Cloning of a Phosphatidic Acid-prefering Phospholipase A$_1$ from Bovine Testis*

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We report the molecular cloning and expression of a phosphatidic acid-prefering phospholipase A$_1$, from bovine testis. The open reading frame encoded an 875-amino acid protein with a calculated molecular mass of 97,576 daltons and a pI of 5.61. The sequence included a region similar to a lipase consensus sequence containing the putative active site serine and also included a potential, coiled-coil-forming region. Expression of the open reading frame in COS1 cells resulted in a 20- to 44-fold increase in phosphatidic acid phospholipase A$_1$ activity over that of control cells. Mutation of the putative active site serine (amino acid 540) demonstrated that it was essential for this increase in enzyme activity. Northern blot analysis revealed at least five different messages with the highest overall message levels in mature testis, but detectable message in all tissues examined. Two possible alternately spliced regions in the open reading frame also were identified. Finally, a search of the database identified six related proteins: a potential counterpart of the phospholipase A$_1$ in Caenorhabditis elegans, two putative lipases in yeast, and three proteins separately encoded by the Drosophila retinal degeneration B gene and its mouse and human homologues.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF045022.

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1 The abbreviations used are: PA, phosphatidic acid; CELG, predicted sequence of an unknown protein from C. elegans; COS1 cells, SV40-transformed African green monkey kidney cells; 5’-RACE, 5’-rapid amplification of cDNA ends; kb, kilobase pair(s); MALDI, matrix-assisted laser desorption ionization; MOPS, 3-(N-morpholino)propanesulfonic acid; ORP, open reading frame; PA-PLA$_1$, phosphatidic acid-prefering phospholipase A$_1$; PCR, polymerase chain reaction; PTP, phosphatidylinositol transfer protein; PLA$_1$, phospholipase A$_1$; RDGBd, RDGBh, and RDGBm: sequences encoded by the retinal degeneration B gene in Drosophila and the corresponding genes in humans and mice; SCERV, predicted sequence of an unknown protein from S. cerevisiae; SPOMB, predicted sequence of an unknown protein from S. pombe; PAGE, polyacrylamide gel electrophoresis; 3′-UTR, 3′-untranslated region.

5468 This paper is available on line at http://www.jbc.org
PA-PLA1 Cloning

Purchased from Avanti Polar Lipids. Organic solvents were from J. T. Baker and were American Chemical Society grade or better. All other reagents were from Sigma, except where indicated. Human infant brain library clone R13928, which corresponded to a human expressed sequence tag, was obtained from Genome Systems, Inc. (St. Louis, MO). The preparation of PA-PLA1 oligonucleotides—PA-PLA1 (35 µg), purified 2000-fold from bovine testis (11), was resolved further by 4–15% gradient SDS-PAGE, then transferred to a polyvinylidene difluoride membrane (Millipore). A major band that had an apparent mass of 110-kDa was identified by Amido Black staining, excised, and digested with either cyanogen bromide or Endo-Lys-C (Promega), as described (12). The resulting peptide fragments were separated by PAGE, dried, eluted with 72% acetic acid, and resolved by high performance liquid chromatography and sequenced by Edman degradation (12).

cDNA Library Screening—Degenerate oligonucleotides were designed from the following peptides: DWHSVDEV (23 bases, 288-fold degeneracy) and NHATHVEF (23 bases, 288-fold degeneracy). Total RNA was prepared from bovine testis using TRIzol Reagent (Life Technologies, Inc.), and poly(A)+ mRNA was prepared from total RNA using a Poly(A) Quick kit (Stratagene). cDNA from bovine testis mRNA was prepared by the Superscript II system (Life Technologies, Inc.). Polymerase chain reaction (PCR) using the above oligonucleotides and cDNA was carried out for 35 cycles of 60 s at 94 °C, 30 s at 55 °C, and 180 s at 72 °C using Taq polymerase (Perkin-Elmer). The resulting 305-base fragment was cloned into the PCR cloning vector using the QuickChange system (Stratagene). The insert was cut out of the purified plasmid with EcoRI and labeled with [32P]dCTP by Random Priming (Boehringer Mannheim). This radiolabeled probe was used to screen a custom-made bovine testis cDNA library, produced with both oligo(dT) and random primers (Stratagene). The resulting 13 clones were sequenced.

