Purification and characterization of *N*-ethylmaleimide-insensitive phosphatidic acid phosphohydrolase (PAP2) from rat liver

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N-Ethylmaleimide-insensitive phosphatidic acid phosphohydrolase (PAP; EC 3.1.3.4) was purified 5900-fold from rat liver. The enzyme was solubilized from membranes with octylglucoside, fractionated with $(\text{NH}_4)_2\text{SO}_4$, and purified in the presence of Triton X-100 by chromatography on Sephacryl S300, hydroxyapatite, heparin–Sephacryl and Affi-Gel Blue. Silver-stained SDS/PAGE indicated that the enzyme was an 83 kDa polypeptide. Sephacryl S-300 gel filtration also produced a second peak of enzyme activity, which was eluted from all of the chromatography columns at a different position from the purified

enzyme. SDS/PAGE indicated that it contained three polypeptides (83 kDa, 54 kDa and 34 kDa), and gel filtration suggested that it was not an aggregate of the purified enzyme. Both forms were sensitive to inhibition by amphiphilic amines, Mn^{2+} and Zn^{2+} , but not by *N*-ethylmaleimide. Purified PAP required detergent for activity, but was not activated by Mg^{2+} , fatty acids or phospholipids. The enzyme was able to dephosphorylate lysophosphatidic acid or phosphatidic acid, and was inhibited by diacylglycerol and monoacylglycerol. No evidence was obtained for regulation of PAP by reversible phosphorylation.

INTRODUCTION

Phosphatidic acid (PA) phosphohydrolase (PAP; EC 3.1.3.4) catalyses the dephosphorylation of PA, to form diacylglycerol. PAP was first identified as an enzyme involved in the glycerolipid-biosynthetic pathway [1], and is now believed to catalyse a regulatory step in the biosynthesis of triacylglycerol, phosphatidylcholine and phosphatidylethanolamine, by controlling the supply of diacylglycerol [2–4]. PAP can be regulated chronically, by hormonal action mediated at the level of enzyme synthesis and breakdown [5], and acutely, by translocation from the cytosol to the endoplasmic reticulum [3], where it is thought to be metabolically active. Translocation on to the endoplasmic reticulum is induced by fatty acids and their CoA esters [3,6] whereas the enzyme can be displaced from the membranes *in vitro* by albumin and by the amphiphilic amine chlorpromazine [3], suggesting that this is a reversible mechanism. This regulatory mechanism has been suggested as a way in which the cell can co-ordinate the rate of esterification with fatty acid supply [3].

More recently, PAP has been shown to play an important role in the phospholipase D signalling pathway [7,8] by dephosphorylating the PA formed by cleavage of phosphatidylcholine. PA, and its derivative lysoPA, act as mitogens on fibroblast and non-fibroblast cell lines, the effect involving stimulation of polyphosphoinositide hydrolysis, activation of protein kinase C, inhibition of adenylate cyclase and stimulation of DNA synthesis [9–11]. Moreover, PA can also directly modulate the activity of several key functional proteins *in vitro*, including protein kinase C [12], GTPase-activating protein [13], phosphatidylinositol-4-phosphate kinase [14], and a novel phospholipid-dependent protein kinase [15]. The reaction product, diacylglycerol, has a major role as an activator of protein kinase C [16]. Therefore, by controlling the relative levels of these two second messengers, PAP could play a pivotal role in cell signalling.

The two functions of PAP appear to be performed by two different enzymes. In rat liver, there is a form of PAP located on the plasma membrane and considered to be involved in cell

signalling, whereas a second form of PAP, present on the microsomes and cytosol, is believed to be involved in glycerolipid synthesis [17,18]. These two enzymes differ in their size and charge [18], have different Mg^{2+} requirements, and can be readily distinguished in cell extracts by a variety of inhibitors, including *N*-ethylmaleimide (NEM) [17]. PAP1, in the cytosol and microsomes, is completely inhibited by NEM, whereas the plasma-membrane enzyme (PAP2) is totally insensitive to this inhibitor. Interestingly, whereas the NEM-insensitive PAP2 is thought to play a signalling role in liver fibrogenesis [19], recent data have correlated the levels of PAP1 to the pathogenesis of fatty liver in humans [20].

Despite the importance of PAP, little information exists on the enzyme polypeptides, and their regulation. PAP1 has not been successfully purified, whereas NEM-insensitive PAP2 has only recently been purified from one tissue (pig thymus), to date [21]. The aim of the work reported here was to purify and characterize the NEM-insensitive form of PAP from rat liver, in order to aid understanding of its role in the phospholipase D signalling pathway, and to clarify the relationship between the thymus and liver enzymes.

