Identification of Essential Residues for Catalysis of Rat Intestinal Phospholipase B/Lipase†

Ting Lu, Masafumi Ito, Urbain Tchoua, Hiroshi Takemori, Mitsuhiro Okamoto, and Hiromasa Tojo*

Department of Molecular Physiological Chemistry, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565-0785, Japan

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ABSTRACT: Intestinal brush border membrane-associated phospholipase B/lipase (PLB/LIP) consists of four tandem homologous domains (repeats 1 through 4) and a COOH-terminal membrane binding domain, and repeat 2 is the catalytic domain that catalyzes phospholipase A_2, lysophospholipase, and lipase activities. We examined the structural basis of the catalysis of PLB/LIP with this unique substrate specificity by site-directed mutagenesis of recombinant repeat 2 enzyme. Ser^{414} and Ser^{459} within the active serine-containing consensus sequence G−X−S−X−G in the best-established lipase family were dispensable for activity. In contrast, substitution of Ala for Ser^{404} almost completely inactivated the three lipolytic activities of PLB/LIP, even though the gross conformation was not altered as determined by CD spectroscopy. Notably, this Ser is located within the conserved G−D−S−L sequence on the NH_2-terminal side in lipolytic enzymes of another group proposed recently. Furthermore, mutagenesis and CD spectroscopic analyses suggested that Asp^{518} and His^{659}, lying within conserved short stretches in the latter group of lipolytic enzymes, were essential for activity. These three essential residues are conserved in the known PLB/LIP enzymes, suggesting that they form the catalytic triad in the active site. These results indicate that PLB/LIP represents a distinct class of the lipase family. PLB/LIP is the first mammalian member of that family. Repeat 2 is equipped with the triad, but not the other repeats, accounting for why only repeat 2 is the catalytic domain. Replacing Thr^{406} with Gly, matching the enzyme's sequence to the lipase consensus sequence exactly, led to a great decrease in secretion and accumulation of inactive enzyme in the cells, suggesting a role of Thr^{406} in the structural stability.

Lipases are ubiquitous lipolytic enzymes and consist of a large superfamily. All structurally characterized triacylglycerol lipases share the α/β hydrolase fold structure irrespective of sequence similarity and use a common catalytic machinery, the Ser−Asp/Glu−His catalytic triad, in which a serine serves as a nucleophile (*1*). Substrate specificities of the members of lipase family, however, vary from enzyme to enzyme, e.g., pancreatic lipases have no significant phospholipase activity, pancreatic lipase-related proteins 2 exhibit both phospholipase A_1 and lipase activities (*2*), and platelet serine-specific phospholipase A_1 are devoid of lipase activity (*3*). The structural bases at the atomic levels for determining these enzymes' substrate specificities including regio- and stereoselectivity and catalytic mechanisms have been studied with great interest in medical, pharmaceutical, and industrial applications of protein engineering of lipases.

We have recently purified and cloned a novel phospholipase B/lipase (PLB/LIP)[1] that catalyzed phospholipase A_2 (PLA_2), lysophospholipase, and lipase activities, but not phospholipase A_1 activity (*4*). It most preferred hydrolysis at the *sn*-2 position of diacylphospholipids and diacylglycerols without strict stereoselectivity, while it apparently exhibited no positional specificity toward triacylglycerol. The enzyme consists of four tandem homologous domains with about 38-kDa molecular masses each (repeats 1 through 4) and a COOH-terminal membrane binding domain, and the catalytic domain is the repeat 2 (*5*). There was no overall sequence similarity to other lipases and PLA_2s. This is the first mammalian enzyme with both triacylglycerol lipase and PLA_2 activities.

Since intensively studied secretory PLA_2s are structurally and mechanistically unrelated to lipases, lipases and PLA_2s were thought to be distinct until recently. However, recent site-directed mutagenesis, chemical modification, and X-ray crystallographic studies demonstrated that like lipases, some of PLA_2 species contain the active site serine, although their three-dimensional folds do not necessarily conform to the α/β hydrolase fold (*6*−*11*). The comparison of PLB/LIP with lipases and these PLA_2s will provide a new insight into the structure−function relationship of lipolytic enzymes. Here we explore essential residues in the catalytic domain of PLB/LIP by site-directed mutagenesis to that end.

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* To whom correspondence should be addressed. Phone: 81-6-6879-3283. FAX: 81-6-6879-3288. E-mail address: htojo@mr-mbio.med.osaka-u.ac.jp.

