Cloning, differential regulation and tissue distribution of alternatively spliced isoforms of ADP-ribosylation-factor-dependent phospholipase D from rat liver

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An alternatively spliced isoform of ADP-ribosylation-factor-dependent phospholipase D (PLD1) was previously shown to occur in rat C6 cells [Yoshimura, Nakashima, Ohguchi, Sakai, Shinoda, Sakai and Nozawa (1996) Biochem. Biophys. Res. Commun. **225**, 494–499] and human HeLa cells [Hammond, Jenco, Nakashima, Cadwallader, Gu, Cook, Nozawa, Prestwich, Frohman and Morris (1997) J. Biol. Chem. **272**, 3860–3868]. However, its complete sequence and the enzymological difference between the two PLD1 isoforms were unclear. Here we report the cloning, complete sequence, enzymological properties and tissue distribution of each of the two alternatively spliced PLD1 isoforms, a and b, from rat liver. The major difference between the two isoforms was the deletion of 38 amino acids in the b isoform, but otherwise the two cDNA sequences were 99.9% identical. The a-isoform sequence was 91% identical with the a form of human PLD1, and the 38-amino-acid deletion in the b form occurred at the same site as in the b form of human PLD1. Both of the rat PLD1 isoforms expressed in the fission yeast *Schizosaccharomyces pombe* were dependent on ADP-ribosylation factor 1 and phosphatidylinositol 4,5-bisphosphate. The a isoform was activated by RhoA in a synergistic manner with ADP-ribosylation factor 1, whereas the b isoform was less responsive to RhoA. Reverse transcription PCR showed that the b form was the predominant PLD1 isoform expressed in rat tissues. The b-form transcript occurred in various rat tissues, including lung, brain, liver, kidney, small intestine and colon, whereas the a-form transcript was only detectable in lung, heart and spleen. Both transcripts were hardly detectable in thymus, stomach, testis and muscle. Thus the two PLD1 isoforms were differently regulated and expressed in rat tissues.

INTRODUCTION

Phospholipase D (PLD), which catalyses the hydrolysis of phosphatidylcholine (PC) to phosphatidic acid and choline [1], has been implicated in the signal transduction, membrane trafficking and mitosis of eukaryotic cells (for reviews, see [2]). Evidence indicates that mammalian tissues contain multiple, distinctly regulated PLD isoforms that are likely to play different physiological roles. Based on the sensitivity to various activators, PLD isoforms can be classified into two large groups, a small G-protein-stimulated form and an oleate-stimulated form [3,4]. Although ADP-ribosylation factor (ARF) [5,6], RhoA [7,8], Ral [9] and Rac1 [10] were reported to be involved in the activation of PLD, most extensively studied are the effects of ARF and RhoA. Siddiqi et al. [11] reported that PLD activity in HL-60 cytosol was activated by ARF, but not by RhoA, whereas PLD activity in HL-60 membranes was synergistically activated by ARF and RhoA. PLD synergistically activated by ARF and RhoA was partially purified from rat brain membranes [12,13]. Thus at least two isoforms, one requiring ARF and the other requiring both ARF and RhoA, are included in the small G-protein-dependent PLDs. A cDNA encoding the latter type of PLD was cloned by Hammond et al. [14], who obtained the cDNA encoding 1074 amino acid residues from HeLa cells by taking advantage of a human-expressed sequence tag encoding a peptide resembling yeast PLD [15]. The enzyme expressed in Sf9 insect cells was dependent on phosphatidylinositol 4,5-bisphosphate (PIP_2) and was synergistically activated by ARF1 and RhoA. The activity was strongly inhibited by oleate, as in the case of the rat brain enzyme [16]. This enzyme was initially designated phospholipase D1 (PLD1), but later it was renamed PLD1a because its splice variant, PLD1b, carrying a 38-amino-acid deletion, was identified in rat [17] and human cells [18].

A second type of mammalian PLD called phospholipase D2 (PLD2) was more recently cloned and characterized from rat brain [19] and mouse embryo [20]. Rat PLD2 was obtained by PCR using degenerate primers corresponding to the conserved regions of eukaryote PLDs [19]. Mouse PLD2 was obtained by hybridization of a human PLD1a (hPLD1a) cDNA fragment [20] as the probe. The deduced amino acid sequences showed significant similarity to hPLD1a, but a large deletion was noted in the middle of the sequence. When the rat and mouse sequences were expressed in the fission yeast *Schizosaccharomyces pombe* [19] or Sf9 insect cells [20] respectively, PLD activity was greatly increased. PLD2 was dependent on PIP_2 and was inhibited by oleate. However, PLD2 activity was not stimulated by small G-proteins, such as ARF and RhoA.

