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Identification of a novel human phosphatidic acid phosphatase type 2 isoform

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Abstract Two human isoforms of membrane associated phosphatidic acid phosphatase have been described (PAP-2a and -2b), and both enzymes have been shown to have broad substrate specificity and wide tissue distribution [Kai et al., J. Biol. Chem. 272 (1997) 24572–24578]. With this report we describe a third isoform, PAP-2c, that we found by searching the database of expressed sequence tags (dbEST) with PAP-2a and PAP-2b sequences. Key structural features described previously in PAP-2a and -2b, including the glycosylation site, putative transmembrane domains, and the proposed catalytic site, are conserved in the novel phosphatase. The kinetics of the three enzymes were compared using as substrates phosphatidic acid, lysophosphatidic acid, and *N*-oleoyl ethanolamine phosphatidic acid. K_m values for each of the substrates, respectively, were (in μM) PAP-2a: 98, 170, 116; PAP-2b: 100, 110, 56; and PAP-2c: 150, 340, 138. Expression of PAP-2c mRNA is more restricted than the two previously described isoforms.

Key words: Phosphatidic acid phosphatase; Lysophosphatidic acid; Enzyme kinetics; PAP-2c

1. Introduction

Phosphatidic acid phosphatases (PAPs) function in phospholipid metabolism by catalyzing the conversion of phosphatidic acid (PA) to diacylglycerol. It has been shown recently that PAPs also catalyze the hydrolysis of additional lipid phosphoric acids, including the bioactive signaling molecules lysophosphatidic acid (LPA), sphingosine 1-phosphate (S1P), and ceramide 1-phosphate (Cer1P) [1,2]. Additionally, many of the products of PAP-mediated hydrolysis (diacylglycerol, sphingosine, and ceramide) are themselves important signaling molecules [3–5]. These observations have attracted attention to the role that PAPs may play in signal transduction. Due to their apparent broad substrate specificity, PAPs are poised to regulate several signaling pathways by simultaneously creating and destroying bioactive lipids.

Initial biochemical characterization of PAP activity in rat liver by Brindley and his associates demonstrated that the enzyme activity could be separated into two components [6,7]. Type 1 (PAP-1) was defined as a cytosolic/endoplasmic reticulum-localized activity that was both dependent on Mg^{2+} and inactivated by the thio-reactive compound *N*-ethylmaleimide (NEM). Conversely, a Mg^{2+} dependent, NEM insensitive membrane-associated activity was classified as type 2 (PAP-2). Further, PAP-1 was reported to translocate to microsomes on stimulation of triacylglyceride synthesis, while PAP-2 was unaffected by lipid metabolism status [6]. Only the type 2 activity, however, is decreased in transformed fibroblasts [8]. Therefore, it was hypothesized that PAP-1 is involved in phospholipid metabolism while PAP-2 is involved in signal transduction.

In 1992, Kanoh reported the partial purification of a 85 kDa PAP-2 from porcine thymus. This activity was not inhibited by lysophosphatidic acid or diacetyl phosphatidate, leading the authors to conclude that the enzyme was specific for PA [9]. The same group reported subsequently that the phosphatase activity arose instead from a 35 kDa membrane-bound, glycosylated protein that was a minor component of their sample [10]. The sequence of an amino-terminal peptide of the 35 kDa porcine PAP-2 was determined and used to clone the mouse ortholog of PAP-2 based on high sequence similarity to the partial cDNA clone *hic*53, a peroxide inducible gene [11]. The peptide sequence of the mouse PAP-2 suggested six putative transmembrane domains and two glycosylation sites [10]. In 1996, Waggoner et al. reported the purification of PAP-2 from rat liver. In contrast to Kanoh's report, this group showed that the rat PAP-2 is not specific for PA, but rather can hydrolyse PA, LPA, S1P, and Cer1P [2]. Subsequently, Dillon et al. showed that the enzyme could hydrolyze diacylglyceride pyrophosphate (DGPP) also [12].

Kanoh's group subsequently cloned two human isoforms of PAP-2: PAP-2a and PAP-2b [1]. PAP-2a shares 83% sequence identity with the original mouse PAP-2 while PAP-2b is 94% identical to rat Dri42, whose gene product has been shown to be localized to the ER and has been implicated in epithelial cell differentiation [13]. Both human clones are also related to the *Drosophila* gene product Wunen, which has been implicated in germ cell migration [14]. PAP-2a and -2b are 57% identical over 253 amino acids and both were shown to be glycosylated at a single site and are predicted by hydropathy analysis to have six transmembrane regions [1]. The membrane topology of Dri42 has been determined by the insertion of Dri42 fusion proteins into microsomal membranes, and this information has been used to predict the extracellular and cytosolic domains of PAP-2a and PAP-2b [1,13].

