Identification of Functional Domains of Rat Intestinal Phospholipase B/Lipase

ITS cDNA CLONING, EXPRESSION, AND TISSUE DISTRIBUTION*

(Received for publication, August 8, 1997, and in revised form, October 23, 1997)

Hiroshi Takemori†, Fyodor N. Zolotaryov‡, Lu Ting‡, Tchoua Urbain‡, Takanori Komatsubara‡, Osamu Hatano§, Mitsuhiko Okamoto‡, and Hiromasa Tojo¶

From the †Department of Molecular Physiological Chemistry, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565 and the ‡Department of Anatomy, Nara Medical University, Shijo-cho, Kashiwara 634, Japan

A cDNA encoding a rat intestinal Ca2+-independent phospholipase B/lipase (PLB/LIP) was cloned from an ileac mucosa cDNA library using a probe amplified by polymerase chain reaction based on the purified enzyme’s sequence. PLB/LIP consists of an NH2-terminal signal peptide, four tandem repeats of about 350 amino acids each, and a hydrophobic domain near the COOH terminus. The enzyme purified previously was found to be derived from the second repeat part. To examine the function of each domain, the full-length PLB/LIP, individual repeats, and a protein lacking the COOH-terminal hydrophobic stretch were expressed in COS-7 cells. The results showed that the second repeat, but not the other repeats, had all the activities (phospholipase A2, lysophospholipase, and lipase) found in the purified natural and expressed full-length enzymes, suggesting repeat 2 is a catalytic domain. The full-length enzyme was mainly present in membrane fractions and efficiently solubilized by treatment with 1% Triton X-100, but not with phosphatidylinositol-specific phospholipase C. Deletion of the COOH-terminal hydrophobic stretch caused the secretion of >90% of synthesized PLB/LIP into culture media. These results suggest the hydrophobic domain is not replaced by a glycosylphosphatidylinositol anchor but serves as a membrane anchor directly. A message of the full-length PLB/LIP was abundantly expressed in the ileum and also, in a smaller, but significant amount, in the esophagus and testis. Immunohistochemistry showed that PLB/LIP is localized in brush border membranes of the absorptive cells, Paneth cells, and acrosomes of spermatid, suggesting its roles related and unrelated to intestinal digestion.

Pancreatic phospholipases A2 (PLA2)1 hydrolyze ester bonds at the sn-2 position of glycerophospholipids and produce fatty acids and lysophospholipids. These steps are prerequisites for lipid absorption by intestinal epithelium cells (4). Lysophospholipid can be directly absorbed, or hydrolyzed by pancreatic lysophospholipase and converted to glycerol 3-phosphate esters and fatty acids.

Until recently, all those processes were believed to proceed in the lumen of alimentary tracts by the action of secretory enzymes mentioned above. However, recent studies suggest the presence of a lipid-hydrolyzing enzyme associated with intestinal brush border membranes (5, 6), named phospholipase B/lipase (PLB/LIP) because this enzyme displayed broad lipo-lytic activities (PLA2, lysophospholipase, and lipase activities) (7). PLB/LIP might participate in terminal digestion, or membrane digestion, of dietary lipids and biliary phospholipids, like well established glycosidases and peptidases in brush border membranes (8). In the preceding paper, we detailed the purification and characterization of the catalytic domain of PLB/LIP after its solubilization from the membrane by autolysis. The purified enzyme consisted of a 14-kDa peptide and a 21-kDa glycosylated peptide and catalyzed PLA2, lysophospholipase, and lipase reactions in a single active site (9). To understand the molecular basis of such a broad enzyme specificity, the structure of the nascent enzyme, the membrane anchoring mode, and its physiological significance, we cloned and sequenced the full-length PLB/LIP cDNA based on information of the NH2-terminal amino acid sequences of the two peptides derived from the purified enzyme. PLB/LIP is translated as a large single peptide of 1450 amino acids containing a signal peptide, four tandem repeats, and the COOH-terminal hydrophobic stretch. The second repeat has the stretch coding the amino acid sequences found in the previously purified enzyme and has full enzymatic activities, as confirmed by the expression of individual repeats in COS-7 cells. Furthermore, to determine whether or not the hydrophobic stretch is responsible for a membrane anchor, a deleted mutant was constructed and expressed in COS-7 cells.

EXPERIMENTAL PROCEDURES

Materials—1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) was obtained from Avanti Polar Lipids, Inc. Trioleoylglycerol (TOG), 1-palmitoyl-sn-glycero-3-phosphocholine (1-palmitoyl-GPC), and diiso-1

The abbreviations used are: PLA2, phospholipase A2; PLB/LIP, phospholipase B/lipase; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; TOG, trioleoylglycerol; 1-palmitoyl-GPC, 1-palmitoyl-sn-glycerol-3-phosphocholine; DFP, diisopropyl fluorophosphate; RT-PCR, reverse-trace DNA-polymerase chain reaction; PCR, polymerase chain reaction; IgG, immunoglobulin G; GPI, glycosylphosphatidylinositol; DTT, dithiothreitol; PBS, phosphate-buffered saline; kb, kilo-base pair(s); bp, base pair(s).

