNA from the mouse
kidney, which was reported to contain hic53 mRNA most abun-
dantly (14). We obtained a single 1212-base pair amplification
product designated mPAP (Fig. 4), which was found to contain
an open reading frame encoding a novel protein of 283 amino
acid residues with a calculated Mr of 31,894. Different from the
hic53 clone, the methionine we identified as the N-terminal
residue of the porcine enzyme should serve as a translational
initiator, since this is encoded by the first ATG sequence fol-
lowing an in-frame stop codon, TGA, which we detected at
nucleotides 74–76 in the 5’-flanking sequence (Fig. 4). It was
also noted that the nucleotide sequence around this initiation
codon completely fulfilled the criteria for eucaryotic initiation
sites (28). Furthermore, as shown in Fig. 5, the mPAP done
encoded at its C terminus an additional 64 residues that were
lacking in the hic53 clone. We noted that the C-terminally
extended portion of mPAP contained the internal sequence of
EEDP(S for the porcine enzyme)HTTLH already determined
for the porcine 35-kDa enzyme. It is thus confirmed that mPAP
codes the mouse homolog of 35-kDa PAP. Despite these
differences, the proteins encoded by mPAP and hic53 are
highly similar to each other as summarized in Fig. 5, sharing
overall 73.5% identical sequence. Furthermore, the total DNA
sequence of mPAP is 95% identical to that of hic53 clone.
Indeed, the Northern blotting analysis (not shown) of several
mouse tissues gave approximately 2-kilobase mRNA expressed
very similarly as described for hic53 (14). At the present stage
we do not know why mPAP encodes a novel protein despite its
high sequence similarity to hic53. However, the PCR amplifi-
cation of the hic53 clone unexpectedly resulted in the identifi-
cation of a novel cDNA encoding the mouse homolog of 35-kDa
PAP, as judged from the conserved amino acid sequences.

In a search of the GenBank™ Data Base, the mouse PAP did
not show significant similarities to the peptide sequences of
known functions. However, we noted that the sequences of gene
products of Caenorhabditis elegans T28D9.3p (NCBI accession
number 861267) and Saccharomyces cerevisiae D9719.9p
(NCBI accession number 927773) share 31.4 and 22.6% iden-
tical sequences, respectively, with that of mouse PAP. The
significance of these similarities remains unclear at present.
In the sequence encoded by mPAP we detected two potential N-
glycosylation sites at Asn142 and Asn276 (Fig. 4). The hydropa-
thy plot of the encoded sequence (Fig. 6) showed that the mouse
PAP is a highly hydrophobic protein containing six hydrophobic

FIG. 7. Transient expression of mPAP in 293 cells. A, 293 cells
were transfected with 2 μg/35-mm dish of mPAP subcloned into pREP9
or pREP9 vector alone. After 3 days, the soluble and membrane frac-
tions were prepared and subjected to the PAP activity assay as de-
scribed under “Experimental Procedures.” The results are means ±
S.D. of three repeated experiments. B, the membrane fractions obtained
from cells after 3 days of transfection with mPAP (●—●) and vector
alone (○—○) were solubilized with 1% each of Triton X-100 and
octylglucoside. The solubilized PAP (3 μg of protein) was subjected to
immunoprecipitation with varying amounts of antipeptide antibody as
described for Fig. 1. The immunoprecipitates were assayed for PAP
activity as described under “Experimental Procedures.” C, 293 cells
after 2.5 days of transfection with mPAP (lanes 2 and 3) or vector alone
(lane 1) were labeled with [35S]methionine for 12 h as described under
“Experimental Procedures.” The labeled cells from 35-mm dishes were
lysed with 500 μl of lysis buffer containing 1% each of Triton X-100 and
octylglucoside, and the aliquots (100 μl) of cell lysates were treated with
5 μg of the antipeptide antibody. The immunoprecipitates were sepa-
rated by SDS-12.5% PAGE, and the labeled proteins were detected
using a BAS2000 analyzer. In some experiments (lane 2), the mPAP-
transfected cells were pretreated with tunicamycin (10 μg/ml) for 3 h
before labeling, and the steady state labeling was done in the presence
of the same amount of tunicamycin. The arrowheads indicate the trans-
lational products of mPAP.
clusters consisting of 17–23 residues. The presence of several putative membrane-spanning domains in the encoded protein can account for the fact that the type 2 PAP is tightly bound to plasma membranes, being solubilized only in the presence of detergents like octylglucoside (10, 11), Triton X-100 (12), and cholate (13). However, further work is required to define the exact membrane-spanning domains of this enzyme molecule.

In order to confirm that the translational product of the mPAP clone indeed possesses PAP activity, the cDNA was subcloned into the pREP9 expression vector and transfected into 293 cells, a human embryonic kidney cell line. The pREP9 expression vector alone was also transfected as a control. As shown in Fig. 7A, the PAP activity measured with the membranes from the mPAP-transfected cells was more than 10-fold greater than that measured in cells transfected with the vector alone, whereas there was no corresponding increase of the soluble PAP activity. Furthermore, immunoprecipitation with the antipeptide antibody of the PAP activity solubilized from the membranes of mPAP-transfected and control cells (Fig. 7B) showed that the mPAP transfection resulted in a 25-fold increase of immunoprecipitable enzyme activity. It was thus finally demonstrated that mPAP encodes a catalytically active PAP molecule.