[1] Abbreviations: $C_{12}E_8$, octaethylene glycol dodecyl ether; GCP, *sn*-glycero-3-phosphocholine; PAF, platelet activating factor; PLA_2, phospholipase A_2; PLB/LIP, phospholipase B/lipase; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Sf, *Spodoptera frugiperda.*

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EXPERIMENTAL PROCEDURES

Materials. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was obtained from Avanti Polar Lipids, Inc. 1-Palmitoyl-*sn*-glycero-3-phosphocholine (GPC) was obtained from Sigma. Triolein and octaethylene glycol dodecyl ether ($C_{12}E_8$) were purchased from Nacalei Tesque, Ltd. (Kyoto, Japan). HPLC-grade acetonitrile was purchased from Katayama Chemical Industries Co., Ltd. (Osaka, Japan).

Preparation of the Wild-Type and Mutant Repeat 2 Enzymes. Repeat 2 enzyme, the catalytic domain of PLB/LIP, was produced as a secretory protein in COS-7 cells transfected with plasmid pSVL#2, which was constructed by inserting a PCR-amplified repeat 2 coding sequence into the *Nhe*I and *Mlu*I sites just after a cDNA sequence encoding the signal peptide of PLB/LIP in pSVL-Nhead as reported (*5*). Transfection by electroporation and cell culture was performed as reported previously (*5*).

We mutated repeat 2 enzyme by two methods. For Ser^{459}-to-Ala (S459A) mutation, the repeat 2 coding sequence of pSVL#2 was divided into two portions at the mutagenic site. The N-terminal part was amplified by PCR with primers F2 (GGgctagcATGAAAGAAGGAACAAAATTCACCTGTCC) and 1412R (CCtttaaaGCCCTTCAAGGAGGGGTT), and the C-terminal part with a mutagenic primer 1412F (GCTGTTGGCACTGGGAAAGA) and R2 (GGTGCGCAGTCAACGACAAGAACCACCACG). The respective products were purified and digested with *Nhe*I and *Dra*I and with *Mlu*I. The two fragments were co-ligated into the *Nhe*I−*Mlu*I sites of pSVL-Nhead. For the other mutants (S404A, S404C, S414A, S429A, S406G, D518A, D518E, D518N, H659A, H665A, H686A), we used Chameleon double-stranded site-directed mutagenesis kit (Stratagene, Ltd.) based on the unique site-elimination mutagenesis procedure (*12*) according to the manufacturer's instructions. A PCR-amplified repeat 2 coding sequence (*5*) was subcloned directly into a pMOS*Blue* vector (Amersham), generating pMOS#2. Appropriate mutagenic primers of 25−30 bp in length, a *Hin*d III selection primer (CACTATAGGGAAAGATTGCATGCCTGC), and pMOS#2 as template were used to produce mutant vectors. All mutations were verified by nucleotide sequencing by the dideoxy method (*13*) using an Applied Biosystem model 373A automatic DNA sequencer. The products were excised with *Nhe*I and *Mlu*I and recloned into pSVL-Nhead. These expression vectors were transfected and cultured by the same method as for the wild-type enzyme.

For production of wild-type enzyme and S404A, D518A, and H659A mutants in a larger amount, we used a baculovirus expression plasmid pAcSG2-#2 that were generated by inserting a *Xho*I−*Bam*HI fragment of pSVL#2 into the *Xho*I and *Bgl*II sites of pAcSG2 (BD Pharmingen). Expression in *Spodoptera frugiperda* (Sf)-9 cells was carried out using a BD BaculoGold baculovirus kit (BD Pharmingen) according to instructions recommended by the manufacturer. Sf-9 cells were cultured in Grace's medium supplemented with yeastolate and lactalbumin hydrolysate and 10% fetal bovine serum (Gibco Laboratories). Expressed enzymes were purified from culture media by sequential chromatographies on a phenyl-Sepharose, a Super QAE-toyopearl and a Cosmogel-QA columns, a modified version of the protocol that was used for purifying rat intestinal PLB/LIP (*4*). Inactive mutants were monitored during purification by enzyme immunoassay or immunoblotting.

Assay for Lipolytic Activities. PLA_2, lysophospholipase, and lipase activities were determined by a nonradiometric HPLC method based on precolumn derivatization with 9-anthryldiazomethane as described previously (*14*). Individual fatty acids released from mixed-acyl glycerophospholipids and trioleoylglycerols were simultaneously determined by this method. Substrate stock solutions used were as follows: mixed micelles of 5 mM POPC and 30 mM cholate; 1-palmitoyl-GPC (5 mM) micelles, and emulsions of 5 mM trioleoylglycerol, 30 mM deoxycholate, and 5% gum arabic. In a typical experiment, the assay mixtures contained 10 mM EDTA, substrate micelles or emulsion (10 μL stock solution), 0.1 M NaCl, 0.1 M Tris/HCl, pH 8.5, and the enzyme sample in a final volume of 50 μL.

