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Purification and Characterization of a Novel Plasma Membrane Phosphatidate Phosphohydrolase from Rat Liver*

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An *N*-ethylmaleimide-insensitive phosphatidate phosphohydrolase, which also hydrolyzes lysophosphatidate, was isolated from the plasma membranes of rat liver. The specific activity of an anionic form of the enzyme (53 kDa, pI < 4) was increased 2700-fold. A cationic form of the enzyme (51 kDa, pI = 9) was purified to homogeneity, but the -fold purification was low because the activity of the highly purified enzyme was unstable. Immunoprecipitating antibodies raised against the homogeneous protein confirmed the identity of the cationic protein as the phosphohydrolase and were used to identify the anionic enzyme. Both forms are integral membrane glycoproteins that were converted to 28-kDa proteins upon treatment with *N*-glycanase F. Treatment of the anionic form with neuraminidase allowed it to be purified in the same manner as the cationic enzyme and yielded an immunoreactive protein with a molecular mass identical to the cationic protein. Thus, the two ionic forms most likely represent different sialated states of the protein. An immunoreactive 51–53-kDa protein was detected in rat liver, heart, kidney, skeletal muscle, testis, and brain. Little immunoreactive 51–53-kDa protein was detected in rat thymus, spleen, adipose, or lung tissue. This work provides the tools for determining the regulation and function of the phosphatidate phosphohydrolase in signal transduction and cell activation.

Phosphatidate phosphohydrolase (PAP)[1] catalyzes the dephosphorylation of PA to DAG. Jamal *et al.* (1) characterized two distinct PAP activities in rat liver. One activity requires Mg^{2+} and is completely inhibited by NEM. In contrast, the other activity has no requirement for Mg^{2+} and is NEM-insensitive. The NEM-sensitive PAP translocates between the cytosol and membrane fractions in response to insulin, glucagon, cyclic AMP, and fatty acids (2). Its regulation and subcellular localization indicate that the NEM-sensitive PAP is primarily involved in synthesis of triacylglycerol and phospholipids *de novo*. The NEM-insensitive PAP is an integral plasma membrane protein whose role in cell metabolism is not well defined. Because of its cellular location (1), it may regulate the relative concentrations of PA and DAG in the plasma membrane (3, 4) and thus participate in signal transduction (5).

The prominent role of DAG as a second messenger became evident when it was identified as an important product formed by agonist-stimulated hydrolysis of phosphatidylinositol bisphosphate. DAG directly activates protein kinase C (6, 7), which phosphorylates numerous target proteins (8). Such effects are involved in the induction of DNA synthesis (9), oocyte maturation (10), and morphological changes in fibroblasts (11). After an initial increase in DAG (from hydrolysis of phosphoinositides), a second, larger increase in DAG mass is generated directly by the action of phospholipase C on phosphatidylcholine (12–14) or indirectly via the sequential actions of phospholipase D and PAP (15–18).

It is likely that the rapid accumulation of PA, which is observed after agonist stimulation, arises from phospholipase D-mediated hydrolysis of plasma membrane phosphatidylcholine (18–23) or via the sequential actions of phospholipase C and DAG kinase (5, 24). In addition to giving rise to DAG (from PAP activity), PA itself is a potent cellular activator. PA stimulates the respiratory burst in neutrophils by activating NADPH oxidase (25) independent of DAG (26, 27). PA also directly activates hepatic monoacylglycerol acyltransferase (28), phospholipase C-γ (29), and phosphatidylinositol-4-phosphate kinase (30). PA has potent mitogenic effects in Swiss 3T3 fibroblasts (31–33), rat fibroblasts (9), and human A431 cells (34). The mechanism of its mitogenic action is not well defined. PA may control intracellular Ca^{2+} levels (35), activate phosphatidate-dependent protein kinases (36), or activate $p21^{ras}$ by inhibiting Ras GTPase activating protein (37, 38) or Ras guanine nucleotide releasing factor (39). When added exogenously to cells, PA decreases adenylate cyclase activity (40) and activates phospholipase D (9, 41). PA may be a precursor for lyso-PA. Lyso-PA, which is released from activated platelets (42), is a potent mitogen for fibroblasts. Treatment of cells with lyso-PA activates tyrosine kinase activity and results in many of the same effects seen with PA (9, 33, 43–45).

The balance in the levels of membrane DAG and PA is important for the appropriate cellular response(s) by cells to extracellular signals. PAP appears to be involved in controlling this balance. For example, in *ras*-transformed fibroblasts, the specific activity of PAP is decreased relative to nontransformed

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[1] The abbreviations used are: PAP, phosphatidate phosphohydrolase; DAG, diacylglycerol; PA, phosphatidate; HAP, hydroxylapatite; QSFF, quaternaryammonium Sepharose fast flow; NEM, *N*-ethylmaleimide; MES, 4-morpholineethanesulfonic acid; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; WGA, wheat germ agglutinin; PAGE, polyacrylamide gel electrophoresis; SSFF, sulfonate Sepharose fast flow.

cells, and agonist-stimulated production of PA relative to DAG is increased (3). Kanoh *et al.* (46) reported the purification of an 83-kDa NEM-insensitive PAP from porcine thymus tissue. In this paper, we describe the purification to homogeneity of a 51–53-kDa NEM-insensitive PAP from rat liver. This enzyme differs kinetically and immunologically from that described in porcine thymus. Immunoprecipitating antibodies generated against PAP purified from rat liver confirm its identity and have been used to characterize PAP as an integral plasma membrane glycoprotein that is expressed in liver and a number of other rat tissues. This work provides the basis for determining the metabolic functions of this enzyme.

EXPERIMENTAL PROCEDURES

Animals and Materials—Male Sprague-Dawley rats (275–325 g) and female New Zealand White rabbits were purchased from Biological Sciences Laboratory Animal Services, Edmonton, Alberta, Canada. Animals were treated in accordance with the guidelines of the Canadian Council on Animal Care. HAP was purchased from Bio-Rad. [^{3}H]PA was prepared from [9,10-^{3}H]palmitic acid, as described (1). Lyso-[^{32}P]PA was synthesized from monooleoylglycerol using diacylglycerol kinase and [γ-^{32}P]ATP (47). *N*-Succinimidyl [2,3-^{3}H]propionate, [γ-^{32}P]ATP, and ECL detection kit were obtained from Amersham Corp. Centricon-10 was from Amicon; Na^{125}I (carrier free) was from ICN Biomedicals. BCA protein detection kit and Iodogen® were from Pierce; *N*-glycanase F and *O*-glycosidase were from Boehringer Mannheim. Protein A-Sepharose, neuraminidase, potato acid phosphatase, PA, and monooleoylglycerol were from Sigma. Ribi adjuvant system was from RIBI Immunochem Research, and phosphoagarose and basic alumina were from Fisher Scientific. QSFF, SSFF, Mono Q, and Mono S were from Pharmacia Biotech Inc., and X-Omat AR film was from Eastman Kodak Co. Anti-thymus PAP antiserum was a gift from Dr. H. Kanoh, and anti-alkaline phosphatase antibody was a gift from Dr. R. Stinson.