Comparison of the PAP-2 sequences with those of vanadium dependent peroxidases reveals striking sequence similarity in regions of the vanadium binding sites and catalytic residues of the peroxidases [15,16]. Wever's group first proposed that these two classes of enzymes share a common active site architecture based on primary structure and similarity between the geometries of vanadate and transition state phosphate

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Abbreviations: PAP, phosphatidic acid phosphatase; PA, phosphatidic acid; LPA, lysophosphatidic acid; NOEPA, *N*-oleoyl ethanolamine phosphatidic acid; S1P, sphingosine 1-phosphate; Cer1P, ceramide 1-phosphate; dbEST, database of expressed sequence tags; NEM, *N*-ethylmaleimide

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[16]. Additionally, Neuwald has proposed a catalytic mechanism of the phosphatases based on the analogous peroxidase mechanism suggested by the crystal structure of the vanadium-peroxidase complex. This putative phosphatase active site is contained in three regions that are highly conserved between the phosphatases and peroxidases, and all of these regions are predicted to be in extracellular loops. This structural prediction indicates that PAP-2s may act as ectophosphatases.

If the PAP-2 enzymes function as ectophosphatases, they may serve as regulators of the strength and duration of signals from phosphorylated lipid mediators at cell surfaces. LPA and S1P signal through G protein-coupled receptors that mediate a wide range of cellular events in response to binding of the lipids [3–5]. Cell surface phosphatases could attenuate these responses by affecting the apparent potency of the ligands.

Obviously, substrate specificity is a critical parameter in defining the physiologic role of signal regulation by PAPs. This study was initiated to define the substrate specificity of the two human isoforms of PAP-2 by analyzing the kinetics of their activity with PA, LPA and *N*-oleoyl ethanolamine phosphatidic acid (NOEPA) as substrates. After sub-cloning the previously described PAP-2a and -2b cDNAs from EST cDNAs, a third isoform was identified in the EST database based on sequence similarity. We present here a description of the novel enzyme, PAP-2c, and a comparison of the activities of the three recombinant enzymes.

2. Materials and methods

2.1. cDNA cloning

Full length clones of human PAP-2a and -2b were found by searching the EST database with the published amino acid sequences using the TBLASTN algorithm [17]. Both of the underlying cDNAs were obtained from the I.M.A.G.E. Consortium [18] (PAP-2a: clone ID 843414; PAP-2b: clone ID 23748). The novel phosphatase cDNA, PAP-2c, was conceptualized from several EST sequences showing high similarity scores with both PAP-2a and -2b. The $5' \rightarrow 3'$ order of the overlapping sequences are as follows (by GenBank accession number): AA429168, Z43618, N75714, AA316437, AA042850, AA458529, R34980. The first clone in the series contained an initiation codon near the initiation codons of the aligned PAP-2a and -2b sequences. This cDNA (clone ID 769745) was acquired from I.M.A.G.E. Consortium and sequenced. All three cDNAs were amplified by PCR using primers flanking the translational open reading frames. The primers were designed to introduce restriction sites at each end of the fragment, and these sites were used for subcloning into the mammalian expression vector, pCR3.1 (Invitrogen).

2.2. Transfection and membrane preparation

Plasmid DNA was transfected into HEK 293 T cells using calcium phosphate transfection protocol [19]. Briefly, a DNA cocktail containing 25 μg DNA and 0.25 M $CaCl_2$ was added to HEPES buffered 2 mM Na_2HPO_4. Subconfluent monolayers of HEK 293 T cells were poisoned with 25 mM chloroquine, and the DNA precipitate was then applied to the cells. After 4 h, the monolayers were washed with PBS and refed media (1:1 Dulbecco's modified essential media: F12 + 10% fetal bovine serum). The cells were harvested 48–72 h after addition of the DNA. The cells were scraped in HME buffer (in mM: 20 HEPES, 5 $MgCl_2$, 1 EDTA, pH 7.4) containing 10% sucrose on ice, and disrupted using a Dounce homogenizer. After centrifugation at $800 \times g$, the supernatant fluid was diluted with HME without sucrose and centrifuged at $100\,000 \times g$ for 1 h. The resulting pellet was rehomogenized and centrifuged a second hour at $100\,000 \times g$. This crude membrane pellet was resuspended in HME with sucrose at 1–5 mg protein/ml, aliquoted, and snap frozen by immersion in liquid nitrogen. The membranes were stored at −70°C until use. Protein concentration was determined spectroscopically by Bradford protein assay.

