Structure and Activity of Rat Pancreatic Lipase-related Protein 2*

(Received for publication, July 8, 1998)

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The pancreas expresses several members of the lipase gene family including pancreatic triglyceride lipase (PTL) and two homologous proteins, pancreatic lipase-related proteins 1 and 2 (PLRP1 and PLRP2). Despite their similar amino acid sequences, PTL, PLRP1, and PLRP2 differ in important kinetic properties. PLRP1 has no known activity. PTL and PLRP2 differ in substrate specificity, bile acid inhibition, colipase requirement, and interfacial activation. To begin understanding the structural explanations for these functional differences, we solved the crystal structure of rat (r)PLRP2 and further characterized its kinetic properties. The 1.8 Å structure of rPLRP2, like the tertiary structure of human PTL, has a globular N-terminal domain and a β-sandwich C-terminal domain. The lid domain occupied the closed position, suggesting that rPLRP2 should show interfacial activation. When we reexamined this issue with tripropionin as substrate, rPLRP2 exhibited interfacial activation. Because the active site topology of rPLRP2 resembled that of human PTL, we predicted and demonstrated that the lipase inhibitors E600 and tetrahydrolipstatin inhibit rPLRP2. Although PTL and rPLRP2 have similar active sites, rPLRP2 has a broader substrate specificity that we confirmed using a monolayer technique. With this assay, we showed for the first time that rPLRP2 prefers phosphatidylglycerol and ethanolamine over phosphatidylcholine. In summary, we confirmed and extended the observation that PLRP2 lipases have a broader substrate specificity than PTL, we demonstrated that PLRP2 lipases show interfacial activation, and we solved the first crystal structure of a PLRP2 lipase that contains a lid domain.

Lipases are ubiquitous enzymes expressed by diverse organisms. They hydrolyze phospholipids and triglycerides to generate fatty acids for energy production or for storage and to release inositol phosphates that act as second messengers. The role of phospholipases in cellular signaling pathways has increased interest in these lipases. Similarly, the central role of triglyceride lipases in energy production and their potential industrial applications have stimulated studies of these essential lipases. As a result, our knowledge about lipases and of the molecular details underlying lipolysis has increased considerably.

Among these contributions was the cloning of cDNAs encoding various lipases. Comparisons of the amino acid sequences predicted from these cDNAs led to the hypothesis that a lipase gene family evolved from a common ancestral hydrolase. At least three members of the lipase gene family are synthesized and secreted by the pancreas. One, the archetype of the family, colipase-dependent pancreatic triglyceride lipase (PTL) has been studied for over 100 years. Giller et al. (2) isolated human pancreatic cDNAs encoding the other two, named pancreatic lipase-related proteins 1 and 2 (PLRP1 and PLRP2), 6 years ago. The primary sequences of the human PLRP1 and PLRP2 have 68 and 65% identity to the primary sequence of PTL with conservation of the catalytic triad and major determinants of tertiary structure. Subsequently, other groups reported the presence of related proteins in the pancreas from several species (3–7).

The best studied of the PTL homologues is PLRP2. These studies provided the first indications that PLRP2 has functional properties different from those of PTL. Mouse PLRP2 was cloned from interleukin-4-activated cytotoxic T-lymphocytes, and rat (r)PLRP2 was cloned as a zymogen granule membrane protein, GP3 (4, 7). The presence of PLRP2 in lymphocytes and on the zymogen granule membrane raised the possibility that PLRP2 has functions other than hydrolyzing dietary fats. For instance, lymphocyte PLRP2 may participate in cell killing, and the PLRP2 on the zymogen granule membrane may mediate granule fusion with the plasma membrane.