EXPERIMENTAL

Materials

Chemicals

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was purchased from ICN Biochemicals. Ecospint A was from National Diagnostics. Leupeptin, pepstatin A and antipain were from the Peptide Institute (Osaka, Japan). Diacylglycerol kinase and octylglucoside were from Calbiochem. All other detergents, soya-bean trypsin inhibitor, NEM, lipids, amphiphilic amines and high-molecular-mass markers were from Sigma. Hydroxyapatite (Bio-Gel HTP) and Affi-Gel Blue F3GA were from Bio-Rad. Sephacryl S-300, heparin–Sephacryl and prepacked Superose 6 and 12 gel-filtration columns were from Pharmacia LKB Biotechnology.

Animals

Wistar rats (170–200 g) were obtained from Newcastle University Comparative Biology Centre. They were fasted for 12 h before being killed by cervical dislocation, after which rat livers were immediately removed, frozen, and stored at -20°C , until use.

Methods

Measurement of PAP activity

PAP activity was measured by monitoring the release of [^{32}P]P_i from [^{32}P]PA. [^{32}P]PA was prepared by the method of Walsh and Bell [22], and delivered as a uniform mixed micelle with Triton X-100, in a Tris/maleate buffer, pH 7.0 [18].

Fractions were incubated in the absence and presence of 4 mM NEM for 15 min, at 37°C , before assay. The NEM-insensitive activity was the activity measured after preincubation with NEM. The assay was carried out at 30°C , with 1 unit of PAP activity being defined as the amount of enzyme which catalyses the release of 1 nmol of P_i/min.

Purification of PAP2

All operations were carried out at 4°C unless otherwise stated.

(1) Preparation of membrane fraction. Rat livers (150 g) were thawed, and homogenized in 2 vol. of buffer A [25 mM Tris/HCl, pH 7.4, 10% (w/v) glycerol, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 0.2 mM EGTA, 1 mM benzamidine, and 5 $\mu\text{g}/\text{ml}$ each of pepstatin A, leupeptin, antipain and soya-bean trypsin inhibitor] for approx. 1 min in a MSE homogenizer. The homogenate was spun at 6000 *g* for 20 min, and the pellet discarded. The supernatant was re-centrifuged at 100 000 *g* for 1 h to collect the membranes, which were then salt-washed twice by resuspending in 10 mM Tris/HCl, pH 7.4, containing 1 mM EDTA and 3 M KCl, and re-centrifuging at 100 000 *g* for 1 h. The membrane fraction was then resuspended in buffer A at 5 mg/ml protein and stored at -80°C until use.

(2) Solubilization of PAP2. The membrane fraction was thawed; phenylmethanesulphonyl fluoride and octylglucoside were added to final concentrations of 1 mM and 1% (w/v) respectively, and the mixture was stirred gently for 1 h. The solution was centrifuged at 100 000 *g* for 1 h, and the solubilized PAP was recovered in the red-brown supernatant. The supernatant was then made 1% (w/v) with Triton X-100 before further purification.

(3) $(\text{NH}_4)_2\text{SO}_4$ precipitation. The solubilized PAP preparation was slowly made to 40% saturation with solid $(\text{NH}_4)_2\text{SO}_4$, and stirred for 45 min, before centrifugation at 10 000 *g* for 20 min. The floating pellet was discarded, and the supernatant adjusted to 70% saturation with $(\text{NH}_4)_2\text{SO}_4$. After stirring for 30 min, the solution was re-centrifuged at 10 000 *g* for 20 min, and the pellet collected and resuspended in approx. 6 ml of buffer B (buffer A containing 1% Triton X-100).

(4) Chromatography. The resuspended $(\text{NH}_4)_2\text{SO}_4$ pellet (40–70% satn.) was loaded on to a Sephacryl S-300 gel-filtration column (2.5 cm $\times$ 65 cm), equilibrated with buffer B. The column was run at 0.5 ml/min, in the same buffer, and 5 ml fractions were collected. Two peaks of PAP activity were detected; one in the void volume (termed PAP2A), and a second (PAP2B) at a V_0/V_0 of approx. 1.30 (see below). The active fractions from each peak were pooled separately for further purification.

The active fractions from the S-300 gel filtration were loaded on to a hydroxyapatite column (2.5 cm $\times$ 8 cm), equilibrated with buffer B. PAP2B passed through the column, where it was collected, and the active fractions were pooled, whereas PAP2A was eluted with 250 mM NaCl.