The analysis of the porcine 35-kDa PAP already demonstrated that the enzyme was N-glycosylated and that the deglycosylated enzyme had a molecular mass of 29 kDa (Fig. 2). In order to investigate the mode of posttranslational modification of the mouse PAP, the mPAP-transfected cells were labeled with [35S]methionine, and the expressed PAP was analyzed by immunoprecipitation. The SDS-PAGE analysis of the immunoprecipitates (Fig. 7C) demonstrated that in the presence of tunicamycin, an inhibitor of protein N-glycosylation, the translational product had a molecular mass of 30 kDa, consistent with the calculated molecular mass of the mouse PAP (Fig. 4). The PAP molecule was precipitated as a 35-kDa protein in cells cultured without tunicamycin. There were several radioactive bands precipitated in common with mPAP-transfected and control cells, the significance of which remains unknown. However, in view of the data obtained for the porcine enzyme treated with N-glycanase (Fig. 2), these data showed that the pig and mouse PAPs are very similar to each other with respect to the molecular size and the mode of N-glycosylation.

**DISCUSSION**

In the present work we first identified the 35-kDa porcine PAP using antipeptide antibody raised against the N-terminal sequence. The analysis of partial amino acid sequences of the porcine enzyme led us to note the highly conserved sequence encoded by the mouse hic53 cDNA clone (14). The PCR amplification of the hic53 cDNA unexpectedly resulted in the identification of the cDNA encoding the mouse homolog of the porcine 35-kDa PAP. Initially we predicted that the mouse PAP might be a splicing isoform of the hic53 gene product, since the N-terminal sequence of the porcine enzyme was conserved as the internal sequence encoded by hic53. However, we found that the PCR-amplified mPAP contained, different from hic53, an open reading frame encoding the mouse 35-kDa PAP with the N-terminal sequence highly similar to that of the porcine counterpart. At the present stage we do not know whether hic53 and mPAP are highly similar but distinct genes. The possibility remains that mPAP is derived from hic53 by an alternative splicing. It is also possible that some artifactual sequencing of the hic53 cDNA might have resulted in the discrepant reading frame. The antibody reactive with the C-terminal portion of the mouse PAP, which is lacking in the hic53 product, would be helpful to clarify the structural and functional relationships between the two gene products. It would be interesting to see if the expression of PAP is also regulated by H2O2 and ras transformation as has been observed for hic53 (14), although it is difficult to assume at present the role of PAP in mediating the growth factor-like action of H2O2. It would be interesting to investigate the level of diacylglycerol and the activation of protein kinase C in cells exposed to H2O2. Interestingly, the fact that the expression of hic53 is decreased upon ras transformation of mouse osteoblastic cells (14) is consistent with the observation that the specific activity of type 2 PAP was decreased in ras transformed fibroblasts (9). The close similarity between the mPAP and hic53 genes, irrespective of their identity, suggests a novel function of PAP in the regulation of cell growth and differentiation.

In the cDNA expression experiments, the mPAP transfection elicited 10- and 25-fold increases of the membrane-bound PAP activities when determined as the total and immunoprecipitable activities, respectively (Fig. 7). The expressed enzyme protein was found to be very similar to the porcine 35-kDa enzyme with respect to the molecular size and the extent of N-glycosylation. The results demonstrated that the 35-kDa glycosylated protein alone can exert the catalytic activity. Furthermore, the immunoprecipitation of the porcine enzyme preparation (Fig. 2) and the expressed mouse enzyme (Fig. 7) confirmed that the PAP activity can be accounted for by a single 35-kDa protein. However, we do not know whether the native type 2 PAP functions as a monomer or as a homooligomer. So far it has been repeatedly recognized that the native type 2 PAP exhibits a very large molecular mass. For example, the porcine 35-kDa enzyme appeared in gel filtration as a 218-kDa protein (10). Similarly, a large molecular size of type 2 PAP was noted for rat liver enzyme (11). Although the presence of detergents in the enzyme preparation prohibited exact data interpretation, the recent work proposed that the rat type 2 PAP would contain a hexameric subunit structure (13) based on the results of gel filtration and glycerol gradient centrifugation of the solubilized enzyme. These results suggest that the porcine and mouse PAPs presently characterized may function as oligomers consisting of the 35-kDa subunit.

Although we succeeded in identifying the cDNA encoding the mouse PAP, there are a number of problems to be addressed in future studies. We already noted that the antipeptide antibody precipitated only partially the PAP activity solubilized from the porcine thymus membranes (Figs. 1 and 3). It is therefore possible that the type 2 PAP consists of several isoforms structurally distinct from the 35-kDa protein. The mouse PAP was found to be a highly hydrophobic protein, the sequence of which did not show significant similarities to those of a number of phosphatases deposited in the data banks. Further work is therefore needed to define the catalytic and regulatory domains of this enzyme. The type 2 PAP has attracted attention because of its putative role in the regulation of cellular signal transduction mediated by phospholipase D (3, 6, 7). The structural knowledge obtained for the first time on the mouse PAP should contribute to further understanding the molecular mechanisms of signal transduction mediated through the lipid second messengers.

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