Pancreatic Lipase Structure−Function Relationships by Domain Exchange[†]

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ABSTRACT: We designed chimeric mutants by exchanging the lid domains of the classical human pancreatic lipase (HPL) and the guinea pig pancreatic lipase related protein 2 (GPLRP2). This latter enzyme possesses naturally a large deletion within the lid domain and is not activated by lipid/water interfaces. Furthermore, GPLRP2 exhibits phospholipase A1 and lipase activities in the same order of magnitude, whereas HPL has no significant phospholipase activity and displays a clear interfacial activation. An HPL mutant [HPL(−lid)] with GPLRP2 mini-lid domain does not display interfacial activation. Its specific activity toward triglycerides is, however, dramatically reduced. A GPLRP2 mutant [GPLRP2(+lid)] with HPL full-length lid domain is not interfacially activated, and its lid domain probably exists under a permanent open conformation. Therefore, the phenomenon of interfacial activation in HPL is not only due to the presence of a full-length lid domain but also to other structural elements which probably allow the existence of stabilized closed and open conformations of the lid. GPLRP2(+lid) phospholipase activity is significantly reduced as compared to GPLRP2, whereas its lipase activity remains at the same level. Therefore, the lid domain plays a major role in substrate selectivity and can be considered as part of the active site. However, the presence of a full-length lid domain is not sufficient to explain the absence of phospholipase activity in HPL since HPL(−lid) does not display any phospholipase activity. We also produced a chimeric GPLRP2 mutant in which the C-terminal domain was substituted by the HPL C-terminal domain. The colipase effects, i.e., anchoring and stabilization of the lipase at the interface, are clearly observed with the chimera, whereas GPLRP2 is insensitive to colipase. The kinetic characterization of this chimera reveals for the first time that the interfacial stability of pancreatic lipases depends on the structure of the C-terminal domain.

In 1990, the two first lipase 3D structures were elucidated. In human pancreatic lipase (HPL; Winkler *et al.*, 1990) as well as in *Rhizomucor miehei* lipase (Brady *et al*., 1990) a surface loop—the lid domain—covers the active site which is inaccessible to solvent. It was postulated that this loop had to undergo a conformational change in the presence of an interface, and this might explain the phenomenon of interfacial activation described by Sarda and Desnuelle (1958), in the particular case of porcine pancreatic lipase. Later on, both lipases were cocrystallized in the presence of either inhibitors (Brzozowski *et al.*, 1991; Derewenda *et al.*, 1992; Egloff *et al.*, 1995a) or mixed micelles (van Tilbeurgh *et al.*, 1993), and the hypothetical movement of the lid domain was then clearly demonstrated. At the same time, we characterized a novel guinea pig pancreatic lipase-related protein (GPLRP2; Hjorth *et al.*, 1993) which displayed atypical kinetic properties such as the absence of interfacial activation. GPLRP2 presents a large deletion in the so-called lid domain that might explain the free access of substrate to the catalytic site. An active site freely accessible to solvent was also observed in the 3D structure of *Fusarium solani* cutinase (Martinez *et al.*, 1992). This esterase, which also hydrolyzes triglycerides, has no lid domain and does not display interfacial activation. Based on all these initial data, the phenomenon of interfacial activation became univocally associated with the existence of a lid domain.

Recently, this clear structure−function relationship became less strict when the primary sequences as well as the kinetic properties of two pancreatic lipases from *Myocastor coypus* (CoPLRP2; Thirstrup *et al.*, 1994) and rat (RPLRP2; Jennens & Lowe, 1995) were determined. These lipases, together with GPLRP2, belong to a novel subfamily of pancreatic lipases which, in contrast to the classical HPL, display no interfacial activation, a high phospholipase activity, as well as a relative insensitivity to colipase. On the contrary to GPLRP2, CoPLRP2 and RPLRP2 possess a full-length lid domain (23 amino acid residues instead of 5 for GPLRP2), and one can thus assume that their lid domain always exists under an open conformation.

The pancreatic lipase is constituted by two structural domains: a large N-terminal domain which contains the catalytic site shielded by the lid domain (Winkler *et al.*,

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Table 1: Primers Used for PCR Mutagenesis[a]

no.	primer sequences and comments
1	cctggatccgctcggc**atgctcccgct;** sense primer, **HPL cDNA 5′-end,** *Bam*HI
2	*acaactaattccagttttaca***tcctggcatttccac;** antisense primer, corresponding to the peptide **VEMPG***CKTGISC*, including *GPLRP2 lid*
3	*tgtaaaactggaattagttgt***aatcacttaagaagc;** sense primer, corresponding to the peptide *CKTGISC***NHLRS**, including *GPLRP2 lid*
4	ccaggatcc**tcagcagggtgtcag;** antisense primer **HPL cDNA 3′-end**, *Bam*HI,
5	gactcgag*tcagctcgg;* sense primer, *GPLRP2 cDNA 5′-end*, *Xho*I
6	**gcaagcagcgaagtctctggtaccttcccagataccatcgatatcaacgatttgagacaagatgttcttcttaca***tccaggcatgtcttt;* antisense primer, corresponding to the peptide *KDMPG***CKKNILSQIVDIDGIWEGTRDFAAC**, including **HPL lid**
7	**tgtaagaagaacatcttgtctcaaatcgttgatatcgatggtatctgggaaggtaccagagacttcgctgcttgc***caaccaccaccggagc;* sense primer, corresponding to the peptide **CKKNILSQIVDIDGIWEGTRDFAAC***NHHRS*, including **HPL lid**
8	cctcaaagctt*tcattaacaagggga;* antisense primer, *GPLRP2 cDNA 3′-end*, *Hin*dIII
9	*ccagct***ctc***ctctccactgtccgtgaagccatgg**ataatgaagcgtgtc;* antisense primer, corresponding to the peptide *TRFIIHGFTDSGE***E***SW*, *Nco*I
10	*ttcattatccatggcttcacggacagtggagag***g***a***g***agctggctatcagacatg*, Sense primer, corresponding to the peptide *FIIHGFTDSGE***E***SWLSDM*, Nco I
11	cctcactcgag*tcattaacaagggga;* antisense primer, *GPLRP2 cDNA 3′-end*, *Xho*I
12	*gctgtgatagtact***t***g***ta***ggatcgatggtggttgcaagc;* antisense primer, corresponding to the peptide *ACNHHRS***YK***YYHS*, *Cla*I
13	*gcttgcaaccaccatcgatcc***tac***a**atactatcacagc;* sense primer, corresponding to the peptide *ACNHHRS***YK***YYHS*, *Cla*I
14	**ttacctccagcgcgcaaa***gttatccgctcctgt;* antisense primer, corresponding to the peptide *TGASDN***FARWRY**, *Bss*HI
15	*acaggagcgagtgataac***tttgcgcgctggaggtat;** sense primer, corresponding to the peptide *TGASDN***FARWRY**, *Bss*HI
16	cctcactcgag**tcagcagggtgtcagcgt;** antisense primer, **HPL cDNA 3′-end**, *Xho*I

[a] Sequences in **bold** and *italics* correspond to **HPL** and *GPLRP2*, respectively. Restriction sites are underlined.