The expression and purification of PLRP2 lipases allowed the enzymatic properties of these enzymes to be characterized. These studies revealed that PLRP2 lipases have enzymatic properties that distinguish them from PTL (4, 5, 7–9). First, PLRP2 has a broader substrate specificity and will hydrolyze triglycerides, phospholipids, and galactolipids. PTL hydrolyzes only triglycerides. Second, they have different behaviors in the presence of bile salts and with colipase. Third, PLRP2 members efficiently hydrolyze monomers of water-soluble, short chain triglycerides, whereas PTL possesses low activity against monomeric substrates. PTL activity increases dramatically against water-insoluble substrates presenting an oil-water interface, a property known as interfacial activation. Clearly, the explanation for these kinetic and functional differences must lie in the structure of these proteins.

The first pancreatic lipase structure to be solved was that of the human enzyme (hPTL) (10). Subsequently, the complex of hPTL with porcine colipase was elucidated, as were the struc-

*This work was supported by National Institutes of Health Grant HD3306002. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†The abbreviations used are: PTL, pancreatic triglyceride lipase; Col, colipase; c, coypu; g, guinea pig; h, human; PLRP1, pancreatic lipase-related protein 1; PLRP2, pancreatic lipase-related protein 2; p, porcine; r, rat; MES, 4-morpholineethanesulfonic acid.

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tures of hPTL-porcine colipase in complex with phospholipid or phosphonate inhibitors (10–13). The structures of horse PTL, hPTL-human colipase complex, and porcine PTL-porcine colipase complex have also been determined (14–16). The structures of hPTL-porcine colipase crystallized in the presence of mixed phospholipid/bile salt micelles or of a C11 phosphonate inhibitor revealed that the lid domain (residues 237–261) covering the active site of hPTL could move away from its closed position (12, 13). The movement creates new contacts with colipase to form the lipid-water interfacial binding site. Another structural element, the β5 loop, undergoes a spatial reorganization and folds back on the core of the protein. These drastic conformational changes, leading to the open conformation, give substrate free access to the catalytic triad.

Comparisons of PLRP2 and PTL family members require high resolution crystal structures, but no structure of a true PLRP2 is available. Withers-Martinez et al. (38) reported the crystal structure of a chimeric lipase with the C-terminal domain of human PTL and the N-terminal domain of guinea pig PLRP2 (gPLRP2). Although inferences about the conformation of PLRP2 can be made from the known PTL and gPLRP2 structure, valid conclusions must be based on the actual PLRP2 structure. In this paper, we report the first crystal structure of a PLRP2 family member that has a lid domain and further characterize the enzymatic properties of this lipase.

MATERIALS AND METHODS

Expression of Rat PLRP2 in Sf9 Cells—We expressed recombinant rat PLRP2 in baculovirus-infected Sf9 cells as described previously with the following modifications (3, 8). The protein was produced in 1-liter spinner flasks containing 350 ml of serum-free medium, EX-CELL 400 (JRH Biosciences, Lenexa, KS), instead of 1 liter of medium. We harvested the medium 3 days instead of 4 days post-infection. The smaller medium volume and shorter culture times gave higher protein yields as initially observed by Bezzine et al. (17).

Purification of Rat PLRP2—We removed cells and debris by centrifuging the medium at 5000 rpm for 10 min in a Beckman J2–21 centrifuge with a JA-20 rotor. The medium was concentrated over a Pall Filtron 10k UltraSette membrane (Filtron Technology Corporation, Northborough, MA) to about 50 ml and dialyzed by repeated dilution with 10 mM Tris-Cl, pH 8.0, and concentrating over the UltraSette membrane. We applied the sample to a 75-ml bed volume DEAE-Blue-Sepharose (Bio-Rad) equilibrated in the Tris buffer. PLRP2 was eluted with a linear NaCl gradient from 0.0 to 0.6 M. Assay with tributyrin in a pH-stat located the PLRP2. We pooled the peak fractions and dialyzed against 10 mM MES, pH 6.2, buffer followed by concentration over an Amicon YM30 membrane (Amicon, Inc., Beverly, MA). The sample was applied to a Pharmacia Mono-S column (5-ml bed volume) (Amersham