5′-Rapid Amplification of cDNA Ends (5′-RACE)—Two antisense oligonucleotide primers were designed: GCACACACACCCGCTCGATA and GTGTGGTGCTACCTGTAAG. Single-stranded cDNA was prepared from bovine testis as above. A stretch of deoxyadenosines persisted in 50 µl of water, then analyzed with a Voyager Elite Biospectrometry instrument. The resulting peptides were separated by C18 reversed phase HPLC (Eastman Kodak Co.). The band migrating with authentic lysophosphatidic acid was scraped and quantitated by scintillation counting in 5 ml of Instagel (Packard). The sequence was changed from TCG (serine) to GCG (alanine). Native PA-PLA1 was produced in COS1 cells following transfection with pBK-CMV alone or pBK-CMV containing the 2700-base insert by means of calcium phosphate precipitation (13). The cells were harvested 55 h after transfection in the following manner (carried out at 4 °C or on ice). The growth medium was removed from the plate, and the cells were washed in 10 ml of phosphate-buffered saline. Following removal of the phosphate-buffered saline, 0.5 ml of extraction buffer (containing 25 mM MOPS, pH 7.2, 5 mM EDTA, 1 mM EGTA, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin, 5 µg/ml aprotinin, and 5 µg/ml pepstatin) was added to the plate. The plate was scraped with a rubber policeman, and the resulting suspension was withdrawn. This procedure was repeated with a second 0.5 ml of extraction buffer, and the supernatant was discarded. The cells were homogenized with the use of a 1-ml, tight-fitting Dounce homogenizer for 20 strokes, and then 10 µl of 0.1 M dithiothreitol were added. A 0.5-ml aliquot of the homogenate was centrifuged at 100,000 g in a TLA 45 rotor (Beckman) for 30 min at 4 °C. The resulting supernatant was transferred to an Eppendorf tube, and the pellet was resuspended in 0.5 ml of extraction buffer by 10 strokes of a Dounce homogenizer. After homogenization, 10 µl of 0.1 M dithiothreitol were added.

PA-PLA Assay of the COS1 Cell Extracts—PA-PLA activity in COS1 cells was determined using the Triton micelle assay, as described previously (10, 11). Briefly, the 100-µl assays contained 50 mM MOPS, pH 7.2, 100 mM KCl, 1 mM EGTA, 14.62 mM Triton X-100, 1.6 mM sn-1,2-alkyl-2-oleoyl PA, and 0.08 mM [3H]-labeled sn-1,2-dioleoyl PA. The sn-1,2-alkyl-2-oleoyl PA and [3H]-labeled sn-1,2-dioleoyl PA were synthesized as described (10, 11). Assays were conducted at 25 °C and were initiated by the addition of enzyme. After 20 min, the assays were terminated by the addition of 0.9 ml of 1:1 chloroform:methanol containing 1% w/v formic acid, 25 µg of oleoyl lysophosphatidic acid, 10 µg of egg PA, and 2 mg/ml butylated hydroxytoluene. The resulting single phase was split by the addition of 0.65 ml of water, the upper phase was removed, and the solvent was evaporated from the lower phase in vacuo (Svant). The pellet was dissolved in 50 µl of 75:25:2 chloroform:methanol:water, and 25 µl were spotted onto a reversed phase C18 thin layer chromatography plate (Whatman). The plate was developed in 98:2 methanol:4 mM ammonium formate for 10 min, then exposed to x-ray film (Eastman Kodak Co.). The band migrating with authentic lysophosphatidic acid was scraped and quantitated by scintillation counting in 5 ml of Instagel (Packard). The sequence was changed to GCC (alanine); in that involving serine 730, the original sequence was changed from TCG (serine) to GCC (alanine). Native PA-PLA1 sequence was used as template. Mutants were generated by PCR using Ffu polymerase under the following conditions: 95 °C for 1 min, followed by 18 cycles at 95 °C for 30 s, 45 °C for 1 min, and 68 °C for 15 min. After amplification, the reaction mix was digested with restriction enzyme DpnI at 37 °C for 1 h to eliminate the parental plasmid. Recombinant clones containing 4 units of BstEII and 10 units of XhoI (New England Biolabs), then incubated overnight at 37 °C, followed by 2 h at 50 °C. The digested PCR reaction was resolved on a 0.8% agarose gel, and the 2700-base band was cut out and purified using the Qiagen gel extraction kit. A 10-µg sample of the pBK-CMV plasmid (Stratagene) was digested and gel-purified in the same manner. The plasmid (20 ng) and PCR product (50 ng) were ligated over-night at 16 °C using 20 units of T4 DNA ligase (New England Biolabs). A 2-µl sample of this ligation mix was transformed into Epicurian Coli XL-1 Blue competent cells (Stratagene) in the absence of isopropyl-1-thio-β-D-galactopyranoside, following the manufacturer’s instructions. The transformed bacteria were plated onto agar plates containing Luria- broth and 50 µg/ml kanamycin (Life Technologies) and were found to contain identical sequence.