2222 This paper is available on line at http://www.jbc.org
propyl fluorophosphate (DFP) were purchased from Sigma.

Amplification of cDNA Fragments by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—Surgically removed rat intestines were washed with distilled water. The ileal mucosa was scraped off and stored at −80 °C until use. The frozen tissues (about 1.0 g) were homogenized in 150 mM potassium phosphate containing 0.1 M Tris-HCl (pH 7.6), 1 mM EDTA, 2% sodium lauryl sarcosinate, and 2% 2-mercaptoethanol. The homogenate was extracted with phenol/chloroform, and RNA in the aqueous phase separated by centrifugation was precipitated with ethanol. One µg of total RNA was reverse-transcribed with 200 units of SuperScript RT (Life Technologies, Inc.) in the presence of 150 ng random hexamers and 200 µM of each dNTP. The reaction mixture was incubated at 37 °C for 45 min, 42 °C for 30 min, and 70 °C for 15 min. The resultant cDNA was digested with EcoRI site of pBluescript KS(−) (Stratagene). A 2.5-kb fragment of the plasmid DNA was ligated into an EcoRI site of pBluescript KS(−) to give a construct named pMOS-RIPLB3. The plasmid DNA was purified from ampicillin-resistant colonies were digested with EcoRI, HindIII, and PstI endonucleases. The fragments were separated on an agarose gel. Four clones named pMOS-RIPLB3, was used for further analysis. To determine its fragment, the plasmid was subcloned by a restriction endonuclease-mediated plasmid self-subclone system (13). Plasmids were ligated into an EcoRI site of pBluescript KS(−), generating pMOS-RPIPLB3. The resultant plasmids were used as the probe DNA for RNA blot analysis and screening of a cDNA library.

Construction and Screening of cDNA Library—A rat ileum mucosa cDNA library was prepared based on the method of Gubler and Hoffman (11) using a λMosElor cloning vector (Amerham). Six µg of poly(A)⁺ RNA, an oligo(dT) primer, and avian myeloblastosis virus reverse transcriptase were used for the first strand synthesis. The second strand was then synthesized by treatment with RNase H and DNA polymerase I, and then the generated cDNA was blunt-ended with EcoRI adaptor (Amerham) by T4 ligase, and further treated with polynucleotide kinase. The DNA greater than 500 bp in length were selected on a spin column. The resultant cDNAs were ligated into an EcoRI site of λMosElor vector, and the vectors were packaged in 10 to 30 plagues. The library was screened without amplification.

For screening, 10⁶ plagues were plated and replicas were made from the resultant plaques (12). Positively hybridizable plaques were screened with a 32P-labeled BamHI cDNA fragment of pICR-RIPLB and 50 strongly hybridized clones were selected and stored at 4 °C. Randomly chosen 20 clones of the 50 were subjected to the second screening, and another 10 clones were isolated by treatment with RNase H and DNA polymerase I, and then the generated cDNA was blunt-ended with T4 DNA polymerase, ligated to EcoRI adaptor (Amerham) by T4 ligase, and further treated with polynucleotide kinase. The DNA greater than 500 bp in length were selected on a spin column. The resultant cDNAs were ligated into an EcoRI site of λMosElor vector, and the vectors were packaged in 10 to 30 plagues. The library was screened without amplification.

For screening, 10⁶ plagues were plated and replicas were made from the resultant plaques (12). Positively hybridizable plaques were screened with a 32P-labeled BamHI cDNA fragment of pICR-RIPLB and 50 strongly hybridized clones were selected and stored at 4 °C. Randomly chosen 20 clones of the 50 were subjected to the second screening, and another 10 clones were isolated by treatment with RNase H and DNA polymerase I, and then the generated cDNA was blunt-ended with T4 DNA polymerase, ligated to EcoRI adaptor (Amerham) by T4 ligase, and further treated with polynucleotide kinase. The DNA greater than 500 bp in length were selected on a spin column. The resultant cDNAs were ligated into an EcoRI site of λMosElor vector, and the vectors were packaged in 10 to 30 plagues. The library was screened without amplification.

Preparation of Antibody and Immunoassay—Polyclonal antibody against a PLB/Lip fragment (amino acids 450–1450). The antigen was produced in E. coli as a fusion protein using T7 RNA polymerase expression system (14). An EcoRI insert of pBS-LIP encoding the peptide was subcloned into EcoRI-cleaved pET28a (+) vector (Novagen), and it was transfected into E. coli strain, BL21(DE3) (Novagen). The expressed proteins were solubilized from a particulate fraction by cell homogenates with an SDS sample buffer containing 1% SDS and 10% mercaptoethanol, and subjected to SDS-PAGE (15, 16).

The protein fusion (about 1.5 mg) was electrophoretically extracted from the gel slices (12). Its aliquot (about 0.4 mg) was emulsified with 2 volumes of complete Freund’s adjuvant and used to immunize Japanese white rabbits (females, body weight of 2.0 kg). Immunoglobulin G (IgG) was purified from 15 ml of antiserum by an IgG purification kit (Amerham).

The freshly prepared intestinal mucosa was homogenized in 5 volumes of HBS buffer (0.25 M sucrose, 1 mM EDTA, 30 mM Tris-HCl (pH 7.4), 2 mM MgCl₂). Testis tissues and sperm collected from epididymis were sonicated in 10 volumes of HBS buffer, and the homogenates were centrifuged at 10,000 × g at 4 °C for 10 min. Supernatants were further centrifuged at 100,000 × g for 45 min at 4 °C, and pellets (membrane fraction) were taken and suspended in 0.1 volume of HBS buffer. All the samples were stored at −80 °C until use. The proteins (20 µg each) were subjected to SDS-PAGE (on 6% or 10% gels), and immunoblotting was performed as described (12). The antigen-antibody complexes on the membranes were further reacted with goat anti-rabbit IgG-horseradish peroxidase conjugate (Cappel). The peroxidase conjugate was developed with a Konica immunostaining kit (Konica, Japan).