| Table I | Data collection and final refinement statistics |
|---------|-----------------------------------------------|
| Data collection | Resolution limit (Å) 10.0 – 1.8 |
| | Data completion all/last shell (l/s[l] > l) 97.5/81.9 |
| | Redundancy 2.4 |
| | Rsym (%) 6.3 |
| Refinement | Resolution limit (Å) 10.0 – 1.8 |
| | Number of reflections 36060 |
| | Number of protein atoms 3799 |
| | Number of water molecules 295 (+1 GlcNAc, 7 EG, 1 Ca) |
| | Final R-factor/R-free (%)b 20.3/24.2% |
| | B-factors (Å2) |
| | Main chains/side chains 19.7/22.0 |
| | water 30.6 |
| | Ca/EG/GlcNAc 9.4/26.9/41.7 |
| | Root mean square deviations from ideal values |
| | Bonds (Å) 0.007 |
| | Angles (°) 1.4 |
| | Improper/dihedral angles (°) 1.3/25.5 |

*R*sym = Σ | L(hk) – <L(hk)> | / | Σ <L(hk)> | ηh; <L(hk)> is the observed intensity of the ith measurement of reflection h, and <L(hk)> ≠ 0 mean intensity of reflection h.

*R* = Σ | Fo –Fc | / | Σ | Fc | Fo and Fc are the observed and calculated structure factor amplitude, respectively.

FIG. 1. Stereo view of the sigma weighted electron density map of the closed lid contoured a 1 σ.
Pharmacia Biotech) attached to an Akta Purifier (Amersham Pharma-
cia Biotech). The column was equilibrated in 50 mM MES, pH 6.2, and
eluted with a linear NaCl gradient from 0.0 to 1.0 M. rPLRP2 eluted
from the column as a symmetrical peak identified by activity against
tributyrin. The peak fractions were pooled, and the pH was adjusted to
8.0 with 1 M Tris-Cl, pH 8.0. The purified protein migrated as a single
band on 10% SDS-polyacrylamide gel electrophoresis and had activity
against tributyrin, trioctanoin, triolein, and phosphotidylcholine as de-

Fig. 2. Structure of rPLRP2. A, stereo view of the Ca trace of rPLRP2. The side chains of the N and C terminus residues as well as those of
the catalytic Ser152 and of the lid residue Trp252 are represented. The ethylene glycol molecules (blue) and the GlcNAc residue linked to Asn254 are
shown. Green, C terminus domain; brown, N-terminal catalytic domain; yellow, catalytic domain following the lid; pink, lid. B, stereo view of the
Ca trace of rPLRP2 superimposed on those of the other known closed pancreatic lipase structures.
Structure/Activity of Pancreatic Lipase-related Protein 2

The activity of rPLRP2 against tributyrin was measured after incubating rPLRP2 with either E600 or tetrahydrolipstatin. A, 100-fold molar excess of tetrahydrolipstatin in the presence of 4 mM sodium taurodeoxycholate in 10% isopropanol. B, 100-fold molar excess of E600 in 5% isopropanol without bile salts. Another aliquot of E600 equivalent to a 100-fold molar excess was again added after the 1-h time point was sampled.

Fig. 3. Interfacial activation of rPLRP2. A, interfacial activation demonstrated in the laboratory of R. Verger. The tripropionin solutions were made in 1% gum arabic as described under “Materials and Methods.” B, interfacial activation demonstrated in the laboratory of M. Lowe. The tripropionin solutions were prepared in 2% gum arabic as described under “Materials and Methods.” C, interfacial activation with p-nitrophenylbutyrate as the substrate. The assay included 20 µg of rPLRP2 and a 5-fold molar excess of pure human colipase was added. C, interfacial activation with p-nitrophenylbutyrate as the substrate. The assay included 20 µg of rPLRP2 and a 5-fold molar excess of pure human colipase. The vertical dashed line in each figure shows the concentration when saturation of the tripropionin or p-nitrophenylbutyrate solutions occurs.

scribed previously (8). rPLRP2 concentrations were determined by the BCA protein assay using purified bovine serum albumin as the standard.