COS1 cells (ATCC 1650) were plated onto 100-mm polylysine plates (Corning) at a density of 500,000 cells/plate. After growing in Dulbecco’s modified Eagle’s medium (Life Technologies) for 24 h at 37 °C and 5% CO2, the cells were transfected with pBK-CMV alone or pBK-CMV containing the 2700-base insert by means of calcium phosphate precipitation (13). The cells were harvested 55 h after transfection in the following manner (carried out at 4 °C or on ice). The growth medium was removed from the plate, and the cells were washed in 10 ml of phosphate-buffered saline. Following removal of the phosphate-buffered saline, 0.5 ml of extraction buffer (containing 25 mM MOPS, pH 7.2, 5 mM EDTA, 1 mM EGTA, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin, 5 µg/ml aprotinin, and 5 µg/ml pepstatin) was added to the plate. The plate was scraped with a rubber policeman, and the resulting suspension was withdrawn. This procedure was repeated with a second 0.5 ml of extraction buffer, and the supernatant was discarded. The cell supernatant was then mixed with 10 µl of 0.1 M dithiothreitol. Two 0.5-ml aliquots of the homogenate were centrifuged at 100,000 g in a TLA 45 rotor (Beckman) for 30 min at 4 °C. The resulting supernatant was transferred to an Eppendorf tube, and the pellet was resuspended in 0.5 ml of extraction buffer by 10 strokes of a Dounce homogenizer. After homogenization, 10 µl of 0.1 M dithiothreitol were added.

Production and Evaluation of PA-PLA1 Mutants—Wild type and mutant forms of PA-PLA1 were produced in the COS1 cell expression system as described above. Cells were harvested 48 h following transfection with the mutant plasmids.
fection, and enzyme activity assays were carried out with the cell homogenates and supernatant and pellet fractions prepared by centrifugation for 30 min at 100,000 g. The homogenates, supernatants, and pellets were analyzed for expressed protein by immunoblotting (15).

Northern Blot Analysis of PA-PLA1 Expression—Human poly(A)1 RNAs were purchased from CLONTECH. Mature bovine testis and newborn calf testis poly(A)1 RNAs were prepared from fresh-frozen tissue as described above. Northern blots (1.2% agarose) were prepared as described (13). A 308-base fragment from base 1242 to 1550 of the cDNA in Fig. 1 was generated by PCR from one of the bovine clones, FIG. 1.

FIG. 1. cDNA and amino acid sequence of bovine testis PA-PLA1, A, the first and second lines indicate the nucleotide and deduced amino acid sequences, respectively. Nucleotides found to be absent in three bovine testis clones are boxed. The region corresponding to the lipase consensus sequence is indicated in white against a black background with the active site serine 540 in bold. The probable coiled-coil-forming region is underlined.