1990), and a small C-terminal domain which is involved in colipase binding (van Tilbeurgh *et al.*, 1992). The structural knowledge on HPL and the sequence comparison between classical pancreatic lipases and PLRP2 represent an interesting system for investigating structure−function relationships within this family of enzymes. They probably share a similar fold but have evolved toward distinct biological functions and substrate selectivities (Carrière *et al.*, 1994a,b). A similar situation is found with hepathic and lipoprotein lipases for which an approach of domain exchange has previously been used for investigating substrate selectivity (Faustinella *et al.*, 1992; Davies *et al.*, 1992; Dugi *et al.*, 1995; Salinelli *et al.*, 1996). In the present study, we produced, purified to homogeneity, and characterized, biochemically and kinetically, chimeric mutants designed by domain exchange between HPL and GPLRP2 in order to investigate the lid function with respect to interfacial activation and substrate selectivity toward triglycerides and phospholipids. We also replaced the GPLRP2 C-terminal domain by the HPL C-terminal domain in order to study the colipase effects and the interfacial stability of pancreatic lipases. In parallel to the present study, the 3D structure of this N-GPLRP2/HPL chimera has been published recently by Withers-Martinez *et al.* (1996).

MATERIALS AND METHODS

DNA Origin. The cDNA clone for HPL was obtained from human placenta mRNA by PCR technology based upon the complete sequence of HPL, including signal peptide (Lowe *et al.*, 1989). A 1411 bp DNA fragment including the complete coding sequence was inserted into the *Bam*HI site of the pBluescript SK II vector (pBSK II, Stratagene, La Jolla, CA). GPLRP2 cDNA was obtained from the total mRNA isolated from guinea pig pancreas as previously described (Hjorth *et al.*, 1993). A 1579 bp DNA fragment including the complete coding sequence was inserted into the *Xba*I site of the pcDV 1 vector (Okayama & Berg, 1983).

DNA Manipulations. Plasmid DNAs were isolated from *Escherichia coli* cultures by alkaline lysis procedure (Sambrook *et al.*, 1989) followed by phenol/chloroform extraction. Before insect cell cotransfection, the baculovirus transfer vectors (pVL1392 and pVL1393) were purified by CsCl-gradient ultracentrifugation. Restriction enzyme digestions and ligations were performed as recommended by the enzyme suppliers. Plasmid DNA was introduced into *E. coli* (ElectroMAX DH10B cells, Life Technologies, Gaithersburg, MD) by electroporation using a Gene Pulser (Bio-Rad). DNA sequencing was carried out by the method of Sanger (1977) using the Sequenase Version 2.0 DNA Sequencing Kit (US Biochemical, Cleveland, OH).

Site-Directed Mutagenesis by PCR. Using internal oligonucleotides in which a specific mutation had been introduced (see Table 1), two PCR products (PCR1 and PCR2) from different sections of the same DNA template were made such that the resultant fragments overlap in sequence. After removal of the primers, these products were mixed, denaturated, and allowed to reanneal. One of the heteroduplex forms consisted of DNA strands that overlapped at their 3′-ends. This overlap region contained the mutation. By amplifying this heteroduplex using primers to the 5′-ends of the strands (PCR3), a mutated DNA fragment was amplified that was the precise joining of the two subsidiary products from PCR1 and PCR2 (Higuchi, 1992). The product from PCR3 was then manipulated with restriction enzymes in order to clone it in a specific vector or to substitute a DNA fragment containing the mutation to a similar fragment of the original template. Chimeric constructions could be obtained by performing PCR1 and PCR2 on two different templates.

(1) HPL(−lid) Mutant: Substitution of HPL Lid Domain by GPLRP2 Mini-Lid. PCR 1 was carried out using HPL cDNA in pBSKII (1411 bp *Bam*HI insert) as template and primers 1 and 2 (Table 1), for 25 cycles of 1 min at 94 °C, 2 min at 45 °C, 3 min at 72 °C. PCR 2 was carried out using HPL cDNA in pBSKII as template and primers 3 and 4 (Table 1), for 25 cycles of 1 min at 94 °C, 2 min at 45 °C, 3 min at 72 °C. PCR 3 was carried out using the products of PCR 1 and PCR 2 as templates and primers 1 and 4 for 30 cycles of 1 min at 94 °C, 2 min at 50 °C, 3 min at 72 °C. The product of PCR 3 (HPL(−lid) DNA) was cloned into the *Bam*HI site of pBSKII vector and sequenced in order to check that only the desired mutation was introduced by PCR. HPL(−lid) DNA (1357 pb) was further subcloned into the *Bam*HI site of the pVL1392 baculovirus transfer vector (Invitrogen, San Diego, CA).

(2) GPLRP2(+lid) Mutant: Substitution of GPLRP2 Mini-Lid Domain by HPL Lid. PCR 1 was carried out using

GPLRP2 cDNA in pcDV1 (1579 bp *Xba*I insert) as template and primers 5 and 6 (Table 1), for 25 cycles of 1 min at 94 °C, 2 min at 50 °C, 3 min at 72 °C. PCR 2 was carried out using GPLRP2 cDNA in pcDV1 as template and primers 7 and 8 (Table 1), for 25 cycles of 1 min at 94 °C, 2 min at 50 °C, 3 min at 72 °C. PCR 3 was carried out using the products of PCR 1 and PCR 2 as templates and primers 5 and 8 for 30 cycles of 1 min at 94 °C, 2 min at 50 °C, 3 min at 72 °C. The product of PCR 3 (GPLRP2(+lid) DNA, *Xho*I/*Hin*dIII fragment) was cloned into the pBSKII vector (*Sal*I/*Hin*dIII) and sequenced. GPLRP2(+lid) DNA (*Sal*I/*Hin*dIII fragment, 1357 pb) was further subcloned into the pIC19R vector (Marsh *et al.*, 1984) for further mutations.

(3) N83E Mutation in GPLRP2(+lid). PCR 1 was carried out using GPLRP2(+lid) DNA in pBSKII (1357 bp *Sal*I/*Hin*dIII insert) as template and primers 5 and 9 (Table 1) for 30 cycles of 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C. PCR 2 was carried out using GPLRP2(+lid) DNA in pBSKII as template and primers 10 and 11 (Table 1) for 30 cycles of 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C. PCR 3 was carried out using the products of PCR 1 and PCR 2 as templates and primers 5 and 11 for 30 cycles of 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C. The product of PCR 3 was digested by *Bcl*I and *Kpn*I. The resulting *Bcl*I/*Kpn*I DNA fragment (600 bp including the mutation) was substituted to the similar fragment of GPLRP2(+lid) in pIC19R and sequenced.

(4) I267Y and E268K Mutations in GPL RP2(+lid). PCR 1 was carried out using GPLRP2(+lid) DNA in pBSKII as template and primers 5 and 12 (Table 1) for 30 cycles of 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C. PCR 2 was carried out using GPLRP2(+lid) DNA in pBSKII as template and primers 13 and 11 (Table 1) for 30 cycles of 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C. PCR 3 was carried out using the products of PCR 1 and PCR 2 as templates, and primers 5 and 11 for 30 cycles of 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C. The product of PCR 3 was digested by *Kpn*I and *Xho*I. The resulting *Kpn*I/*Xho*I DNA fragment (600 bp including the mutations) was substituted to the similar fragment of GPLRP2(+lid) in pIC19R, and sequenced.

(5) GPLRP2(+lid) Mutant with N83E, I267Y, and E268K Mutations. The *Bcl*I/*Kpn*I and *Kpn*I/*Xho*I DNA fragments obtained previously were substituted to the similar fragments of GPLRP2(+lid) DNA in pIC19R. GPLRP2(+lid) DNA (1445 bp *Sma*I/*Bgl*II DNA fragment) was further subcloned into the pVL1393 baculovirus transfer vector (Invitrogen, San Diego, CA).