Immunoblotting. After 72-h culture of COS-7 cells transfected with the expression vectors, cells and culture media were separately collected. Cells harvested from one culture plate were homogenized in 0.8 mL of 20 mM Tris/HCl containing 0.9% NaCl and 0.1% Triton X-100, sonicated for 1 min, and centrifuged at 43000*g* for 30 min. Five microliters of the resultant supernatant and the culture medium were subjected to SDS−polyacrylamide gel electrophoresis (SDS−PAGE) according to Laemmli (*15*) on 12% gels. Immunoblotting was performed as reported (*16*), and immunoreactive proteins were visualized by a Konica immunostain kit (Konica).

Enzyme Immunoassay for PLB/LIP. The plates were coated with 10 μg/mL of IgG purified from rabbit antiserum raised against amino acids 450−1450 of PLB/LIP (*5*) at 4 °C overnight. Enzyme immunoassay with Fab′-peroxidase-conjugates was carried out as reported (*17*). We used 100 μL of the culture medium and the cell extracts for assays. The peroxidase activity was measured with *o*-phenylenediamine as a substrate.

Circular Dichroism (CD) Spectroscopy. For CD measurements, repeat 2 enzyme, and S404A, D518A, and H659A mutants expressed in Sf-9 cells and purified as described above were used. Protein concentrations were determined by a molar extinction coefficient at 280 nm calculated based on the numbers of Tyr and Trp per molecule. Spectra were accumulated 10 times in 20 mM Tris-HCl containing 50 mM NaCl and 0.1% $C_{12}E_8$ with a JASCO J-600 spectrometer equipped with a cuvette of 2-mm path length.

RESULTS

Candidates for Catalytically Essential Residues of PLB/LIP. We previously identified repeat 2 as the catalytic domain of PLB/LIP for lipolysis (*4*, *5*). DFP, an irreversible inhibitor for PLA_2s with an active serine and for lipases, inhibited purified PLB/LIP (*4*) and recombinant repeat 2 enzyme (*5*). Irreversibility of inhibition was judged kinetically and by isolating the inactivated enzyme by reverse phase HPLC (data not shown). This suggested that the catalysis of PLB/LIP could rely structurally on the chymotrypsin-like catalytic triad involving an active serine (*18*, *19*). For comparative purpose, we could divide the lipase superfamily into two groups, families 1 and 2, based on sequence and structural similarity. In the best-established lipase family (family 1), the serine is present within the conserved pentapeptide

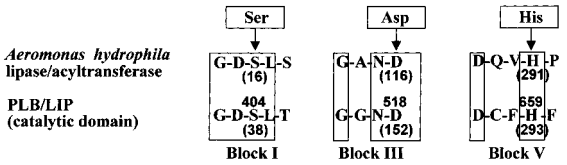

FIGURE 1: Sequence similarity around catalytic triad residues between a family 2 enzyme and rat PLB/LIP. Similarity shown is not extensive, but confined in short sequences around the catalytic triad residues (Ser, Asp, and His). *A. hydrophila* enzyme, a representative of family 2 enzymes, has been most extensively studied, and its catalytic triad residues are located in the motifs indicated as blocks I, III, and V (*23*). The amino acid sequence of repeat 2 was aligned with these motifs. The numbers in parentheses indicate the numbering of amino acid sequence starting from the NH_2 terminus of the mature *Aeromonas* enzyme or of the PLB/LIP catalytic domain, and the numbers in the upper parts of sequences indicate the numbering starting from the initiation Met of the nascent PLB/LIP.

sequence G−X−S−X−G located in the mid-portion of amino acid sequences (*1*), although in some lipases one of Gly residues at both terminals of the motif can be replaced by an amino acid residue with a small side chain (Sm) including Ala and Ser. The repeat 2 domain of PLB/LIP (*5*) contains two serines, Ser^{414} and Ser^{459}, in this conserved sequence. Hereafter, we will use the numbering of amino acid sequence starting from the initiation Met of the nascent protein, although expressed enzymes comprised of the repeat 2 domain only. Mammalian family 1 lipases can be further grouped into at least four subfamilies (pancreatic lipase, carboxyl ester lipase, hormone-sensitive lipase, and acidic lipase families) according to sequence similarity, and the other catalytic triad partners (Asp/Glu and His) are usually located in the motifs characteristic of each subfamily (*20*−*22*); PLB/LIP did not, however, contain such motifs.