Northern blot analysis of PA-PLA1 expression. B, the Northern blots shown are of poly(A)1 RNAs from the testes shown in A. T and A, newborn calf testis and adult bovine testis, respectively; P, adult bovine prostate; R, adult bovine renal cortex; with the molecular weight markers shown in the left hand margin.
gel-purified (Qiagen), then randomly primed to about 10^9 cpm/mg (Boehringer Mannheim). The Northern blots were pre-hybridized for 3 hours at 68°C in 10 mM Tris, pH 7.5, 1 M NaCl, 10% dextran sulfate (Pharmacia Biotech Inc.), and 1% SDS. The probe (5 x 10^7 cpm) was mixed with 10 mg/ml sheared salmon sperm DNA (0.5 ml), boiled for 5 min, then put on ice for 5 min. This solution was mixed with 50 ml of prehybridization solution that had been previously equilibrated to 62°C. The blots were transferred to this mixture and incubated at 62°C overnight. They were then washed with 0.1 SSC (5Prime-3Prime Inc.) plus 1% SDS three times for 20 min each at 25°C, followed by two 20-min washes at 62°C. The blots were then exposed to x-ray film (Kodak).

Other Methods—DNA masses were estimated by absorption of 260 nm light, using the correction factor of 50 ng/ml per optical density unit. Homology searches were conducted using BLAST (National Center for Biotechnology Information). Alignments were made with CLUSTALW (16) and the program Align, adapted by W. R. Pearson from Ref. 17. Similar regions in sequences were identified with the help of Block Maker (18). The regions shown in Figs. 4–6 were those identified by both the MOTIF program and the GIBBS program. Coiled-coil-forming regions were predicted using the COILS program (19).2

RESULTS

Cloning of PA-PLA_2 cDNA from Bovine Testis—Material that had an apparent molecular mass of 110-kDa, prepared from 2000-fold purified bovine testis PA-PLA_2, was used to obtain peptide sequences (see “Experimental Procedures”). Four peptides were obtained from Endo-Lys-C digestion and two from cyanogen bromide digestion. Two of the peptide sequences were used to design oligonucleotides, which were used in the amplification by PCR of a 300-base fragment from bovine testis. This fragment, which also contained the DNA sequence for a third peptide, was used to screen a bovine testis cDNA library. Thirteen clones were obtained and partially sequenced from the 5' and 3' ends. The two clones that contained the most sequence in the 5' and 3' directions, respectively, were fully sequenced to reveal an ORF of 2293 bases with 2496 bases of 3' untranslated

PA-PLA_2 Cloning

5471

2 BLAST software is available via the World Wide Web (http://www.ncbi.nlm.nih.gov/BLAST/). Block Maker is also available via the World Wide Web (http://blocks.fhcrc.org/). COILS is also available via the World Wide Web (http://ulrec3.unil.ch/software/COILS_form.html).
regions (3’-UTR). Analysis of the other clones revealed one other with 2496 bases of 3’-UTR, three clones with only the first 800–900 bases of 3’-UTR, and four clones with fewer than 100 bases of 3’-UTR. All of the above clones contained 20–45 adenines at their 3’ ends. The four additional clones did not contain poly(A) sequences, and thus were probably the results of random priming. Three clones contained identical 123-base deletions in the ORF (corresponding to His343–Ser382). Fig. 1).