The enzyme from hydroxyapatite was loaded on to a heparin-Sepharose column (1.5 cm $\times$ 8 cm), equilibrated with buffer B. The column was washed with 45 ml of buffer B before the enzyme was eluted with 120 ml of a linear NaCl gradient (0.05–1 M) in buffer B, at 0.5 ml/min. The eluate was collected in 30 $\times$ 4 ml fractions. The active fractions were pooled for further chromatography.

Enzyme from heparin-Sepharose were loaded on to Affi-Gel Blue F3GA (0.5 cm $\times$ 4 cm), equilibrated with buffer B. The column was washed with 20 ml of buffer B before the enzyme was eluted with 20 ml of a linear NaCl gradient (0.05–3 M) at 0.5 ml/min; 1 ml fractions were collected and the active fractions pooled (10 ml total volume) and stored at -80°C .

Analytical techniques

Before analytical gel filtration, purified PAP was concentrated in a Centricon-30 concentrator (Amicon). The native molecular mass of the enzyme was estimated by gel filtration on Superose 12 and Superose 6, pre-equilibrated with buffer A containing 0.1% Triton X-100, in a Pharmacia FPLC system. A flow rate of 0.25 ml/min was used, and 0.5 ml fractions were collected.

After each chromatography step, samples of PAP were subjected to SDS/PAGE on 10% resolving gels, under reducing and non-reducing conditions. The protein samples were precipitated with chloroform/methanol, before electrophoresis, to remove Triton X-100 [23]. Proteins were detected by silver staining [24].

Protein concentrations were determined by the method of Bradford [25], with BSA as the standard.

RESULTS

Purification of PAP2

In rat liver, PAP1 and PAP2 activities are present in approximately equal quantities [26]. However, although most of the PAP1 is cytosolic, all of the NEM-insensitive PAP2 enzyme is membrane-associated [17,18]. Therefore, the first step in the purification was to prepare a crude membrane fraction, as described under 'Methods'. Treatment of the liver membranes with hypotonic buffer (10 mM Tris/HCl, pH 7.4), 1–10 mM EDTA, or 0–3 M NaCl, or KCl, failed to release the PAP2 (results not shown), suggesting that it is an integral membrane protein. The enzyme was, however, solubilized by treating membranes with detergents (Triton X-100, CHAPS, deoxycholate, octylglucoside). Although all of the detergents tested could solubilize the enzyme to some extent, the results indicated that the concentration, and type, of detergent used had an important influence on both the percentage of PAP solubilized and the specific activity of the solubilized enzyme (results not shown). Optimal solubilization of PAP2 was observed with 1% (w/v) octylglucoside. Surprisingly, approx. 110% of initial enzyme activity was recovered after solubilization, possibly because PAP2 is more accessible to the PA substrate when solubilized, or perhaps due to the removal of an inhibitory component present in the membrane.

Before further purification, 1% Triton X-100 was added to the enzyme solution. The enzyme was then subjected to the purification procedure described under 'Methods'. PAP2 was purified essentially as in [21], but with several modifications.

Gel filtration on Sephacryl S-300 gave two peaks of activity: PAP2A, containing approx. 35% of the enzyme activity, eluted in the void volume of the column; and PAP2B, containing the remainder of the activity, eluted with a V_0/V_0 of approx. 1.30

Figure 1 Elution profile of rat liver PAP2 from Sephacryl S-300

After $(\text{NH}_4)_2\text{SO}_4$ precipitation, PAP2 was loaded on a Sephacryl S-300 gel-filtration column ($2.5 \text{ cm} \times 65 \text{ cm}$) pre-equilibrated with buffer B and eluted at 0.5 ml/min in the same buffer; 5 ml fractions were collected, and assayed for PAP2 activity (●) and protein content (▲), as described in the Experimental section. The elution profile of PAP2 produced during a typical purification is shown. Two peaks of PAP activity were detected: one in the void volume (fractions 34/35, determined with Blue Dextran), which contained approx. 35% of the enzyme activity (termed PAP2A), and a second (PAP2B), which contained the remainder of the PAP2 activity, at a V_e/V_0 of approx. 1.30. The active fractions from each peak were pooled separately for further purification.

Table 1 Purification of PAP2A and PAP2B from rat liver

PAP2A and PAP2B were solubilized and purified from rat liver as described in the text. The results are the summary of a typical purification of PAP2. Peaks A and B are indicated.

