Identification of hepatic peroxisomal phospholipase A_2 and characterization of arachidonic acid-containing choline glycerophospholipids in hepatic peroxisomes

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Abstract Recently, a sequence encoding a novel mammalian calcium-independent phospholipase A_2 (iPLA$_2\gamma$) was identified in the human genome and subsequently cloned and expressed in Sf9 insect cells. Unexpectedly, expression studies in recombinant systems demonstrated the usage of multiple translation initiation codons resulting in different polypeptides. Herein, we demonstrate that hepatic iPLA$_2\gamma$ is localized to rat liver peroxisomes, possesses a molecular mass of 63 kDa and that peroxisomal membranes are highly enriched in arachidonic acid-containing phospholipids. Collectively, these results provide the first demonstration of iPLA$_2\gamma$ in mammalian tissue and suggest the possibility that iPLA$_2\gamma$ can contribute to lipid second messenger generation by hydrolysis of peroxisomal arachidonic acid-containing phospholipids.

Key words: Phospholipase A_2; iPLA$_2\gamma$; Peroxisome; Arachidonic acid; Phospholipid; Phosphatidylcholine

1. Introduction

Phospholipases A_2 are critical enzymes in cellular lipid metabolism which regulate cellular energy storage, membrane function and the generation of lipid second messengers [1,2]. Recently, we described the genomic organization, mRNA sequence and enzymatic activity of a novel calcium-independent phospholipase A_2 (i.e. iPLA$_2\gamma$, (GenBank accession no. AF263613)) [3]. Analysis of the message sequence of iPLA$_2\gamma$ identified several potential translation initiation sites located at residues 226, 526, 589 and 886, resulting in 88, 77, 74 and 63 kDa protein products, respectively [3]. Accordingly, we cloned and expressed iPLA$_2\gamma$ in Sf9 cells utilizing a baculovirus construct designed to drive the expression of the 88 kDa isoform through usage of the ATG translation initiation codons at residue 226. However, this construct failed to produce the anticipated 88 kDa protein product and instead produced protein products utilizing ATG translation initiation codons at residues 526 and 886, resulting in 77 and 63 kDa polypeptides, respectively [3].

Sequence analysis demonstrated that iPLA$_2\gamma$ contained a C-terminal peroxisomal localization sequence (-SKL) and thus was predicted to be predominantly present in the peroxisomal compartment [3]. Peroxisomes fulfill a variety of lipid anabolic and catabolic functions in eukaryotic cells [4–8]. Since phospholipases A_2 catalyze the hydrolysis of a variety of lipids, including phospholipids [9], lysophospholipids [10] and acyl CoA derivatives [11], and thereby participate in the regulation of lipid metabolic flux and signaling, we reasoned that iPLA$_2\gamma$ may regulate lipid metabolic flux within the peroxisomal compartment. The present study was undertaken to identify and characterize the iPLA$_2\gamma$ protein naturally occurring in mammalian tissues. Specifically, we sought to determine the isoforms of iPLA$_2\gamma$ expressed in mammalian tissues (i.e. 88, 77, 74 or 63 kDa isoforms), confirm its anticipated subcellular localization in the peroxisomal compartment and gain insight into its potential physiological functions by analysis of peroxisomal membrane lipids using electrospray ionization mass spectrometry (ESI-MS).

We now report that iPLA$_2\gamma$ predominantly exists as a 63 kDa polypeptide in rat liver and that the 63 kDa iPLA$_2\gamma$ isoform predominantly localized to the peroxisomal compartment. We further demonstrate that choline- and ethanoamine-glycerophospholipids in hepatic peroxisomes contain a high level of arachidonic acid-containing species. Collectively, the demonstration of iPLA$_2\gamma$ in the peroxisomal compartment in close spatial proximity to arachidonic acid-containing phospholipids suggests a potential role for iPLA$_2\gamma$ in cellular lipid homeostasis and lipid second messenger generation.

2. Materials and methods

2.1. Materials

Horseradish peroxidase (HRP)-linked protein A and ECL™ Western blotting detection reagents were purchased from Amersham Biotech (Piscataway, NJ, USA). Gelman acrodisc CR polytetrafluoroethylene (PTFE) syringe filters (0.2 μm) were from Gelman Science (Ann Arbor, MI, USA). BCA (bicinchoninic acid) protein assay kits

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Abbreviations: ESI-MS, electrospray ionization mass spectrometry; MS/MS, tandem mass spectrometry; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; iPLA$_2\gamma$, calcium-independent phospholipase $A_2\gamma$

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were obtained from Pierce (Rockford, IL, USA). Antibodies against PMP70, calnexin, and porin were from Affinity Bioreagents (Golden, CO, USA), BD Transduction Laboratories (Lexington, KY, USA) and Molecular Probes (Eugene, OR, USA), respectively. The antibody against iPLA$_2\gamma$ was developed against peptide CENIPLDESRNEKLDQ and prepared as previously described [3]. The internal standards for ESI-MS were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Most other chemicals were from Sigma (St. Louis, MO, USA). Sprague–Dawley rats used for these experiments were obtained from Charles River Laboratory (Wilmington, MA, USA).