Lipase Assays—1,2-rac-Didecanoyl glycerol (dicaprin) was purchased from Sigma. 1,2-sn-Didecanoyl phosphatidylcholine, 1,2-sn-didecanoyl phosphatidylethanolamine, and 1,2-sn-didecanoyl phosphatidylglycerol were purchased from Fluka (Paris, France). 3-Monogalactosyl-1,2-rac-didecanoyl glycerol was prepared by chemical synthesis and is a generous gift from Professor G. C. Ortaggi (Roma). A rPLRP2 solution 0.5 mg/ml was used for kinetic experiments using the monolayer technique as well as for the interfacial activation experiments, using tripropionin as substrate. Bulk phase assays were done by the pH-stat method as described (5, 8, 18). The conditions for inhibitor assays are given in the figure legends. Tetrahydrolipstatin was kindly provided by Dr. Hans Lengsfeld from Hoffmann-LaRoche.

Kinetic Experiments on Monolayers—Before each utilization, the Teflon trough used to form the monomolecular film was cleaned with water, then gently brushed in the presence of distilled ethanol, washed again with tap water, and finally rinsed with double-distilled water (19, 20). The aqueous subphase was composed of 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 21 mM CaCl2, and 1 mM EDTA for all lipases. The buffer was prepared with double-distilled water and filtered through a 0.45-µm Millipore filter. Any residual surface-active impurities were removed before each assay by sweeping and suction of the surface. Kinetic experiments were performed with a KSV-2200 barostat (KSV-Helsinki) and a “zero-order” Teflon trough (20). The trough was equipped with a mobile Teflon barrier, which was used to compensate for the substrate molecules removed from the film by enzyme hydrolysis (monododecanoyl glycerol, decaenoic acid, lyso-dodecanoyl phospholipids, and dodecanolic acid are soluble in water), thereby keeping the surface pressure constant. The latter was measured using a Wilhelmy plate (perimeter 3.94 cm) attached to an electromicrobalance, which was connected in turn to a microprocessor controlling the movement of the mobile barrier. The reactions were performed at room temperature (20 °C). The subphase of the reaction compartment was continuously agitated with a 2.0-cm magnetic stirrer moving at 250 rpm. The rPLRP2 solution (10–60 µM) at 0.5 mg/ml) was injected through the film over the stirrer with a Hamilton syringe. The surface area of the reaction compartment was 31 cm², and the volume was 55 ml. The length of the reservoir compartment was 30 cm, and the width was 17.6 cm.

Interfacial Activation of rPLRP2—The TC₃ solutions were systematically prepared by mixing three times 30 s in a Waring blender, a given amount of TC₃ in 15 ml of 1% gum arabic in water (w/v) (21). Before each assay, 5 ml of the TC₃ gum arabic solution was added to 10 ml of pure water in the thermostated (37 °C) pH-stat vessel. Deionized water, purified with a Millipore Super Q system, was used throughout all the experiments. Lipase activity was recorded with either a TTT 80 pH-stat (Ratiometer) equipped with a 250-µl syringe containing 0.1 N NaOH or a VIT 90 pH-stat (Radiometer) equipped with an automatic burette containing 0.05 ± NaOH. Activity was measured potentiometrically at pH 7.0, but at pH 8.0 the spontaneous hydrolysis of TC₃ reaches relatively high levels. The assay was carried out on a mechanically stirred solution of substrate in the reaction vessel. Spontaneous hydrolysis was recorded in the pH-stat mode for 2 min before lipase injection, and this background value was subtracted from the activity measurement. One international lipase unit is the amount of enzyme catalyzing the release of 1 µmol fatty acid/min. Each assay contained a 5-fold molar excess of pure colipase. We checked that bovine serum albumin (final concentration, 1%) had no detectable catalytic activity on a TC₃ solution (7.7 mM) or on a TC₅ emulsion (15.33 mM). Interfacial activation assays with p-nitrophenylbutyrate were done as described (22). Crystallization, X-ray Data Collection, and Processing—Small crystals of rPLRP2 were obtained at room temperature using the hanging drop vapor diffusion method, by mixing 2 µl of protein (18 mg/ml) in 0.2 M NaCl, 0.1 M Tris-HCl, pH 8.5) and 2 µl of the Hampton screen 1 solution 36 (Hampton Research, Laguna Hills, CA) containing 8% polyethylene glycol 8000 and 0.1 mM Tris-HCl, pH 8.4. Crystals were improved by diminishing the polyethylene glycol and protein concentra-
tions by a factor of two. Larger crystal were obtained by macroseeding using a 4:1 mixture and 2% polyethylene glycol 8000.

