Articles

Evidence for a Pancreatic Lipase Subfamily with New Kinetic Properties†

Kenneth Thirstrup,‡ Robert Verger,§ and Frédéric Carrière*,‡

Novo-Nordisk A/S, Novo Allé, DK-2880 Bagsvaerd, Denmark, and Laboratoire de Lipolyse Enzymatique ERS 26-CNRS, 31, Chemin Joseph Aiguier, F-13402 Marseille Cedex 9, France

Received September 1, 1993; Revised Manuscript Received November 15, 1993®

ABSTRACT: Several new members of the pancreatic lipase family have been reported recently, and amino acid sequence comparison reveals that this family can now be divided into three subgroups: (1) "classical" pancreatic lipases, (2) related proteins 1 (RP1), and (3) related proteins 2 (RP2) (Giller, T., *et al.* (1992) *J. Biol. Chem. 267*(23), 16509–16516). Whereas "classical" pancreatic lipases are well characterized with respect to kinetic properties, i.e., interfacial activation and dependence on colipase in the presence of bile salts, the two latter subfamilies have been poorly investigated so far. The kinetic behavior of a lipase from guinea pig pancreas differs, however, from that of "classical" lipases (Hjorth, A., *et al.* (1993) *Biochemistry 32*, 4702–4707). This enzyme is highly homologous to RP2 lipases with the exception of a deletion in the so-called lid domain that regulates access to the active center of pancreatic lipases. We have now characterized a novel lipase from coypu (*Myocastor coypus*) pancreas. This enzyme, also belonging to the RP2 subfamily, possesses a full-length lid domain, but its kinetic properties are very similar to those of the guinea pig enzyme: (1) a high phospholipase activity, (2) the absence of interfacial activation, and (3) the absence of a colipase effect at high bile salt concentrations. Since both guinea pig and coypu pancreas produce a classical pancreatic lipase and no measurable phospholipase A2 activity, it is suggested that RP2 enzymes act as real phospholipases under physiological conditions. In fact, all RP2 lipases from other species might share phospholipase activity and fulfill new biological functions.

Pancreatic lipases are genetically classified in the lipase superfamily which includes hepatic lipase, lipoprotein lipase, and the drosophila yolk proteins (Kirchgessner *et al.*, 1989; Hide *et al.*, 1992). Until recently the pancreatic lipase family was considered to be distinct and well characterized with respect to kinetic properties, i.e., its activity on an insoluble substrate, triglycerides, through the phenomenon of "interfacial activation" (Sarda & Desnuelle, 1958) and its dependence on a small protein cofactor, colipase, in the presence of micellar concentrations of bile salts (Maylié *et al.*, 1971; Borgström & Erlanson, 1971). Several new members of the pancreatic lipase family have now been cloned and sequenced. In the mouse cytotoxic T lymphocytes, a pancreatic-like lipase has been shown to be expressed under interleukin-4 stimulation (Grusby *et al.*, 1990). In addition to the classical and well-characterized pancreatic lipase, two new human pancreatic lipase related proteins, HPL-RP1 and HPL-RP2, have been shown to be expressed in human pancreas (Giller *et al.*, 1992). Whereas HPL-RP1 displayed no enzymatic activity under the reported assay conditions, HPL-RP2 showed lipolytic activity insensitive to a micellar concentration of bile salts and slightly increased in the presence of colipase in contrast to that of the classical human pancreatic lipase (HPL). Finally, GP-3, a glycoprotein linked to the secretory granule membranes of rat pancreatic acinar cells, has been reported to belong to the pancreatic lipase family (Wishart *et al.*, 1993). Sequencing of these new lipases revealed that the pancreatic lipase family could be divided into three subgroups according to the primary structure comparison. Using the nomenclature of Giller *et al.* (1992), these subgroups are (1) classical pancreatic lipases, (2) RP1 pancreatic lipases, and (3) RP2 pancreatic lipases as shown in Figure 1.