Assay of PAP, Lyso-PAP, and Protein—PAP activity was measured as described for PAP-2 (47). Lyso-PAP activity was determined by the release of $^{32}P_i$ from lyso-[^{32}P]PA (47). Where stated, 5 mM NEM was included in a 10-min incubation at 37 °C prior to the addition of substrate. Protein was quantitated using the BCA assay and bovine serum albumin as a standard.

Purification of PAP: Isolation and Extraction of Plasma Membranes, HAP, and Ion Exchange Chromatography—Typically, 24 rats were sacrificed by cervical dislocation. The livers were chilled immediately and washed free of blood *in situ* by retrograde perfusion through the inferior vena cava with ice-cold Buffer A (20 mM sodium phosphate, pH 6.5, 300 mM sucrose, and 1 mM EDTA). Samples and solutions were maintained at 4 °C during all of the purification procedures except where stated. Because PAP is found predominantly in plasma membranes (1) this fraction was isolated (48) with modifications. Perfused livers were weighed and homogenized with five to six strokes of a loose fitting Teflon-glass Potter-Elvehjem homogenizer at 600 rpm in Buffer A containing 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 2.3 μl/ml aprotinin, and 166 μg/ml benzamidine. The pooled homogenate was diluted to 20% (w/v) and centrifuged at 3,800 × g_{max} for 10 min. The pellet containing large sheets of plasma membranes was washed and then homogenized 3 times in Buffer A (containing protease inhibitors) with a Waring blender on low setting for 5 s to generate small membrane vesicles. This mixture was centrifuged at 1,400 × g_{max} for 10 min, the pellet washed once and discarded. The combined supernatants were then centrifuged at 4,800 × g_{max} for 10 min. The pellets were washed once and then discarded. The combined supernatant was then centrifuged at 168,000 × g_{max} for 20 min.

The pelleted plasma membranes were homogenized in 5 mM sodium phosphate, pH 6.5, containing 1.5 M NaCl and 0.1% Triton X-100, incubated for 1 h and centrifuged for at least 2 h at 142,700 × g_{max} to remove peripheral membrane proteins. After the supernatant was decanted, the fluffy reddish upper layer of the pelleted protein was collected and resuspended in 200 ml of 10 mM sodium phosphate, pH 6.5, and 100 mM NaCl. An equal volume of acetone (−20 °C) was added slowly with constant agitation to delipidate the sample. The procedure was carried out at −17 °C in an ice-salt bath, the temperature of the protein mixture not rising above −4 °C. Acetone extraction did not decrease PAP activity and resulted in more stable PAP activity during subsequent chromatography. The mixture was incubated for 30 min at −20 °C prior to centrifugation at 350 × g_{max} and −10 °C for 20 min. Pelleted proteins were resuspended gently in 300 ml of 5 mM sodium phosphate, pH 6.5, 200 mM NaCl, and 10% Triton X-100 by incubating for 3–6 h on a slowly rotating wheel, after which any undissolved pellet was resuspended by gentle homogenization. The Triton extract was then dialyzed twice against 4 liters of 5 mM sodium phosphate, pH 6.5, containing 1% Triton X-100 and was clarified by centrifugation (105,000 × g_{max} for 15 min). The sample was applied immediately to a freshly poured 240–260-ml HAP column (XK-50 column, Pharmacia) equilibrated in 5 volumes of 5 mM sodium phosphate, pH 6.5, containing 1% Triton X-100, at a flow rate 600 ml/h. The column was washed to equilibrium, and PAP was eluted in about 1,100 ml of equilibration buffer containing 400 mM NaCl. The eluate was dialyzed twice against 16 liters of 5 mM sodium phosphate, pH 6.5, and 0.1% Triton X-100.

The dialyzed HAP eluate was incubated with 30 ml of swollen QSFF resin for 90 min, and unbound material was separated by filtration. The filtrate was incubated with 30 ml of swollen SSFF resin for 90 min, and any unbound material was separated by filtration. Washed QSFF and SSFF resins were poured into separate XK-16 columns (Pharmacia), and each column was eluted with 5 mM sodium phosphate, pH 6.5, 2 mM EDTA, 0.1% Triton X-100, and 0.5 M NaCl at a flow rate of 60 ml/h. At pH 6.5, PAP activity bound specifically to either QSFF (anionic PAP) or SSFF (cationic PAP). Lack of binding to QSFF was not a result of overloading since no more activity bound during a second incubation with QSFF (prior to SSFF).

Purification of Anionic PAP—The sample eluted from QSFF was dialyzed against 4 liters of 5 mM sodium phosphate, pH 6.5, 2 mM EDTA, and 0.1% Triton X-100 and then applied to a 15-ml phosphoagarose column (CB-30 column, Pharmacia) at a flow rate of 60 ml/h. Anionic PAP was eluted in 6–8 ml with 360 mM NaCl in the same buffer. Gradient elution resulted in no greater purification. The eluted sample was dialyzed 3 times against 2 liters of 10 mM BisTris, pH 6.5, 2 mM EDTA, and 0.1% Triton X-100 and was applied to a Mono Q HR 5/5 column. Proteins were fractionated by fast protein liquid chromatography at a flow rate of 60 ml/h using a 20 mM/ml gradient between 0 and 360 mM NaCl in 10 mM BisTris, pH, 6.5, 2 mM EDTA, and 0.1% Triton X-100.