2.2. Isolation of peroxisomes

Peroxisomes were isolated from rat liver by differential and density gradient centrifugation [12]. Briefly, 5 g of rat liver was homogenized in three volumes (w/v) (15 ml) of homogenization buffer (0.25 M sucrose, 5 mM MOPS, pH 7.4, 1 mM EDTA and 0.1% (v/v) of ethanol, 0.2 mM dithiothreitol containing protease inhibitors (phenylmethylsulfonyl fluoride (0.2 mM), leupeptin (1 μg/ml), aprotinin (1 μg/ml) and phosphoramidon (15 μg/ml)). Liver was cut into pieces and homogenized at 1000 rpm with a single stroke using a Potter-Elvehjem homogenizer. The homogenate was centrifuged at $100\times g$ for 10 min to remove cellular debris. The pellet was re-homogenized and again centrifuged at $100\times g$ for 10 min. The supernatants were combined and centrifuged at $1000\times g$ for 10 min. The resultant pellet was resuspended in homogenization buffer and centrifuged similarly. The pellet was used as the nuclear fraction. The combined supernatants were pooled and centrifuged at $3000\times g$ for 20 min to produce the heavy mitochondrial fraction. The supernatant was centrifuged at $25\,300\times g$ to pellet a light mitochondrial fraction. The pellet was resuspended in homogenization buffer and again centrifuged at $25\,300\times g$ for 15 min. The supernatant of $25\,300\times g$ was centrifuged at $100\,000\times g$ to produce a microsomal pellet and cytosolic fraction. The light mitochondrial pellet was resuspended in 2.5 ml of homogenization buffer. The resuspension was layered on top of a discontinuous Nycodenz gradient (2 ml of 56% (w/v), 4 ml of 45% (w/v) and 16 ml of 30% (w/v) Nycodenz) prepared in the gradient buffer (homogenization buffer without sucrose). After centrifugation at $35\,000\times g$ for 1 h, the peroxisomal fraction present at the interface between the 30% and 45% Nycodenz layers was aspirated and used for subsequent experiments.

2.3. Western blotting

The relative enrichment and degree of contamination of the peroxisomal fraction was determined by comparing the intensity of marker proteins from different organelles by Western blot analysis. Proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [13] and transferred to Immobilon-P membranes in 10 mM CAPS buffer (pH 11) containing 10% methanol. Powdered milk (5% (w/v)) was used to block the non-specific binding sites prior to incubation with primary antibody. HRP-linked secondary antibodies or HRP-linked protein A were used in combination with an ECL detection system to visualize immunoreactive bands.

2.4. Phospholipid extraction

Phospholipids were extracted from isolated peroxisomes by a modified method of Bligh and Dyer [14]. Internal standards including 14:1-14:1 PtdCho (phosphatidylcholine; 40 nmol/mg protein), 15:0-15:0 PtdEtn (phosphatidylethanolamine; 25 nmol/mg protein) and 14:0-16:0 phosphatidylglycerol (6 nmol/mg protein) were added to the chloroform extract for quantitation of choline-containing, ethanolamine-containing and anionic phospholipids, respectively [15,16]. The lipid extracts were dried under a nitrogen stream, dissolved in chloroform, filtered with PTFE syringe filters and dried again under a N_2 stream. The final lipid residue was resuspended in 0.2 ml of 1:1 chloroform/methanol prior to ESI-MS analysis.

2.5. ESI-MS of phospholipids

ESI-MS analyses were performed on a Finnigan TSQ-7000 spectrometer equipped with an electrospray ion source, as previously described [16,17]. Diluted samples were infused into the ESI source at the flow rate of 1 μl/min with a Harvard syringe pump. Anionic phospholipids were quantitated in the negative ion mode by comparison with the internal standards. After rendering the solution basic with LiOH in methanol (50 nmol/mg of protein), ethanolamine glycerophospholipids were quantitatively analyzed in the negative-ion mode, and choline glycerophospholipids were quantified as their lithium adducts in the positive-ion mode by comparisons with internal standards. Data were corrected for the ^{13}C isotope effects as previously described [18].