Recently a cDNA clone encoding a guinea pig pancreatic (phospho)lipase, GPL, has been sequenced and expressed at our laboratory (Hjorth *et al.*, 1993). The recombinant enzyme (as well as native) was found to differ from other pancreatic lipases in that (1) it is not interfacially activated, (2) its activity is apparently unaffected by the presence of bile salts and/or colipase using tributyrin as substrate, and (3) it exhibits phospholipase A1 and lipase activities of the same order of magnitude. The amino acid sequence of GPL is highly homologous to that of other known pancreatic lipases, with the exception of a deletion in the so-called lid domain that regulates access to the active center (Winkler *et al.*, 1990; van Tilbeurgh *et al.*, 1993). We have then proposed that this deletion was directly responsible for the anomalous behavior of that enzyme. From primary sequence comparison analysis, it appeared that GPL (from now on called GPL-RP2), even with a shortened lid domain, was closely related to the new lipases belonging to the RP2 subfamily (67 ± 5% identities, whereas identities toward RP1 and "classical" lipases are 53 ± 1% and 52 ± 7%, respectively). These RP2 lipases could also exhibit nontypical enzymatic behavior as already suggested by the preliminary characterization of HPL-RP2 (Giller *et al.*, 1992).

Here we report the characterization of a novel pancreatic lipase from the coypu (*Myocastor coypus*), a species genetically related to the guinea pig (Beintema & Campagne, 1987).

† This research was carried out with the financial support of the BRIDGE-T-lipase programme of the European Communities under Contract No. BIOT-CT90-0181 (SMA).

* To whom correspondence should be addressed. Present address: Laboratoire de Lipolyse Enzymatique du CNRS 31, Chemin Joseph Aiguier, F-13402 Marseille Cedex 9, France.

‡ Novo-Nordisk A/S.

§ Laboratoire de Lipolyse Enzymatique ERS 26-CNRS.

® Abstract published in *Advance ACS Abstracts*, February 1, 1994.