Purification of Cationic PAP—Samples eluted from SSFF were dialyzed 4 times against 2 liters of 10 mM 2-amino-2-methyl-1-propanol, pH 10, and 0.1% Triton X-100 and then applied to a Mono Q HR 5/5 column equilibrated in the same buffer. PAP activity was eluted with a 20 mM/ml gradient between 0 and 120 mM NaCl (Peak 1), and the ionic strength was then held constant for 15 ml at a flow rate of 60 ml/h. A second peak of PAP activity with a lower specific activity eluted with a step to 400 mM NaCl (Peak 2). Fractions containing Peak 1 were pooled and dialyzed 3 times against 2 liters of 20 mM Tris, pH 8, and 0.05% Triton X-100. Deoxycholate (0.2% final concentration) was added to the dialyzed sample and incubated in batch with WGA agarose for 3 h. Unbound material was separated from the WGA agarose by filtration in a Poly-Prep column (Bio-Rad). Nonspecifically-bound proteins were removed using 20 mM Tris, pH 8, 0.05% Triton X-100, 0.2% deoxycholate, and 0.5 M NaCl. PAP was then eluted using 20 mM Tris, pH 8, containing 0.05% Triton X-100, 0.2% deoxycholate, and 0.5 M *N*-acetylglucosamine at a flow rate of approximately 6 ml/h. The eluted sample was applied to a 2-ml column of HAP (CB-10 column, Pharmacia) equilibrated in 5 mM sodium phosphate, pH 6.5, and 0.1% Triton X-100. After washing the column extensively, PAP was eluted with 0.5 M sodium phosphate, pH 6.5, containing 0.1% Triton X-100 and dialyzed against 2 liters of 10 mM sodium phosphate, pH 6.5, and 0.1% Triton X-100.

Radiolabeling Protein—Protein samples were radiolabeled by two methods. First, 0.5 mCi of *N*-succinimidyl-[2,3-^{3}H]propionate (102 Ci/mol) in toluene was dried under N_2. To this was added a 1.5-ml sample containing 0.87 mg of protein in 100 mM sodium borate, pH 8.5, and 0.1% Triton X-100. This mixture was incubated for 3 h on ice with constant agitation, and then the reaction was quenched with excess glycine. The ^{3}H-labeled protein was dialyzed twice against 4 liters of 100 mM sodium borate, pH 8.5, and 0.1% Triton X-100 before use. Second, protein was labeled with 0.5 mCi of Na^{125}I (carrier-free) in a tube containing 100 mg of Iodogen® for 3–5 min according to the manufacturer's instructions. Excess tyrosine was added to quench the reaction, and the sample was dialyzed exhaustively in 100 mM sodium phosphate, pH 6.5, 100 mM NaCl, and 0.1% Triton X-100 prior to use.

Antibody Production and Purification—Polyclonal antibodies were generated in rabbits by repeated subcutaneous injections using protein-Ribi adjuvant system emulsions. Antibodies were purified using Protein A-Sepharose, dialyzed extensively against 10 mM sodium phosphate, pH 7.4, containing 100 mM NaCl and stored at −70 °C (49, 50). To prepare Ab-A92, anionic PAP (0.25 mg of protein in 5 mM sodium phosphate, pH 6.5, and 0.1% Triton X-100) that was purified as Peak 1 on Mono Q chromatography at pH 6.5, was incubated with 5 units of neuraminidase for 1.5 h at 37 °C and then applied to Mono Q and Mono

TABLE I

Purification of phosphatidate phosphohydrolase from rat liver

PAP was purified from pooled rat liver homogenate as described in the text. PAP activity and protein concentrations were determined as described under "Experimental Procedures." Samples of homogenates were incubated with NEM before measuring the PAP activity. The results are averages of three purifications. Anionic PAP refers to the form of the enzyme that was initially separated from the total by QSFF chromatography. Cationic PAP refers to the form of the enzyme that bound to SSFF. SA, specific activity.

Form of PAP	Purification procedure	PAP		Protein		SA	Purification
		Activity	Yield	Mass	Yield		
		nmol/min	%	*mg*	%	*nmol/min/mg*	*-fold*
Total	Homogenate	194,300	100	90,820	100	2.1	1
	Plasma membrane extract	25,120	12.9	1,260	1.39	19.9	9.3
	HAP eluate	10,820	5.57	102	0.112	106	49.6
Anionic	QSFF eluate	5,224	2.69	26.1	0.029	199	93.2
PAP	Phosphoagarose eluate	2,865	1.47	6.1	0.007	470	219
	Mono Q at pH 6.5 Peak 1	1,487	0.765	0.26	0.0003	5,719	2,673
Cationic	QSFF unbound	4,743	2.44	75.5	0.083	62.8	30
PAP	SSFF eluate	1,931	0.994	17.3	0.019	112	52.2
	Mono Q at pH 10 Peak 1	783	0.403	3.18	0.003	246	115
	WGA/HAP eluate	28.6	0.015	0.287	0.0003	99.7	46.6

S columns in tandem. Material that passed through the Mono Q column but was retained on Mono S was eluted with 0.5 M NaCl and used for antibody production. This sample was not homogeneous for the PAP protein. A second antibody (Ab-D503) was generated against homogeneous cationic PAP. Some of Ab-D503 was affinity-purified against anionic PAP. To make an affinity column, anionic PAP (1.32 mg of protein) was bound to a matrix using the Pierce ImmunoPure® Ag/Ab Immobilization kit, according to the manufacturer's instructions, except that the buffer contained 0.1% Triton X-100. About 90% of the protein was covalently linked to the column. Immunospecific antibody was eluted from the affinity column with 10 mM sodium phosphate, pH 6.5, containing 150 mM NaCl and 2 M potassium thiocyanate (51). Affinity-purified Ab-D503 was dialyzed twice against 2 liters of 10 mM sodium phosphate, pH 7, and 150 mM NaCl at 4 °C and then concentrated using a Centricon-10 concentrator.

Collection of Tissue Samples, Immunoprecipitation, and Western Blot Analysis—Rat tissues were obtained by freeze-clamping dissected organs from anesthetized rats prior to sacrifice. To minimize postmortem changes in PAP, samples stored at −70 °C were thawed in 10 mM sodium phosphate, pH 6.5, containing 200 mM sucrose, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 2.3 μl/ml aprotinin, and 166 μg/ml benzamidine and homogenized on ice.

For immunoprecipitation, samples were incubated at 4 °C for 2–3 h with antibody in 100–200 μl of Buffer B (100 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, and 0.1% Triton X-100). Aliquots were taken for analysis of PAP activity, and immunoreactive protein was precipitated with Protein A-Sepharose. The pellet was washed 4 times with 1 ml of incubation buffer prior to analysis of PAP activity. For analysis of immunoprecipitates by SDS-PAGE, samples were denatured immediately prior to electrophoresis on 5–15% gradient gels (52) by heating at 95 °C for 3 min in 2 × Laemmli sample buffer containing 5 M urea. All other samples were denatured in the presence of 8 mg/ml dithiothreitol. Proteins were either visualized by silver staining (53) or transferred to polyvinylidine difluoride membranes for Western blot analysis (54). Blotting was performed at room temperature in 10 mM HEPES, pH 7.8, 150 mM NaCl, 1 mM EDTA, 0.5% Tween 20, and 0.25% gelatin. Immunoreactive proteins were detected using enhanced chemiluminescence.