Purification step	Enzyme activity (units)	Protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Homogenate	2170	1205.0	1.8	1.0	100.0
Membrane fraction	1850	411.0	4.5	2.5	85.2
Solubilized fraction	2080	264.0	7.9	4.4	95.8
$(\text{NH}_4)_2\text{SO}_4$ pellet	1248	63.4	19.7	10.9	57.5
S-300 gel filtration	A 382	8.42	45.4	25.2	17.6
	B 652	14.7	44.3	24.6	30.0
Hydroxylapatite	A 248	0.09	2722	1512	11.4
	B 220	0.11	1990	1106	10.1
Heparin–Sephacryl	A 186	0.04	4650	2583	8.6
	B 84	0.015	5598	3111	3.9
Affi-Gel Blue	A 140	0.03	4670	2594	6.5
	B 53	0.005	10660	5889	2.4
Q-Sepharose	B 40	0.004	10000	5556	1.8

(Figure 1). The two peaks were separately pooled for further purification on hydroxylapatite.

Under the conditions used, PAP2B passed straight through the hydroxylapatite column. Although there was a relatively low recovery of enzyme activity, a large purification was obtained, as most of the proteins bound to the column (Table 1). In contrast, PAP2A was retained by the hydroxylapatite, and was eluted by 250 mM NaCl, in buffer B. A high recovery of PAP2A was obtained from this step, with a good purification (Table 1).

The active PAP preparations were further purified on heparin–Sephacryl. PAP2B was eluted by 250 mM NaCl, whereas 650 mM NaCl was required to elute PAP2A. Although only a small purification was produced by this column (Table 1), it was essential for the production of pure PAP.

Figure 2 Elution profile and SDS/PAGE analysis of rat liver PAP2A from Affi-Gel Blue F3GA

After heparin–Sephacryl chromatography, PAP2A was loaded on an Affi-Gel Blue F3GA column ($0.5 \text{ cm} \times 4 \text{ cm}$), pre-equilibrated with buffer B, at 0.5 ml/min . The column was washed with 20 ml of buffer B, and the enzyme was eluted by a 20 ml linear NaCl gradient (---; 0.05 – 3 M), at 0.5 ml/min ; 1 ml fractions were collected and assayed for PAP2 activity (●). The elution profile of PAP2A produced during a typical purification is shown in (a). Samples ($50 \mu\text{l}$) of selected fractions were analysed by SDS/PAGE on a 10% resolving gel, and the proteins were detected by silver staining (b). The 57 kDa band observed in each lane of the polyacrylamide gel is due to keratin contamination [43].

The final purification step involved chromatography on Affi-Gel Blue F3GA. PAP2B was eluted from this column by 1.75 M NaCl, with a good yield of enzyme activity and a small increase in purity (Table 1). In contrast, PAP2A was eluted from Affi-Gel Blue F3GA at 2.25 M NaCl (Figure 2), with no net increase in purity (Table 1).

SDS/PAGE of PAP2B, followed by silver staining, showed that the major protein band present represented an 83 kDa subunit, under both reducing and non-reducing conditions (Figure 3). Moreover, this band was eluted with the PAP activity in the fractions from the Affi-Gel Blue column. Indeed, further chromatography on Q-Sepharose produced no net purification (Table 1), whereas the enzyme activity still co-eluted with the 83 kDa band (results not shown), strongly suggesting that it represented PAP2. A 5900 -fold purification of PAP2B was produced, with a 2.4% yield of enzyme activity (Table 1).

SDS/PAGE of PAP2A indicated that three polypeptides were present (83 kDa , 54 kDa and 34 kDa), all of which were eluted with the enzyme activity from the Affi-Gel Blue column (Figure 2). Further chromatography on Q-Sepharose, Superose 6 and Superose 12 columns failed to separate the three protein bands, which continued to be eluted with the PAP activity from each column. Although PAP2A was not purified to homogeneity, a 2600 -fold purification was obtained, with a 6.5% yield of enzyme activity (Table 1).

Figure 3 Analysis by SDS/PAGE of fractions from purification scheme

After each purification step, the pooled enzyme fractions from the PAP2B preparation were subjected to SDS/PAGE on a 10% acrylamide resolving gel, and the proteins detected by silver staining; 1.2 units of PAP activity were loaded per lane. Lane 1, S-300 gel filtration; lane 2, hydroxyapatite; lane 3, heparin-Sepharose; lane 4, Affinity-Blue; lane 5, Q-Sepharose. Molecular-mass standards are shown in the end lanes.