0006-2960/94/0433-2748$04.50/0

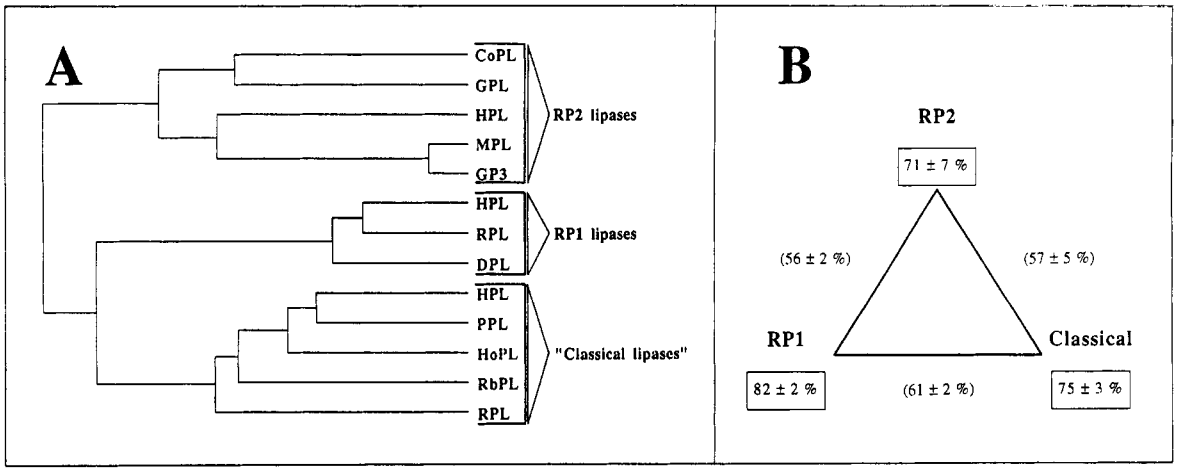


FIGURE 1: Amino acid sequence comparison within the pancreatic lipase family and its subdivision into three subfamilies. (A) Dendrogram of sequence alignment. "Classical" pancreatic lipase includes human (HPL, Winkler *et al.*, 1990; Lowe *et al.*, 1989), porcine (PPL, De Caro *et al.*, 1981), horse (HoPL, Kerfelec *et al.*, 1992), rat (RPL, Sims *et al.*, GenBank Accession No. M58369, 1991), and rabbit (RbPL, Aleman-Gomez *et al.*, 1992) pancreatic lipases. RP1 subfamily includes dog (DPL-RP1, Kerfelec *et al.*, 1986), rat (RPL-RP1, Wicker-Planquard & Puigserver, 1992), and human (HPL-RP1, Giller *et al.*, 1992) pancreatic lipases. RP2 subfamily includes mouse (MPL-RP2, Grusby *et al.*, 1990), human (HPL-RP2, Giller *et al.*, 1992), guinea pig (GPL-RP2, Hjorth *et al.*, 1993), rat (GP3-RP2, Wishart *et al.*, 1993), and coypu (CoPL-RP2, this work). (B) Percentages of amino acid identities within the three subfamilies (framed numbers) and in between the subfamilies (numbers in parentheses). The multisequence alignment was made by use of the program CLUSTAL from the PC-GENE software package.

This lipase (CoPL-RP2), belonging to the RP2 subfamily, possesses a full-length lid domain; its kinetic characteristics are, however, similar to those of GPL-RP2. Both enzymes challenge the classical distinction between lipases, esterases, and phospholipases. From the detailed enzymatic characterization of GPL-RP2 and CoPL-RP2, and the preliminary characterization of the other RP2 lipases, it is suggested that all RP2 lipases share new kinetic properties and fulfill new biological functions.