RESULTS

Purification of anionic and cationic forms of PAP is summarized in Table I. Greater than 90% of the activity eluted from HAP was recovered from the QSFF and SSFF columns. Approximately equal amounts of activity bound at pH 6.5 to QSFF (anionic PAP) and SSFF (cationic PAP). Anionic PAP eluted from Mono Q at pH 6.5 in a biphasic manner (Fig. 1). A sharp peak of activity eluted between 80 and 120 mM NaCl (Peak 1), and a second broad peak eluted between 200 and 300 mM NaCl (Peak 2). Overall recovery of activity from Mono Q chromatography at pH 6.5 was typically 90% of the applied activity, 30–50% of which was in Peak 1. Although Peak 1 contained PAP at the highest specific activity (Table I), the sample contained at least five major protein bands (approximately 78, 67, 62, 53, and 47 kDa) when visualized with silver staining after SDS-PAGE (Fig. 2, *lane 2*). Neuraminidase treatment of this sample followed by chromatography on Mono S at pH 6.5 failed to effect a significant purification of the enzyme, however antibodies (Ab-A92) were raised against this material. Ab-A92 was instrumental in the identification and purification of cationic PAP from the SSFF-binding material. As described below, desialated anionic PAP is identical, or very similar, to cationic PAP (Fig. 2, *lane 5*).

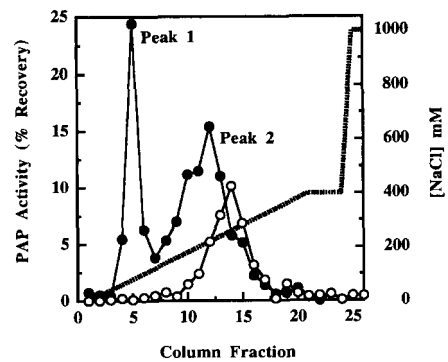

FIG. 1. **Fractionation of anionic PAP using Mono Q chromatography at pH 6.5.** Phosphoagarose-eluted material was fractionated on Mono Q at pH 6.5. Anionic PAP activity (●) consistently eluted in a biphasic manner from the column with a gradient of NaCl. *Peak 1* contained 30–50% of the eluted activity and represents the fraction containing the highest specific activity of PAP. *Open symbols* (○) represent the elution profile of PAP that eluted from phosphoagarose and was incubated in 6.4 M urea at room temperature for 15 min prior to chromatography on Mono Q at pH 6.5 in the presence of 10 mM sodium phosphate, pH 6.5, 2 mM EDTA, and 6.4 M urea. The *dashed line* represents the NaCl gradient used to elute the column.

Cationic PAP activity eluted from Mono Q at pH 10 in a biphasic manner (Fig. 3*A*). Overall recovery of cationic PAP activity from the Mono Q column at pH 10 was typically 75% of the applied activity, 50% of which was in Peak 1. Analysis of cationic PAP by SDS-PAGE and Western blot with Ab-A92, revealed that Peak 1 contained only one major immunoreactive protein (Fig. 3*B*) and fewer silver-stained proteins compared with Peak 2 (not shown). Binding of cationic PAP to Mono Q at pH 10 indicated an isoelectric point between pH 6.5 and 10, and PAP activity eluted from a chromatofocusing column around pH 9. Chromatofocusing was not useful in the purification since the activity eluted in a broad peak. Anionic PAP did not elute from the chromatofocusing column between pH 7 and 4 but was recovered in a 1 M salt wash; thus the pI of anionic PAP was less than pH 4.

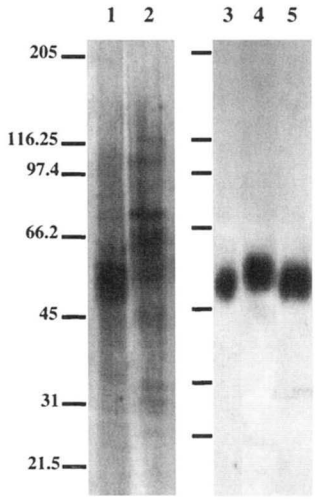

FIG. 2. **Analysis of purified cationic and anionic PAP.** Purified cationic PAP (*lanes 1* and *3*) and anionic PAP (Peak 1 eluted from Mono Q at pH 6.5, *lanes 2* and *4*) were analyzed by SDS-PAGE under reducing conditions. *Lane 5* is neuraminidase-treated anionic PAP, further purified by sequential chromatography on Mono Q at pH 10, WGA, and HAP concentrating chromatography. *Lanes 1* and *2* were silver stained. *Lanes 3–5* were analyzed by Western blot with affinity-purified Ab-D503. A Western blot of the same samples with preimmune antibody showed no immunoreactive bands (results not shown). Migration of molecular mass markers (kDa) is indicated at the sides of the figure.

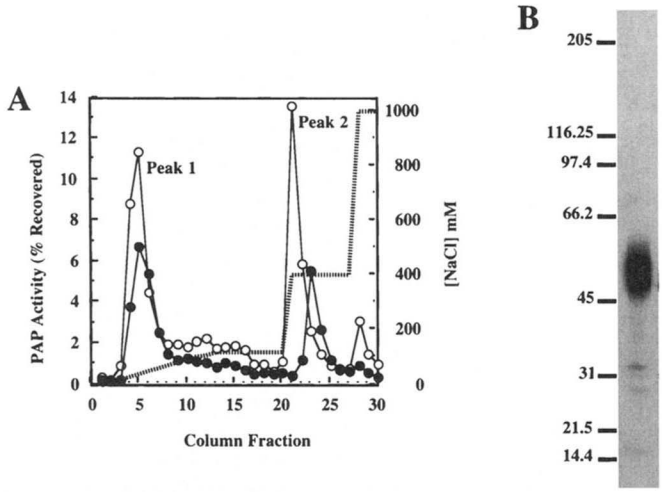

FIG. 3. **Fractionation of cationic PAP using Mono Q chromatography at pH 10.** *A*, cationic PAP activity (●) consistently eluted in a biphasic manner from the Mono Q column at pH 10 with a gradient of NaCl. These peaks are designated as *Peak 1* and *Peak 2*, respectively. Neuraminidase treated-anionic PAP (○) in the unbound fraction after Mono Q chromatography at pH 6.5 was dialyzed to pH 10 against 10 mM 2-amino-2-methyl-1-propanol, pH 10, and 0.1% Triton X-100 and then reapplied to a Mono Q column equilibrated at pH 10. After washing, the sample was eluted with a gradient of NaCl (*dashed line*). *B*, a sample of material from Peak 1 eluted from Mono Q chromatography at pH 10 was separated by SDS-PAGE under reducing conditions and analyzed by Western blot using Ab-A92. Migration of molecular mass markers (kDa) is indicated at the side of the figure.