The crystals were soaked in a synthetic mother liquor containing 33% ethylene glycol as cryoprotectant and were subsequently cryo-cooled using the Oxford equipment (Oxford Cryosystems, Oxford, UK). X-ray diffraction data were collected to 1.80 Å resolution on a 30-cm Mar-research imaging plate at 0.970 Å wavelength on beamline DW32 in LURE (Orsay, France). The data were processed using the DENZO software package (23). The rPLRP2 crystallizes in the space group P21 with cell dimensions $a = 57.4 \, \text{Å}$, $b = 79.1 \, \text{Å}$, $c = 60.9 \, \text{Å}$, and $\beta = 102.1^\circ$. Specific volume calculations yielded one molecule/asymmetric unit, with a $V_m$ of 2.7 Å$^3$/Da and a solvent content of approximately 54% (24).

**FIG. 5.** Monolayer activity of PLRP2 lipases and PTL against various substrates. Activities were determined in a monolayer trough as described under “Materials and Methods.” The substrate legend is given in the figure. n-gPLRP2, guinea pig PLRP2 isolated from pancreas; gPLRP2, recombinant guinea pig PLRP2; hPTL, human PTL isolated from pancreas; cPLRP2, recombinant coypu PLRP2; rPLRP2, recombinant rat PLRP2.

**TABLE II**
Activities of lipases against various triglycerides in bulk phase assays
All assays done in pH-stat with excess colipase.

| Lipase | Tripropionin$^a$ | Tributyrin | Triolein | Dicaprin$^b$ |
|-------|-----------------|------------|----------|-------------|
| hPTL  | 7000            | 8000$^c$   | 1600$^c$ | 170         |
| rPLRP2| 224             | 3900       | 1200     | 130         |
| gPLRP2| 1000            | 1700$^c$   |          |             |
| cPLRP2| 850             | 2000$^d$   |          |             |

$^a$ Measured in gum arabic with no taurodeoxycholate.
$^b$ Measured in 4 mM taurodeoxycholate.
$^c$ Taken from Ref. 39.
$^d$ Taken from Ref. 5.
A total number of 36,060 unique reflections were indexed using the SCALEPACK program with an R-factor on intensities of 6.3%, a data set multiplicity of 2.4 and a completeness of 97.5%, between 10.0 and 1.8 Å (Table I) (25).

**Structure Determination**—The structure was solved with the molecular replacement method using the AMoRe program (25). The closed form of the classical human pancreatic lipase was used as the search model. The rotation function of the two bodies, performed with the N-terminal domain without the lid and the C-terminal domain, yielded only one significant solution for the entire molecule (correlation coefficient of 0.50 and R-factor of 0.39 between 10.0 and 3.5 Å resolution). The structure was refined using the X-PLOR program (26). After performing 12 cycles of slow cooling protocol starting at 2000 K and manual replacement and adjustments using the Turbo-Frodo program, the R-factor had decreased from 45 to 24.6% (R-free 31.8%) (27). The water molecules located in the ($F_o - F_c$) and ($F_c - F_o$) maps numbered 295, and one GlcNac sugar bound to Asn334 was identified. Seven molecules of ethylene glycol were modeled in the density. The R-factor calculated with 36,060 reflections between 10.0 and 1.8Å resolution was 20.3% and the final R-free factor calculated with 5% reflections was equal to 24.2% (Table I). The Ramachandran plot and the electron density map (see Fig. 1) further demonstrate the quality of the model. Coordinates have been deposited in the Protein Data Bank with the accession number 1bu8.