2.3. Radiolabeling lipids

^{32}P-labeled phosphatidic acid, lysophosphatidic acid and *N*-oleoyl ethanolamine phosphoric acid were prepared by reacting dioleoyl glycerol (Sigma), mono-olein (NuChek Prep), or *N*-oleoyl ethanolamine (prepared as described by Lynch et al. [21]), respectively, with [γ-^{32}P]ATP in the presence of diacylglycerol kinase (Sigma) as described by Walsh et al. [20]. The method used here differs from the published protocol in that the reaction size was increased 10-fold and the reaction was allowed to proceed for 18 h. The labeled products were purified by normal phase HPLC using a Varian Microsorb MV, 4.6 mm × 250 mm, 5 μm silica column. Lipids were eluted using the following gradient profile: isocratic solvent A ($HCCl_3$/MeOH/H_2O/NH_4OH: 77/21.65/0.85/0.5) from 0–5 min, linear transition from solvent A to solvent B ($HCCl_3$/MeOH/H_2O/NH_4OH: 59/34.5/6/0.5) from 5–15 min, and isocratic solvent B from 15 min onward. The collected fractions were then washed with 0.1% HCl and MeOH and the organic phase was retained and dried.

2.4. Assay for PAP activity

Assays for phosphatase activity were carried out according to the protocol described by Kanoh et al. [1], using a constant Triton X-100:labeled lipid ratio of 50:1. The final specific activity of the lipids was adjusted to 10–50 Ci/mol. Briefly, 10 μg of membrane protein was prewarmed to 37°C in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 1 mg/ml fatty acid-free bovine serum albumin. Reactions were started by the addition of the radiolabeled lipid-Triton X-100 mixture, kept at 37°C for 5 min, and stopped by the addition of 100 μl 0.1 N HCl in methanol. 200 μl of $CHCl_3$ and 200 μl of 1 M $MgCl_2$ were then added, and the reactions were vortexed, centrifuged briefly, and a fraction of the aqueous layer was added to scintillation fluid for counting.

2.5. Northern blot

A multiple tissue Northern blot was purchased from Clontech Laboratories and hybridized with radiolabeled cDNA according to the manufacturer's instructions. PAP-2c cDNA was radiolabeled by priming DNA synthesis with random hexamers in the presence of α-[^{32}P]dATP and α-[^{32}P]dCTP (3000 Ci/mmol). The radiolabeled DNA was separated from unincorporated nucleotides on a G-50 Sephadex spun column.

3. Results and discussion

3.1. Construction of PAP-2c

We searched the dbEST with the published sequences of PAP-2a and PAP-2b [1] and identified and retrieved full length cDNAs encoding both enzymes. We also found a set of human EST sequences that were significantly similar, but not identical, to both PAP-2a and PAP-2b. From these sequences, we conceptualized a putative third human isoform of PAP-2. We obtained the cDNA of the first sequence in our contig from the I.M.A.G.E. Consortium [18], and found it encoded a full translational open reading frame (864 bp → 288 aa) of the hypothetical protein, which we named PAP-2c (Fig. 1A). PAP-2c is 58% identical to PAP-2a and 52% identical to PAP-2b. The three major regions of conservation with the peroxidase proteins, including the amino acids implicated in the phosphatase catalytic site, are conserved in the novel isoform, as are the consensus site for *N*-linked glycosylation and six putative transmembrane domains (Fig. 1B,C).