Recoveries of PAP activity from the WGA agarose column were 20–40% of that applied, when assayed immediately after chromatography. Routine analysis for protein in these column fractions could not be made because of the relatively high concentrations of detergent and reducing sugar; therefore, this chromatographic step is not included in Table I. To generate a quantifiable sample, the WGA-agarose-eluted material was concentrated, and the deoxycholate and *N*-acetylglucosamine were removed using HAP. After HAP-concentrating chromatography, no protein was detected (by silver staining of SDS-PAGE), and no PAP activity was present in the unbound material. PAP activity was recovered in the phosphate-eluted fraction, and only one major protein band was detected by silver staining after SDS-PAGE (Fig. 2, *lane 1*), indicating that the eluted PAP was essentially homogeneous. Purified cationic PAP migrated on SDS-PAGE with an apparent molecular mass of 51 kDa, independent of the presence or absence of 100 mM dithiothreitol in the solubilization buffer, indicating that the enzyme is a single polypeptide containing no sulfhydryl-linked subunits. Overall recovery of enzyme activity from the last two chromatographic steps was only 4% (partly due to intrinsic instability); however, this procedure yielded purified PAP. Loss of enzyme activity may also be attributed to the use of WGA-agarose (55, 56). When the last steps in the purification (WGA/HAP) were performed with radiolabeled protein, only one radiolabeled protein of 51 kDa was detected by autoradiography of the purified fraction after SDS-PAGE (results not shown).

The ability of anionic PAP and homogeneous cationic PAP (Table I) to hydrolyze lyso-PA was evaluated. Optimum reaction rates of lyso-PA hydrolysis were obtained between 20 and 100 μM lyso-PA with V_{max} values for anionic and cationic PAP that were 34 and 29% of that for PA, respectively.

Characterization of Ab-A92 and Ab-D503—To characterize the antibodies generated against anionic and cationic PAP, we evaluated the ability of Ab-D503 (raised against cationic PAP) to immunoprecipitate anionic PAP activity and that of Ab-A92 (raised against neuraminidase-treated anionic PAP) to immunoprecipitate cationic PAP activity. Both antibodies recognized either form of the enzyme in solution and immunoprecipitated PAP activity in a dose-dependent fashion (Fig. 4). Affinity-purified Ab-D503 also immunoprecipitated PAP activity from an anionic sample (results not shown). When PAP was assayed in the presence of either antibody, over the range of antibody used, activity was inhibited up to 20%. Therefore, PAP activity assayed in the pellet underestimates the amount of immunoprecipitated PAP. Purified Ab-A92 immunoprecipitated PAP activity from an anionic PAP or neuraminidase-treated anionic PAP sample in a dose-dependent fashion (results not shown). Likewise, Ab-D503 immunoprecipitated PAP activity from a cationic PAP sample in a dose-dependent fashion (results not shown).

Characterization of the Anionic and Cationic Forms of PAP—Like the elution of Peak 2 from Mono Q at pH 6.5, Peak 2 from Mono Q at pH 10 eluted with a gradient between 200 and 300 mM NaCl (results not shown). To determine whether Peak 1 and Peak 2 that eluted from the Mono Q at pH 6.5 represented chemically distinct proteins (over and above the distinct ionic forms), we took advantage of an observation that when anionic PAP was incubated with 6.4 M urea, there was an initial loss of 50% of activity, but the remaining activity was quantifiable for up to 3 h. PAP that had been purified from phosphoagarose, was incubated in 6.4 M urea and then chromatographed on Mono Q at pH 6.5 in a buffer containing 6.4 M urea. PAP activity eluted in one peak with a gradient between 180 and 260 mM NaCl (Fig. 1). Moreover, when samples of Peak 1 and Peak 2 (eluted from Mono Q at pH 6.5) were incubated and chromatographed separately in urea, the elution profile of PAP activity was essentially coincident (results not shown). Peaks 1 and 2 from Mono Q chromatography at pH 6.5 contained a protein of equivalent molecular mass that cross-reacted with Ab-D503, suggesting that the same or closely related proteins were responsible for PA hydrolysis (Fig. 5*A*, *lanes 1* and *2*).

All measurable PAP activity was lost when a cationic sample of PAP was incubated with urea, consequently it was not possible to assess cationic PAP in the same manner. However, Peak 2 material from Mono Q chromatography at pH 10 con-

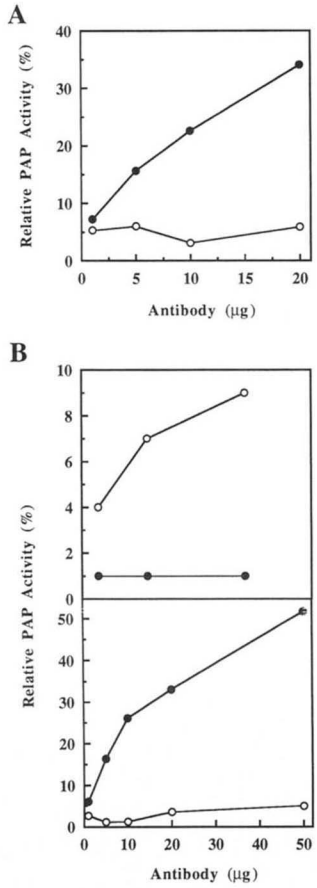

FIG. 4. **Dose-dependent immunoprecipitation of PAP activity.** *A*, samples of cationic PAP; *B*, samples of anionic PAP (*upper panel*) or desialated (neuraminidase-treated) anionic PAP (*lower panel*) were immunoprecipitated with preimmune antibody (○) or Ab-D503 (●). PAP activity of the pelleted material is expressed as a percentage of the sum of the immunoprecipitated and nonimmunoprecipitated activity.