Recently, a database search for sequence similarity to *Aeromonas hydrophila* lipase/acyltransferase prompted Upton and Buckley (*23*) to propose another class of the lipase family (family 2), although 3D structures of enzymes in this class have not yet been determined. This includes bacterial and plant proteins that share five short sequence blocks arranged in the same order (blocks I through V from NH_2- to COOH-terminals). Block I contains the active serine in a conserved G−D−S−L−S sequence (*23*, *24*), which is consistent with the family 1 consensus sequence. Unlike family 1 lipases, the motif is located in the NH_2-terminal part of amino acid sequences. Recent site-directed mutagenesis study on the *Aeromonas* enzyme (*25*) suggested that the catalytic triad partners Asp and His were present in blocks III and V, respectively (Figure 1). Notably, this order of the catalytic triad residues in sequences is the same in both families. We found that the repeat 2 enzyme contains Ser^{404}, Asp^{518}, and His^{659} in the places conforming to the family 2 specifications (Figure 1). Moreover, its amino acid sequence could align with block II that contains semi-conserved three Gly residues (Gly^{428}, Gly^{433}, and Gly^{434} of PLB/LIP), but alignment with block IV was difficult. There was no overall homology between PLB/LIP and the *Aeromonas* enzyme.

An Essential Serine Residue for PLB/LIP Catalysis. We individually replaced the candidates for active site serine residues of repeat 2 enzyme (Ser^{404}, Ser^{414}, and Ser^{459}) with alanine by site-directed mutagenesis as described under Experimental Procedures. Ser^{429} in the G−X−S−X−Sm sequence similar to the lipase consensus sequence was also changed to alanine.

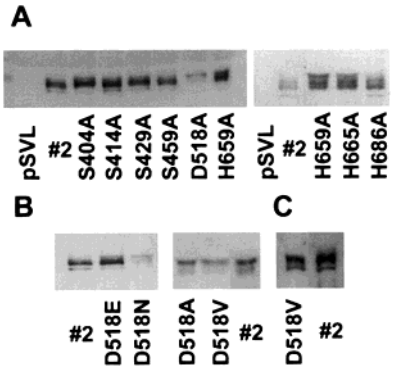

FIGURE 2: Immunoblot analyses of PLB/LIP mutants secreted from COS-7 cells into culture media. pSVL, pSVL vector alone; #2, the wild-type repeat 2 enzyme. The sample volume applied was 10 μL of culture media (A and B). In panel C, the same volumes of repeat 2 enzyme and D518V mutant were partially purified and concentrated on a QAE-toyopearl column (4.6 × 15 mm) and then analyzed by immunoblotting using 10-μL aliquots of the column effluents.

We expressed the wild type and mutant enzymes of the secretory form in COS-7 cells, and immunoblotting analysis showed that levels of the wild-type enzyme in culture media tended to be rather lower than those of serine mutants (Figure 2); Enzyme activities were normalized to protein concentrations determined by a specific enzyme immunoassay with anti-PLB/LIP antibody (*17*). Mutant with a Ser^{404}-to-Ala mutation (S404A) completely lacked PLA_2 activity (comparable with or less than the levels of endogenous PLA_2 activity of 0.2 nmol min^{-1} mL^{-1} of culture medium), whereas the other serine mutants S414A, S459A, and S429A exhibited 106, 10, and 30% of the specific activity of wild-type repeat 2 enzyme (Table 1). Mutation of Ser^{404} to Cys with a similar molecular volume also led to inactivating PLA_2 activity. Since PLB/LIP has lipase and lysophospholipase activities as well as PLA_2 activity, we checked these activities and obtained similar results: S404A mutant lacked the lipolytic activities, but the other Ser mutants retained them (Table 1). The ratios of lysophospholipase to PLA_2 (0.44, 0.46, 0.45, and 0.64 for the wild type, S414A, S429A, and S459A, respectively) and those of lipase to PLA_2 (0.19, 0.17, 0.17, and 0.31, for wild type, S414A, S429A, and S459A, respectively) were rather similar to one another. These results suggested that Ser^{404} was essential for the catalysis of PLB/LIP and further supported the fact that three lipolytic activities were catalyzed by a single active site, in agreement with the results of a previous inhibition study (*4*).