Expression of PLB/LIP in COS-7 Cells—To construct the expression vector, pMOS-RIPLB3 was partially digested by BamHI and the full-length 4.5-kb cDNA fragment containing the signal peptide on both sides was isolated by agarose gel electrophoresis and cloned into the BamHI site of a simian virus 40 (SV40) expression vector (PROMEGA Corp., Madison, WI). This vector, named pSVL-RIPLB3, had an extra ATG at NcoI site in the adaptor sequence preceding the initiating ATG of RIPLB cDNA. This was removed by replacing an Xhol fragment of pSVL-RIPLB3 with a fragment from the initiator ATG to XmlI site (nucleotide position 333) that was amplified by PCR using the pMOS-RIPLB3 as a template and primers, Nfor (Fig. 3b) and L331 (CATCTGTCATGGCTTCGACAGGC). The resultant vector was named pSVL-RIPLB.

Expression plasmids for repeats 1, 2, 3, or 4, named pSVL#1, pSVL#2, pSVL#3, or pSVL#4, respectively, were constructed by linking a cDNA fragment encoding the NH₂-terminal peptide of PLB/LIP including the signal peptide with a cDNA encoding each repeat to meet requirements for secretable proteins. We first constructed pSVL-Nhead that contains a cDNA encoding amino acids 1–40 of PLB/LIP and a synthetic polylinker consisting of Nhel and DraI sites, a stop codon, and a MluI site in this order. The insert was amplified by PCR using primers Nfor and Nrev (Fig. 3), and pSVL-RIPLB as template, and subcloned directly into a pMOS-Blue vector (Amerham). The Apol-BamHI fragment excised from this vector was substituted for an Apol-BamHI fragment of pSVL-RIPLB, generating pSVL-Nhead.

pSVL#1 containing amino acids 1–357 of PLB/LIP was generated by replacing an Apol-DraI fragment of pSVL-Nhead with an Apol-PstI fragment of pSVL-RIPLB (Fig. 3). Expression vectors for the other repeats were constructed as follows. Fragments encoding repeats 2, 3, and 4 were first amplified by PCR using the pSVL-RIPLB template, sense primers (F2, F3, and F4, respectively) with a 5’-terminal Nhel site, and antisense primers (R2, R3, and R4, respectively) with a 5’-terminal DraI site, then the cloned fragments were inserted into Nhel or DraI sites of pSVL-Nhead vector, and ligated with a MluI site, and the resultant fragments were finally cloned into pSVL-Nhead that had been previously digested by the same restriction endonucleases. An expression vector lacking the COOH-terminal hydrophobic stretch (pSVL-AC) was constructed by replacing the 3’-side BamHI fragment of pSVL-RIPLB (nucleotides 3701–3737) with that of pSVL#4 (nucleotides 3701–4259).

COS-7 cells (4 × 10⁶ cells) suspended in 0.4 ml of saline G were transfected with the plasmids (50 µg) in the presence of 250 µg of carrier DNA by electroporation using a single pulse (125 microfarads at 400 V) as reported (16). The efficiency of transfection estimated by enzyme activity recovered medium and cell fractions depended on cell density (16); the cell density of 60–70% confluence was optimal. The transfected cells were plated on two 9-cm dishes containing Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. The dishes were incubated at 37 °C under an atmosphere of 5% CO₂, 95% air. The medium was changed once every 24 h. The medium was recovered for enzyme assay, and the cells were harvested for immunoblot and Northern blot analyses after 72 h.

Polyclonal Anti-PLB/LIP Serum—A rat ileum mucosa cDNA library was prepared based on the method of Gubler and Hoff-
troline as substrates, respectively, as described in the preceding paper (9). The lipolytic activities of PLB/LIP toward exogenous substrates were expressed as the difference between the activities in the presence and absence of the substrates and as nanomoles of free fatty acid released/1 min. Protein concentration was measured by a Bio-Rad protein assay system.

RT-PCR Analysis—Total RNA (1 µg) prepared from the ileum and poly(A)-rich RNA (1 µg) prepared from esophagus or testis were reverse-transcribed as described above. Transcripts (4 µl) were mixed with a pair of primers (50 pmol each), NfI and R2 or P3 and R4, in 5 µl of 10 X Taq polymerase buffer supplied by company (Takara), 4 µl of 10 m$m$ dNTP solution, 5 µl of dimethyl sulfoxide, and H$_2$O to give the final volume of reaction mixture, 50 µl. The mixture was overlaid with 30 µl of mineral oil to prevent evaporation. Taq polymerase (2.5 units) was added to the reaction mixture, and the chain reaction was conducted as follows: denaturation at 94 °C for 40 s, annealing at 60 °C for 1 min, and polymerization at 72 °C for 4 min with 30 cycles.