**RESULTS AND DISCUSSION**

**Overall Structure of rPLRP2**—The structure of rPLRP2 has been refined at the highest resolution observed among the pancreatic lipases. The current model consists of 3505 protein atoms, one GlcNac connected to Asn334, one calcium ion, seven molecules of ethylene glycol, and 295 water molecules. One protein segment, located between residues 405 and 411, was found to have no interpretable electron density and was therefore removed from the model. In the Ramachandran plot (Procheck software) of the final model, all of the main chain dihedral angles but one (Ser152) fell within the allowed regions (90.5% in the most favorable regions, and 9.3% in additional allowed regions) (28). The active site Ser152 has the conformation found in other lipases and in the α/β hydrolase fold family and is therefore located in a "generously allowed region" (28, 29). The glycosylated Asn334 is conserved in all PLRP2 lipases except the coypu. This glycosylation site was not present in the structure of the gPLRP2/hPTL chimera because the C-terminal domain originated from hPTL. The PLRP1 glycosylation site at position 138 is not present in PTL or in PLRP2 lipases (30). Another potential glycosylation site in classical lipases is located at position 166 but is only partially conserved (14) and is not present in PLRP1 or PLRP2 lipases. Due to cryocooling, high resolution, and low B-factors, seven molecules of cryoprotectant can be observed in the electron density map. The ethylene glycol molecules are stabilized mainly by hydrogen bonds and in part by hydrophobic interactions. Hydrogen bonds are established with Arg side chains (four cases) and with main and side chains of various semi-polar and polar residues. Hydrophobic interactions involve aromatics (four cases) and aliphatic residues (two cases).

The rPLRP2 structure belongs to the α/β hydrolase fold family of proteins (29). The protein consists of two main domains, a globular N-terminal domain and a β-sandwich C-terminal domain. This structure closely resembles the structure of hPTL and the other members of the pancreatic lipase fold family (see Fig. 2A) (10). The core of the N-terminal domain of the molecule consists of a tightly packed β-sheet surrounded by five helices. The rPLRP2 lid, which adopts a closed conformation, has an electron density of excellent quality and does not show any sign of disorder or particular flexibility (Fig. 1). Its B-factors display the same pattern as in other closed pancreatic lipases (data not shown). The lid is located between the two sides of the bridged cysteine residues 237–261. Together with the β5 loop, it adopts the same conformation as that observed in the closed structure of hPTL (10). The active site is located at the bottom of a hydrophobic cleft that is covered by the lid. The catalytic triad (Ser152, His363, and Asp176) includes the nucleophile belonging to the usual consensus sequence G-X-(nucleophile)-X-G (Fig. 2A).

The structure of rPLRP2 has been superimposed on all the known pancreatic lipase structures: hPTL, hPTL bound to porcine colipase (hPTL-pCol), hP-L-pCol in complex with a C11 phosphonate inhibitor (hPTL-pCol-C11), horse pancreatic lipase, gPLRP2, dog pancreatic PLRP1, and porcine pancreatic lipase bound to porcine colipase (pPTL-pCol). The N-terminal catalytic domains of the various molecules superimpose well (within 1.0 Å), apart from the mobile loops (the lid and the β5 loop), which switch between the closed and open conformations. The degree of structural homology of rPLRP2 compared with the other lipases found in the closed conformation (dog PLRP1, hPTL, and horse PTL) is remarkable (Fig. 2B). As described previously, the C-terminal domain orientations of the structures included in the comparisons were found to differ due to rotations of a few degrees occurring around hinge residue 337 (Fig. 2B) (11-14).