The 2293-base ORF, predicted to encode a polypeptide of 85,907 daltons, contained no initiation methionine or upstream stop codons. Therefore, 5’-RACE was used to extend the ORF to its 5’ end. Two different gene-specific RACE primers were used, and clones from both of the RACE products were analyzed. The RACE products from both gene-specific primers added an additional 551 bases of 5’ sequence. In the reading frame that coded for all six sequenced peptides (located at positions Ser348–Ser364, Gly388–Pro405, Ile441–Lys463, Pro627–Pro639, Lys702–Thr713, and Ile750–Lys758, respectively), the full sequence contained a methionine codon downstream of one stop codon (Fig. 1). The sequence surrounding the methionine codon, ACAGCATGA, fit reasonably well with the Kozak consensus (20). The calculated mass of the 875-residue polypeptide encoded by this ORF was 97,576 daltons. This mass was smaller than the apparent mass of the 110-kDa band observed upon analyzing the purified bovine testis PA-PLA1 by SDS-PAGE. However, it agreed well with the mass of 97,637 ± 890 daltons (n = 11) obtained by MALDI (data not shown). This suggested that the full-length ORF for PA-PLA1 had been obtained. In addition, the calculated pi of 5.61 was consistent with the purified enzyme’s precipitation properties during acetic acid titration (data not shown).

Upon further inspection of the sequence, two potentially interesting regions were identified. The first region, Ser538–His540–Ser542 (Fig. 1, black area), resembled the consensus sequence containing the active serine nucleophile present in most lipases: GXXG (21, 22). This suggested that serine 540 in the polypeptide encoded by the ORF might correspond to the conserved serine residue in lipases. However, the Ser538–Ser540–His542 region of PA-PLA1 sequence contained an initial serine instead of the highly conserved, initial glycine found upstream of the active serine in all but one of 40 lipases. The second potentially interesting region of the PA-PLA1 sequence, Arg580–Ser582 (Fig. 1, underlined), appeared to be a coiled-coil-forming region. It yielded a probability score of 0.817 when analyzed with COILS version 2.1 using the non-weighted MTIDK matrix and the 28-residue window (data not shown). The significance of this region remains to be determined. However, as mentioned earlier, PA-PLA1 appears to form a homotetramer in solution (11), and coiled-coil-forming regions have been shown to promote oligomerization including tetramerization of other proteins (23).

Expression of PA-PLA1 in COS1 Cells—The putative PA-PLA1 ORF was PCR-amplified from bovine testis cDNA. The PCR product was cloned into the pBK-CMV plasmid alone (pBK-CMV), pBK-CMV containing the entire WT PA-PLA1 ORF (pBK-CMV WT), pBK-CMV containing the PA-PLA1 S730A mutant (S730A), or pBK-CMV containing the PA-PLA1 S540A mutant (S540A). A, whole-cell homogenates were analyzed for PA-PLA activity with 32P-labeled dioleoyl-PA in a Triton micelle assay. Results shown represent means of duplicate measurements. B, Western blot of PA-PLA1 expression in each of the transfected cells with a polyclonal antibody to a peptide corresponding to Thr589–Ser607 of the PA-PLA1 sequence. Note that total cell homogenate and high speed supernatant and pellet fractions were analyzed and that the bulk of the PA-PLA1 protein identified in the homogenate was recovered in the supernatant fraction. Similar results were found in two different experiments.
rather than a glycine residue is present in the corresponding position in PA-PLA1, as mentioned earlier. Accordingly, we constructed a S540A point mutant of PA-PLA1 and transfected it into COS1 cells. In addition, we did parallel experiments with a S730A point mutant and the wild type construct. The results of these experiments revealed that (a) cells that expressed the S540A mutant contained no more detectable PA-PLA1 activity than the mock-transfected cells, whereas cells that expressed wild type PA-PLA1 or the S730A construct contained high levels of PA-PLA1 activity (Fig. 2A); and (b) cells that separately expressed the three constructs produced similar levels of soluble PA-PLA1 protein, as determined by Western blotting (Fig. 2B). Comparable results have been reported for other lipases containing the conserved serine nucleophile in the GX\_S\_GX sequence (e.g. Refs. 25–27). Therefore, the data suggest that serine 540 is the active site nucleophile in PA-PLA1.