Figure 4 Determination of the molecular mass of native PAP2 by gel-filtration

Purified PAP2B, PAP2A and marker proteins (●) were analysed in Buffer A, containing 0.1% Triton X-100, on a Superose 6 gel-filtration column, as described in the text. Molecular-mass standards used were Blue Dextran (3000 kDa), thyroglobulin (669 kDa), apoferritin (443 kDa), β -amylase (200 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa) and carbonic anhydrase (29 kDa). The molecular mass of each protein was plotted against V_e/V_0 .

Characterization of PAP

Both forms of PAP2 were assayed under a variety of different conditions during characterization of the purified enzyme. All subsequent results, unless otherwise stated, were obtained for both forms.

Native PAP2B was eluted with an apparent molecular mass of 265 kDa from Superose 12 (results not shown) and Superose 6 columns (Figure 4). In contrast, native PAP2A was eluted from the Superose 12 column in the void (exclusion size 300 kDa; results not shown), and from the Superose 6 column with an apparent molecular mass of 290–300 kDa (Figure 4). Hence it appears unlikely that PAP2A is an aggregate of PAP2B, and suggests that there may be two isoenzymes of PAP2 in rat liver, with different native molecular masses.

The 83 kDa polypeptide of PAP2B was routinely observed as a somewhat broad band on SDS/PAGE (Figure 3). One possible cause of this is limited proteolysis, occurring during purification. In support of this was the observation of a rapid loss of activity

Figure 5 Effect of cations on the activity of PAP2

Purified PAP2B was diluted 20-fold in EDTA-free buffer B, and 0.5 ng (20 μ l) was assayed in the presence of various concentrations of MgCl₂, CaCl₂, MnCl₂, CoCl₂ and ZnCl₂. The results are expressed as a percentage of the control activity, and are averages $\pm$ S.E.M. of four independent experiments.

during and/or after purification on hydroxyapatite which could be largely, but not completely, prevented in the presence of a cocktail of protease inhibitors (Table 1). Moreover during storage, breakdown of the 83 kDa protein to smaller fragments was observed. Direct evidence for limited proteolysis came from sequence analysis. N-terminal sequence determination of PAP2B indicated the presence of two amino acid residues at each cycle, present in approximately equal amounts (result not shown). Inspection of the data indicated that this corresponded to a single sequence, in which the first three residues were deleted from approx. 50% of the polypeptide. The N-terminal sequence of PAP2B showed little similarity to that of any sequenced gene/protein in the PC Gene database (IntelliGenetics) or the Genbank/EMBL data bank, with the exception of a CD36-related class B scavenger receptor [27]. However, it is extremely unlikely that the 83 kDa polypeptide identified here as PAP2B is actually the scavenger receptor, since there are significant differences in their amino acid sequences. Furthermore, the scavenger receptor has a predicted molecular mass of 57 kDa, whereas the polypeptide studied here has a molecular mass of 83 kDa, as determined by SDS/PAGE.

Several enzymological properties of purified PAP2 from pig thymus have been reported previously [21]. The rat liver enzyme was similarly investigated. Purified rat liver PAP2 requires detergent for optimal activity (results not shown). Triton X-100 and deoxycholate (2–4 mM) both activate the enzyme approx. 10–12-fold. Higher concentrations (8–10 mM) of CHAPS activate the enzyme to the same extent. In contrast, 10 mM octylglucoside only activates the purified PAP2 approx. 5-fold. Complete removal of detergent from the purified enzyme, on a Q-Sepharose column, resulted in the total loss of PAP2 activity, suggesting that detergent is required for stability (results not shown).

Unlike rat liver PAP1 [3,17], and the PAP purified from yeast membranes [28,29], purified rat liver PAP2 does not require Mg²⁺ for optimal activity (Figure 5). However, Mn²⁺, Zn²⁺, and Co²⁺ all inhibit the NEM-insensitive enzyme, whereas Ca²⁺ has no effect (Figure 5). Purified PAP2 has a broad pH optimum

Figure 6 Effect of triacylglycerol, diacylglycerol, monoacylglycerol and lysoPA on PAP2 activity

Purified PAP2B (10 ng) was assayed against 0.5 mM [32 P]PA substrate in the presence of increasing concentrations of non-radioactive triacylglycerol, diacylglycerol, monoacylglycerol or lysoPA. The results are averages of four independent experiments, and are expressed as a percentage of the control activity $\pm$ S.E.M.