The present paper reports the complete sequences and expression in the fission yeast *S. pombe* of two alternatively spliced rat PLD1 isoforms, rat PLD1a (rPLD1a) and rPLD1b. The

Abbreviations used: PLD, phospholipase D; PC, phosphatidylcholine; ARF, ADP-ribosylation factor; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLD1, phospholipase D1; PLD1a(b), phospholipase D1a(b); PLD2, phospholipase D2; hPLD1a(b), human phospholipase D1a(b); rPLD1a(b), rat phospholipase D1a(b); PE, phosphatidylethanolamine; GTP[S], guanosine 5′-[γ-thio]-triphosphate; RT-PCR, reverse-transcription PCR; ORF, open reading frame.

The nucleotide sequence data reported appears in the DDJB Nucleotide Sequence Databases under the accession numbers AB000778 (rPLD1a) and AB000779 (rPLD1b).

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major difference was the deletion of 38 amino acids in the rPLD1b isoform, but otherwise the two cDNA sequences were 99.9 % identical. The studies of the expressed enzymes showed that rPLD1a required ARF1, RhoA and PIP_2 for full activity, whereas rPLD1b was rather insensitive to RhoA, being highly activated by ARF1 and PIP_2. We also show that the tissue distribution of mRNAs encoding rPLD1a and rPLD1b are markedly different. Although rPLD1a mRNA was expressed only in rather confined tissues, such as lung, heart and spleen, rPLD1b mRNA occurred more widely in rat tissues.

EXPERIMENTAL

Construction of a rat liver cDNA library

Total RNA was extracted from a male rat liver using TRIZOL reagent (Life Technologies, Grand Island, NY, U.S.A.) according to the manufacturer's instructions. Poly(A)$^+$ RNA was obtained from total RNA using Oligotex-dT30 Super (Japan Roche, Tokyo, Japan). Oligo(dT)-primed cDNA was synthesized using a cDNA synthesis kit (Life Technologies). cDNAs were ligated to the λZAPII arms (Stratagene, La Jolla, CA, U.S.A.) and packaged using the GigaPack ll Gold packaging extracts (Stratagene).

Screening of the rat liver cDNA library

A human-expressed sequence tag (GenBank R93485) [21] containing a partial sequence of hPLD1 [14] was used as the screening probe. Plaques formed from a rat liver λZAPII cDNA library were transferred to Hybond N+ membranes (Amersham International, Amersham, Bucks., U.K.) and hybridized with the digoxigenin-labelled R93485 insert at 68 °C overnight in a solution containing 5 × SSC (1 × SSC = 0.15 M NaCl/0.015 M sodium citrate), 1 % (v/v) blocking reagent (Boehringer Mannheim, Mannheim, Germany), 0.02 % (v/v) SDS and 0.1 % (v/v) sodium *N*-lauroylsarcosine. The filters were washed twice with 2 × SSC in 0.1 % SDS at room temperature and twice in 0.1 × SSC in 0.1 % SDS at 50 °C. Positive phages were located using a digoxigenin-labelled nucleic acid detection kit (Boehringer Mannheim) according to the manufacturer's instructions. Plasmids were obtained from the isolated phages by excision *in vivo*. Clone DL1 thus obtained (Figure 1) was cleaved with *Bam*HI (one site within the insert and the other site in the vector) and the excised 500 bp fragment was used as the probe to screen a rat liver λZAPII cDNA library for the full-length cDNA under similar screening conditions. Both strands of the cDNA obtained (DL2e and DL2i) were sequenced using a DNA sequencing kit (Perkin-Elmer, Foster, CA, U.S.A.) on a 373A DNA sequencer (Perkin-Elmer) after subcloning into pBluescript II (Stratgene).