EXPERIMENTAL PROCEDURES

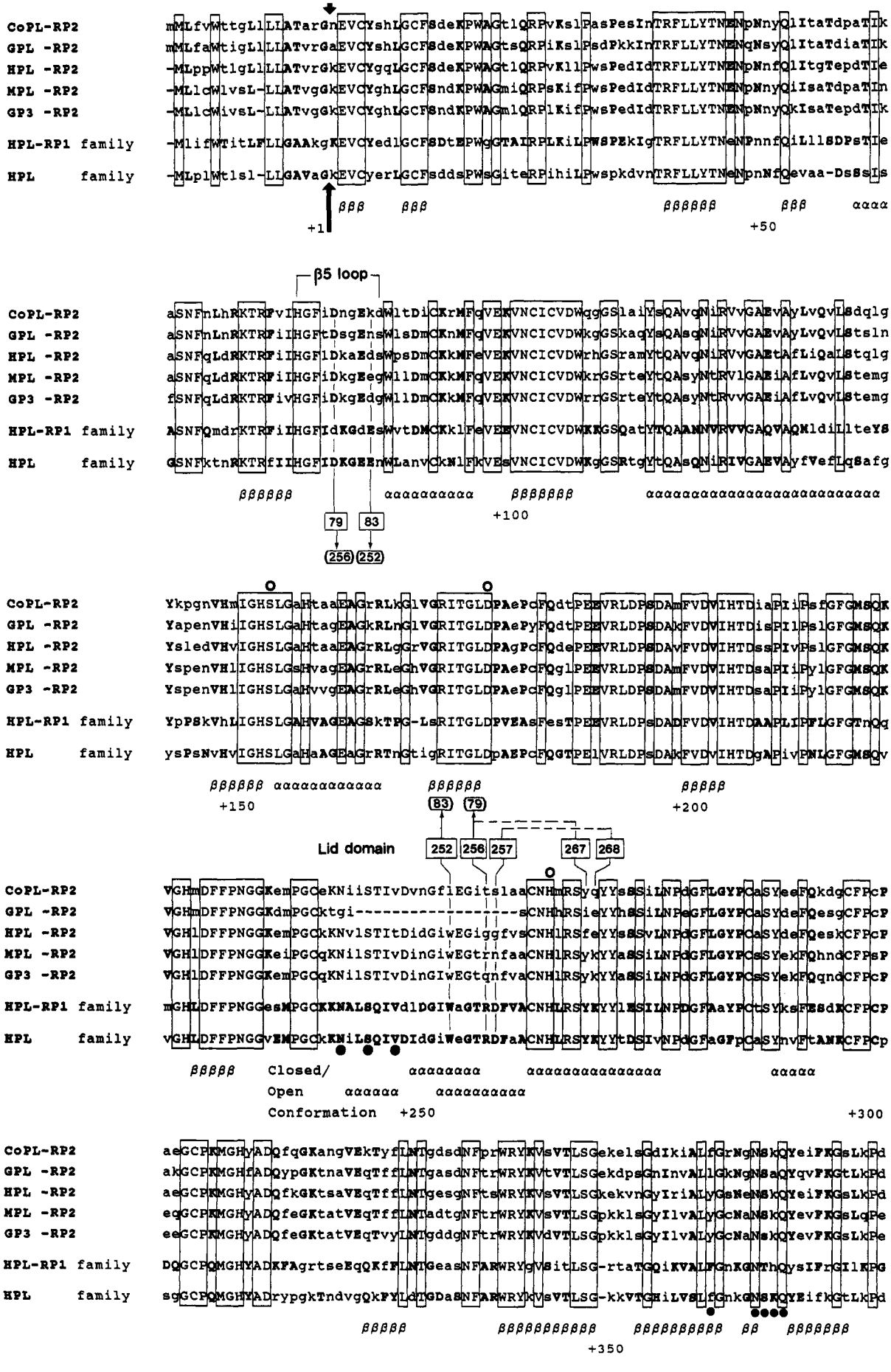
Cloning and Expression of CoPL-RP2. Coypu pancreases were obtained from the Zoo of Copenhagen and from Mr. Linas Prapuolenis (Danish Lithuanian joint venture, Vilnius, Lithuania). Poly(A)$^+$ RNA was prepared by the guanidinium/CsCl method (Chirgwin *et al.*, 1979) and purified by chromatography on oligo(dT) cellulose (Aviv & Leder, 1972). A cDNA plasmid library was constructed starting from 5 μg of mRNA essentially in accordance with previously described procedures (Houamed *et al.*, 1991). After ligation of *Bst*XI linker (Invitrogen, San Diego, CA) to the double-stranded cDNA, a size fractionation was performed on a 0.8% seaKem GTG agarose gel to isolate cDNA with sizes ranging from 800 to 2300 bp. The cDNA was then electroeluted in a dialysis bag and purified by phenol/chloroform extractions before ethanol precipitation. The purified cDNA was ligated into the pLN86 cloning vector and introduced into ElectroMAX DH10B cells by electroporation as described by the supplier (Life Technologies, Gaithersburg, MD). The pLN86 vector was constructed by expanding the multiple cloning site of the pVEGT plasmid (Houamed *et al.*, 1991) with a *Bst*XI recognition site. Prior to cDNA library screening, PCR was run on first strand material synthesized from pancreatic poly(A)$^+$ RNA using the following primers: TAACCATCGATGTGAACTGCATCTGTGTGGACTGG and TAACCATCGATTCAGCATAGTGACCCAT (*Cla*I cloning sites underlined). These oligonucleotides are homologous to sequences highly conserved in all pancreatic lipases. A 650-bp PCR fragment was subcloned, sequenced, and identified as a partial RP2 pancreatic lipase sequence. The primer TACCCCTGTGCATCCTATGAG synthesized from the partial sequence was used in the screening of the cDNA library. It was labeled with [γ-^{32}P]ATP, and hybridization to immobilized clones was performed for 16 h at 55 °C in 5 × SSC. After washing with 6 × SSC at 55 °C, 15 independent clones that hybridized to the probe were identified and characterized by restriction enzymes and southern blotting. Two were found to be full-length clones (2.1-kb insert), and one of them was fully sequenced and classified as the coypu pancreatic lipase related protein 2 (CoPL-RP2). The full-length CoPL-RP2 cDNA was subcloned into the pVL1392 baculovirus transfer vector (Invitrogen, San Diego, CA), and the expression of the recombinant protein in the baculovirus system was done as previously described (Thirstrup *et al.*, 1993).