ranging from 5 to 9, with maximum activity at pH 6–7 (results not shown). This is in direct agreement with the results of Jamal et al. [17] for NEM-insensitive PAP in rat liver membranes. Purified PAP2 displayed saturation kinetics, when assayed in the presence of increasing amounts of substrate (result not shown). Lineweaver–Burk analysis gave a K_m of approx. 0.032 mM PA and a V_{max} of 12.6 μ mol/min per mg of protein for homogeneous PAP2B. PAP2 activity was also measured as a function of the surface concentration of PA, expressed as mol% of PA, in Triton X-100/PA mixed micelles. A hyperbolic curve was produced, suggesting that rat liver PAP obeys surface dilution kinetics. At a bulk concentration of 0.5 mM PA, a K_m value of 0.31 mol% was obtained (results not shown).

In order to determine the phospholipid requirements of PAP2, 0.001–0.5 mM phosphatidylcholine, phosphatidylinositol, phosphatidylserine or cardiolipin was added to the standard assay mixture, along with the substrate, and sonicated before use. None of the added phospholipids altered the PAP activity, at the concentrations used, suggesting that PAP2 does not have a phospholipid requirement (results not shown). Similarly, fatty acids (0.001–1 mM stearate, palmitate, oleate, linoleate and arachidonate) had no significant effect on the activity of the enzyme.

A number of substrate analogues were tested for their ability to modulate PAP2 activity. Glycerol 3-phosphate (0–5 mM; result not shown) or triacylglycerol (0–2 mM; Figure 6) had no effect on rat liver PAP2. Diacylglycerol, on the other hand, significantly inhibited the rat liver enzyme (Figure 6), suggesting it may act as a feedback inhibitor in the cell. Interestingly monoacylglycerol, and to a larger extent lysoPA, were both inhibitors of the liver PAP2 (Figure 6), suggesting that the enzyme may dephosphorylate lysoPA. Indeed, purified PAP2 dephosphorylates [32 P]lysoPA in a linear manner in both time- and protein-concentration-dependent assays, with the purified enzyme being only 50% more active against the dioleoyl-PA substrate than 1-oleoyl-lysoPA (results not shown).

In most PAP studies, the enzyme is assayed against either radiolabelled dipalmitoyl [17] or dioleoyl [18] PA. Purified

Table 2 Effect of acyl-chain length of PA on PAP2 activity

PAP2A (0.28 unit) and PAP2B (0.1 unit) were assayed under standard assay conditions against a range of PA substrates, containing different acyl chains. The results are averages of four independent experiments and are expressed as a percentage ($\pm$ S.E.M.) of the activity against dioleoyl (18:1, 18:1) PA.

PA substrate	Relative PAP2A activity (%)	Relative PAP2B activity (%)
Dihexanoyl (6:0, 6:0)	119.2 $\pm$ 8.3	615 $\pm$ 98.0
Didecanoyl (10:0, 10:0)	86.6 $\pm$ 1.0	82.1 $\pm$ 3.0
Dimyristoyl (14:0, 14:0)	67.1 $\pm$ 4.5	50.0 $\pm$ 10.5
Dipalmitoyl (16:0, 16:0)	117.1 $\pm$ 3.5	67.9 $\pm$ 7.1
Distearoyl (18:0, 18:0)	112.7 $\pm$ 3.8	114.3 $\pm$ 3.0
Dioleoyl (18:1, 18:1)	100 $\pm$ 2.1	100 $\pm$ 1.5
β -Arachidonyl- γ -stearoyl (18:1, 20:4)	52.3 $\pm$ 0.8	112 $\pm$ 2.6

Figure 7 Effect of amphiphilic amines on PAP2 activity

Purified PAP2B (5 ng) was assayed in the presence of various concentrations of sphingosine, chlorpromazine, desipramine or propranolol. The results are averages of four independent experiments, and are expressed as a percentage of the control activity $\pm$ S.E.M.

PAP2A and PAP2B were assayed against a range of PA substrates, to determine whether altering the length or saturation of the acyl chain affected PAP activity. PAP2A was active against all of the PA species tested (Table 2). The enzyme was found to be least active against intermediate-length saturated acyl groups, and arachidonate-containing PAs, which are thought to predominate in cell-signalling diacylglycerols. PAP2B was also active against all of the PA species tested (Table 2). Although this enzyme was relatively inactive against intermediate-length saturated acyl chains, it was found to be extremely active against C_6 acyl groups, and twice as active as PAP2A against arachidonate-containing PAs.