Expression of rPLD1a and rPLD1b in *S. pombe*

The *Apa*I fragments containing the entire open reading frames (ORFs) of rPLD1a (3.6 kbp) and rPLD1b (3.5 kbp) were isolated from DL2e and DL2i and were subcloned into the *Apa*I site of pREP4KS vector to yield pREP4KS-rPLD1a and pREP4KS-rPLD1b respectively. pREP4KS was a derivative of the *S. pombe* expression vector, pREP4 [22], containing the *ura*4$^+$ marker and thiamin-repressible promoter. *S. pombe* strain TKP1 (*h*$^{*-}$ *ade6-704 leu1-32 ura4-D18*) was used as the host for transformation. Edinburgh minimal medium and YES medium were used for culture [23]. Where required, adenine, L-leucine, uracil and thiamin were added at concentrations of 100, 100, 50 and 5 mg/l respectively. Yeast cells were grown aerobically at 32 °C and were transformed by the lithium acetate method as described [24]. Standard procedures for *S. pombe* manipulation were as described [23].

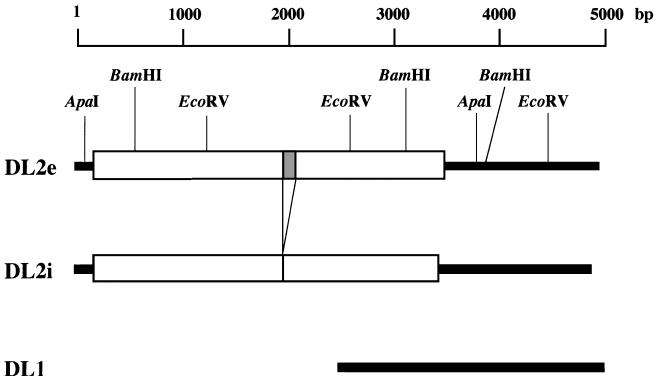

Figure 1 Restriction map of rPLD1a and rPLD1b cDNA

The open box and thick line indicate the coding and non-coding sequences respectively. The stippled box indicates the deleted region in DL2i.

Preparation of *S. pombe* extract and cell fractions

Yeast cells grown in Edinburgh minimal medium containing thiamin were washed three times with the thiamin-free medium, cultured in 100 ml of the same medium for 24 h at 32 °C and then harvested by centrifugation at 2000 ***g*** for 5 min. The cells were washed with water and suspended in 1 ml of extraction buffer containing 50 mM Hepes/NaOH, pH 7.0, 1 mM EDTA, 1 mM EGTA, 0.1 mM dithiothreitol, 2 μM (*p*-amidinophenyl)methanesulphonyl fluoride and 300 mM sucrose. The cells were disrupted by vigorously vortexing for 1 min four times with 1.5 g of glass beads (diameter, 0.3 mm) at 4 °C. The beads and cell debris were removed by centrifugation at 2000 ***g*** for 10 min and the supernatant was centrifuged at 100000 ***g*** for 60 min to separate the cytosolic and membrane fractions. The membrane fraction was suspended in 0.2 ml of the extraction buffer. Samples were kept at 4 °C until use.

PLD assay

PLD activities in the cell extracts were determined under the assay conditions essentially as described [5] by measuring the transphosphatidylation activity in the presence of ethanol [25–27]. The standard assay mixture, containing 50 mM Hepes/NaOH, pH 7.5, 200 mM NaCl, 3 mM $MgCl_2$, 2 mM $CaCl_2$, 3 mM EGTA, 1 mM dithiothreitol, 400 mM ethanol, 140 μM phosphatidylethanolamine (PE), 12 μM PIP_2 and 8.6 μM 1-palmitoyl-2-[1-^{14}C]palmitoyl-PC (130 d.p.m./pmol) (Amersham International) in a total volume of 100 μl, was incubated for 60 min at 37 °C. PC, PIP_2 and PE were added as mixed micelles as described [5]. Guanosine 5′-[γ-thio]-triphosphate (GTP[S]), ARF1 and RhoA were added as indicated. The reaction was stopped with 100 μl of 1 M HCl and the lipid product was extracted with 1 ml of chloroform/methanol (2:1, by vol.) and washed with 0.5 ml of 170 mM NaCl. The lower phase was isolated, concentrated in a centrifugal vaporizer (Tokyo Rika, Tokyo, Japan) and separated on a Silica Gel 60 TLC plate (Merck, Darmstadt, Germany) with chloroform/methanol/acetic acid (13:3:1, by vol.) as the developing solvent. The area containing phosphatidylethanol was scraped and the radioactivity was counted. Recombinant ARF1 was prepared from *Escherichia coli* expressing human ARF1 and yeast myristoyl-CoA: protein *N*-myristoyltransferase [28], as described by Randazzo et al. [29]. Isoprenylated RhoA was prepared from Sf9 cells expressing human RhoA, as described by Mizuno et al. [30].