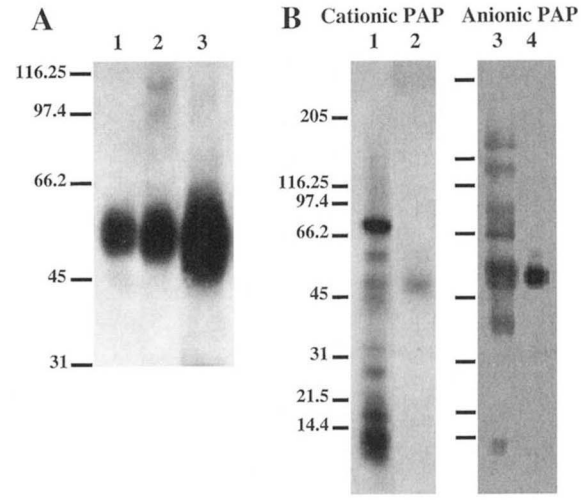

FIG. 5. **Western blot analysis and immunoprecipitation of radiolabeled protein from partially purified cationic and anionic PAP.** *A*, samples from chromatographic fractions were separated by SDS-PAGE under reducing conditions and analyzed by Western blot with Ab-D503. *Lane 1*, Peak 1 from Mono Q at pH 6.5; *lane 2*, Peak 2 from Mono Q at pH 6.5; *lane 3*, Peak 2 from Mono Q at pH 10. *B*, cationic PAP (Peak 1 eluted from Mono Q at pH 10), radiolabeled with [2,3-^{3}H]propionate (*lane 1*) and radioiodinated anionic PAP (Peak 1 eluted from Mono Q at pH 6.5 (*lane 3*) were immunoprecipitated with Ab-A92 (*lane 2*), or Ab-D503 (*lane 4*), separated by SDS-PAGE under nonreducing conditions and analyzed by autoradiography. Migration of molecular mass markers (kDa) is indicated at the sides of the figure.

tained a protein that cross-reacted with Ab-D503 (Fig. 5*A*, *lane 3*) and migrated on SDS-PAGE with a molecular mass identical to the major immunoreactive protein band in Peak 1 (Fig. 3*B*).

To test whether PAP exists in a complex with other proteins, partially purified samples of anionic and cationic PAP were radiolabeled using two different methods and then incubated with Ab-D503 or Ab-A92. Only one protein band was immunoprecipitated from either sample (Fig. 5*B*). The immunoprecipitated radiolabeled proteins migrated on SDS-PAGE with an apparent molecular mass identical to the corresponding protein bands detected by Western blot analysis or silver staining (Figs. 2, 3, and 5*A*). Less than 10% of the radioactivity was immunoprecipitated with Ab-A92 from the crude radiolabeled cationic sample.

Cationic PAP was shown to be a glycoprotein, containing complex polysaccharide by virtue of its ability to bind specifically to WGA agarose (Table I). The anionic form of PAP had similar lectin-binding characteristics (results not shown). Although anionic and cationic PAP were not immunologically distinguishable, anionic PAP consistently migrated on SDS-PAGE with an apparent molecular mass approximately 2 kDa larger than cationic protein (Fig. 2). Incubation of anionic PAP with neuraminidase did not alter enzyme activity significantly but caused PAP to bind to Mono S rather than Mono Q at pH 6.5. Also, when neuraminidase-treated anionic PAP was chromatographed on Mono Q at pH 10, PAP activity eluted from the column in a manner similar to the cationic form of the protein (Fig. 3*A*). Neuraminidase treatment of cationic PAP does not affect its chromatographic properties on Mono Q at pH 10 (results not shown). Subsequent purification of the neuraminidase-treated anionic PAP with WGA agarose and HAP after Mono Q chromatography at pH 10 yielded an immunologically cross-reactive protein (with Ab-D503) that co-migrated on SDS-PAGE with the purified cationic PAP (Fig. 2, *lane 5*).

To determine whether PAP, as purified, was a phosphoenzyme, samples of cationic and anionic PAP were incubated with acid phosphatase and/or neuraminidase and then examined by SDS-PAGE and Western blot analysis (Fig. 6). Anionic PAP migrated slightly above cationic PAP. The band shifts in the phosphatase-treated samples indicated that anionic PAP could be a phosphoprotein. The smaller change in the migration of the phosphatase-treated cationic PAP makes the same conclusion equivocal for that form of the protein. The band shift of anionic PAP seen with neuraminidase treatment was intermediate to that seen with phosphatase treatment alone or with neuraminidase and phosphatase (Fig. 6). Neuraminidase had no effect on the migration of cationic PAP. The calculated molecular mass of dephosphorylated and desialated anionic PAP was identical to the phosphatase-treated cationic PAP. The phosphorylation state of anionic PAP did not affect its elution profile on Mono Q at pH 10 (results not shown). The results in Figs. 2 and 6 indicate that the primary difference between the two forms of PAP is the degree of sialation of the polysaccharide side chain(s).

Cationic PAP probably contains only *N*-linked polysaccharides since neither *O*-glycosidase (Fig. 7) nor neuraminidase (Fig. 6) altered its migration on SDS-PAGE. The apparent molecular mass of anionic PAP may be decreased slightly by *O*-glycosidase. Treatment with *N*-glycanase F caused both cationic and anionic PAP to shift to an apparent molecular mass of 28 kDa (Fig. 7). Analogous results were found when samples of anionic and cationic PAP were labeled with ^{125}I, treated with *N*-glycanase F, immunoprecipitated, and analyzed by autoradiography after SDS-PAGE (results not shown). To determine whether a fully glycosylated protein was required for expression of PAP activity, nondenatured anionic PAP was incubated with *N*-glycanase F at 37 °C. Although the specific activity of

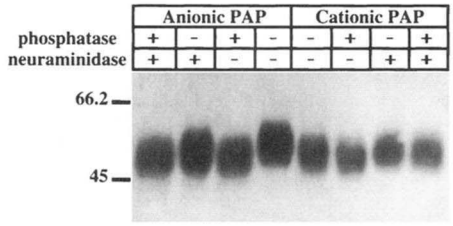

FIG. 6. **Treatment of PAP with acid phosphatase and neuraminidase.** Anionic and cationic PAP samples containing 1 μg of protein were incubated for 2 h at 37 °C with or without 0.2 units of neuraminidase in a volume of 22 μl with 10 mM sodium phosphate, pH 6.5, and 0.1% Triton X-100. Citrate-MES buffer (pH 4.8, final concentration of 50 mM) and 2 units of potato acid phosphatase (if indicated) were added, and the samples were incubated for an additional 4 h. The samples were then denatured and analyzed by SDS-PAGE under reducing conditions and Western blot with affinity-purified Ab-D503. Migration of known molecular mass markers (kDa) is indicated at the side of the figure.