The activity of the three PAP isoenzymes was assayed using membranes harvested from HEK 293 T cells that had been transfected with the phosphatase DNAs. Membranes from cells transfected with each of the cloned PAP-2s had several fold higher activity than vector-DNA transfected cells (Fig. 2). These data demonstrate that our copies of the two previously reported PAP-2 cDNAs, as well as the cDNA for the putative

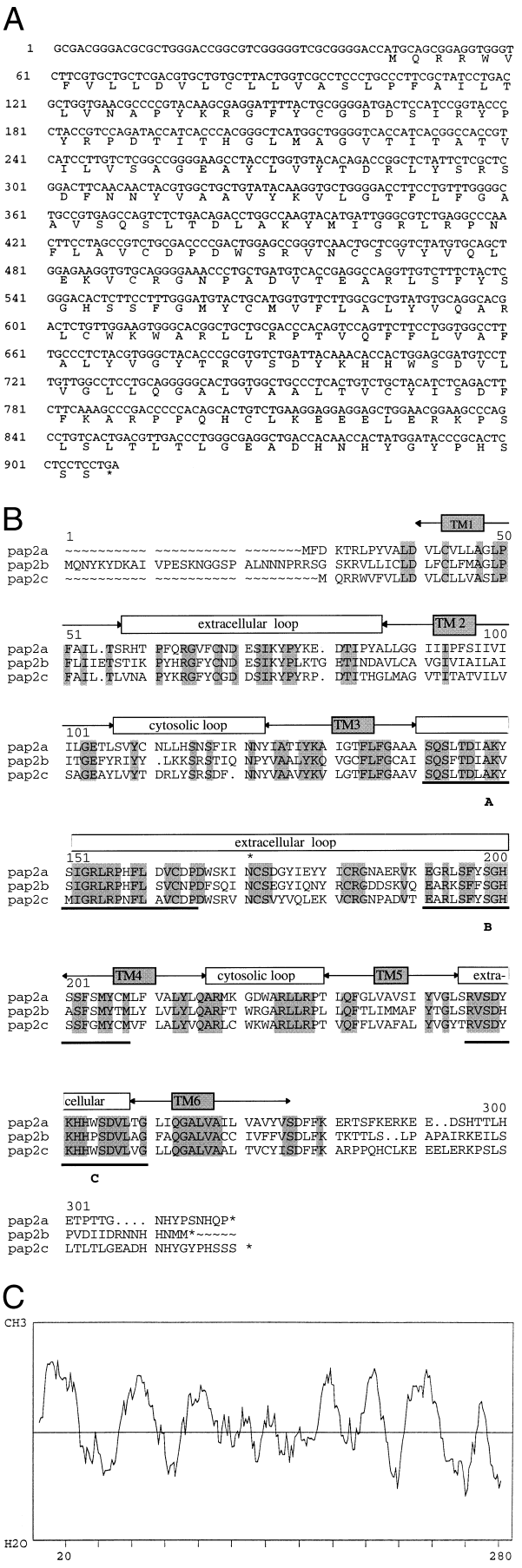

Fig. 1. A: DNA and conceptualized protein sequence of PAP-2c. B: Alignment of the three human PAP-2 protein sequences. Conserved residues are shaded, and the three major regions of similarity to the peroxidase family are labeled A, B, and C. *: glycosylation site. The alignment was constructed using the PILEUP algorith supported by the Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, Wisconsin. C: Hydropathy plot of PAP-2c protein sequence. A Kyte-Doolittle plot of the PAP-2c protein sequence (window size: 9) suggests six transmembrane domains. ←

third isoform, encode functional phosphatases in our assay system.

Confirming Kanoh's observation of the purified porcine PAP activity, we observed complete inhibition of all three isoforms with 5 mM sphingosine. Mono-oleoyl glycerol also inhibited PAP-2 activity, but to a lesser degree than sphingosine. However, generic phosphatase inhibitors, including sodium pyrophosphate, *para*-nitrophenyl-phosphate, and β-D-glycerol phosphate were unable to inhibit the activity of PAP-2a, -2b or -2c at concentrations up to 5 mM (data not shown).

3.2. Enzyme kinetics

Brindley's group reported broad substrate specificity in the PAP-2 they isolated from rat liver. Phosphatidic acid, lysophosphatidic acid, sphingosine 1-phosphate, and ceramide 1-phosphate were all hydrolyzed by the purified enzyme with K_m values between 0.4 and 4.0 μM [2]. Likewise, Kanoh et al. showed that recombinant human PAP-2a and PAP-2b are not specific for PA. When assayed at a single substrate concentration (200 μM) PAP-2a was most active with PA and LPA as substrates, displaying weak activity toward ceramide 1-phosphate and no activity toward sphingosine 1-phosphate. PAP-2b showed the highest activity with PA and S1P as substrates and had much lower activity when LPA or Cer1P were substrates [1]. To characterize the substrate specificity of the human isoforms of PAP, we developed kinetic profiles of the previously tested recombinant enzymes, as well as the novel PAP-2c, using ^{32}P-labeled PA, LPA and NOEPA as substrates.