Reverse transcription PCR (RT-PCR)

Total RNA (0.1 μg) was reverse-transcribed and amplified by PCR using a one-step RT-PCR kit (Toyobo, Osaka, Japan) according to the manufacturer's instruction. RT was carried out at 60 °C for 30 min and PCR amplification was performed at 94 °C for 1 min, 56 °C for 1 min and 60 °C for 1.5 min for 40 cycles using primers: 5′-GGACGGTGGGATGACAATGAGC-3′ (sense primer, nucleotide positions 1648–1669 of rPLD1a), 5′-ATGCGCTGTGGCTTGAGACTTT-3′ (antisense primer, nucleotide positions 2358–2379 of rPLD1a), or a pair of primers for glycerol 3-phosphate dehydrogenase supplied by the manufacturer.

RESULTS

Cloning of rat PLD1a and PLD1b cDNAs

In order to isolate PLD1 cDNA from rat liver, we screened a rat liver λZAPII cDNA library using an expressed sequence tag (GenBank R93485) for human PLD1a [14] identified by a BLAST search [31] through the DNA databases. After screening of 1×10^5 plaques, one positive clone, designated DL1, was obtained (2.5 kbp, Figure 1), of which the 5′ sequence was then used for a second round of cDNA cloning. Ten clones (DL2a–DL2j) were obtained by screening of 2×10^5 plaques and analysed by restriction enzymes and nucleotide sequencing. Clone DL2e with the largest insert (5.0 kbp, Figure 1) contained a single large ORF capable of encoding 1074 amino acids with a calculated relative molecular mass of 123785. The predicted amino acid sequence was 91 % identical with the entire sequence of hPLD1a [14]. Thus the encoded protein was designated rPLD1a. Restriction analysis revealed that four of the clones obtained above carried a deletion of about 100 bp between the two adjacent *Eco*RV sites in the ORF. The largest of the four, DL2i (4.9 kbp, Figure 1), contained a single large ORF encoding 1036 amino acids with a calculated relative molecular mass of 119355. The major difference between DL2i and DL2e was a deletion of 38 amino acid residues in the ORF of DL2i (Figure 2). The site of the 38-amino-acid deletion was located at the same position as seen in hPLD1b [18]. Thus DL2i was thought to be the species variant of hPLD1b and was designated rPLD1b. Direct sequence comparison between rPLD1b and hPLD1b could not be made