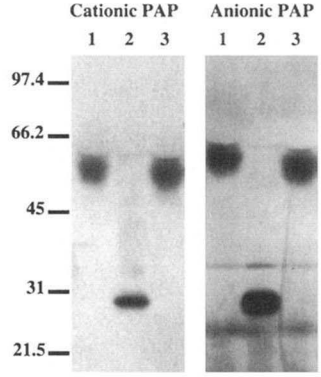

FIG. 7. **Treatment of PAP with endo- and exoglycosidases.** Purified cationic and anionic PAP were denatured by heating for 3 min at 95 °C in the presence of 12% methanol and then cooled, buffered with 50 mM sodium phosphate, pH 6.0, containing 0.5% Triton X-100, 0.1% bovine serum albumin, and incubated without glycosidase (*lanes 1*), with 0.1 unit of *N*-glycanase F (*lanes 2*), 2 milliunits of *O*-glycosidase (*lanes 3*) overnight at 37 °C. Samples were then denatured in 2 × Laemmli buffer, separated by SDS-PAGE under reducing conditions, and analyzed by Western blot with Ab-A92 (cationic PAP) or Ab-D503 (anionic PAP). Neither Ab-A92 nor Ab-D503 showed immunoreactivity with *N*-glycanase F or *O*-glycosidase. Migration of molecular weight markers (kDa) is indicated at the side of the figure.

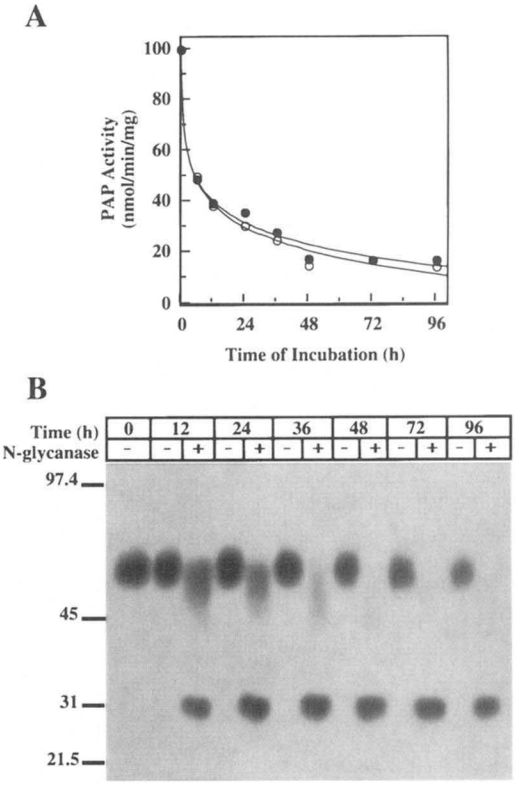

FIG. 8. **Time course incubation of anionic PAP with *N*-glycanase F.** *A*, anionic PAP, which had not been denatured, was incubated at 37 °C in the presence (●) or absence (○) of *N*-glycanase F for up to 96 h. At the indicated times, aliquots were removed from the incubation and were assayed for PAP activity. Values represent the average of two independent experiments. *B*, aliquots from the same incubation of anionic PAP were also analyzed by SDS-PAGE under reducing conditions and Western blot with affinity-purified Ab-D503. Migration of molecular mass markers (kDa) is indicated at the side of the figure.

PAP declined, there was no difference between control and glycanase-treated PAP activity at any time (Fig. 8*A*), despite the fact that by 48 h the enzyme had been fully deglycosylated (Fig. 8*B*). From this experiment, it appears that PAP does not have to be fully glycosylated to remain catalytically active *in vitro*; however, activity in both the control and *N*-glycanase-treated samples was not stable to extended incubation at 37 °C.

Tissue Distribution of PAP—NEM-insensitive PAP activity has been characterized in rat liver (1, 57, 58), heart, brain, and adipose tissue (59–61) and fibroblasts (3), as well as rabbit kidney cells (62), human neutrophils (63, 64), and porcine thymus (46). Therefore we screened a variety of rat tissues for PAP activity and for the PAP protein by Western blot analysis. NEM-insensitive PAP activity was present in all of the tissues examined (Fig. 9*A*). The specific activity of PAP was lowest in skeletal muscle and greatest in brain, kidney, and spleen. An intense immunoreactive protein with an apparent molecular mass of 51–53 kDa was present in seven of the 11 tissues tested (Fig. 9*B*). Brain contained two major immunologically cross-reactive proteins of 51 and 86 kDa. A strongly immunoreactive 17-kDa protein in both thymus and spleen was also present to a lesser extent in kidney, lung, and testis. Thymus, spleen, and liver also contained a cross-reactive 36-kDa protein.

DISCUSSION

During the purification of PAP, two ionically distinct forms of the protein were identified and characterized. The specific activity of anionic PAP (53 kDa, pI < 4) was increased 2,700-fold. This degree of purification is an underestimate since the total PAP activity was distributed between cationic and anionic forms, each of which was further separated into two peaks of activity. The specific activity of the 51-kDa cationic PAP (pI = 9) was lower than that of anionic PAP (at least in part due to instability); however, cationic PAP was purified to homogeneity. In addition to appearing essentially homogeneous by silver staining, cationic PAP elicited antibody production against only one protein, attesting to the homogeneity of the purified sample. Only one protein from anionic PAP and one protein from cationic PAP was immunoprecipitated using Ab-D503, and PAP activity co-purified with the immunoreactive proteins. Antibodies generated against either form of PAP immunoprecipitated PAP activity from any PAP sample and cross-reacted on a Western blot with a 51–53-kDa protein. Neuraminidase treatment of anionic PAP decreased the apparent molecular mass of the protein by about 2 kDa and produced an enzyme having essentially the same characteristics as cationic PAP. It is concluded that cationic PAP is probably a desialated form of anionic PAP. In support of this conclusion, the molecular masses of both forms of PAP are decreased to 28 kDa by treatment with *N*-glycanase F, and both deglycosylated proteins cross-react with either Ab-A92 or Ab-D503.