Immunohistochemical Analysis—Rat ileum and testis were fixed in 4% parafomaldehyde/PBS (pH 7.4) for 2 h. Frozen sections (6 µm) prepared on glass slides were stained immunohistochemically (17). Prior to incubation with the primary antiserum, the sections were treated with Gomori's oxidation mixture (0.5% potassium permanganate, 0.25% sulfuric acid) (18). The primary antiserum was diluted 1:300. For the controls, the preimmune serum was incubated with the antiserum for 12 h at 4 °C. The cells and culture media were separately recovered and the former was further fractionated into cytosol and membrane fractions as described under “Experimental Procedures.” Expression was assessed in these fractions by enzyme assay and immunoblot analyses. The cells transfected with the recombinant plasmid revealed greatly enhanced PLA$_2$, lysophospholipase and lipase activities, compared with cells transfected with the pSVL vector alone. This confirmed that the full-length PLB/LIP exhibits the same substrate specificity as the enzyme purified from rat intestine (9) as a single polypeptide protein. These activities were mainly localized in membrane fractions as verified by immunoblot analysis (Fig. 4, a and b), confirming PLB/LIP is a membrane-bound protein (9). The enzyme produced by COS-7 cells apparently migrated as two bands on a 6% gel, and their estimated molecular masses (~200 and 220 kDa) were larger than that calculated from the predicted amino acid sequence data (161 kDa). This may be due in part to the difference in the degree of glycosylation.

Membrane Anchoring Mode of PLB/LIP—The COOH-terminal segment, residues 1421–1443, is the only stretch that is hydrophobic and long enough to meet requirement for membrane anchoring. To determine whether the stretch plays a role in binding of the enzyme to membrane, an expression vector (pSVL-DC) that lacked the COOH-terminal 42 amino acid residues (Fig. 3c) was constructed and introduced into COS-7 cells. Immunoblot analysis indicated that about 90% of the expressed truncated enzyme was recovered in culture media (Fig. 4a), in contrast with membrane localization of the full-length PLB/LIP. In that culture medium, PLA$_2$ and lysophospholipase activities were unexpectedly not observed, but a substantial activity of lipase was detectable. The truncated protein, however, restored phospholipid hydrolyzing activities to the level as comparable as lipase activity, when dithiothreitol (DTT) was added to the reaction mixtures to the final concentration of 4–20 mM. This apparent activation of PLA$_2$ and lysophospholipase activities occurred only in the truncated enzyme released in culture media. DTT (4 mM) treatment had little effect on the activities of the membrane-bound PLB/LIP.

There are two possibilities that the COOH-terminal hydro-
phobic stretch can serve as a direct membrane anchor or as a signal for attaching a glycosylphosphatidylinositol (GPI) moiety to the COOH-terminal end after the cleavage of that stretch (22). As in the case of PLB/LIP in brush border membranes (9), phospholipase C treatment (25 and 100 munits/ml) did not solubilized PLA2 activity in membrane fractions of full-length PLB/LIP-expressing COS-7 cells, but treatment with 1% Triton X-100, a poor solubilizer for GPI-anchored proteins (23), did solubilize 72% of the activity. This suggests that the hydrophobic stretch functions as a direct membrane anchor.

Location of the Catalytic Domain—The enzyme purified from rat intestine had an apparent molecular weight of 35,000 with PLA2, lysophospholipase and lipase activities. It consisted of the 14-kDa and 21-kDa peptides, and their amino acid sequences were derived from the second repeat of PLB/LIP, suggesting the second repeat is a catalytic domain. To determine which domains are responsible for the activities observed in the expressed full-length and purified natural PLB/LIP, we constructed four expression vectors, each of which contained a nucleotide sequence encoding a NH2-terminal signal peptide and the respective repeats (Fig. 3c), and transfected them into COS-7 cells. The antibody used in this study (raised against the PLB/LIP peptide, which lacked the first repeat) immunoreacted with the second and third repeats well, but did not with the first or the fourth repeat. We estimated the level of expression of each repeat in the cells by Northern blot analysis using the full-length PLB/LIP cDNA as a probe. As shown in Fig. 5a, transcripts of 2.0 kb were found at comparable levels in the cells transfected with each vector. The culture media and harvested cells were examined for the enzyme activities (Fig. 5b).

All the activities found in the purified enzyme were present in the culture media of repeat 2-expressing cells, but not in the media of cells transfected with the other constructs. No activity was detected in the cell homogenates except for a trace amount of enzyme activities in repeat 2-expressing cell fractions (data not shown). This may preclude impaired secretion of expressed proteins. Furthermore, dithiothreitol had no effect on the enzyme activities of all culture media (data not shown). DFP is a potent inhibitor that inhibited PLA2, lysophospholipase, and lipase activities of the purified PLB/LIP to a comparable extent (9). It similarly inhibited these three activities of the expressed full-length PLB/LIP by 99, 88, and 94%, respectively, and of the repeat 2 enzyme by 99, 97, and 91%, respectively, compared with those in the absence of inhibitors. These results suggested
that repeat 2 is the catalytic domain with a single active site responsible for all the enzymatic activities tested.

Distribution of PLB/LIP—The distribution of PLB/LIP among various rat tissues was determined by Northern analysis using rat PLB/LIP cDNA as a probe. Fig. 6a showed that two kinds of transcripts of about 4.6 kb and larger than 6.0 kb in length were readily detected in the ileum. The shorter transcript was also present in esophagus and testis in a small amount. Further examination of intestinal segments showed that a large amount of message existed in the ileum and a trace amount in jejunum, but not in duodenum (Fig. 6b). No difference was found in the levels of the message between upper and lower portions of esophagus. The message was exclusively expressed in the esophageal mucosa (Fig. 6c).