(6) N-GPLRP2/C-HPL Chimera Mutant: Ligation of GPLRP2 N-Terminal Domain to HPL C-Terminal Domain. PCR 1 was carried out using GPLRP2 cDNA in pcDV1 (1579 bp *Xba*I insert) as template and primers 5 and 14 (Table 1) for 30 cycles of 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C. PCR 2 was carried out using HPL cDNA in pBSKII (1411 bp *Bam*HI insert) as template and primers 15 and 16 (Table 1) for 30 cycles of 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C. PCR 3 was carried out using the products of PCR 1 and PCR 2 as templates, and primers 5 and 16, for 30 cycles of 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C. The product of PCR 3 was digested by *Nco*I and *Xho*I. The resulting *Nco*I/*Xho*I DNA fragment (1254 bp including GPLRP2 mini-lid and HPL C-terminal domain) was substituted to the similar fragment of GPLRP2(+lid) DNA in the pIC19R vector and sequenced. The chimera DNA (1382 bp *Sma*I/*Bgl*II insert) was further subcloned from the pIC19R vector to the pVL1393 baculovirus transfer vector.

Production of Mutants Using the Baculovirus Expression System. The production of recombinant baculoviruses and the expression of mutants were performed as previously described by Thirstrup *et al*. (1993) and following instructions from the Baculovirus Expression System Manual, from Invitrogen Corp. (version 1.5.5). The baculovirus tranfer vectors containing the mutant DNA were purified by CsCl-gradient ultracentrifugation and then used for cotransfection with AcMNPV DNA (*Autographa californica* virus) into Sf9 cells (*Spodoptera frugiperda*) grown in THM-FH medium (Summers & Smith, 1987) at 27 °C. Pure recombinant baculoviruses were obtained after two rounds of plaque purification, and their titers were further amplified by two additional cycles of Sf9 cell infection. To ensure that recombinant virus stocks were free of wild-type virus, the corresponding virus DNAs were purified and subjected to a PCR reaction with reverse and forward primer annealing upstream and downstream the polyhedrin gene. The last infection cycle was performed using Sf9 cells grown in serum-free culture medium (SF-900 II from Gibco BRL) in order to eliminate serum proteins from the virus stock used for expression. The mutants were expressed in Sf9 cell suspensions grown to a concentration of 8×10^5 cells/mL in 250 mL of SF-900 II medium using 1000 mL screw-capped Erlenmeyer flask on an orbital shaker set at 80 rpm. The recombinant baculovirus was added to the cells at a multiplicity of infection close to 1 (one plaque-forming unit per cell). Sampling of culture medium was performed each day for 7 days in order to check cell viability and measure lipase production. After centrifugation to remove cells and debris, lipolytic activity was assayed using tributyrin assay. In all cases, the recombinant protein was secreted into the culture medium and was the major protein observed by electrophoresis on 12% SDS−polyacrylamide gels (data not shown).

Purification of Mutants. All the mutants were purified following the one-step procedure reported previously for the purification of human pancreatic lipase expressed in insect cells (Thirstrup *et al*., 1993). We decided to harvest the cultures of recombinant baculovirus-infected Sf9 cells after 3 days, in order to avoid proteolysis of the recombinant protein due to intracellular proteases released into the medium during cell lysis. The cells were pelleted by centrifugation at 10 000 rpm for 10 min, and the supernatant was lyophilized during 24 h. The dry material was dissolved in a few milliliters of distilled water and dialyzed overnight against 10 mM MES buffer. The pH of the MES buffer was adjusted at 5.5, 6.5, and 6.5 for the purification of GPLRP2(+lid), HPL(−lid), and N-GPLRP2/C-HPL chimera, respectively. Prior to chromatography, the solution was passed through a 0.8 μm Millipore filter. Using FPLC (Pharmacia), cation exchange chromatography was performed on a Mono S HR 5/5 column equilibrated in 10 mM MES buffer. After sample injection, a linear NaCl concentration gradient was applied, and GPLRP2(+lid), HPL(−lid), and N-GPLRP2/C-HPL chimera were eluted at 130, 120, and 90 mM NaCl, respectively. The flow rate was adjusted to 1 mL/min, and the pressure was maintained between 20 and 25 bars. The protein elution profile was recorded

spectrophotometrically at 280 nm, and the lipase activity wasmeasured potentiometrically in all fractions collected using tributyrin as substrate.

Analysis of Mutants. Prior to N-terminal sequencing, samples of the purified GPLRP2(+lid), HPL(−lid), and N-GPLRP2/C-HPL chimera eluted from the Mono S column were run through an HPLC column. Each sample was injected onto a Vydac 214TD54 reverse-phase C4 HPLC column (0.46 × 25 cm) equilibrated at 30 °C at a flow rate of 1.5 mL/min with 0.1% TFA in 20% (v/v) acetonitrile. The concentration of acetonitrile in the eluting solvent was raised to 70% (v/v) over 25 min. Absorbance was measured at 280 nm. GPLRP2(+lid), HPL(−lid), and N-GPLRP2/C-HPL chimera eluted at retention times of 14.2, 14.0, and 13.9 min, respectively, corresponding to 48.4%, 48.0%, and 47.8% of acetonitrile, respectively. For each separation, the material eluting in the main peak (≥95% of the total proteins) was collected, concentrated by vacuum centrifugation, and submitted to automated Edman analysis using an Applied Biosystem Model 470A gas-phase sequencer (Thim *et al.*, 1987).

Amino acid and carbohydrate composition analyses were carried out as previously described (Thirstrup *et al.*, 1993).

The N-GPLRP2/C-HPL chimera and GPLRP2(+lid) mutants were analyzed by electrospray mass spectrometry (ESMS) on API III LC/MS/MS system (Perkin-Elmer Sciex Instrument, Thornhill Canada) as reported for the recombinant human pancreatic lipase (Thirstrup *et al.*, 1993).

Mass spectrometry analysis of HPL(−lid) mutant was performed using a prototype laser desorption mass spectrometer (LDMS, ABI AB Uppsala, Sweden) at Odense University (Department of Molecular Biology) in collaboration with Dr. Henrik R. Nielsen. The matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) instrument was run in the positive mode with an acceleration voltage of 24 kV. It was equipped with a nitrogen laser (wavelength 355 nm), and the flight tube was ∼0.7 m. The sample (10 pmol) in water was mixed with a matrix consisting of α-cyano-2-hydroxycinnamic acid (20 μg/μL) in 70% acetonitrile before analysis.

Lipolytic Activity Measurements. The hydrolysis rates of emulsified triglycerides were measured potentiometrically in a thermostated vessel (37 °C) by using the pH-stat technique (TTT 80 Radiometer, Copenhagen) with mechanically stirred emulsions of either triproprionin or tributyrin, or trioctanoin (Fluka, Paris, France). The exact composition of the reaction media is given in the legends of figures.

The hydrolysis of emulsified phospholipids from egg yolk was measured by the pH-stat technique (De Haas *et al.*, 1968). The assay conditions consisted of a mechanically stirred emulsion of 5 mL of substrate solution (one homogenized egg yolk in 100 mL of 4 mM $CaCl_2$, filtered through a cheese cloth and adjusted to pH 8.0) added to 10 mL of 20 mM sodium deoxycholate.

Under the above assay conditions, one lipase or phospholipase unit (U) corresponds to 1 μmol of fatty acid released per minute.