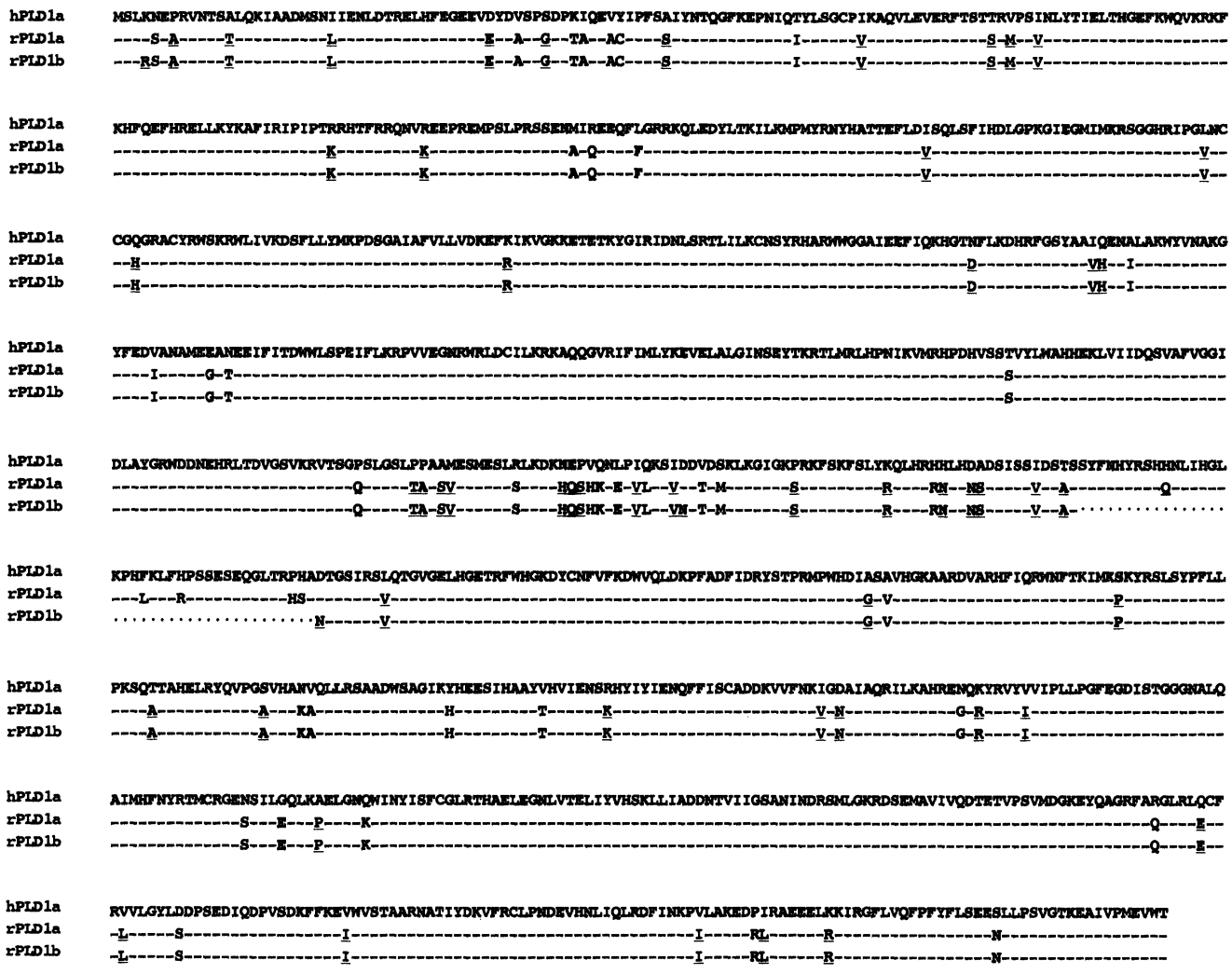

Figure 2 Alignment of amino acid sequences of PLD isoforms

The deduced amino acid sequences of rPLD1a and rPLD1b are aligned to that of hPLD1a. Identical amino acids are designated by broken lines. Gaps are indicated by dots and conservative substitutions of amino acids are underlined.

Table 1 Expression of rPLD1a and rPLD1b in *S. pombe* cells

S. pombe TKP1 cells were transformed with pREP4KS-rPLD1a (rPLD1a), pREP4KS-rPLD1b (rPLD1b) or pREP4KS (control) and then cultured for 24 h in the absence of thiamin to induce rPLD1a and rPLD1b. The cytosol and membrane fractions were prepared and 0.5 μg of each fraction was used for PLD assay with and without 1 μM ARF1, as described in the Experimental section. Data are representative of five experiments.

		Activity (nmol/min per mg of protein)	
Cell	Fraction	No ARF1	ARF1
Control	Cytosol	0.04	0.08
	Membrane	0.03	0.05
rPLD1a	Cytosol	0.04	0.25
	Membrane	0.07	1.48
rPLD1b	Cytosol	0.05	0.23
	Membrane	0.10	1.68

Table 2 Stimulation of rPLD1a or rPLD1b by GTP[S], PIP_2, ARF1 and RhoA

S. pombe TKP1 cells expressing rPLD1a or rPLD1b were disrupted and fractionated, and then 0.5 μg of the membrane fraction was assayed for PLD activity as described in the Experimental section, except that the reaction mixture was modified as follows: the basic mixture was prepared by removing PIP_2 from the standard reaction mixture, supplemented as indicated, and used for the assay. PIP_2, ARF1 and RhoA were added at final concentrations of 12, 1 and 0.05 μM respectively. Data are representative of two experiments.

Addition			Activity (nmol/min per mg of protein)	
ARF1	RhoA	PIP_2	rPLD1a	rPLD1b
+	+	+	1.97	1.04
−	+	+	0.25	0.18
+	−	+	0.75	0.90
+	+	−	0.29	0.08
+	−	−	0.12	0.04
−	+	−	0.04	0.05
−	−	+	0.11	0.09
−	−	−	0.03	0.02

because the complete sequence of the latter has not been reported. As shown in Figure 2, three amino acid substitutions were noted in rPLD1b when compared with rPLD1a. One of the substitutions was found at the junction site and the other two were due to single nucleotide substitutions. Overall, the two cDNA sequences showed three nucleotide mismatches in the 4371 bp overlap. The reason for the mismatches was unknown.