**PA-PLA1 Expression in Human Tissues**—Northern blot analysis of poly(A)\_1 RNA from human tissues revealed five PA-PLA1 message lengths of approximately 2.8, 3.0, 3.6, 5.3, and >10 kb (Fig. 3, A–C). Overall expression was highest in testis, which was the only tissue in which the 2.8- and 3.0-kb messages were seen, but in which the >10-kb message was absent. Consistent with this finding, the 13 bovine testis clones contained 3' untranslated regions of various lengths, predicted to correspond to mRNA lengths of the four smaller messages but not to the >10-kb message (data not shown). Moreover, mature bovine testis contained high PA-PLA1 message levels, whereas only trace amounts of message (5.3 kb) were present in newborn bovine testis (Fig. 3C) in agreement with our previous findings with regard to PA-PLA1 activity (10).

All of the other human tissue mRNAs examined contained various amounts of the 3.6-, 5.3-, and >10-kb bands, with the highest levels in brain, spleen, and lung. When RNA from individual human brain regions was examined, the 3.6- and 5.3-kb messages were expressed relatively evenly (Fig. 3B). However, the >10-kb message was found only in cerebellum and fetal brain.

**Possible PA-PLA1 Homologues**—When the translated PA-PLA1 ORF was used to search the non-redundant protein sequence data base, only six sequences with appreciable similarity to PA-PLA1 were identified; none of them corresponded to known lipases. Three of these sequences, CELG (ORF from Caenorhabditis elegans (C. elegans), accession no. 1208846); SPOMB (ORF from Schizosaccharomyces pombe, accession no. 2094857), and SCERV (ORF from Saccharomyces cerevisiae, accession no. 1078029) corresponded to unknown proteins identified during genome sequencing of the respective organisms. The three other sequences, RDGBd (accession no. Y08035), RDGBm (accession no. 2347141), and RDGBh (accession no. 2245317), corresponded to sequences encoded by a Drosophila gene, identified in a mutant screen for retinal degeneration (28), and its mouse and human homologues (29, 30).

The CELG sequence closely resembled the sequence of PA-PLA1, though it corresponded to a smaller protein (765 amino acids as compared with 875 amino acids). When the two protein sequences were compared with Align, they showed 29% identity and yielded a global alignment score of 945. When a BLAST search was done using the PA-PLA1 sequence, the CELG sequence yielded the highest score (P(N) = 1.6e−79). When the two sequences were compared with Block Maker, encoding 308 bases of the bovine testis PA-PLA1 ORF, as described under “Experimental Procedures.” B, a poly(A)\_1 mRNA human blot of different brain sections was probed as in A. C, a poly(A)\_1 mRNA bovine blot of mature and newborn testis was probed as in A. Below each blot is the same blot probed with human 32P-labeled human actin cDNA.
regions of similarity, identified in PA-PLA1, and the putative lipases (Fig. 5, regions 5 and 6). The functional significance of these regions remains to be determined. It may be noteworthy that a sequence in RDGBd, Gly\(^{152}\)-Asp-Ser-Met-Gly, not identified by the Block Maker search, resembled the lipase consensus sequence but contained an aspartic acid residue that could potentially place it in a subgroup of lipases distinct from that of PA-PLA1 and the three putative lipases from \textit{C. elegans} and yeast (31). However, the corresponding sequences in RDGBm and RDGBh, e.g. Gly\(^{363}\)-Asp-Gly-Val-Gly in RDGBh, lacked a corresponding serine residue. Therefore, the functional importance of the putative lipase sequence in RDGBd has to be questioned.

**DISCUSSION**

In this study, we cloned and sequenced the cDNA for bovine testis PA-PLA\(_1\). In support of this conclusion, the sequences of six regions in the putative ORF corresponded to those of peptides isolated from digests of the purified bovine testis enzyme, the calculated molecular mass of the ORF (97,576 daltons) agreed well with the molecular mass of purified bovine testis PA-PLA1, determined by MALDI, expression of the ORF in COS1 cells was accompanied by a 20–40-fold increase in PA-PLA1 activity, and serine 540, which is located in a region of the ORF that resembles a conserved sequence in lipases, was shown to be required for PA-PLA1 activity.