2.6. Protein assay

Protein concentration was determined utilizing a BCA protein assay kit using bovine serum albumin as standard according to the manufacturer's instruction.

3. Results

3.1. Characterization of peroxisomes

Since Northern analysis of mammalian tissues identified the presence of iPLA$_2\gamma$ message in multiple tissues, including heart, brain, liver, and pancreas [3], we initially attempted to identify the molecular mass(es) of the expressed polypeptides from Western blot analysis of the membrane fractions derived from these tissues. However, no immunoreactive bands could be visualized in crude membrane preparations due to the low abundance of iPLA$_2\gamma$. Accordingly, we purified rat hepatic peroxisomes by established procedures (i.e. Nycodenz discontinuous gradient centrifugation) [12]. To determine the purity of the peroxisomal preparation, Western blot analyses were performed to demonstrate the relative enrichment of peroxisomes and degree of cross contamination by other subcellular organelles using antibodies against the subcellular markers PMP70 (peroxisomes) [19,20], porin (mitochondria) [21] and calnexin (endoplasmic reticulum) [22,23] (Fig. 1). The high intensity of PMP70 and the corresponding relatively low intensities of calnexin and porin in the peroxisomal fraction indicated that highly purified peroxisomes were obtained and used in our studies (Fig. 1).

3.2. Detection of iPLA$_2\gamma$ in mammalian peroxisomes

To determine the subcellular localization of iPLA$_2\gamma$ and to identify the molecular mass(es) of its isoforms in mammalian tissues, proteins of each fraction were separated by SDS–PAGE and analyzed by Western blotting. Probing with antibody directed against the iPLA$_2\gamma$ peptide CENIPLDESRNEKLDQ (735–750) [3] demonstrated that a 63 kDa protein was present in the isolated rat liver peroxisomes (Fig. 2) with no observable signal in the other fractions. This represents the first identification of the mass of naturally occurring iPLA$_2\gamma$

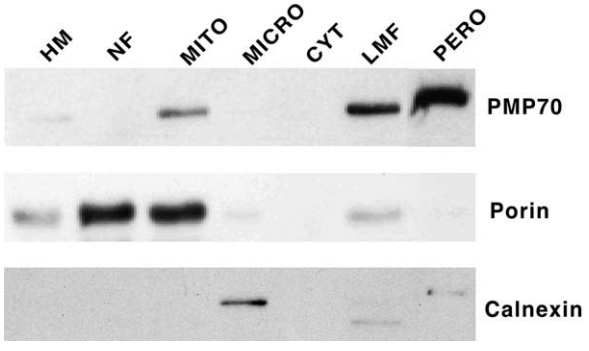

Fig. 1. Demonstration of the purity of isolated peroxisomes. Equal amounts of protein (20 μg) from rat liver homogenate (HM), nuclei (NF), mitochondria (MITO), microsomes (MICRO), cytosol (CYT), light mitochondrial fraction (LMF) and peroxisomes (PERO) were separated by SDS–PAGE, transferred to Immobilon-P membranes, probed with antibodies against PMP70, porin and calnexin, and visualized by enhanced chemiluminescence detection.

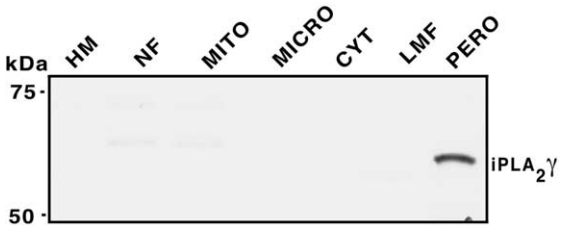

Fig. 2. Characterization of iPLA$_2$γ by Western blotting. Equal amounts of proteins (20 μg) from individual subcellular fractions were loaded onto SDS polyacrylamide gel, electrophoresed and transferred to Immobilon-P membranes. The blot was probed with the antibody directed against iPLA$_2$γ, demonstrating a robust signal which co-purified with the peroxisomal marker PMP70.

(i.e. that present in non-recombinant systems) and the first demonstration that iPLA$_2$γ is localized in the peroxisomal compartment.