Expression and characterization of rPLD1a and rPLD1b

We next sought to express cDNAs for rPLD1a and rPLD1b using the *S. pombe* expression system, which has been used to express several mammalian cDNAs, for example for protein kinase C [32], phosphatidylinositol 3-kinase [33] and more recently rat PLD2 [19]. The *Apa*I fragments containing the entire ORFs of rPLD1a (3.6 kbp) and rPLD1b (3.5 kbp) were ligated into the pREP4KS *S. pombe* expression vector containing the thiamin-repressible promoter [22] to yield pREP4KS-rPLD1a and pREP4KS-rPLD1b respectively. *S. pombe* TKP1 cells (*h*$^{*-}$ *ade*6-704 *leu*1-32 *ura*4-*D*18) were transfected with either vector alone or with either one of the constructs, and then induced to express the cloned cDNAs in thiamin-free medium. The transformants were disrupted by vortexing with glass beads, fractionated into the cytosol and membrane fractions and then assayed for PLD activity by measuring the transphosphatidylation [25–27] using labelled PC mixed with PIP_2–PE micelles in the presence of ethanol [5]. The phosphatidylethanol formed was separated by TLC and the radioactivity was counted. As shown in Table 1, marked PLD activities were detectable in the membrane fractions of the pREP4KS-rPLD1a and pREP4KS-rPLD1b transfectants in the presence of ARF1. When ARF1 was omitted from the assay mixture, activity decreased to a level of only 5–6 %. Thus both of the PLDs expressed from pREP4KS-rPLD1a and pREP4KS-rPLD1b were almost completely dependent on ARF1. When the cytosol fraction was used for assay, the formation of phosphatidylethanol decreased markedly. The addition of the membrane fraction from the control cells to the cytosol of rPLD1a and rPLD1b transfectants did not increase the PLD activities of the cytosols (results not shown), thereby excluding the possibility that the cytosols require the membrane component for activity. Thus the major portion of the expressed PLD activity was localized in the membrane fraction of the transfectants, although neither the rPLD1a nor the rPLD1b sequence contained any extended hydrophobic amino acid stretch thought to be membrane-associated or membrane-spanning, as examined by the method of Kyte and Doolittle [34]. PLD activity in the membrane fraction of the vector control was as low as 3 % of the levels of the transfectants, thus confirming that both cDNAs encoded ARF1-dependent PLD.

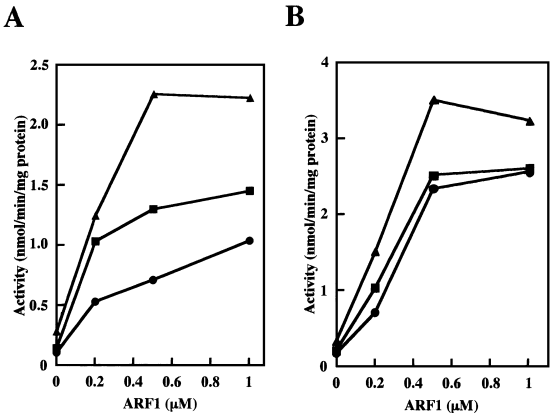

Figure 3 Effects of ARF1 and RhoA on rPLD1a and rPLD1b

The membrane fraction (0.5 μg) of *S. pombe* TKP1 cells expressing rPLD1a (**A**) or rPLD1b (**B**) was assayed for PLD activity using various concentrations of ARF1 in the presence of 0 (●), 0.01 (■) or 0.05 μM RhoA (▲), as described in the Experimental section. Data are representative of two experiments.

Responses of rPLD1a and rPLD1b to ARF and RhoA

We next examined the enzymological properties of rPLD1a and rPLD1b using the membrane fractions of the transfectants. In both cases, PLD activities were dependent on PIP_2 (Table 2). GTP[S] was added to the assay as it was definitely required for activity. Both rPLD1a and rPLD1b were markedly stimulated by ARF1. When RhoA, in addition, was added to the assay containing GTP[S], ARF and PIP_2, rPLD1a activity increased 2.6-fold, whereas rPLD1b activity was increased only 16 %. Thus rPLD1b was less sensitive to RhoA than its counterpart rPLD1a. To confirm this, we examined the effect of increasing concentrations of ARF1 on both enzymes at different, fixed concentrations of RhoA. As shown in Figure 3, RhoA stimulated