Lipase and phospholipase activities were also measured by the monolayer technique (Ransac *et al.*, 1991) using monomolecular films of either 1,2-dicaprin or various phospholipids as substrates (1,2-didodecanoylphosphatidylcholine; 1,2-didodecanoylphosphatidylethanolamine; 1,2-didodecanoylphosphatidylglycerol; Fluka, Paris, France). The lipid monolayer was spread over an aqueous phase made of 10 mM Tris-HCl buffer, pH 8.0, 100 mM NaCl, 21 mM $CaCl_2$, and 1 mM EDTA, placed in a zero order trough. In all cases, the enzyme was injected into the reaction compartment (volume, 51 mL; surface, 31 cm^2). For each substrate, the enzymatic activity was measured at various surface pressures with the barostatic mode, and the hydrolysis rate was expressed in mole of substrate hydrolyzed per cm^2 of the reaction compartment, per minute, and for a theoritical enzyme concentration of 1 M in the reaction compartment. This calculation does not take into account the enzyme partitioning between the bulk phase and the lipid monolayer.

The colipase effect was studied using pure procolipase B (Val1−Ser93) from porcine pancreas which was checked for the absence of proteolytic degradation as reported by Rugani *et al.* (1995).

Identification of the Proteolytic Cleavage of GPLRP2-(+lid) Produced in Aspergillus orizae. The GPLRP2(+lid) mutant was first produced in *Aspergillus orizae* as described previously for GPLRP2 (Hjorth *et al.*, 1993). After purification, the reduced protein was analyzed by electrophoresis on SDS−polyacrylamide gels (12%) using a Bio-Rad Mini Protein II cell, and the gels were either stained with Coomassie blue or submitted to electrophoretic transfer onto a glassybond membrane. The Coomassie staining revealed the presence of two protein bands at 30 and 20 kDa, instead of the expected single band at 50 kDa. The N-terminal sequences of the corresponding polypeptides transferred onto the glassybond membrane were AEVCYSHLGC for the 30 kDa fragment (N-terminal part of GPLRP2) and RDFAAC-NHLR for the 20 kDa fragment, revealing that the GPLRP2-(+lid) mutant was totally and specifically cleaved by an *Aspergillus* protease at a T255−R256 peptide bond present in the lid domain.

RESULTS

Construction of the Pancreatic Lipase Mutants. Using HPL and GPLRP2 cDNAs as templates, chimeric DNA constructions were obtained by the polymerase chain reaction overlap extension method (Higuchi, 1992). These DNA constructions were further subcloned into baculovirus expression vectors, and the corresponding recombinant proteins were produced in baculovirus-infected insect cells. As shown in Figure 1, three chimeric mutants were obtained: (1) a human pancreatic lipase mutant—HPL(−lid)—in which the HPL lid domain (C^{237}KKNILSQIVDIDGIWEGTRDF-AAC^{261}) was replaced by GPLRP2 mini-lid domain (CKT-GISC); (2) a guinea pig pancreatic lipase related protein 2 mutant—GPLRP2(+lid)—in which the mini-lid domain of GPLRP2 was replaced by the HPL lid domain and three point mutations (N83E, I267Y, E268K) were introduced; (3) a chimeric pancreatic lipase—N-GPLRP2/C-HPL chimera—resulting from the fusion of the N-terminal domain of GPLRP2 and the C-terminal domain of HPL.

The reason why we introduced three additional point mutations in GPLRP2(+lid) was to allow the interactions of the lid domain with the β5-loop as well as with the protein core (see Figure 1) as they are observed in the open conformation of the HPL 3D structure (van Tilbeurgh *et al.*, 1993). These interactions are two salt bridges (R256−D79, D257−K268) and two hydrogen bonds (W252−E83, R256−Y267). The amino acid residues involved in these stabilizing interactions are strictly conserved in all classical pancreatic

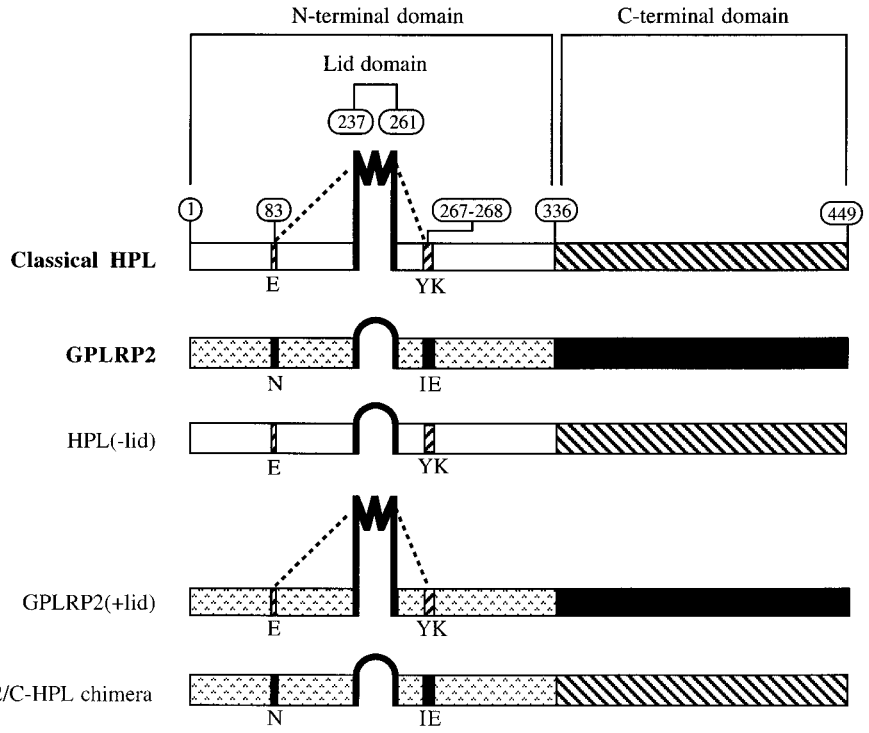

FIGURE 1: Schematic representation of the primary structures of the classical HPL, GPLRP2, and their chimeric mutants. HPL amino acid residues are numbered according to Winkler *et al*. (1990). In HPL, the lid domain is the polypeptide included between C237 and C261. Amino acid residues E83, Y267, and K268 are involved in stabilizing interactions between the protein core and the lid domain in its open conformation (van Tilbeurgh *et al*., 1993).

lipases, whereas they are often mutated in PLRP2. This observation suggests a key function of these residues for locking the lid of classical pancreatic lipase under its open conformation (Carrière *et al*., 1994). In GPLRP2, only D79 is conserved, whereas W252, R256, and D257 do not exist in the mini-lid. Residue 83 from the β5-loop is N instead of E in HPL. Residues 267 and 268 from the protein core are I and E, respectively, instead of Y and K in HPL (see Figure 1).

Expression, Purification, and Structural Analysis of the Pancreatic Lipase Mutants. The three mutants were expressed using the baculovirus expression system as reported previously for HPL (Thirstrup *et al*., 1993), with yields reaching 10−40 mg of recombinant protein/L of insect cell suspension cultures. The cells (Sf9 from *Spodoptera frugiperda* ovarian tissues) were cultivated in serum-free medium, and each recombinant protein secreted into the medium was purified in one step using cationic exchange chromatography (Figure 2A,C,E). As in the case of the recombinant HPL purification, we harvested the culture medium at day 3 post-infection in order to avoid any undesirable proteolysis due to intracellular proteases released in the medium when cells start to lyse between days 3 and 4. This precaution was particularly necessary in the case of HPL(−lid) production. From SDS−PAGE analysis of the proteins present into the culture medium, we observed that the electrophoretic band corresponding to HPL(−lid) increased up to day 3 and completely disappeared at day 4 (data not shown). Under similar conditions, we observed that recombinant HPL was rather resistant to proteolysis (Thirstrup *et al*., 1993). We concluded that the replacement of HPL lid domain by a shorter loop (GPLRP2 mini-lid) increases the sensitivity to insect cell proteases.