The Other Partners of Catalytic Triad. The sequence of the repeat 2 domain has the motifs characteristic of family 2 enzymes, that is, blocks III and V which contain the putative catalytic triad residues Asp^{518} and His^{659}, respectively (Figure 1). We changed Asp^{518} to Ala, Val, Glu, or Asn by site-directed mutagenesis and then expressed in COS-7 cells. D518V mutant was prepared because Val is similar to Asp in the side-chain length. Levels of Asp mutants secreted into culture media were lower than the wild type enzyme except for D518E mutant (Figure 2). When the wild type enzyme and D518V mutant were partially purified and concentrated on a small QAE-toyopearl column (4 × 15 mm), their staining patterns on immunoblotting were the same (Figure

Table 1: Specific Activities of Wild Type and Mutant PLB/LIPs[a]

constructs	specific activity[b] (μmol min^{-1} mg^{-1} PLB/LIP)		
	PLA_2	lysophospholipase	lipase
wild type			
repeat 2	586 ± 33	260 ± 34	112 ± 13
repeat 2 (purified)	470	234	53.3
serine mutants			
S404A	−0.03 ± 0.03	0.003 ± 0.06	0.019 ± 0.01
S404A (purified)	0.016	0.02	0.014
S404C	−0.27 ± 0.06	*d*	*d*
S414A	1110 ± 10	517 ± 28	193 ± 28
S429A	476 ± 5.8	214 ± 16	80.5 ± 4.7
S459A	228 ± 7.7	145 ± 12.1	69.6 ± 1.2
aspartate mutants			
D518A	0.15± 0.09	−0.013 ± 0.25	0.056 ± 0.019
D518A (purified)[c]	0.001	0.002	<0.001
D518V	−0.83 ± 0.69	−2.2 ± 2.3	0.06 ± 0.02
D518E	39.3 ± 2.4	13.1 ± 0.9	7.1 ± 0.2
D518N	9.31 ± 1.68	5.66 ± 0.26	5.41 ± 0.11
histidine mutants			
H659A	−0.02 ± 0.02	−0.04 ± 0.15	0.02 ± 0.01
H659A (purified)	0.034	0.026	0.012
H665A	7.48 ± 0.24	7.67 ± 0.38	1.5 ± 0.08
H686A	499 ± 6	316 ± 17	155 ± 10

[a] PLB/LIP concentrations in culture media of COS-7 cells were determined by a specific enzyme immunoassay, and those of enzymes purified from culture media of Sf-9 cells were determined by amino acid analysis with γ-amino butyric acid as an internal standard (*4*). Lipolytic activities were assayed as described in Experimental Procedures. Data are presented as means and SD from triplicate determinations. [b] Endogenous lipolytic activities were subtracted in COS-7 cell cultures: 0.20 ± 0.08, 2.37 ± 0.53, and 0.04 ± 0.02 nmol min^{-1} mL^{-1} of culture media for POPC, 1-palmytoyl-GPC, and TOG, respectively. [c] Values comparable with background levels on incubation for 80 min. [d] Not determined.

2C). Specific activities were again calculated with protein concentrations determined by enzyme immunoassay to normalize variations of secreted protein levels. D518A and D518V mutants exhibited virtually no PLA_2, lysophospholipase, and lipase activities, that is, comparable with or less than those (0.2, 2.4, and 0.04 nmol min^{-1} mL^{-1} of medium, respectively) secreted from cells transfected with pSVL alone (Table 1). In contrast, D518E and D518N mutants exhibited respective low, but significant specific activities of PLA_2 (6.7 and 1.6%), lysophospholipase (5.0 and 2.2%), and lipase (6.3 and 4.8%), as compared with those of the wild-type enzyme. These results suggested that Asp^{518} was essential for activity and that these polar residues more or less mimicked the function of the Asp residue.

Similarly, we replaced the putative catalytic His^{659} by Ala. The order of catalytic triad residues in sequence (Ser−Asp−His) was the same in the lipase families (*1*, *25*). Since PLB/LIP contains additional two His residues (His^{665} and His^{686}) near the block V region on the COOH-terminal side of family 2 enzyme sequences, we replaced each of these His residues by Ala. Levels of His mutants in culture media were rather greater than that of the wild-type enzyme (Figure 2A). Specific activities were calculated as in Ser and Asp mutants. H659A mutant exhibited virtually no lipolytic activities (Table 1). Replacement of His^{686} by Ala did not significantly affect the specific activities of PLA_2, lysophospholipase, and lipase (85.2, 122, and 138% of those of the wild-type enzyme, respectively). However, those of H665A mutant decreased to 1.3−3.0% of those of the wild-type enzyme, which was still significant, as compared with those of H659A mutant (comparable with a background level). These results indicate that His^{659} is essential for activity.

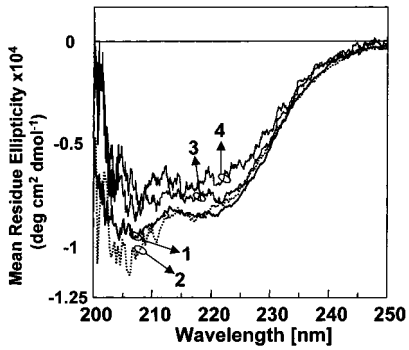

FIGURE 3: CD spectra of wild type and mutant enzymes expressed in Sf-9 cells and purified from culture media. 1, the wild-type enzyme; 2, S404A mutant; 3, D518A mutant; 4, H659A mutant. The helical content of the wild-type enzyme was estimated by calculating the weighted average of the helical fractions at 13 specific wavelengths (210−240 nm) and an empirical equation between helix and β-strand contents provided an estimate of β-strand content as reported previously (*40*).