To ascertain that the 4.6-kb transcripts present in esophagus and testis were identical to that in intestine, the size of transcripts in these tissues was analyzed by RT-PCR. Because the full-length mRNA was too large to be amplified by PCR, we amplified two fragments from the message separately; one corresponded to repeats 1 and 2, and the other to repeats 3 and 4 (Fig. 7) as described under “Experimental Procedures.” Since the amount of messages in esophagus and testis seemed to be less than 2% of that in the ileum, poly(A)-rich fractions prepared from the former two tissues were used for reverse transcription. As shown in Fig. 7 (a and b), single bands of the expected sizes (2164 or 2109 bp, respectively) in all three tissues were detected at the identical position. To eliminate a possibility that these PCR bands were amplified from genomic DNA contaminated into the RNA fractions, a negative control reaction was performed with 1 mg of rat genomic DNA. No specific DNA fragment was amplified from genomic DNA under these conditions (data not shown). The PCR products were cloned into plasmid vectors, and their nucleotide sequences were determined up to about 300 bp from both the termini. The cDNAs of esophagus and testis were found to have sequences identical to that of the intestine cDNA. Furthermore, digestion of the PCR products of all tissues by restriction enzymes gave the identical fragment patterns on agarose gel electrophoresis (Fig. 7). These results strongly suggest that the 4.6-kb transcripts expressed in esophagus and testis are identical to the intestinal PLB/LIP mRNA.

Next, we examined levels of protein expression in the mRNA-expressing tissues by immunoblot analysis using SDS-PAGE under the reducing conditions. As shown in Fig. 8, the immunoblot of freshly prepared ileal mucosa preparations revealed at least three strongly stained bands corresponding to the molecular masses of 200, 130, and 90 kDa. PLB/LIP was not detectable in testis homogenate, but a faint band of 200 kDa, which is the same as the largest one of the three bands found in ileum homogenate, was detectable in membrane fractions of testis (Fig. 8). When membrane fractions of sperm collected from epididymis were used for the analysis, the single band appeared more clearly. This band was not detectable using preimmune serum (not shown), suggesting that the PLB/LIP protein is expressed in the membrane fractions of sperm (and its precursor). Comparison of the sizes of PLB/LIP in freshly prepared extracts of the ileum, sperm membrane, and COS-7 cells suggested that proteolytic cleavage was considerably limited in the latter two extracts.

We investigated the localization of PLB/LIP in the ileum and testis by light microscopy using anti-PLB/LIP IgG. In the ileum, absorptive cells located in the apical region of villi were diffusely but strongly stained by anti-PLB/LIP IgG (Fig. 9c). Examination on higher magnification showed that the brush border membranes were more intensely stained than the cytoplasm (Fig. 9c). No immunopositive reactions were observed in
control sections stained with the antibody preabsorbed with E. coli extracts containing the PLB/LIP fragment as antigen (Fig. 9b). Secretory granules of Paneth cells were also stained (Fig. 9c). In testis, spermatocytes and spermatids were stained with the antibody, being visualized as round and crescent bodies, respectively (Fig. 9c), but not with preimmune serum (Fig. 9f).

The combination of the immunofluorescent and phase-contrast figures showed that PLB/LIP proteins may be localized in the acrosome.

FIG. 4. Effects of deletion of a COOH-terminal hydrophobic stretch on subcellular localization of PLB/LIP expressed in COS-7 cells. a, Immunoblotting of PLB/LIP in membrane (Mem), culture medium (Med), and cytosol (Cyt) fractions of COS-7 cells transfected with the wild-type pSVL-RIPLB, deleted pSVL-ΔC (refer to Fig. 3c), or vector alone using the polyclonal anti-PLB/LIP IgG after SDS-PAGE on a 6% gel. The protein amounts applied to Mem and Cyt lanes were 10 times as much as to Med lanes. b, lipolytic activities were determined for each fraction as described under “Experimental Procedures.” The substrate used were POPC, 1-palmitoyl-GPC, and TOG (1 mM each). The activities in culture media of deleted mutant expressing cells were assayed in the presence and absence of 4 mM DTT.

FIG. 5. The second repeat is a catalytic domain. a, Northern blot analysis of PLB/LIP mRNA extracted from cells transfected with the same constructs as in Fig. 3c. Upper panel, total RNA (20 μg) was electrophoresed, blotted onto a nylon membrane, and hybridized with the [32P]dCTP-labeled PLB/LIP cDNA fragment that had been prepared by PCR amplification from the pMOS-RIPLB3 plasmid with Nfor and R4 primers (corresponding to almost full-length RIPLB, see Fig. 3a). Lower panel, ribosomal RNAs were stained by ethidium bromide. b, lipolytic activities in each culture medium were tested as in Fig. 4b. As a positive control, we used the culture medium of COS-7 cells transfected with pSVL-ΔC, because the expressed full-length enzyme was not secreted into the medium. Only the lipase activity was detected in this medium in the absence of DTT as in Fig. 4b.