Following the purification on Mono S column, the proteins recovered from the main fractions containing lipolytic activity were subjected to N-terminal amino acid sequence analysis, which revealed that all three mutants were produced under their mature forms and that the signal peptides were correctly cleaved (Table 2). The 20 first amino acid residues of HPL-(−lid) were found identical to those of HPL (Lowe *et al*., 1989; Winkler *et al*., 1990), whereas the N-terminal sequences of GPLRP2(+lid) and the N-GPLRP2/C-HPL chimera were identical to that of GPLRP2 (Hjorth *et al*., 1993). In each case, the detection of a single amino acid sequence and mass spectrometry analysis (see below) ruled out any proteolytic degradation of the protein.

The molecular mass of each mutant was experimentally determined using mass spectrometry and amino acid and carbohydrate analysis. Using laser desorption mass spectrometry (Figure 2B), the molecular mass obtained for HPL-(−lid) is very similar to the theoretical molecular mass (Table 2) including both the polypeptide chain (HPL with the GPLRP2 lid domain) and a glycan chain linked to Asn166. The molecular mass of the glycan chain (1038 Da) corresponds to 2 *N*-acetylglucosamine, 3 mannose, and 1 fucose residues, as determined by carbohydrate analysis. This short glycan chain is classically obtained with Sf9 insect cells and was previously observed in the case of the recombinant HPL (Thirstrup *et al*., 1993). We checked that recombinant and native HPL display identical kinetic properties (Thirstrup *et al*., 1993), even though the native enzyme possesses a longer glycan chain (Fournet *et al*., 1987). In fact, the glycosylation site is remote from the active site and is unlikely to play a role in catalysis (Hermoso *et al*., 1996). Using electrospray mass spectrometry (Figure 2D,F), the molecular masses obtained for both GPLRP2(+lid) and the N-GPLRP2/C-HPL chimera are very similar to the theoretical molecular masses of their respective polypeptide chains only (Table 2). This result indicates that the potential glycosylation site ($\underline{N}^{334}\underline{FT}$)

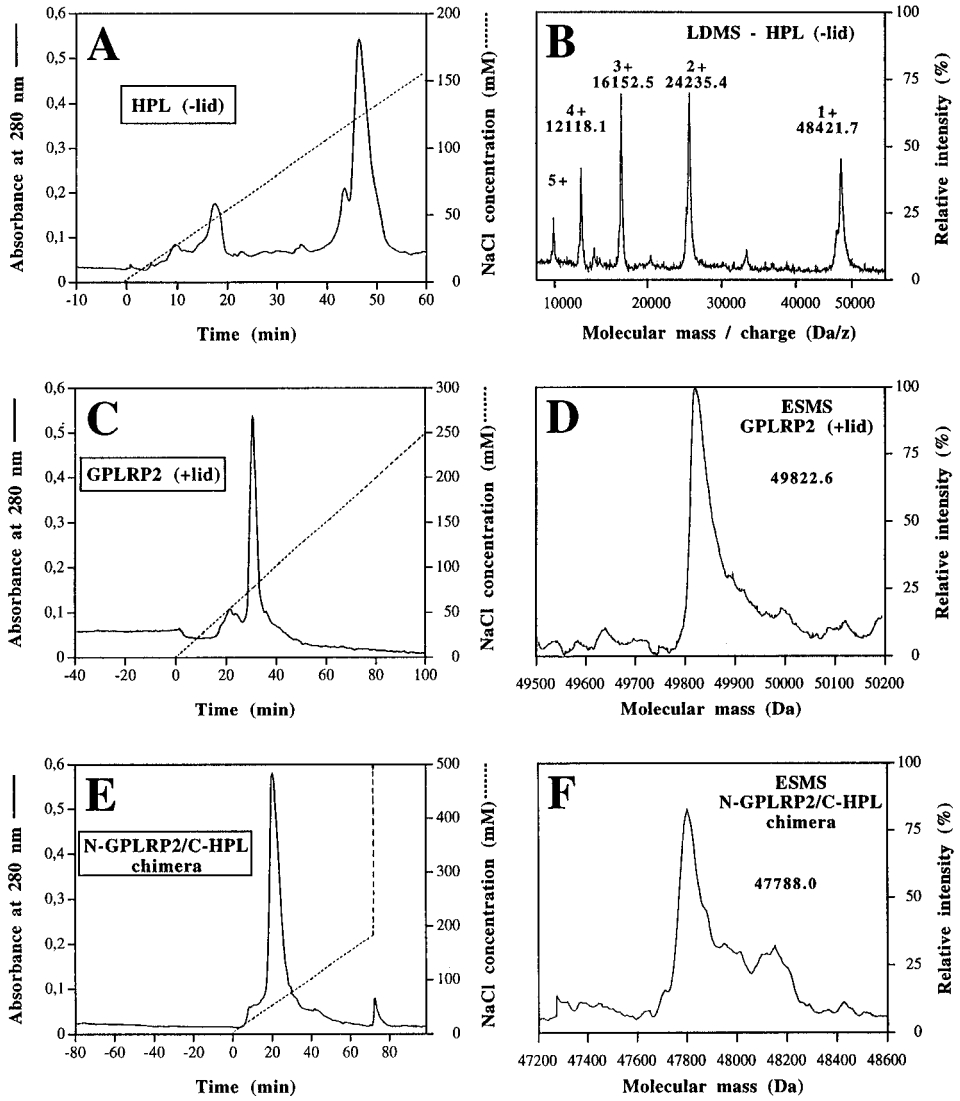

FIGURE 2: Purification of recombinant HPL(−lid), GPLRP2(+lid), and N-GPLRP2/C-HPL chimera expressed in baculovirus-infected insect cells. Pure proteins were obtained using a single purification step of cation exchange chromatography on a Mono S HR 5/5 column. Their elution, using a NaCl concentration gradient, was recorded by measuring absorbance at 280 nm (panels A, C, E). The purified proteins were analyzed by mass spectrometry. The average molecular mass of HPL(−lid) (48 453 ± 19 Da) was experimentally determined using laser desorption mass spectrometry (LDMS). The mass spectrum (panel B) displays a series of multiple protonated molecular ions (+1 to +5). The GPLRP2(+lid) and N-GPLRP2/C-HPL chimera molecular masses were determined using electrospray mass spectrometry (ESMS) and reconstructed mass spectra (panels D and F).

Table 2: Structural Analysis of the Pancreatic Lipase Mutants Purified from the Insect Cells Culture Media[a]

mutant	calcd molecular mass (Da)			exptl molecular mass (Da)	N-terminal sequence analysis
	polypeptide	glycan chain	total		
HPL(−lid)	47 417	1038	48 455	48 453 ± 19	KEV(C)YERLG(C)FSDDSPWSGI
GPLRP2(+lid)	49 827	n.d.	n.d.	49 823 ± 10	AEV(C)YSHLG(C)FSDEKPWAGT
N-GPLRP2/C-HPL chimera	47 792	n.d.	n.d.	47 788 ± 10	AEV(C)YSHLG(C)FSDEKPWAGT

[a] The cysteine residues in parentheses are deduced from the DNA sequence and correspond to gaps in the N-terminal amino acid sequence analysis. n.d. ≡ not determined.

at the junction between the N- and the C-terminal domains of GPLRP2(+lid) is not used. In the case of the N-GPLRP2/C-HPL chimera, the absence of glycosylation was expected due to the disappearance of the above-mentioned N-glycosylation site. The N^{334}FT sequence is replaced by N**FA** in the chimera.