Specific Activities and CD Spectroscopy of Wild Type and Mutant PLB/LIPs Purified. To examine whether expressed proteins folded properly, the gross conformation of wild-type repeat 2 enzyme was compared with those of S404A, D518A, and H659A mutants by CD spectroscopy at 200−250 nm. For this purpose, these proteins were expressed in Sf-9 cells, and then they were purified to homogeneity as described under Experimental Procedures. Levels of secretion of the wild-type, S404A, D518A, and H659A proteins in Sf-9 cell expression were similar to one another because the respective yields of proteins purified to homogeneity from 100-mL culture media were 13, 23, 36, and 27 μg. Purified Ser^{404}, Asp^{518}, and His^{659} mutants almost completely abolished the three lipolytic activities (Table 1), confirming the results of an expression study with COS-7 cells. All CD spectra were similar to one another with some variations probably caused by the errors in protein concentration determinations and showed a pattern characteristic of α-helical content (Figure 3): the helical and β-strand contents of the wild-type enzyme were estimated to be 28.1 ± 1.2 and 37.8%, respectively, as described in the legend of Figure 3. These results suggested that introducing the mutations into the enzyme did not cause significant conformational changes. We concluded that Ser^{404}, Asp^{518}, and His^{659} were essential for the catalysis of PLA_2, lysophospholipase, and lipase reactions by PLB/LIP.

Effect of Replacement of Thr^{406} with Gly on Activity and Secretion. All structurally resolved family 1 lipases show the α/β hydrolase fold structure, and the active serine is located within the lipase consensus G−X−S−X−G motif described above. The presence of Gly or Sm at the both termini of this motif may allow the torsion angles at the active Ser to maintain the ϵ-conformation, or the "nucleophilic elbow", forbidden usually for amino acid residues other than Gly; in PLB/LIP Thr^{406} replaces this Gly. We therefore changed Thr^{406} to Gly, which meets the specification for family 1 lipase, and examined its effect on enzyme activity and secretion from COS-7 cells. This mutation greatly suppressed secretion of the mutant from COS-7 cells, leading to retention of an appreciable amount of mutant with a low PLA_2 specific activity in cells (Figure 4). The secreted mutant

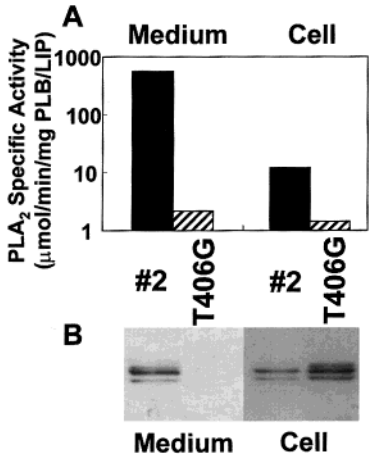

FIGURE 4: Effect of substitution of Gly for Thr[406] on enzyme activity and secretion from COS-7 cells. (A) PLA_2 activities in media and cells transfected with vectors containing repeat 2 or T406G mutant cDNA. Culture media and cells were collected and analyzed as described under Experimental Procedures. The protein concentrations were determined by enzyme immunoassay. (B) Immunoblot analyses of culture media and cell fractions.

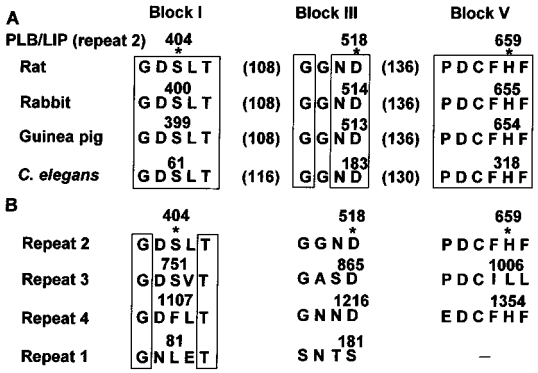

FIGURE 5: Alignment of short amino acid sequence stretches around the catalytic triad residues in blocks I, III, and V. (A) Comparison of repeat 2 domain sequences of rat, rabbit (*41*), and guinea pig (*42*) PLB/LIPs, and the sequence of a putative *C. elegans* homologue (accession number, Z68132). Numbers in parentheses indicate the number of residues between the conserved blocks. (B) Comparison of sequences of repeat 2 and the other repeats in rat PLB/LIP. (−) This block is deleted in repeat 1.

proteins were detectable by enzyme immunoassay, but not by immunoblotting: the PLA_2 specific activity of the mutant was also very low (0.2% of that of the wild type, Figure 4A).