NOEPA is an LPA mimetic with an ethanolamine rather than a glycerol backbone, making it metabolically more stable (i.e. it cannot be acylated) and more water soluble. NOEPA has been shown previously by us to be indistinguishable from LPA with respect to its activity in LPA functional assays [21]. We hypothesized that NOEPA is a naturally occurring lipid

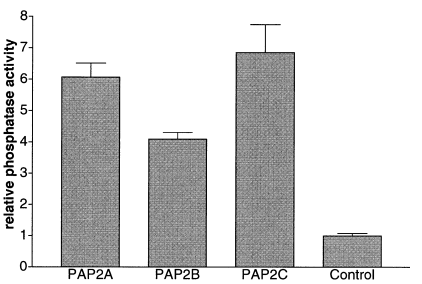

Fig. 2. The phosphatase activity of each membrane sample is shown relative to the activity in vector transfected cells. The data shown here are representative of several experiments, each performed in triplicate. Similar data were also obtained using LPA and NOEPA as substrates.

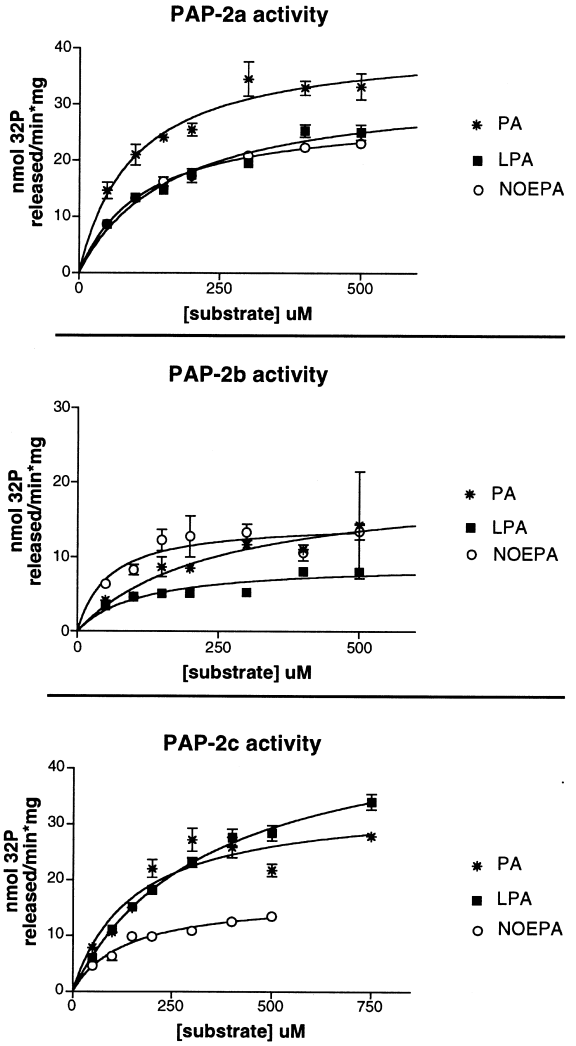

Fig. 3. The kinetic behavior of each of the three PAP-2 isoforms were determined with PA, LPA, and NOEPA as substrates. Each concentration was assayed in triplicate. The results were analyzed by Prism Graphpad, using a non-linear regression.

signaling molecule. This hypothesis predicts that NOEPA, like known lipid phosphoric acid mediators, must have a mechanism for its destruction. PAP-2 is a likely candidate to perform this function based on its activity toward the structurally similar compound LPA. If the ethanolamine backbone were acylated with a polyunsaturated fatty acid (e.g. arachidonic acid) the product of its hydrolysis by PAP-2 would be anandamide, the putative physiologic ligand for the G protein-coupled cannabinoid receptors, CB_1 and CB_2 [22].