 DISCUSSION

Using a probe amplified by RT-PCR based on the knowledge of partial amino acid sequences of the previously purified PLB/LIP (9), we isolated and sequenced its full-length cDNA. The NH2-terminal amino acid sequences of the small and large fragments of purified PLB/LIP were found in complete accordance with the second repeat part of the deduced amino acid sequence. COS-7 cells transfected with the full-length cDNA produced the active enzyme that exhibited the same substrate specificity, i.e. PLA2, lysophospholipase, and lipase activities, as the purified enzyme (9). These results demonstrated that the isolated cDNA indeed encodes the functional rat intestinal PLB/LIP.

The amino acid sequence of PLB/LIP showed 67.9% similarity to that of rabbit AdRab-B protein that had been isolated from an adult intestine specific cDNA library (21). Both proteins had the primary structural arrangement similar to lactose-phlorizin hydrolase, a well characterized hydrolase associated with brush border membranes: a signal peptide, four internal repeats, and a COOH-terminal transmembrane anchor. This suggests AdRab-B is a rabbit counterpart of rat PLB/LIP, although their substrate specificities were different from each other (9). Pind and Kuksis (24) reported a purifica-
tion of detergent solubilized PLA2/lysophospholipase from rat intestinal brush border membranes. The use of SDS-PAGE at the final purification step gave an inactive enzyme, which renatured under appropriate conditions. That enzyme may be identical to PLB/LIP, because the amino acid composition reported was similar to that calculated from the amino acid sequence deduced in this study.

The putative signal sequences of rat PLB/LIP and AdRab-B were similar to each other. The method of von Heijne predicted cleavage between Gly19-Ala20 for AdRab-B (21); in rat PLB/LIP, the corresponding cleavage at Gly21-Pro22 generates a new NH2-terminal Pro, which may make this cleavage unlikely, because the occurrence of Pro at the NH2 terminus is extremely rare. Other candidates for a signal sequence cleavage site are the peptide linkage between residues 28 and 29 or that between residues 29 and 30. It, however, remains to be clarified which

**FIG. 7.** RT-PCR analysis of PLB/LIP transcript. Poly(A)-rich RNA (1.0 μg) from the testis (Tes) and esophagus (Eso) or 0.1 μg of total RNA from the intestine (Int) was reverse-transcribed with random hexanucleotides. The resultant single strand cDNAs were used for PCR amplification of cDNA encoding repeats 1 and 2 with primers F3 and R4 (a) and of that encoding repeats 3 and 4 with primers F3 and R4 (b). After purification by phenol extraction, aliquots of the former products were digested with EcoRI or BclI, while those of the latter were digested with XbaI or BamHI (see Fig. 3). The digests were separated on a 1.2% agarose gel. All digestions gave two fragments of the following lengths: 796 and 1369 bp for EcoRI digestion, 582 and 1582 bp for BclI digestion, 870 and 1239 bp for XbaI digestion, 570 and 1541 bp for BamHI digestion. The sizes of markers are: 23.13, 9.24, 6.56, 4.36, 2.32, 2.02, and 0.56 kb for λ/HindIII; 1419, 517, 396, 214, and 75 bp for pUC19/HindIII. Asterisk denotes a nonspecific product, which was also amplified from rat genomic DNA.

**FIG. 8.** Immunoblot analysis of PLB/LIP in the ileum, testis, and sperm. Ileum (3 mg) and testis (20 mg) homogenates and sperm membrane fractions (20 μg) were subjected to SDS-PAGE on a 12% gel, blotted to a PVDF membrane, and detected with 500-fold diluted anti-PLB/LIP serum. Prestained proteins (New England Biolabs) were used as size markers.

**FIG. 9.** Immunofluorescence localization of PLB/LIP in rat ileum (a–d) and testis (e and f). Thin cryosections of the ileum (a, c, and d) or testis (e) were reacted with anti-PLB/LIP antibody, while the control sections were reacted with the antibody preabsorbed with the corresponding antigen expressed in E. coli (b), and with preimmune serum (f) (see "Experimental Procedures"). a, the PLB/LIP protein located at the apical portion of the villi (V) and in Paneth cells (P). c and d, pictures of higher magnification showing brush border membranes (B) and Paneth cells, respectively. c, the high level of accumulation of the PLB/LIP protein was observed on the brush border membranes, but not in the nucleus (N) and on the basement membrane side. d, there were also immunopositive signals for PLB/LIP in granules of Paneth cells. c, the heads of spermatocytes (Sc) and spermatid (St) were immunopositive. L, lumen; C, crypt. White bars in a, c, d, and e are 60, 10, 10, and 60 μm in length, respectively.
bonds are processed in vivo.

The results presented in this and the preceding paper (9) demonstrated that the repeat 2 is the catalytic domain that catalyzes PLA₂, lyso-phospholipase, and lipase activities. Comparable inhibition of these activities of purified (9), expressed full-length and repeat 2 enzymes by DFP suggested the involvement of a single active site with broad substrate specificity. This raises further questions about the functional roles of the other repeats. The primary structural similarity between repeat 2 and the other repeats, especially repeats 3 and 4 (Fig. 2c), implies the presence of another active site with different specificity in these repeats, like lactase-phlorizin hydrolase, which has the same structural arrangement as PLB/LIP and two distinct active sites in homologous repeat 3 and 4 (25). However, we have not yet obtained direct evidence for this. Alternatively, repeats other than repeat 2 may play roles in regulating the enzyme action of repeat 2. To address these problems, we will need the structural and functional identification of active site catalytic residues.