Amphiphilic amines have been described as potent inhibitors of PAP in a number of different cell types [17,21,26,30–36]. Homogeneous rat liver PAP2 is also inhibited by these molecules (Figure 7), suggesting that inhibition is through direct interaction with the enzyme itself, as suggested by the data of Kanoh et al.

[21]. Sphingosine was the most potent PAP2 inhibitor, with chlorpromazine and desipramine inhibiting to a lesser extent. Propranolol only partially inhibited the purified enzyme. It may be significant that the most potent inhibitor (sphingosine) is also the most hydrophobic. Interestingly, the cell-permeant ceramide *N*-acetylsphingosine had no effect on purified PAP2 (result not shown), suggesting that the increase in PAP2 activity observed by Gomez-Munoz et al. [37] is not by direct activation of the enzyme by the ceramide. The polyamines spermine, spermidine and putrescine had no effect on purified PAP2 in this assay system (results not shown). PAP2A and PAP2B were both completely insensitive to the cysteine-modifying reagent NEM, and were unaffected by 200 μ M tetrahydrolipstatin, eliminating the possibility that hepatic lipase was contributing to the enzyme activity [20]. NaF was able to inhibit PAP2 partially (40% inhibition at 10 mM NaF), whereas the arginine-modifying reagent phenylglyoxal had no effect (results not shown).

Five different protein kinases were tested for their ability to phosphorylate and regulate PAP2 [cyclic-AMP-dependent protein kinase, AMP-activated protein kinase, Ca^{2+} /calmodulin-dependent protein kinase II, protein kinase C, and MAP kinase (42 and 44 kDa isoenzymes)]. None of these kinases brought about any observable change in the activity of PAP2 (results not shown). Furthermore, incubation of purified PAP2 with kinase and [γ - ^{32}P]ATP, followed by SDS/PAGE and autoradiography, indicated that the 83 kDa PAP2 polypeptide (or any other minor contaminant) was not phosphorylated, by any kinase tested, whereas, under identical conditions, known protein substrates of each kinase were (results not shown).

DISCUSSION

Over the years, a number of unsuccessful attempts have been made to purify PAP from rat liver [38,39]. We report here the successful purification of the PAP2 isoenzyme from this tissue.

SDS/PAGE analysis of PAP2B indicates that an 83 kDa protein accounts for this activity (Figure 3). This is supported by the fact that the N-terminal sequence of PAP2B does not match that of any other protein purified and sequenced to date. Moreover, the observation that the 83 kDa polypeptide is co-eluted with the PAP activity during all of the chromatography steps used, and is present in both the PAP2A and PAP2B purification preparations, provides strong evidence that this polypeptide represents the rat liver PAP2. However, molecular cloning and expression studies will be necessary to confirm this.

The native enzyme exhibits an apparent molecular mass of 265 kDa on gel filtration in the presence of Triton X-100 (Figure 4). Assuming that each Triton X-100 micelle contains one protein molecule, and that each micelle is 90 kDa in size [40], then the native PAP would have a molecular mass of 175 kDa, and is likely to be a dimer. The second peak of PAP activity (PAP2A) detected during purification was eluted from all of the chromatography columns at a different position from PAP2B, and does not appear to be an aggregate of this enzyme. It is therefore conceivable that there are actually two isoenzymes of PAP2 in rat liver. SDS/PAGE analysis of PAP2A indicated the presence of three polypeptides (83 kDa, 54 kDa and 34 kDa), any one of which could account for the enzyme activity. It seems likely that the 83 kDa polypeptide catalyses the reaction, but further work is necessary to establish this, and whether the other polypeptides are proteolytic fragments, regulatory subunits, or tightly bound contaminants.

Characterization of the purified enzyme revealed a number of important properties of PAP2. First of all, purified PAP2 is totally inactivated by removal of detergent, and strongly activated

by increasing the detergent concentration in the assay, suggesting that it is probably an integral membrane protein. Moreover, although the purified enzyme is inhibited by Zn^{2+} and Mn^{2+} , it does not require Ca^{2+} or Mg^{2+} for activity (Figure 5), and is completely unaffected by NEM, confirming that it corresponds to the enzyme described previously in rat liver membranes [17].

Surprisingly, we could detect no phospholipid requirement for the purified PAP2, suggesting that it is not regulated in the same manner as protein kinase C [16] or diacylglycerol kinase [41]. This lack of lipid requirement could also indicate that detergent can effectively substitute for phospholipids during assay of PAP, or that the purified enzyme is actually tightly bound to phospholipid. Likewise, saturated and unsaturated fatty acids had no detectable effect on purified PAP2. This suggests that the oleate-stimulated increase in the conversion of phosphatidic acid into diacylglycerol, demonstrated by Siddiqui and Exton [42] in rat liver membranes, was probably due to an enhanced delivery of phosphatidic acid to the membrane-bound enzymes, and not to activation of PAP2.