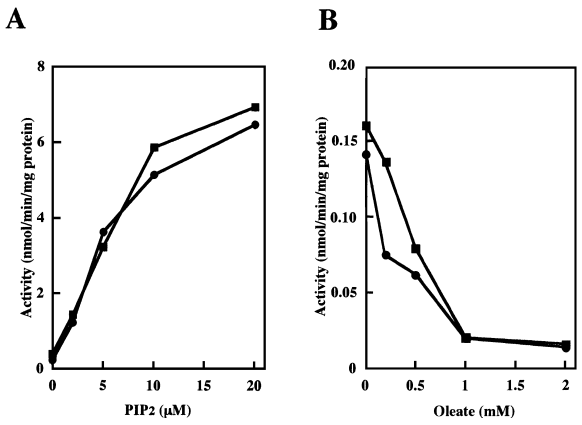

Figure 4 Effects of PIP_2 and oleate on rPLD1a and rPLD1b

The membrane fraction (0.5 μg) of *S. pombe* TKP1 cells expressing rPLD1a (●) or rPLD1b (■) was assayed for PLD activity in the presence of increasing concentrations of PIP_2 (**A**) or oleate (**B**). Data are representative of two experiments.

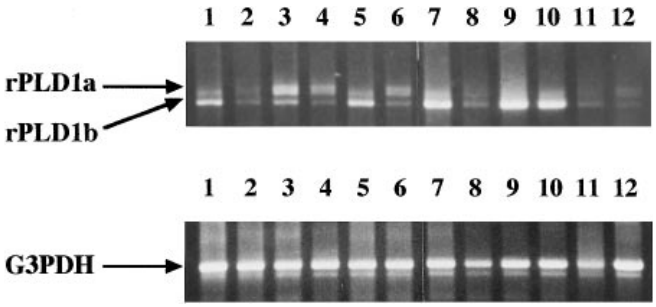

Figure 5 Tissue distribution of rPLD1a and rPLD1b mRNA

Total RNA was isolated from various tissues of a male rat and 0.1 μg was analysed by RT-PCR using a set of primers for rPLD1 isoforms or glycerol-3-phosphate dehydrogenase (G3PDH). Lanes: 1, brain; 2, thymus; 3, lung; 4, heart; 5, liver; 6, spleen; 7, kidney; 8, stomach; 9, small intestine; 10, colon; 11, testis; 12, muscle. Data are representative of two experiments.

rPLD1a in a synergistic manner with ARF1. In contrast, RhoA stimulated rPLD1b to a much lesser extent. Although the sensitivities of rPLD1a and rPLD1b to RhoA were distinguishable, their responses to PIP_2 [35] and oleic acid [16] were essentially the same (Figure 4). Micromolar concentrations of PIP_2 efficiently stimulated both rPLD1a and rPLD1b activities. Oleic acid was inhibitory to both enzymes at concentrations less than 1 mM, as reported for hPLD1 isoforms [14,18].

Tissue distribution of rPLD1a and rPLD1b transcripts

We designed a pair of specific PCR primers to distinguish rPLD1a and rPLD1b transcripts and used them to examine the tissue distribution of both mRNAs by the RT-PCR technique. As shown in Figure 5, one or two PCR bands were observed in most of the rat tissues examined, but three bands were seen in lung. We identified the top band as rPLD1a and the bottom band as rPLD1b by using the PCR fragments produced from rPLD1a and rPLD1b cDNA as references. The rPLD1b band was clearly visible in brain, liver, kidney, small intestine and colon, but the rPLD1a band was not present in these tissues. In heart and spleen, a faint rPLD1a band could be seen, but the rPLD1b band was not detectable. In lung tissue, both rPLD1a and rPLD1b bands were faintly detectable. The entity forming the second band from the top observed in brain, lung, heart and spleen was not studied further. Transcripts for the two PLD1 isoforms were hardly detectable in thymus, stomach, testis and muscle in rat.