In rabbit lactase-phlorizin hydrolase, repeats 1 and 2 serve as parts of the propeptide that is intracellularly removed to give the mature brush border membrane form. Its precursor protein is not a zymogen but by itself is as active as the mature enzyme. In rat PLB/LIP, the intestinal enzyme was partially degraded into proteins in size ranging from 90 to 200 kDa, but the testis enzyme has the same size as the full-length PLB/LIP expressed in COS-7 cells, suggesting proteolytic cleavage did not occur in that tissue. The similar cleavage has been reported in rabbit intestinal AdRab-B (21). It is unknown at present whether this proteolysis merely reflects greater abundance of proteases in the intestinal mucosa than in the testis or whether the difference in the degree of degradation is relevant to tissue-specific roles of this enzyme. The full-length PLB/LIP is again not a zymogen because it exhibited as high a lipolytic activity as the enzyme purified from rat intestine when enzyme concentrations were estimated by immunoblot analysis (Fig. 4a).

We purified the 35-kDa catalytic domain of PLB/LIP with a nick at the peptide linkage between Arg⁵²⁸ and Phe⁶⁲⁹ that was released from brush border membrane by the action of endogenous protease(s). Although the NH₂-terminal amino acid sequences of its small and large fragments were included in the second repeat, its exact COOH-terminal processing site remained to be clarified. PLB/LIP has 14 potential N-glycosylation sites; repeat 2 has a single site at Asn⁶⁰⁶ near its COOH-terminal end. The results of concanavalin A-peroxidase stain on PVDF membrane consistently indicated that the COOH-terminal large peptide of the purified PLB/LIP was indeed glycosylated (9). The apparent molecular mass of the large peptide was about 21 kDa, which agreed with that calculated from amino acid sequence data (20.7 kDa), assuming that the processing occurs at the peptide bond just after the Lys⁷¹⁰ that is present in the COOH-terminal KR/NS sequence conserved among all repeats (Fig. 2a). These considerations suggested that the processing site of the purified enzyme is present at the COOH-terminal side of Asn⁶⁰⁶.

In a previous study on rabbit AdRab-B, it was inferred by analogy with lactase-phlorizin hydrolase that the COOH-terminal hydrophobic domain is responsible for its membrane anchoring (21). To address the problem experimentally, we created a PLB/LIP mutant that lacks the COOH-terminal 42 amino acids including the hydrophobic stretch. The wild-type PLB/LIP was mainly found in the membrane fractions of COS-7 cells, whereas the truncated mutant was released into the culture media. Triton X-100 treatment, but not phosphatidylinositol-specific phospholipase C treatment, efficiently solubilized PLB/LIP from COS-7 cell membrane and brush border membrane fractions. These results provide evidence that the COOH-terminal hydrophobic domain does not serve as a signal to direct glycospholipid attachment, but directly as a membrane anchor. Since there exist phospholipase C-resistant GPI-anchored proteins (26), conclusive proof for the enzyme’s anchoring mechanism must, however, await structural determination of detergent-solubilized enzyme.

Surprisingly, virtually no PLA₂ and lyso-phospholipase activities were detectable in the culture medium of the cells expressing the deleted mutant-enzyme, despite the fact that both lipase activity and immunoreactivity were appreciably detectable. DTT treatment of the mutant, however, restored the lost activities. This can be explained as follows. The interaction between repeat 2 and another repeat(s), presumably via disulfide bridge formation, might cause the truncated mutant to undergo a conformational change, leading to selectively decreasing the binding affinity for and/or catalytic efficiency to phospholipids, of which the binding mode to the enzyme (9) and the surface quality (1) appear to differ from those of triacylglycerol. It is clear that further detailed kinetic and structural studies are required to solve this problem.

A recent study indicated that the COOH-terminal tail of lactase-phlorizin hydrolase contains the consensus sequence for the phosphorylation site by protein kinase A, and this site was indeed phosphorylated by protein kinase A in vitro and in tissue culture (27). This confirmed the intracellular disposition of this tail and suggested its possible physiological significance. In rat PLB/LIP, the corresponding tail does not contain the consensus sequence for a protein kinase A site. The intracellular regulation mechanism mediated by protein kinase A may not operate in the rat PLB/LIP.

Northern blot and RT-PCR analyses suggested that PLB/LIP mRNA was expressed not only in ileal mucosa but also in esophageal mucosa and testis (Figs. 6 and 7). However, rabbit AdRab-B mRNA was not detected in the testis. This may be due to species difference or due to the difference in sensitivity of the method used. The RT-PCR products of these tissues were found to be identical to one another by partial sequencing and restriction fragment length analysis. Immunoblot and immumohistochemical analyses provided further evidence for the localization of PLB/LIP protein (Figs. 8 and 9). In the ileum, a large amount of PLB/LIP existed in the brush border membrane on the apical side of villi; in contrast, secretory PLA₂s are synthesized in and secreted from cells in the bottom regions of gastrointestinal mucosa: gastric chief cells for group I PLA₂ (32) and Paneth cells for group II PLA₂ (29). A similar localization of rabbit AdRab-B has been reported (21). These results support the idea that PLB/LIP acts on the brush border membrane to facilitate the absorption of digested lipids.