Significantly, purified PAP2 is able to dephosphorylate both PA and lysoPA. This suggests that PAP2 may well have a role in destroying the signalling properties of lysoPA [11], as well as dephosphorylating PA. In this respect, it is notable that the reaction products, monoacylglycerol and diacylglycerol respectively, both inhibit the enzyme to a significant extent, suggesting that the enzyme may be regulated, at least in part, by feedback inhibition.

PAP2A and PAP2B were both active against all PA substrates tested (Table 2). However, the two forms of PAP2 gave different activity profiles against these substrates. PAP2A was least active against intermediate-length acyl chains and arachidonate-containing PAs. In contrast, whereas PAP2B was also least active against intermediate-length acyl chains, it was twice as active as PAP2A against arachidonate-containing PAs, and 5 times as active against C_6 acyl-chain PAs. Although the full significance of these results is not clear, the different activity profiles suggest that PAP2A and PAP2B represent different isoenzymes of PAP.

It has been suggested [35] that the natural amphiphilic amine sphingosine may modulate diacylglycerol levels by activating diacylglycerol kinase and inactivating PAP. Our results confirm that sphingosine is a potent inhibitor of the purified rat liver PAP2 (Figure 7), and may well have a role in modulating its activity.

In view of its likely role in signal transduction, the possibility of the regulation of PAP2 by phosphorylation was investigated. However, none of the five protein kinases tested was apparently able to phosphorylate, or regulate the activity of, purified PAP2. It has been suggested that NEM-insensitive PAP could play an important role in regulating the activity of the MAP kinase pathway by mediating the level of PA in the cell, so controlling the activity of the GTPase-activating protein and thereby the GTP:GDP ratio of ras [13]. Therefore, these results suggest that MAP kinase does not self-regulate by altering the cellular PA levels, through modulation of NEM-insensitive PAP activity. Our results also suggest that protein kinase C does not regulate diacylglycerol levels by directly phosphorylating NEM-insensitive PAP. It is possible, however, that protein kinase C could indirectly control diacylglycerol levels through sphingosine metabolism, as noted above [35,36]. Although the results presented here indicate that NEM-insensitive PAP activity is not regulated by any of the kinases tested, each of which has a relatively broad substrate specificity, it remains possible that its activity is controlled by a specific protein kinase.

The general properties of the rat liver NEM-insensitive PAP2 are very different from those of the purified yeast enzyme.

Whereas the yeast enzyme requires Mg^{2+} for activity, and is inhibited by NEM and triacylglycerol [28], PAP2 appears to be unaffected by these factors. Moreover, whereas the yeast enzyme is a 91 kDa polypeptide band as seen on SDS/PAGE [28], the rat liver enzyme activity is accounted for by an 83 kDa polypeptide. Indeed, the yeast enzyme appears more similar to PAP1, described in [17], than to the isoform characterized here.

The enzymes from rat liver and pig thymus share several key properties. Firstly, PAP2 from both tissues appears to be an 83 kDa band on SDS/PAGE, which is completely insensitive to NEM, and does not require Mg^{2+} for activity. Purified PAP2 from each tissue has a similar specific activity. The thymus enzyme has a specific activity of 15800 units/mg of protein assayed at 37 °C [21], whereas the rat liver enzyme has a specific activity of 10600 units/mg of protein when assayed at 30 °C (Table 1). Furthermore, homogeneous PAP2 was purified approx. 2300-fold from both rat liver and pig thymus membranes. Finally, both enzymes are inhibited by Zn^{2+} , Mn^{2+} and amphiphilic amines, are not activated by fatty acids or phospholipids, and have similar K_m values. There are, however, also a number of important apparent differences between the thymus and liver enzymes. First of all, the two proteins give a different apparent molecular mass on gel filtration. The reason for this is not known, but could be due to differences in oligomeric structure or co-purification with phospholipids. The enzymes also respond differently to Ca^{2+} , diacylglycerol and monoacylglycerol. A more crucial difference is that the liver enzyme has significant activity against lysoPA. Thus, although the liver and thymus enzymes have many similarities, there are a number of significant differences between them, which may have important consequences in their regulation. Further biochemical and molecular studies will be necessary to elucidate the molecular basis for these differences.

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