3.3. ESI-MS analysis of phospholipid molecular species in rat liver peroxisomes

Lipid extracts from rat liver peroxisomes were quantitatively analyzed by ESI-MS and ESI tandem mass spectrometry (MS/MS) as previously described [16,17]. The presence of phosphatidylserine (PtdSer) molecular species was verified by identifying the neutral loss of 87 in the negative-ion mode, and peaks corresponding to phosphatidylinositol (PtdIns) were verified by the product ion of *m*/*z* 241 in the negative-ion mode (data not shown). Choline containing lipids (PtdCho and sphingomyelin) were verified by MS/MS through demonstrating the neutral loss of 183 in the positive-ion mode (Fig. 3). Quantitation of each phospholipid class was accomplished by comparisons with internal standards. As summarized in Table 1, purified peroxisomes contain 105 nmol of phospholipid/mg protein. The predominant phospholipid classes are choline-(PtdCho) and ethanolamine-containing (PtdEth) glycerophospholipids. PtdCho is the most abundant class, representing 60% of the total phospholipids. PtdEth represents 38% of total phospholipids. Thus PtdCho and PtdEth collectively constitute ~98% of the phospholipids in hepatic peroxisomes. The low anionic phospholipid content of peroxisomes (2%) is quite unusual amongst intracellular membrane fractions we and others have examined [16,24]. Moreover, detectable amounts of ether lipids were not observed. No detectable sphingomyelin or cardiolipin was observed, attesting to the low plasma membrane or mitochondrial contamination in the peroxisomal preparations utilized

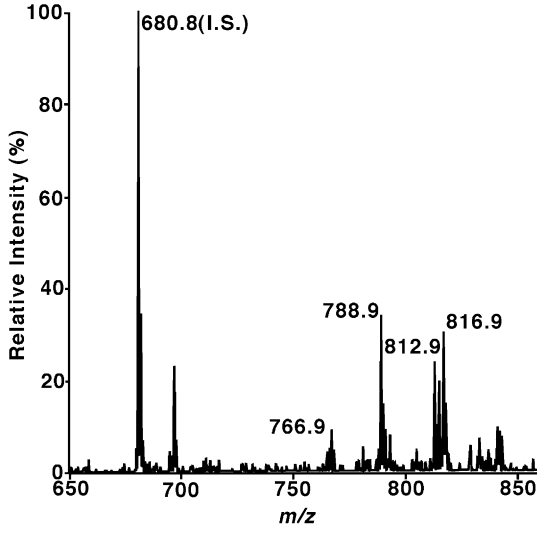

Fig. 3. Positive-ion EIS-MS/MS spectra of choline glycerophospholipids in lipid extracts of rat liver peroxisomal fraction. Lipid extracts were prepared and analyzed in the positive-ion mode by ESI-MS/MS employing the neutral loss of 183 as described in Section 2. The internal standard is 14:1-14:1 PtdCho (*m*/*z* of $[M+Li]^+$ is 681). Lithiated PtdCho molecular species were identified by product-ion ESI-MS and are tabulated in Table 1.

and further underscoring its purity [25]. The predominant molecular species of PtdCho and PtdEth were quite similar, which is also unusual for most mammalian membrane fractions we have examined [16,24]. The presence of highly unsaturated fatty acids (i.e. arachidonic acid and cervonic acid) was observed in both classes. The dominant PtdCho molecular species are 18:0-20:4, 16:0-20:4 and 18:1-20:4, accounting for 66% of the total choline-containing phospholipids (Fig. 3 and Table 1). No disaturated fatty acid-containing species were observed in any of the phospholipid classes. PtdIns was the major anionic phospholipid species present and 18:0-20:4 PtdIns was the only detectable molecular species present.

4. Discussion

The initial insights leading to the identification of a second calcium-independent phospholipase A$_2$ (i.e. iPLA$_2$γ) in mammalian tissues were gleaned from the sequencing of the human genome which identified a lipase consensus sequence present on chromosome 7. Analysis of 5′ and 3′ neighboring sequences provisionally identified a 40 kDa protein product spanning from physical markers 79 671 to 109 912 on chromosome 7.

Table 1
Phospholipid content of rat liver peroxisomes

PtdCho	nmol/mg protein	mol%	PtdEtn	nmol/mg protein	mol%	Anionic phospholipids	nmol/mg protein	mol%
16:0-18:1	5.09 ± 1.00	4.8	16:1-18:1	4.16 ± 0.88	4.0	PtdSer		
16:0-20:4	19.18 ± 4.00	18.3	16:0-20:4	5.57 ± 0.62	5.3	18:1-22:6	0.60 ± 0.06	0.6
18:1-18:1/18:0-18:2	3.49 ± 0.26	3.3	18:1-18:1/18:0-18:2	2.92 ± 0.25	2.8			
16:0-22:6	8.88 ± 1.50	8.5	16:0-22:6	7.02 ± 1.25	6.7	PtdIns		
18:1-20:4	7.10 ± 1.00	6.8	18:1-20:4	5.40 ± 1.21	5.1	18:0-20:4	1.50 ± 0.33	1.4
18:0-20:4	15.54 ± 1.80	14.8	18:0-20:4	9.72 ± 1.88	9.3			
18:0-22:6	4.07 ± 0.40	3.9	18:0-22:6	4.72 ± 0.38	4.5			
Total	63.35	60.4	Total	39.51	37.6	Total	2.10	2.0

Lipids were extracted from rat liver peroxisomes and quantitatively analyzed by ESI-MS as described in Section 2. Results are reported as the mean ± S.E.M. The first number in each pair refers to the number of carbon atoms in the fatty acid chain. The number after the colon refers to the number of double bonds.