The curves describing the kinetic profiles of PAP-2a, -2b, and -2c with PA, LPA, and NOEPA as substrates are shown in Fig. 3 and the derived V_{max} and K_m values are given in Table 1. The V_{max} for each isoform is dependent on the transfection efficiency, so these values cannot be used for meaningful comparison between the isoforms. They can be used, however, to compare the relative ability of the different isoforms to hydrolyze each substrate. For both PAP-2a and PAP-2b, the maximum activity of the enzymes are approximately 30% higher when PA is the substrate than when LPA is substrate. In PAP-2c transfected cells, however, the maximum activity is higher when LPA is used as a substrate. The maximum phosphatase activity when NOEPA is the substrate is remarkably lower than LPA or PA in PAP-2c transfected cells.

The affinity of the enzymes for each substrate, indicated by K_m values, is independent of the enzyme concentration and can therefore be compared between the different isoforms. All three recombinant isoforms have similar affinities for PA ($K_m \sim 100$–150 μM). LPA, however, appears to be a more selective substrate, with K_ms of 170 μM, 110 μM, and 340 μM for PAP-2a, -2b, and -2c, respectively. Remarkably, all three PAP-2 isoforms have a higher affinity for NOEPA than its structural analog, LPA.

The ability of the phosphatase to distinguish between LPA and NOEPA while the receptor responsible for mediating LPA signaling does not is potentially useful in further development of lipid phosphoric acid medicinal chemistry. The structural elements necessary for receptor binding may not be necessary for recognition by the phosphatase and vice versa. It may be possible, then, to design a functional LPA mimetic that cannot be hydrolyzed by one or more of the PAP-2 isoforms, a useful tool in investigating the role of both LPA and PAP-2.

3.3. PAP-2 mRNA expression

In Kanoh's characterization of human PAP-2a and -2b, Northern blot analysis showed expression of both mRNAs in a wide range of tissues. Of the 16 tissues tested, PAP-2a mRNA was detected in all except placenta, thymus, and leukocytes, with particularly high expression in prostate, while PAP-2b mRNA was expressed ubiquitously [1]. The high expression of PAP-2a in prostate is intriguing in view of the recent report that expression of PAP-2a is androgen dependent in the prostatic adenocarcinoma cell line, LNCaP [23].

In contrast, when the cDNA of PAP-2c was hybridized to a Northern blot panel of human poly(A)$^+$ RNA, it revealed a more restrictive expression pattern (Fig. 4). RNA of approximately 1.4 kb was detected in extracts of brain and pancreas, and a somewhat smaller ($\sim$1.3 kb) and more abundant mRNA was found in placenta. The EST cDNAs for human PAP-2c were derived from the following tissue libraries: pregnant uterus, fetal liver spleen, infant brain, ovary tumor, placenta and pancreas. The narrower expression pattern of PAP-2c may imply a more specialized role than the other two isoforms.

Finally, the kinetic data reported here, while useful in comparing the relative activity of the enzymes and their affinities for different substrates, has limited physiological relevance due to the presentation of the lipids as Triton X-100 micelles. Therefore, we are working currently to define PAP-2 activity with the substrates loaded onto albumin, as the bioactive

Table 1
K_m and V_{max} values for the three PAP isoforms

Substrate	PAP-2a		PAP-2b		PAP-2c	
	Km (uM)	Vmax (nmol/min*mg)	Km (uM)	Vmax (nmol/min*mg)	Km (uM)	Vmax (nmol/min*mg)
PA	98	41	100	13	150	34
LPA	170	33	110	9.2	340	49
NOEPA	116	29	56	15	138	17

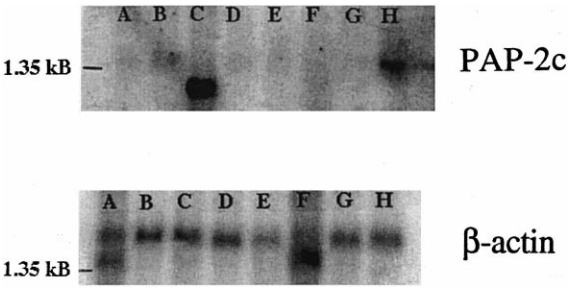

Fig. 4. Northern blot hybridization with PAP-2c DNA was performed with a CLONTECH multiple human tissue blot. Lanes contain 2 μg poly(A)$^+$ RNA from: A, heart; B, brain; C, placenta; D, lung; E, liver; F, skeletal muscle; G, kidney; H, pancreas. β-Actin cDNA was used as a control hybridization probe.

lipids may be presented to the surfaces of cells in vivo. Our characterization is also being expanded to include sphingosine 1-phosphate and other lipid signaling molecules.

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