Interfacial Activation of the Pancreatic Lipase Mutants. The effect of the substrate aggregation state on the lipolytic activity of the three mutants was investigated using triproprionin as substrate. For HPL(−lid), GPLRP2(+lid), and the N-GPLRP2/C-HPL chimera, the variations in the initial rates of hydrolysis with substrate concentration are quasi-hyperbolic, and no sharp increase in the activity was seen above triproprionin solubility (12 mM), in contrast to what was observed with HPL (Figure 3A,B). The N-GPLRP2/C-HPL chimera displays a specific activity of 1200 U/mg at 60 mM triproprionin similar to that observed with GPLRP2 (Figure 3A). In contrast, the specific activity of GPLRP2-(+lid) is reduced by a factor of 20 as compared to GPLRP2 (Figure 3B). Even more drastically, the specific activity of HPL(−lid) is reduced by a factor of 400 at 60 mM triproprionin as compared to that of HPL (Figure 3B).

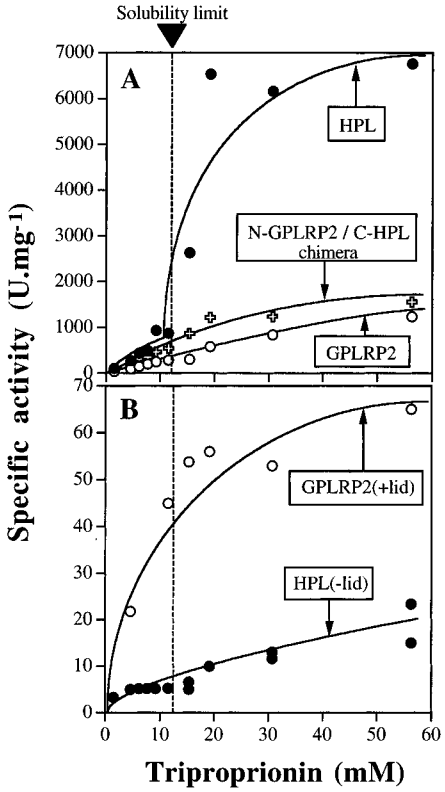

FIGURE 3: Interfacial activation. The initial rates of hydrolysis as a function of the triproprionin concentration were measured by the pH-stat technique, using recombinant HPL, GPLRP2, HPL(−lid), GPLRP2(+lid), and N-GPLRP2/C-HPL chimera. Each assay was performed in 15 mL of a 2% gum arabic solution containing variable amounts of triproprionin. Triproprionin solubility under the present experimental conditions (37 °C, pH 8.0) is 12 mM. The amounts of recombinant enzymes added into the reaction vessel were 1, 4, 60, 80, and 4 μg for HPL, GPLRP2, HPL(−lid), GPLRP2(+lid), and N-GPLRP2/C-HPL chimera, respectively. Since HPL, HPL(−lid), and N-GPLRP2/C-HPL chimera are sensitive to interfacial denaturation in the absence of bile salts (see Table 4), the enzyme samples were first incubated with colipase at a molar excess of 5, in order to stabilize the lipolytic activities above the triproprionin solubility and, thus, to record linear kinetics.

Table 3: Activity of Pancreatic Lipases and Mutants on Egg Yolk Phospholipids and Tributyrin Using the pH-Stat Technique

enzyme	phospholipase act. on egg yolk ($U \cdot mg^{-1}$)	lipase act. on tributyrin[d] ($U \cdot mg^{-1}$)	phospholipase to lipase act. ratio
GPLRP2	570[a]	1690	0.34
GPLRP2(+lid)	45 ± 5	600	0.07
N-GPLRP2/C-HPL chimera	549 ± 17	1550	0.35
HPL	0	12800	0
HPL(−lid)	0	100	0
porcine PLA_2	700[b]	0	∞
CoPLRP2	180[c]	2000	0.09

[a] From Hjorth *et al.*, 1993. [b] From De Haas *et al.*, 1968. [c] From Thirstrup *et al.*, 1994. [d] Optimal conditions for measuring HPL activity with the pH-stat method: 0.28 mM Tris buffer, 1.4 mM $CaCl_2$, 150 mM NaCl, 0.5 mM NaTDC, pH 7.5, + colipase with HPL and HPL(−lid). Colipase had no effect on the phospholipase activity, whatever the enzyme or mutant tested (data not shown).

Phospholipase Activity of the Pancreatic Lipase Mutants. HPL(−lid) as well as HPL do not display any phospholipase activity when using the pH-stat technique (Table 3). As expected, the N-GPLRP2/C-HPL chimera displays a specific activity similar to that of GPLRP2. The phospholipase activity of GPLRP2(+lid) is 13-fold lower as compared to that of GPLRP2. Moreover, the comparison of the phospholipase to lipase activity ratio reveals that, in the case of GPLRP2(+lid), this ratio is reduced to 0.07 instead of 0.34 for GPLRP2 (Table 3).

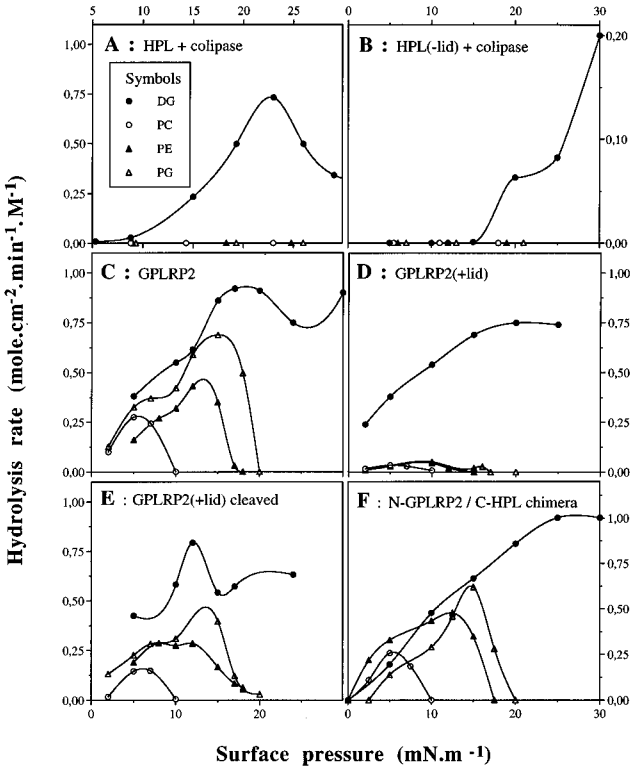

FIGURE 4: Hydrolysis of 1,2-didodecanoyl phospholipid and 1,2-didecanoylglycerol monomolecular films at variable surface pressures. Lipase and phospholipase activities were measured using the monolayer technique (see Materials and Methods) and various substrates: 1,2-didecanoylglycerol (DG), 1,2-didodecanoylphosphatidylcholine (PC), 1,2-didodecanoylphosphatidylethanolamine (1,2-PE), and 1,2-didodecanoylphosphatidylglycerol (PG). The data obtained with HPL (panel A) and GPLRP2 (panel C) are from De la Fournière *et al.* (1994) and Hjorth *et al.* (1993), respectively, and have been reproduced with permission. The data concerning HPL(−lid) (panel B), GPLRP2(+lid) (panel D), the cleaved GPLRP2(+lid) (panel E), and the N-GPLRP2/C-HPL chimera (panel F) have been obtained in the course of the present study. No effect of colipase was observed with the N-GPLRP2/C-HPL chimera, contrary to what is observed in bulk assays (Figure 5).