**Active Site Structure of rPLRP2**—We have proposed a model for the putative binding of a triglyceride at the hPTL-pCol-C11 active site crevice, based on the hPTL-pCol-C11 complex (13). One acyl chain of the triglyceride, the leaving fatty acid, was assumed to bind at the position of the C11 conformer 1, whereas a second acyl chain was taken to bind at the position of the C11 conformer 2 (Fig. 3A). We superimposed and compared the closed structure of rPLRP2 with the open structure of hPTL-pCol-C11, to investigate, on the basis of our model, whether the binding of a triglyceride to rPLRP2 was compatible with the active site structure. To make these comparisons valid, a model of the open rPLRP2 has been built. The lid and the β5 loop were taken from the open hPTL structure and grafted onto the core of the PLRP2 enzyme, and the lid residues were substituted according to the rPLRP2 sequence. No residues were found to be substituted between hPTL and rPLRP2 in a 10 Å radius around the nucleophilic Ser152 O-. Consequently, the C11 phosphonate inhibitor positioned into the open rPLRP2 model exhibits the same protein contacts as in the classical lipase.

hPTL has two colipase binding sites. One is located at the C-terminal domain and was found to bind colipase when the catalytic domain is in the closed or in the open conformation (11). A second site appears only when lipase opens, yielding an interaction of colipase with the open lid (12). We have investigated the likelihood of both sites with a model of open PLRP2. The three lid domain residues involved in the interaction with colipase are conserved. Among the 12 C-terminal residues interacting with colipase, only four are substituted compared with hPTL: Phe460 → Tyr, Ile461 → Leu, Tyr463 → Asn, and Glu441 → Asp (31). The I401L substitution does not alter the interaction. The E441D substitution may abolish the interaction with colipase Arg65, but very limited changes, such as side chain torsion, could restore the ion pair. The stacking interaction of Tyr403 with Arg65 is also lost but is readily replaced by a hydrogen bond between Asp403 and Arg. The fourth substituted residue, Tyr403, clashes slightly with colipase Gha45. This unfavorable interaction of the tyrosine hydroxyl group can be easily turned to a favorable hydrogen bond through side chain torsion of these two surface residues. To summarize, most interactions between colipase and PTL would be conserved in rPLRP2, the two unfavorable interactions yielding from substitutions can be relaxed easily, and no new unfavorable interactions appear. This conclusion is consistent with kinetic data showing that rPLRP2 does interact with colipase.
Finally, the biantennary saccharide is located at position 334, between the N- and the C-terminal domains, on the enzyme face opposite to the catalytic center. Despite this location, it should not interfere with lid opening or with colipase binding.

Interfacial Activation—Because it was previously reported that rPLRP2 and cPLRP2 displayed no interfacial activation, we expected structural differences between hPTL and rPLRP2, particularly in the lid domain (5, 8). The results contradicted our expectations because the lid domain was neither disordered nor in an open conformation. In fact, the closed position of the lid in rPLRP2 closely resembled the position of the lid domain in hPTL (10). The good electron density and closed conformation suggested that rPLRP2 should show interfacial activation.

Because of our findings, we reexamined rPLRP2 for interfacial activation using the recently validated method with tripropionin (21, 32). The use of tripropionin overcomes the difficulties that accompany the poor water solubility of tributyrin, the substrate utilized in previous studies of PLRP2 and interfacial activation (5, 6, 8). The experiment was replicated independently in two different laboratories under slightly different conditions (Fig. 3, A and B). At concentrations below the solubility limit of tripropionin, rPLRP2 had little activity. The activity increased considerably above the solubility limit of the substrate. Similar kinetics were found with another substrate, p-nitrophenylbutyrate, that has been used to demonstrate interfacial activation for other lipases (Fig. 3C). These results clearly show that rPLRP2 possesses interfacial activation preferring aggregated substrates over monomeric substrates as does PTL. The observation of interfacial activation on tripropionin restores the validity of the classical explanation: closed lid means interfacial activation. Although open lids or disordered lid structure have been observed in other lipases, even in the absence of inhibitor, and appear to violate this principle, these structures were obtained in the presence of less polar solvent or in detergent, which may simulate an interface (33–36).