With knowledge of the sequence of PA-PLA1 in hand, several important questions can now be addressed. For example, one set of questions concerns the structural basis of PA-PLA1 activity. Are other specific amino acids in addition to serine 540 required for catalysis? What is the basis for the enzyme’s substrate specificity? What regions of the PA-PLA1 sequence influence the enzyme’s association with the membrane lipid bilayer? It may be possible to address these questions by taking advantage of some of the other observations made in the course of this study.

Catalysis by several known lipases has been shown to require the presence of a “catalytic triad” of amino acids: the conserved serine nucleophile, a histidine residue, and an aspartic acid residue (see, for example, Ref. 32). The results of the data base search with Block Maker that are shown in Fig. 5 may provide clues concerning the location of potential catalytic triad histidine and aspartic acid residues in PA-PLA1, and the putative lipases from \textit{C. elegans} and yeast. Several of the regions of similarity that are shown contain aligned residues of histidine and aspartic acid that could be candidates for
catalytic triad function, and it should be possible to examine the functional significance of these residues by experimentation with point mutants of PA-PLA1.

As mentioned earlier, experiments with mixed micelles have suggested that PA-PLA1 may have both a substrate-binding site for PA, involved in catalysis, and multiple binding sites for PA involved in enzyme activation (10, 11). Experiments with well defined, unilamellar liposomes, currently under way in FIG. 5.

Sequence similarities between two proteins encoded by unidentified yeast genes and between these proteins, PA-PLA1, and the unidentified protein from C. elegans. Seven regions of similarity between the sequences of PA-PLA1, CELG, SCERV, and SPOMB, identified by Block Maker, are shown (boxed sections of regions 1–7); note that the location of each region is indicated as in Fig. 4. Region 4 contains sequences corresponding to the conserved sequence in lipases, shown in white on a black background. Sequences outside the box correspond to regions of similarity identified in a separate search involving SCERV and SPOMB only.

FIG. 6. Sequence similarities between RDGBd, RDGBh, RDGBm, PA-PLA1, CELG, SPOMB, and SCERV. A block diagram of the structure of RDGBd is shown to indicate the positions of two regions of similarity between the sequences of RDGB proteins, PA-PLA1, and the three putative lipases identified by Block Maker. PITP refers to the position of the PITP-like region of RDGBd. Note that the regions of similarity identified by Block Maker are located downstream of the PITP-like region in the RDGB proteins and correspond approximately to regions 8 and 9 in PA-PLA1 and CELG and to region 5 and an upstream region in SPOMB and SCERV. Note also that the precise location of each region is indicated as in Fig. 4.
our laboratory, have supported this possibility. However, the amino acids or domains that contribute to the PA-binding sites have yet to be identified. If expression studies of the ORFs from *C. elegans* and yeast confirm that they encode lipases, it might be possible to identify the relevant PA-binding sites in PA-PLA₁ by carefully comparing the properties and sequences of the four enzymes.

The experiments with unilamellar liposomes that have done so far have also provided evidence that non-substrate lipids influence the ability of PA-PLA₁ to interact with PA. Therefore, it is possible that the enzyme may bind to liposome surfaces in additional, yet-to-be-identified ways. Domains that might be responsible for this binding remain to be identified, but the regions of sequence similarity, regions 5 and 6, shown in Fig. 5, might be good candidates for study because of their similarity to two regions identified in the putative membrane association domains of the RDGB proteins (Fig. 6).

Another set of questions concerns the relation between PA-PLA₁ and other lipases: Does PA-PLA₁ belong to a special family of lipases? What defining structural and functional characteristics do members of this family share? How do members of the family differ from one another? Evidence related to the first of these questions is already beginning to accumulate.

The sequence of PA-PLA₁ definitely differs from the sequences of many other lipases, even though the region that surrounds serine 540 in PA-PLA₁ resembles a conserved region in many of these lipases. For example, PA-PLA₁ lacks further sequence similarity to types I–IV phospholipase A₂ (33, 34), a phosphatidylserine-specific PLA₁ from rat platelets (35), lecithin:cholesterol acyltransferase (36), lysophospholipases (27, 37), and triacylglycerol lipases (38, 39). However, the sequence of PA-PLA₁ does resemble that of CELG, SCERV, and SPOMB, as shown in Fig. 5; and SCERV and SPOMB appear to correspond to different, but closely related family members. We are currently attempting to prepare and express the cDNA that corresponds to SCERV to examine the catalytic properties of the putative SCERV lipase by direct experimentation.