Using the monolayer technique (Figure 4), we studied the variation in phospholipase activity with surface pressure, using three different phospholipid films as substrates: 1,2-didodecanoylphosphatidylcholine (PC), 1,2-didodecanoylphosphatidylethanolamine (PE), and 1,2-didodecanoylphosphatidylglycerol (PG). Furthermore, we compared the phospholipase activities with the lipase activity on 1,2-didecanoylglycerol (DG) under similar experimental conditions.

HPL(−lid) as well as HPL do not hydrolyze phospholipid films but only DG (Figure 4A,B), and no significant activity of HPL(−lid) is observed below 15 $mN \cdot m^{-1}$, whereas HPL is already active below 10 $mM \cdot m^{-1}$.

GPLRP2(+lid) hydrolyzes DG at a similar rate as GPLRP2 (Figure 4D,C), but the activity of the former enzyme on all phospholipids tested is drastically reduced. In a first attempt to produce GPLRP2(+lid) in *A. orizae* (see Materials and Methods), we obtained a protein which was totally and specifically cleaved within the lid domain at the T255−R256 peptide bond. This protein was fully active, however, and

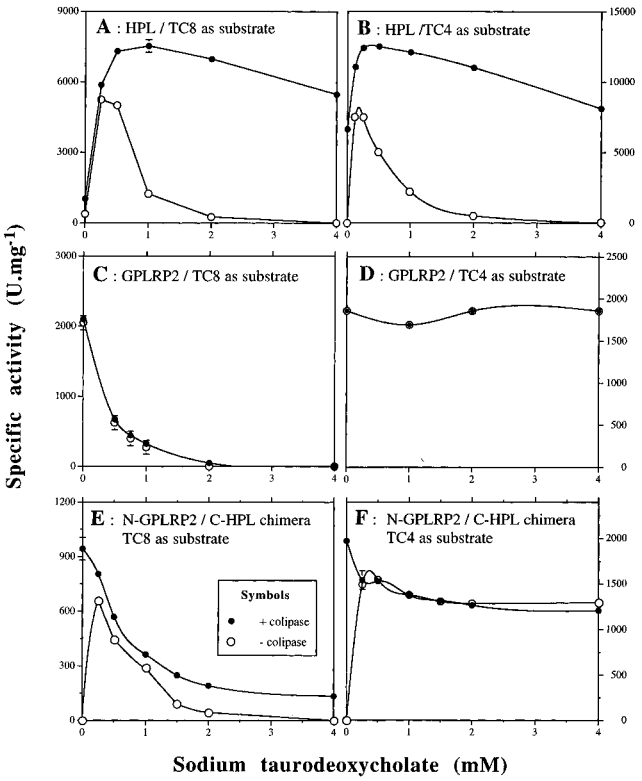

FIGURE 5: Influence of bile salts and colipase on the lipolytic activity of the N-GPLRP2/C-HPL chimera, GPLRP2, and HPL. The effect of variable sodium taurodeoxycholate (NaTDC) concentrations on the initial rates of lipolysis has been studied using the pH-stat technique, with both trioctanoin (TC8, panels A, C, E) and tributyrin (TC4, panels B, D, F) as substrates, in the absence or presence of colipase. The assay (pH 7.5, 37 °C) was performed in a total volume of 15 mL containing 0.5 mL of trioctanoin or tributyrin, 14.5 mL of 0.28 mM Tris-HCl buffer, 150 mM NaCl, 1.4 mM $CaCl_2$, and variable concentrations of NaTDC. Colipase was added at a molar excess of about 2. In the total absence of bile salt and colipase, both HPL and the N-GPLRP2/C-HPL chimera display no activity and are irreversibly denaturated. The data obtained with HPL (panels A and B) are from Thirstrup *et al.* (1993, 1994), and the data obtained with GPLRP2 (panels C and D) are from Hjorth *et al.* (1993) and have been reproduced with permission.

showed a phospholipase to lipase activity ratio comparable to that obtained with GPLRP2 (Figure 4E).

Finally, the N-GPLRP2/C-HPL chimera (Figure 4F) displays hydrolysis patterns which are very similar to those observed with GPLRP2 or the cleaved GPLRP2(+lid), whatever the substrate.

Effect of Bile Salts and Colipase on the Lipolytic Activity of HPL, GPLRP2, and the N-GPLRP2/C-HPL Chimera. We investigated the effect of sodium taurodeoxycholate (NaTDC) on the activity of HPL, GPLRP2, and the N-GPLRP2/C-HPL chimera in the presence or absence of colipase, using trioctanoin (totally insoluble medium chain triglyceride) and tributyrin (partly water-soluble short chain triglyceride) as substrates.

As established earlier by many authors, we confirmed that colipase substantially restored, in the presence of bile salts, the highly depressed HPL catalytic activity (Figure 5A,B). In contrast, colipase had absolutely no effect on GPLRP2 activity, as revealed with either trioctanoin (Figure 5C) or tributyrin (Figure 5D) as substrates.

Colipase reduces the inhibition of the N-GPLRP2/C-HPL chimera, at high bile salt concentrations, as evidenced when using only trioctanoin as substrate (Figure 5E).

Table 4: Catalytic Activity of Pancreatic Lipases and Mutants on Tributyrin in the Absence of Bile Salts[a]

enzyme	sp act. ($U \cdot mg^{-1}$) in the absence of colipase or when colipase is added after enzyme	sp act. ($U \cdot mg^{-1}$) when colipase is added before enzyme
HPL[a]	0	6600
HPL(−lid)	0	35
GPLRP2[b]	2000	2000
GPLRP2(+lid)	590	590
N-GPLRP2/C-HPL chimera	0	1970

[a] From Thirstrup *et al.*, 1993. [b] From Hjorth *et al.*, 1993.

With tributyrin as substrate, the N-GPLRP2/C-HPL chimera (Figure 5F) as well as GPLRP2 (Figure 5D) are not at all inhibited at high bile salt concentrations, probably due to the high catalytic activity of these two lipases acting on isotropic tributyrin solutions.

In absence of bile salts and colipase, the N-GPLRP2/C-HPL chimera is irreversibly and rapidly inactivated at the surface of trioctanoin (Figure 5E) or tributyrin (Figure 5F) droplets.

A similar inactivation was observed with HPL (Figure 5A,B) and other pancreatic lipases (Gargouri *et al.*, 1995), but not with GPLRP2, which displays a high and stable activity on short or medium chain triglyceride emulsions in the absence of bile salts and colipase (Figure 5C,D). We have summarized, in Table 4, the kinetic properties of HPL, GPLRP2, and their respective mutants using tributyrin as substrate. These data indicate that all the lipases possessing the C-terminal domain of HPL are rapidly inactivated at the water/triglyceride interface. This phenomenon is prevented by a prior addition of colipase (Table 4).