PAP in the partially purified samples did not associate with other cellular proteins (Fig. 5*B*), therefore this is not an explanation for its biphasic elution profile from Mono Q. It is possible

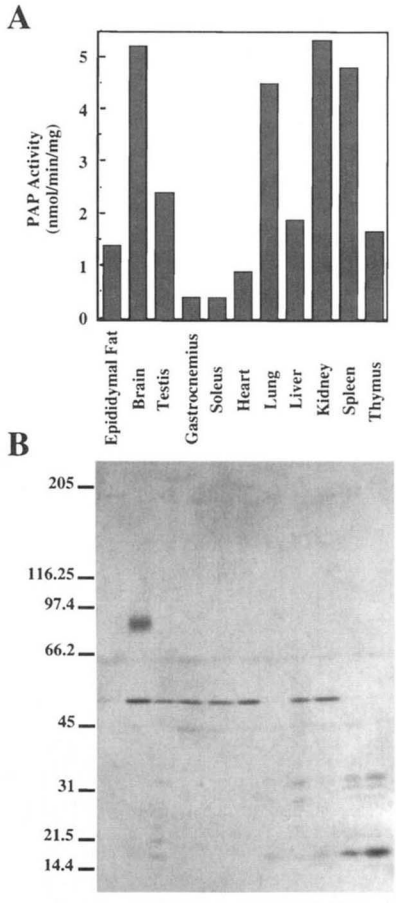

FIG. 9. **Tissue distribution of PAP activity and immunologically cross-reactive protein.** *A*, homogenates of rat tissues obtained by freeze-clamping dissected organs from an anesthetized animal were screened for PAP activity. The PAP assay included a preincubation in the presence of NEM. *B*, samples from tissue homogenates containing 50 μg of protein were analyzed using SDS-PAGE under reducing conditions and by Western blot with affinity-purified Ab-D503 as described under "Experimental Procedures." Migration of molecular mass markers (kDa) is indicated at the side of the figure.

that self-association or aggregation of PAP may alter its chromatographic behavior. The presence of an approximately 112-kDa immunoreactive protein on Western analysis in Peak 2 from Mono Q at pH 6.5 (Fig. 5*A*, *lane 2*) may represent a dimer of PAP (65). Self-association or aggregation may also explain our lack of ability to employ nondenaturing PAGE or size exclusion chromatography in the analysis or purification of PAP.

N-linked polysaccharide constitutes about 46% of the total mass of PAP. Several other mammalian glycoproteins have been purified that are as much as half polysaccharide by weight. For example, polysaccharide contributes 54% of the mass of the β-subunit of the porcine gastric H^+/K^+-ATPase (66), 47–50% of an angiotensin receptor from human myometrium (67), 52% of a major lysosome-associated protein (68, 69), and 37% of contactinhibin (70). The polysaccharide portion of a glycoprotein may be required for proper folding during synthesis, appropriate cellular localization, stability, or biological function (71). Complete deglycosylation of native anionic PAP does not affect its activity toward PA, but the activity of desialated PAP appears to be inherently less stable than that of the anionic form of the enzyme.

It is unclear whether the purification of two ionic forms of PAP from liver homogenate is artifactual or if it reflects the presence of distinct ionic forms *in vivo*. The absence of a doublet of immunoreactive proteins at 51–53 kDa in the Western blot analysis of tissue homogenates could indicate that only one form of the protein exists in the intact liver. However, the appearance of a single tight band in the homogenate, compared with a relatively diffuse one in purified fractions, indicates that PAP migrates differently in a complex mixture of proteins. Thus, two forms of a glycoprotein differing in mass by 2 kDa may not be resolved under these conditions. Two-dimensional gel electrophoresis could not be used to address this issue because PAP precipitated when we attempted isoelectric focusing. If two ionic forms of PAP are present *in vivo*, the difference may be functionally significant. Different states of sialation affect the clearance of circulating glycoproteins from the blood (72), and increased sialation of contactinhibin reduces its ability to mediate contact inhibition of cell proliferation (70).

Band shift experiments indicate that PAP is a phosphoprotein. Further evidence for the phosphorylation of PAP was obtained by labeling cultured rat hepatocytes with $^{32}P_i$ and immunoprecipitating a radiolabeled 51–53 kDa band with affinity-purified Ab-D503.[2] Phosphorylation does occur on amino acid residues,[2] but it could also occur on residues of the polysaccharide (73, 74). Phosphorylation-dependent regulation of PAP has been implicated in previous work (3, 5) and could explain, at least in part, the difference in specific activity or stability of the two ionic forms of PAP. Phosphorylation may also explain the lack of correlation between the specific activity of PAP and the intensity of the immunoreactive 51–53-kDa proteins in the various tissues (Fig. 9). However, the relative abundance of PAP may be tissue-specific, or tissues may express different isoforms of PAP, for which our antibodies have different affinity. The 86-kDa protein in brain and the 17- and 36-kDa proteins in thymus and spleen may be tissue-specific isoforms of PAP. The existence of tissue-specific isoforms is supported by the observation that lyso-PA does not inhibit PAP purified from pig thymus (46) and that anti-thymus 83-kDa PAP antiserum failed to immunoprecipitate PAP activity from rat liver samples, nor did it cross-react with any protein bands on a Western blot of rat liver homogenate (results not shown). In contrast, purified rat liver PAP hydrolyzed lyso-PA with a V_{max} of about 30% that for PA, and the two substrates are mutually competitive.[3] There also may be other forms of PAP that do not cross-react well with our antibodies (for example, in lung or adipose tissue). Furthermore, differential regulation of PAP in adipose tissue, compared with heart and liver, is indicated in insulin-resistant rats (59). The reasons for the lack of correlation between the activity and band intensity (Fig. 9) will be clarified when sequence information is known and physical modifications of PAP in the various tissues have been characterized.

PAP has also been purified from yeast (75, 76), but this enzyme resembles the NEM-sensitive and Mg^{2+}-requiring PAP in liver. Neither Ab-A91 nor Ab-D503 immunoprecipitates NEM-sensitive PAP activity from liver microsomes or cytosol (results not shown), confirming that the two enzymes are distinct proteins (1, 58). Purified hepatic PAP is not alkaline phosphatase; since the pH optimum for PAP is 6.5, it does not require Mg^{2+}, and antibodies to alkaline phosphatase do not interact with PAP (results not shown).

An NEM-insensitive "ecto-PAP" in neutrophils (63) may be the same protein we have purified from rat liver. If the active site of PAP in the plasma membrane is oriented externally, it could degrade exogenous PA and lyso-PA, thus mitigating the effects of these lipids on cell signaling and cell division (5, 9, 33, 34). Alternatively, PAP activity oriented internally could de-

[2] C.-N. Wang, D. W. Waggoner, and D. N. Brindley, unpublished observations.

[3] D. W. Waggoner and D. N. Brindley, unpublished observations.

grade PA present in the inner leaflet of the plasma membrane, thus altering the balance of PA and DAG and their actions on intracellular target proteins (3, 5).

In this paper we describe the purification and characterization of a novel 51–53-kDa NEM-insensitive PAP from rat liver. This enzyme is an integral plasma membrane glycoprotein that is expressed in rat liver, brain, kidney, testis, heart, and skeletal muscle. Immunoprecipitating antibodies directed against purified PAP protein will be useful tools for further characterization and investigation into the regulation of PAP and of its proposed role in signal transduction.

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