If PA-PLA₁ does indeed belong to a special family of lipases, splice variants of PA-PLA₁ might well be included. The three bovine testis cDNAs that contained the 123-base deletion shown in Fig. 1 may correspond to such splice variants. The 40 amino acids that would be eliminated by the deletion would normally be located between regions 2 and 3 identified by Block Maker (Fig. 4), but their functional significance is unknown. We have also obtained evidence for the existence of a second type of PA-PLA₁ splice variant. We sequenced a cDNA clone from human infant brain, which corresponded to an expressed sequence tag (accession no. R13928), and found that it contained a 64-base deletion in another region of the ORF. Further work with these variants might suggest functions for the deleted regions.

If splice variants of PA-PLA₁ that have different substrate specificities are present in mammalian cells, an apparent discrepancy between the results of an earlier study (10) and the expression patterns of human PA-PLA₁ mRNA that are shown in Fig. 3 (A and C) might conceivably be explained. We previously found high PA-PLA₁ activity in high speed supernatant fractions from homogenates of mature bovine testis and brain, but little or no PA-PLA₁ activity in corresponding fractions from newborn testis, liver, spleen, heart, kidney, and blood (10). In good agreement with the measurements of enzyme activity in bovine tissues, expression of PA-PLA₁ mRNA was highest by far in human testis, strong in brain, and very low in liver. However, unexpectedly, there also appeared to be expression of human PA-PLA₁ mRNA in the spleen and kidney (Fig. 3A) as well as in the heart (data not shown). One possible explanation of this unexpected result could be that the majority of the message detected in the spleen, kidney, and heart was due to PA-PLA₁ splice variants, and that the enzymes that corresponded to these splice variants had substrate specificities that differed from that of PA-PLA₁ and so escaped detection in our assay system.

The presence of splice variants of PA-PLA₁ might also account, at least in part, for the multiple message sizes for PA-PLA₁ observed in this study. The >10-kb message is of particular interest because it was absent from testis but strongly present in lung, spleen, cerebellum, and fetal brain (Fig. 3, A and B). Do these different transcripts correspond to particular splice variants, such as the ones described above? Another possibility, not mutually exclusive with the first, is that differences in message lengths depend on differences in the 3′-untranslated regions of the messages, which confer different properties to the transcripts, such as variations in RNA half-life, translational control, or cellular localization (40, 41).

A different set of questions concerns the possible biological functions of PA-PLA₁ and its close relatives. Answers to many of these questions will have to await the accumulation of further information about the PA-PLA₁ lipase family. However, it may be possible to address questions related to the biological function of PA-PLA₁ itself in the near future. As mentioned earlier, we have been focusing attention on PA-PLA₁ because experiments with mixed micelle systems and unilamellar liposomes have shown that it hydrolyzes and is activated by PA, and because it is present in high levels in the mature testis and brains. This has suggested that it may function in phospholipase D- or diacylglycerol kinase-dependent signaling systems required for spermatogenesis and neuronal interactions. Now that the bovine testis PA-PLA₁ has been sequenced and an antibody to the enzyme has been prepared, it may be possible to obtain further clues concerning the biological role of the enzyme using the tools of molecular biology and immunocytochemistry.

In summary, this paper announces the cloning of the cDNA for a bovine testis PA-PLA₁. Knowledge of the primary structure of PA-PLA₁ will be of great utility in the characterization of its enzymatic properties and physiologic function. In addition, several different splice variants of the protein appear to exist, suggesting modulations of its function in different cells or sub-cellular regions. Finally, the PA-PLA₁ ORF contains regions that are similar to regions in six other ORFs, suggesting interesting possibilities for further experimentation.

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PA-PLA₂ Cloning

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