Finally, immunohistochemistry showed that, in addition to the absorptive cells, Paneth cells and acrosomes of spermatids were immunopositive, but to a lesser extent, to anti-PLB/LIP antibody, suggesting PLB/LIP’s roles other than intestinal digestion. Paneth cells contain a variety of antibacterial materials including group II PLA₂, which can kill certain strains of intestinal bacteria directly or with the aid of neutrophil bactericidal/permeability-increasing protein (29–31). PLB/LIP with broad specificity toward polar head groups of phospholipids actively hydrolyzes phosphatidylglycerol, the major component of bacterial cell membrane phospholipids, like group II PLA₂ (32, 33). It is interesting to examine whether PLB/LIP in Paneth cells participates in an antibacterial defense mechanism and where it is destined for after synthesis, since Paneth cells lack brush border membranes.
REFERENCES

1. Carey, M. C., Small, D. M., and Bliss, C. M. (1983) *Annu. Rev. Physiol.* **45**, 651–677
2. Rinderknecht, H. (1986) *The Exocrine Pancreas*, (Go, V. L. W., ed) pp. 163–183, Raven Press, New York
3. Carriere, F., Laugier, R., Barrowman, J. A., Douchet, I., Priymenko, N., and Verger, R. (1993) *Scand. J. Gastroenterol.* **28**, 443–454
4. Carriere, F., Barrowman, J. A., Verger, R., and Laugier, R. (1993) *Gastroenterology* **105**, 876–888
5. Diagne, A., Mitjavila, S., Fauvel, J., Chap, H., and Douste, B. L. (1987) *Lipids* **22**, 33–40
6. Pind, S., and Kuksis, A. (1989) *Lipids* **24**, 357–362
7. Gassama-Diagne, A., Rogalle, P., Fauvel, J., Willson, M., Klaebe, A., and Chap, H. (1992) *J Biol Chem.* **267**, 13418–13424
8. Senzena, G. (1986) *Annu. Rev. Cell Biol.* **2**, 255–313
9. Tojo, H., Ichida, T., and Okamoto, M. (1998) *J. Biol. Chem.* **273**, 2214–2221
10. Sanger, F., and Kuksis, A. (1991) *Biochem. Cell Biol.* **69**, 346–357
11. Mayor, S., Menon, A. K., and Cross, G. A. (1999) *J. Biol. Chem.* **265**, 6174–6181
12. Keller, P., Senzena, G., and Shaltiel, S. (1995) *FEBS Lett.* **368**, 563–567
13. Thomas, J. R., Dwek, R. A., and Rademacher, T. W. (1990) *Biochemistry* **29**, 5413–5422
14. Harwig, S. S., Tan, L., Qu, X. D., Cho, Y., Eisenhaeuer, P. B., and Lehrer, R. I. (1995) *J. Clin. Invest.* **95**, 603–610
15. Tojo, H., Ono, T., Kuramitsu, S., Nagamizu, H., and Okamoto, M. (1993) *J. Lipid Res.* **34**, 837–844
16. Nonaka, Y., Takemori, H., Halder, S. K., Sun, T., Ohta, M., Hatano, O., Takakusu, A., and Okamoto, M. (1995) *Eur. J. Biochem.* **229**, 249–256
17. Hatano, O., Takayama, K., Inai, T., Waterman, M. R., Takakusu, A., Omura, T., and Morohashi, K. (1994) *Development* **120**, 2787–2797
18. Kurubuchi, S., and Tanaka, S. (1997) *Cell. Tissue Res.* **288**, 485–496
19. Benet, S. M. (1984) *Nature* **309**, 179–182
20. Rapoport, T. A. (1991) *FASEB J.* **5**, 2792–2798
21. Bolli, W., Schmid-Chanda, T., Semenza, G., and Mantei, N. (1993) *J. Biol. Chem.* **268**, 12901–12911
22. Thomas, J. R., Dwek, R. A., and Rademacher, T. W. (1990) *Biochemistry* **29**, 5413–5422
23. Hooper, N. M., and Turner, A. J. (1988) *Biochem. J.* **250**, 865–869
24. Pind, S., and Kuksis, A. (1991) *Biochem. Cell Biol.* **69**, 346–357
25. Wacker, H., Keller, P., Falchetto, R., Legler, G., and Semenza, G. (1992) *J. Biol. Chem.* **267**, 18744–18752
26. Mayor, S., Menon, A. K., and Cross, G. A. (1999) *J. Biol. Chem.* **265**, 6174–6181
27. Keller, P., Semenza, G., and Shaltiel, S. (1995) *FEBS Lett.* **368**, 563–567
28. Tojo, H., Ono, T., Fujita, H., and Okamoto, M. (1990) *Histochemistry* **94**, 135–140
29. Minami, T., Tojo, H., Shinomura, Y., Matsuawara, Y., and Okamoto, M. (1993) *Biochim. Biophys. Acta* **1170**, 125–130
30. Elsbach, P., Weiss, J., Fransen, R. C., Beckerlarge-Quagliata, S., Schneider, A., and Harris, L. (1997) *J. Biol. Chem.* **254**, 11090–11099
31. Harwig, S. S., Tan, L., Qu, X. D., Cho, Y., Eisenhaeuer, P. B., and Lehrer, R. I. (1995) *J. Clin. Invest.* **95**, 603–610
32. Tojo, H., Ono, T., Kuramitsu, S., Kagamiyama, H., and Okamoto, M. (1988) *J. Biol. Chem.* **263**, 5724–5731
33. Tojo, H., Ono, T., and Okamoto, M. (1993) *J. Lipid Res.* **34**, 837–844