Based on those initial insights, we identified the predicted complete amino acid sequence of iPLA$_2\gamma$ [3]. Accordingly, we constructed a baculovirus expression vector designed for the expression of a predicted 88 kDa protein product. Remarkably, infection of Sf9 cells with this vector led to the production of ~77 and 63 kDa protein products with no evidence for the predicted 88 kDa polypeptide [3]. However, naturally occurring (i.e. non-recombinant) iPLA$_2\gamma$ protein has not previously been identified and thus the types of iPLA$_2\gamma$ translated in mammalian cells were not known. In the present study, we demonstrated for the first time that iPLA$_2\gamma$ is present in rat liver peroxisomes and possesses a molecular weight of 63 kDa. The localization of iPLA$_2\gamma$ to the peroxisomal compartment was not unanticipated because iPLA$_2\gamma$ contains a C-terminal SKL peroxisomal targeting sequence [3].

The functional role of iPLA$_2\gamma$ in peroxisomal lipid metabolism is unknown at present. Since the directed regulation of the flux of fatty acids in the peroxisomal compartment into either lipid oxidative or synthetic pathways seems essential to cellular energy storage and utilization [4–8], the enzymes that direct the flow of these metabolites into anabolic or catabolic pathways are candidate enzymes for regulatory determinants of obesity. Considering the significant roles which phospholipases A$_2$ play in cellular function in many systems [1,2] and the localization of iPLA$_2\gamma$ to the peroxisomal fraction, we propose that iPLA$_2\gamma$ may participate in the regulation of cellular energy storage and utilization. Indeed, Hajra et al. demonstrated that approximately half of cellular triacylglycerides originated through initial lipid anabolic synthetic steps in the peroxisomal compartment [8]. Thus, it seems likely that the 63 kDa iPLA$_2\gamma$ isoform contributes to and regulates the flux of aliphatic chains into cellular triacylglycerol pools.

Having purified peroxisomes in hand, we exploited recent advances in ESI-MS to gain insight into the potential roles of iPLA$_2\gamma$ function in mammalian tissues from the molecular species analysis of peroxisomal membrane lipids. The results of ESI-MS analysis were remarkable for the high content of choline glycerolphospholipids containing arachidonic acid in peroxisomal membranes. Approximately 66% of peroxisomal choline glycerophospholipids contained arachidonic acid, which is higher than that from multiple other subcellular fractions we have analyzed [16,24]. Since recombinant iPLA$_2\gamma$ actively catalyzes the hydrolysis of arachidonic acid-containing choline glycerophospholipids at the *sn*-2 position [3], and those lipids are in close spatial juxtaposition to iPLA$_2\gamma$, this raises the intriguing possibility that peroxisomal membranes represent a potential source for arachidonic acid released during cellular perturbations. Thus, iPLA$_2\gamma$ is also a candidate enzyme potentially contributing to the production of biologically active eicosanoids. The recent identification of chiral mechanism-based inhibitors which discriminate iPLA$_2\beta$ activity from iPLA$_2\gamma$ activity can thus be utilized to assess the role(s) of each enzyme in cellular lipid metabolism [26]. Finally, the mechanisms through which lipid precursors originating in the peroxisomal compartment are trafficked to their subsequent intracellular destinations has not been defined. Since no ESI-MS evidence for the accumulation of lysophosphatidic acid, alkyl-DHAP or alkyl glycerophosphates were observed, the present results are consistent with the notion that peroxisomal lipid synthetic precursors are transported (either actively or passively) to their sites of final lipid covalent assembly faster than they accumulate in the peroxisomal membrane [8].

In summary, the present study identifies iPLA$_2\gamma$ in rat liver as a 63 kDa protein product, demonstrates the predominant peroxisomal localization of this polypeptide and identifies arachidonic acid-containing choline glycerophospholipids in close spatial proximity to iPLA$_2\gamma$, thereby rendering this pool a potential contributor to eicosanoid release in mammalian cells after stimulation.

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