DISCUSSION

The 3D structure of GPLRP2 has not been resolved yet, but it is assumed from sequence comparison (64% amino acid identity) that both GPLRP2 and HPL share a similar fold. We have built up models of GPLRP2 3D structure based on the closed conformation of HPL structure (Hjorth *et al.*, 1993) as well as on its open conformation (Carrière and van Tilbeurgh, unpublished data). From these models, it can be seen that the active site serine is freely accessible to solvent due to the large deletion present in GPLRP2 lid domain. Within a 10 Å radius sphere from the oxygen γ of the active site serine, there are only a few residue changes in GPLRP2 as compared to HPL. The hydrophobic amino acids lining the active site groove are all conserved (Y114, P180, I209, P211, L213, F215) except V210 in HPL, which is replaced by L in GPLRP2. From these earlier observations, we proposed that the large deletion in the GPLRP2 lid domain might be responsible for both the absence of interfacial activation and the high phospholipase activity. The large size lid domain of HPL might behave as a substrate selectivity filter, whereas its active site would be potentially functional for hydrolyzing both triglycerides and phospholipids.

In order to test the previous hypothesis, we produced chimeric mutants by exchanging the lid domains of the classical HPL and GPLRP2.

On the Role of the Lid Domain in the Phenomenon of Interfacial Activation. By substituting the lid domain of HPL

by the GPLRP2 "mini-lid", we suppressed interfacial activation but we also reduced drastically the enzyme turnover (Figure 3B and Table 3). Several residues from the HPL lid domain are involved in one of the two acyl chain binding sites of HPL (van Tilbeurgh *et al*., 1993; Egloff *et al*., 1995a). The low turnover number of HPL(−lid), with respect to triglyceride hydrolysis, might result from the absence of one acyl chain binding site and a weaker stabilization of the substrate in the binding cleft. It has been reported by Jennens and Lowe (1994) that several deletions within HPL lid domain suppress the phenomenon of interfacial activation and decrease the interfacial binding to tributyrin. Surprisingly, the activity of their HPL lid mutants is not as drastically decreased as in our case for HPL(−lid). A careful examination of their experimental assay conditions reveals very high specific activities (230 000 U/mg for HPL and 60 000−120 000 U/mg for the lid mutants), which had never been reported so far.

The GPLRP2(+lid) mutant, in which the GPLRP2 mini-lid domain was substituted by the HPL lid domain, showed a reduced specific activity of 20-fold as compared to GPLRP2, using triproprionin as substrate (Figure 3B). It is clear that GPLRP2(+lid) does not display interfacial activation. Therefore, this latter phenomenon is not only controlled by the presence of a large size lid domain. This was also the conclusion drawn previously from the kinetic characterization of CoPLRP2 (Thirstrup *et al.*, 1994). The phenomenon of interfacial activation in HPL is linked to the ability of the lid domain to undergo a conformational change from an open to a closed conformation in the presence of a lipid/water interface. These conformations are probably stabilized by structural elements which are only present in the homologous protein core of HPL.

On the Role of the Lid Domain in Substrate Selectivity. Using both bulk and monolayer assays, we observed that the phospholipase to lipase activity ratio is significantly reduced in GPLRP2(+lid) as compared to GPLRP2 (Table 3, Figure 4). A lower phospholipase activity versus lipase activity was also reported in the case of CoPLRP2 (Table 3; Thirstrup *et al.*, 1994). Therefore, the lid domain plays an important role in substrate selectivity. This functional role is supported by the proteolytic cleavage of GPLRP2(+lid) at the T255−R256 peptide bond within the lid domain. The degraded enzyme remains fully active, and a phospholipase to lipase activity ratio comparable to that obtained with GPLRP2 is restored (Figure 4).

However, the presence of a large size lid domain in HPL is not the only reason for the absence of phospholipase activity since HPL(−lid) is inactive on phospholipids. Alternatively, the presence of a mini-lid domain in GPLRP2 is not per se directly responsible for the phospholipase activity of this latter enzyme. Apart from the lid domain, there are probably some other subtle structural differences between HPL and GPLRP2 that modulate their respective substrate selectivities.

On the Role of the C-Terminal Domain of Pancreatic Lipases in the Colipase Effect. HPL shares with other lipases a common tertiary structure termed the α/β hydrolase fold (Ollis *et al.*, 1992). This structural homology is however restricted to the catalytic N-terminal domain of HPL which has a size similar to that of fungal lipases. The C-terminal domain, on the other hand, is only found within the pancreatic lipase gene family which also includes lipoprotein lipase and hepatic lipase (Kirchgessner *et al.*, 1989). It has been clearly shown by X-ray crystallography that a specific function of the HPL C-terminal domain is to bind colipase (van Tilbeurgh *et al.*, 1992), the specific cofactor of classical pancreatic lipases. Colipase also interacts with the lid in its open conformation (van Tilbeurgh *et al.*, 1993). The molecular interactions between the two proteins are now well documented (Egloff *et al*., 1995b). It was predictable that these interactions could not exist with GPLRP2 due the large deletion in the lid domain as well as several key mutations in the C-terminal domain (Carrière *et al*., 1994a). We demonstrated experimentally that colipase does not reactivate GPLRP2 in the presence of bile salts using trioctanoin as substrate (Figure 5C; Thirstrup *et al*., 1994).

The chimeric construction made of the GPLRP2 N-terminal domain and the HPL C-terminal domain resulted in the partial resaturation by colipase of the depressed catalytic activity on trioctanoin, in the presence of bile salts (Figure 5E). At high bile salt concentrations, the colipase effect on the N-GPLRP2/C-HPL chimera is however not as high as with HPL (Figure 5A). This might result either from a weaker interaction between the chimera and colipase since the short lid domain of the chimera cannot interact with colipase, or from a reduction of the interfacial binding site of the lipase−colipase complex. Colipase normally counteracts the bile salt inhibitory effect by anchoring the lipase at the water/lipid interface. The hydrophobic fingers of colipase together with the open lid domain form the interfacial binding site of the HPL−colipase complex (van Tilbeurgh *et al.*, 1992, 1993). The affinity of the chimera-colipase complex for a bile salt covered water/triglyceride interface is probably reduced due to the presence of a short lid domain.

A clear colipase effect was also observed with HPL(−lid) in the presence of bile salts (data not shown). Therefore, the colipase effects observed with both the N-GPLRP2/C-HPL chimera and HPL(−lid) can only be attributed to a specific interaction between colipase and the HPL C-terminal domain. The role of the C-terminal domain in colipase binding has however been questioned recently by Jennens and Lowe (1995), from the kinetic characterization of a deletion mutant made of the N-terminal domain of HPL only. This mutant is still active in the presence of bile salts and colipase. The lack of control experiments, however, i.e., without colipase, does not allow a quantification of the well-established anchoring effect of colipase.

On the Role of the C-Terminal Domain of Pancreatic Lipases in Interfacial Stability. The importance of the C-terminal domain in the stability of pancreatic lipases at lipid/water interfaces is highlighted by an unexpected result from the kinetic characterization of the N-GPLRP2/C-HPL chimera (Table 4). In the absence of bile salts and colipase, GPLRP2 as well as GPLRP2(+lid) are both resistant to surface denaturation when using either tributyrin or trioctanoin as substrate. On the contrary, HPL, the N-GPLRP2/C-HPL chimera, and HPL(−lid) undergo a rapid and irreversible inactivation which is thought to result from the unfolding of these enzymes at high interfacial energy (Rietsch *et al*., 1977). Thus, all the lipases possessing the C-terminal domain of HPL are rapidly denaturated, except in the presence of colipase. This denaturation process probably starts by the unfolding of the C-terminal domain, which can

alternatively be stabilized by interactions with colipase. This is the first time that the involvement of the C-terminal domain in the interfacial stability of pancreatic lipases is demonstrated, whereas the stabilizing effect of colipase was already studied (Borgström & Erlansson, 1973; Canioni *et al.*, 1977; Verger *et al.*, 1977).

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