DISCUSSION

This site-directed mutagenesis study provides evidence that Ser[404], Asp[518], and His[659] are essential for activity of PLB/LIP, suggesting that these three residues form a chymotrypsin-like catalytic triad in its active site. These essential residues also lie in the sequences conserved within the known rabbit and guinea pig PLB/LIP enzymes and a distantly related *Caenorhabditis elegans* homologue (Figure 5A), consistent with their important roles in the catalysis of PLB/LIP. The conserved stretches differed from those of family 1 enzymes, but resembled those of bacterial and plant family 2 enzymes (Figure 1). PLB/LIP is the first mammalian member of the latter category. In family 2 enzymes, efforts of estimating the catalytic triad residues have relied largely on sequence similarity (*23*, *26*). A site-directed mutagenesis study on *A. hydrophila* lipase/acyltransferase provided an experimental support for this (*25*). The results of this study further supported the fact that the active site Ser, Asp, and His of family 2 enzymes are located in blocks I, III, and V, respectively.

All family 1 enzymes conform to the α/β hydrolase folds, the essential features of which seem to be the order of the catalytic triad in sequence, the presence of the nucleophilic elbow, and at least five parallel β sheets ($\beta3-\beta7$) forming a structural core (*1*, *27*). PLB/LIP also contains such a Ser−Asp−His linear sequence. Its catalytic Ser[404] is, however, located in the NH_2-terminal part of the catalytic domain, in contrast to family 1 enzymes with the corresponding Ser located in the mid-portion of the sequences. The latter results from a structural requirement for the presence of the essential Ser in a nucleophilic elbow between a central $\beta5$ and helix C, which is the best-conserved structure in the α/β hydrolase folds (*1*). Hence, the presence of the Ser in the NH_2-terminal parts of family 2 enzymes and PLB/LIP might be incompatible with features of the α/β hydrolase fold. Similar locations of the catalytic Ser were reported in *Streptomyces scabies* esterase (*28*) and platelet activating factor (PAF) acetylhydrolase type Ib (*10*), of which folds are novel. Conclusive distinction whether PLB/LIP belongs to a subfamily of or a family distinct from the family 1 category should await the experimental determination of its tertiary structure.

The active Ser was located in the G−X−S−X−G consensus in family 1 enzymes, and PLB/LIP's catalytic domain contains such serines at positions 414 and 459; their mutations did not, however, cause a significant loss of activity. This motif is not unique to esterases and lipases but occurs in proteins with no hydrolytic activity (*29*). We then turned to Ser404 lying in the sequence G−D−S−L conserved among family 2 enzymes. Its mutation to Ala led to a decrease of all lipolytic activities of PLB/LIP by $\sim10^5$, which was comparable with the extent of a decrease in the k_{cat} value reported in replacement of the active Ser[195] of trypsin by Ala (*30*). This with the help from secondary structural data by CD spectrometry (Figure 3) demonstrated that Ser[404] plays an important role in the catalysis of PLB/LIP.

In family 1, a structural need to maintain the nucleophilic elbow results in the prevalence of Gly in the both ends of the motif containing the catalytic Ser (*29*). PLB/LIP has Thr[406] with a larger molecular volume in place of the second Gly in the motif, but the mutation of Thr[406] to Gly for its sequence to match the G−X−S−X−G consensus caused great inhibition of its secretion from COS-7 cells and accumulation of the inactivated enzymes in the cells. Similar results have been reported in the *A. hydrophila* enzyme of the family 2 type (*31*), which has a Ser as the Thr[406] equivalent. These results suggest that in family 2 enzymes the second amino acid following the catalytic Ser might be important for precise folding of proteins. In this connection, type Ib PAF acetylhydrolase with a bulky Val replacing the Gly takes a conformation around the catalytic Ser different from the ϵ-conformation (*10*).