DISCUSSION

The present paper reports the complete sequences, characterization and tissue distribution of cDNAs for two isoforms of rat ARF-dependent PLD, rPLD1a and rPLD1b. The major difference between the two isoenzymes was a deletion of 38 amino acid residues in the middle of the rPLD1b sequence, but otherwise the two cDNA sequences were 99.9 % identical with each other, indicating that they are produced by alternative splicing. The presence of the splice variants of ARF-dependent PLD had been reported in rat C6 [17] and human HeLa [18] cell lines. By performing RT-PCR of rat C6 cells, Yoshimura et al. [17] obtained two PCR bands for PLD1, with one band being 114 bp shorter than the other. Hammond et al. [18] isolated cDNA encoding 'long form' (hPLD1a) and 'short form' (hPLD1b) of PLD1 in a HeLa cell cDNA library. The long-form PLD1 was shown to correspond to the previously obtained PLD1 [14], and the short form was the splice variant lacking 38 amino acids. They provided evidence that the short form also encoded ARF-dependent PLD activity by expressing the clone in Sf9 cells. However, the complete sequence of the short-form PLD1 was not reported. In the present work, we showed that, except for the 38-amino-acid deletion, the sequence of the rat short-form variant was essentially identical with that of the rat long-form PLD1. Furthermore, when expressed in *S. pombe*, the two isoforms showed different responses to RhoA, with rPLD1a being more strongly stimulated by RhoA while rPLD1b was much less stimulated by RhoA. Both isoforms, however, were similarly stimulated by ARF. These findings are consistent with the results of Provost et al. [36], who examined PLD activities in various rat tissues and showed that rat tissues contained different PLD isoforms with variations in the relative responses to ARF and RhoA, with the response to ARF being greater than that to RhoA in most tissues. However, they also showed that PLD activity associated with rat liver plasma membranes exhibited a greater response to RhoA than ARF [36]. An interesting possibility would be that there may be a third PLD1 splice variant with a greater response to RhoA than to ARF.

A rather unexpected finding was that PLD1a was the minor isoform expressed in rat tissues, although this was the first cloned mammalian PLD [14]. Among the tissues examined, only lung, heart and spleen showed a detectable PLD1a band on RT-PCR. The other tissues did not show any clear PLD1a band. PLD1b was the predominant PLD1 isoform in most of the rat tissues examined, such as brain, liver, kidney, small intestine and colon. Provost et al. [36] showed that PLD activities in lung and spleen were more responsive to RhoA than those in the other tissues. They did not examine the heart tissue, but their results are consistent with the present observation of the tissue distribution of PLD1a mRNA. Furthermore, the present data show that thymus, stomach, testis and skeletal muscle do not contain any detectable levels of mRNA for PLD1 isoforms. Among these tissues, stomach and testis were found to contain PLD2 [19], but thymus and skeletal muscle were deficient. The failure to detect both PLD1 and PLD2 in muscle was unexpected, since rat muscle microsomes were previously reported to contain considerable ARF- and RhoA-stimulated PLD activity [36].

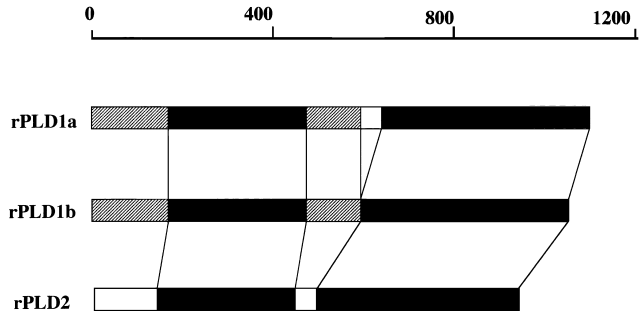

Figure 6 Sequence comparison

The conserved regions of rPLD1a, rPLD1b and rPLD2 were searched for by the MACAW program [37]. The black and hatched boxes represent the conserved regions in all three sequences and in two PLD1 sequences respectively.

Another, as yet unidentified, PLD isoform might be present in these tissues.

The sequences of rPLD1a and rPLD1b obtained here and the rPLD2 cloned previously [19] resemble one another considerably. The three enzymes were similarly activated by PIP_2 and inhibited by oleic acid. However, their responses to small G-proteins were different. rPLD1a was sensitive to RhoA as well as ARF1. In contrast, rPLD1b was less sensitive to RhoA, but fully dependent on ARF1. rPLD2 was not affected by either ARF1 or RhoA [19]. As shown in Figure 6, the most remarkable difference among the three sequences is the successive deletions of amino acids in the middle regions of the sequences. Thus, the simplest explanation for the differential responses of these PLD enzymes to small G-proteins would be that the site for RhoA stimulation lies, at least partly, in the 38-residue region, which is present in rPLD1a, but not in rPLD1b, and the site for ARF stimulation in the 140-residue region, which is present in rPLDb, but not in rPLD2.

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