Inhibition of rPLRP2 by E600 and Tetrahydrolipstatin—The conformation of the rPLRP2 active site and the conserved Ser-His-Asp catalytic triad suggested that it should be inhibited by lipase inhibitors like E600 (diethyl p-nitrophenyl phosphate) and tetrahydrolipstatin (37). To determine whether these compounds inhibit rPLRP2, we incubated the lipase with both inhibitors for various lengths of time and measured activity against tributyrin (Fig. 4). Both inhibitors effectively reduced the activity of rPLRP2. The inhibition suggests that the catalytic mechanism of rPLRP2 is similar to that of PTL. If rPLRP2 participates in fat digestion, its activity should be effectively decreased by tetrahydrolipstatin. Finally, these results suggest that obtaining the structure of rPLRP2 in the open form may be possible in the presence of an inhibitor as previously done with PTL.

Substrate Specificity—Bulk phase assays had previously demonstrated that PLRP2 lipases have a broader substrate specificity than does PTL (5, 6, 8). We extended these observations by measuring activity against various substrates using the monolayer technique (Fig. 5). We compared the activities against three different phospholipase substrates, 1,2-didodecanoylphosphatidylcholine, 1,2-didodecanoylphosphatidylethanolamine, and 1,2-didodecanoylphosphatidylglycerol; one lipase substrate, 1,2-dicaprin; and one galactolipase substrate, monogalactosyldiglyceride. This is the first use of a galactolipid substrate in the monolayer assay. Furthermore, we compared the activity of rPLRP2 to those of cPLRP2, cPLRP2, and hPTL. All four lipases had activity against 1,2-dicaprin. hPTL was not active against the phospholipid or galactolipid substrates. In contrast, all three of the PLRP2 lipases showed activity against phospholipids as previously reported. Like cPLRP2, rPLRP2 shows a clear preference for 1,2-didodecanoylphosphatidylethanolamine and 1,2-didodecanoylphosphatidylglycerol over 1,2-didodecanoylphosphatidylcholine (5). The activity of rPLRP2 and cPLRP2 against 1,2-didodecanoylphosphatidylcholine was quite low compared with the other two phospholipid substrates. Both rPLRP2 and gPLRP2 but not hPTL showed activity against monogalactosyldiglyceride. In addition to confirming the activity of PLRP2 lipases against galactolipids, this result demonstrates the utility of the monolayer assay for measuring galactolipase activity.

Although rPLRP2 activity against these various substrates could be easily measured in the monolayer assay, rPLRP2 had lower activity than did the other lipases. This finding is consistent with the results of the bulk phase assay with galactolipids where rPLRP2 had decreased activity compared with gPLRP2 (9). The lower activity of rPLRP2 against 1,2-dicaprin was surprising. In bulk phase assays, the specific activity of rPLRP2 compares favorably with that of hPTL (Table II). The explanation for this difference was not examined, but the finding may indicate that rPLRP2 is more sensitive to denaturation by the monolayer than are the other lipases or that rPLRP2 may partition itself less favorably in the monolayer system than other lipases. Direct comparisons of bulk phase phospholipase activity of rPLRP2 and the other PLRP2 lipases have not been done. The monolayer data indicate that rPLRP2 has lower activity against phospholipids then do other members of the PLRP2 family.

The current rPLRP2 structure does not explain the different activities of rPLRP2 and hPTL against phospholipids and galactolipids. There were no differences in the residues or positions of the residues around the active sites of rPLRP2 and hPTL to explain the substrate preferences. We observed differences in the lid domain and in the β5 loop between the two enzymes, but they do not obviously explain the substrate differences when compared with the open, active form of hPTL. It will be necessary to solve the open structure of rPLRP2 before differences in the active sites become apparent. Possibly, residues away from the active site will affect substrate specificity as found in the serine proteases.

Concluding Remarks—In this paper, we report the first structure for a member of the PLRP2 family and demonstrate that rat PLRP2 does show interfacial activation. Additionally, we confirm and extend the observations that PLRP2 lipases possess broader substrate specificities than do the closely homologous pancreatic triglyceride lipases. These studies represent the beginning of investigations that will contribute to understanding the molecular mechanisms underlying lipolysis.

Acknowledgments—We thank Frédéric Carrière for critically reading the manuscript and for the generous gift of coypu PLRP2 and Josiane de Caro for performing the inhibition studies with tetrahydrolipstatin and E600.

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