PLB/LIP's amino acid sequence contains the motif unique to family 2 enzymes that includes the putative catalytic Asp (block III in Figure 1). As expected, D518A and D518V mutants expressed in COS-7 cells almost completely lost the three lipolytic activities. Using the purified D518A mutant,

we verified its intact structural integrity by CD spectroscopy, and confirmed the essentiality of Asp^{518} for PLB/LIP catalysis (Table 1 and Figure 3). Secretion levels on mutation of Asp^{518} to uncharged amino acids were lower than those of the wild-type enzyme and D518E mutant with the negative charge retained at the mutated site (Figure 2), like in an expression of the *Aeromonas* enzyme in *Escherichia coli* system (*25*). A similar effect of mutation at the triad acid on secretion has often been reported in family 1 enzymes (*32*−*34*). This suggests structural roles of the catalytic acid in the folding and/or stability of protein. Since the CD data suggested that folding of the D518A mutant purified from culture media was not impaired (Figure 3), Asp^{518} would take part in fine-tuning the positioning of the catalytic His and in stabilizing the active site geometry. The lifetime of a heterologously expressed protein would depend on the nature of host cells as well as on its structural stability itself. In Sf-9 cell expression, secretion levels of D518A proteins were similar to those of the wild type, S404A, and H659A proteins in contrast to in COS-7 cell expression.

The PLB/LIP mutant with a conservative replacement of Asp^{518} with Glu retained the lipolytic activities by ~6% of the wild-type enzyme (Table 1). The effect of exchange of the catalytic acid between Asp and Glu on activity varied from lipase to lipase (*34*−*37*). Differences in the fine structure around the catalytic triad residues may be responsible for how a change in the side chain length of the catalytic acid by one carbon atom affects activity (*34*).

Homology of the PLB/LIP's sequence with block V of family 2 was weak (Figure 1), but His^{659} in this region was found to be catalytically essential, because a H659A mutant with its structural integrity intact as revealed by CD spectroscopy had almost no activity (Table 1 and Figure 3). The assignment of this region as block V and of His^{659} as a catalytic triad residue is therefore reasonable. In light of the conserved sequence order of the triad (Ser−Asp−His), we mutated His residues (His^{665} and His^{686}) near or on the COOH terminal side of His^{659}. As expected, mutation of His^{686} to Ala did not affect the lipolytic activities; the corresponding mutation at His^{665} led to an ~80-fold loss of lipolytic activities, but less than in His^{659} mutants (an ~14 000-fold loss, Table 1). This suggests that His^{665} is not essential for activity, but plays some roles in catalysis.

PLB/LIP consists of homologous 4 tandem repeats, but only repeat 2 is the catalytic domain (*5*). This can be explained by the fact that repeat 2 domain of rat PLB/LIP is equipped with the catalytically essential Ser, Asp, and His residues, but the other repeats lack one (repeats 3 and 4) or all (repeat 1) of these residues (Figure 5B). In contrast, repeat 3 of the guinea pig enzyme and repeats 3 and 4 of the rabbit enzyme also have all triad residues. However, a chemical modification study of rabbit PLB/LIP with diisopropyl fluorophosphate, a serine-modifying irreversible inhibitor, indicated that the inhibitor reacted with a Ser^{400} in the G−D−S−L−T sequence of repeat 2, but not with the equivalent serines in the other repeats (*38*). The reason for this is unknown at present, but optimal arrangement and orientation of the triad and oxyanion hole residues at the transition state should be required for catalysis. Since respective overall similarity between repeat 2 and either repeat 3 or repeat 4 is at most ~50% in these enzymes, subtle changes in the active site geometry might lead to poorer efficiency of catalysis. Alternatively, domain−domain interaction may affect activity; for example, the interaction, presumably via disulfide bridge, between domains perturbed substrate specificity in a PLB/LIP mutant lacking its COOH-terminal membrane-binding domain (*5*).

Parallel loss of its lipolytic activities on mutation at the catalytic residues (Table 1) strongly suggested that PLB/LIP uses the catalytic triad in a single active site to display its broad substrate specificity including PLA_2, lysophospholipase, and lipase activities. All known lipases and PLA_2s,[2] except for PLA_2s of the secretory type, use the same catalytic machinery, i.e., the catalytic triads. They can, however, presumably use substrate binding subsite structures at the active site for determining substrate selectivity and, in some cases, regulatory domains to pursue a broad spectrum of physiological functions. PLB/LIP is expressed in rat ileum and sperm (*4*, *5*). We recently studied the functional relationship between the intestinal PLB/LIP and pancreatic lipolytic enzymes using WBN/Kob rats with pancreatic insufficiency (*17*) and found that the PLA_2 activity of PLB/LIP compensated for the depletion of pancreatic PLA_2 in WBN/Kob rats. In the sperm, we have not yet obtained evidence for its physiological significance. It is important to determine which substrate is relevant to the physiological function of an enzyme with broad substrate specificity.

[2] Cytosolic Ca^{2+}-dependent PLA_2 does not use His as a general base, but presumably uses an Asp forming the Ser-Asp dyad (*11*), indicating that it is a relative of enzymes with the catalytic triad (*39*).

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