Purification of CoPL-RP2. A highly pure recombinant lipase, CoPL-RP2, was obtained by one-step cationic exchange chromatography as previously described for recombinant HPL (Thirstrup *et al.*, 1993). The purity was analyzed by SDS–PAGE, HPLC, mass spectrometry analysis, and N-terminal amino acid sequencing.

Other Enzymes and Colipases. Recombinant HPL was expressed using the baculovirus system and purified as described (Thirstrup *et al.*, 1993). Recombinant GPL-RP2 was expressed in *Aspergillus oryzae* and purified as described (Hjorth *et al.*, 1993). Porcine procolipase (1–93) was a generous gift from Louis Sarda (ERS 26 CNRS, Marseille). Recombinant coypu colipase was expressed in the baculovirus system (Thirstrup *et al.*, unpublished results).

Lipase Activity Measurements. Lipase activities were measured potentiometrically in a thermostated vessel (37 °C) by using the pH-stat (TTT 80 Radiometer, Copenhagen). The assay conditions consisted of a mechanically stirred emulsion of 0.5 mL of tributyrin or trioctanoin (Fluka, Paris, France) in 14.5 mL of buffer (see captions to Figures 3 and 4 for details). One lipase unit = 1 μmol of fatty acid released/min.

Phospholipase Activity Measurements. The hydrolysis of emulsified phospholipids by CoPL-RP2 or GPL-RP2 was measured by the pH-stat technique according to the classical egg yolk assay (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


CoPL-RP2
GPL -RP2
HPL -RP2
MPL -RP2
GP3 -RP2
HPL-RP1 family
HPL family
β5 loop
Lid domain
Closed/
Open
Conformation

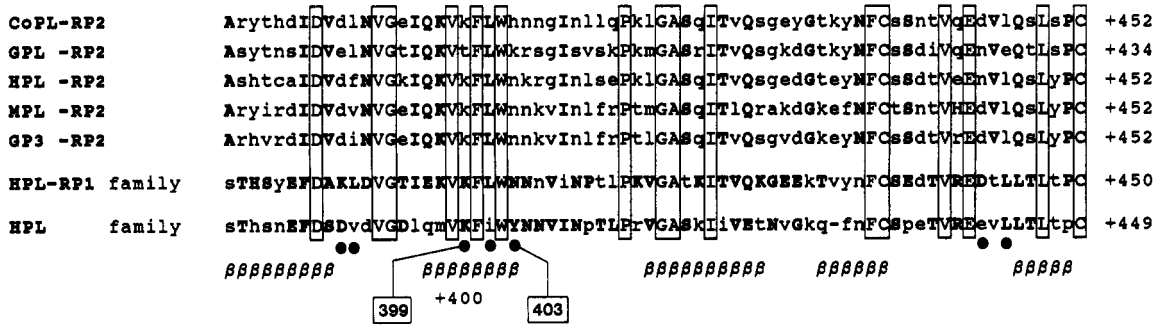


FIGURE 2: Amino acid sequence alignment of pancreatic lipases. Abbreviations used are the same as in the caption below Figure 1. All five members of the RP2 subfamily are shown, while HPL-RP1 and HPL are used as representative members of their respective subfamilies. The numbering of the amino acids (below the alignment) is based on that of HPL (Winkler *et al.*, 1990), and the number at the end of each sequence is the size of the mature protein. An arrow indicates the cleavage of the signal peptide. Framed residues in capital letters indicate conservation within the three subfamilies whereas residues in bold capital letters indicate conservation within one subfamily. Residues in small letters are not conserved within a subfamily. Lightface capital letters are used exclusively for the RP2 subfamily in the lid domain when residues are conserved, except in GPL-RP2. Open circles (○) indicate the three residues in the catalytic triad (Ser152, Asp176, and His263). Closed circles (●) indicate residues involved in colipase binding in HPL (van Tilbeurgh *et al.*, 1992, 1993). β-Strand segments and α-helices are underlined according to Winkler *et al.* (1990) for the closed conformation and van Tilbeurgh *et al.* (1993) for the open conformation of HPL.

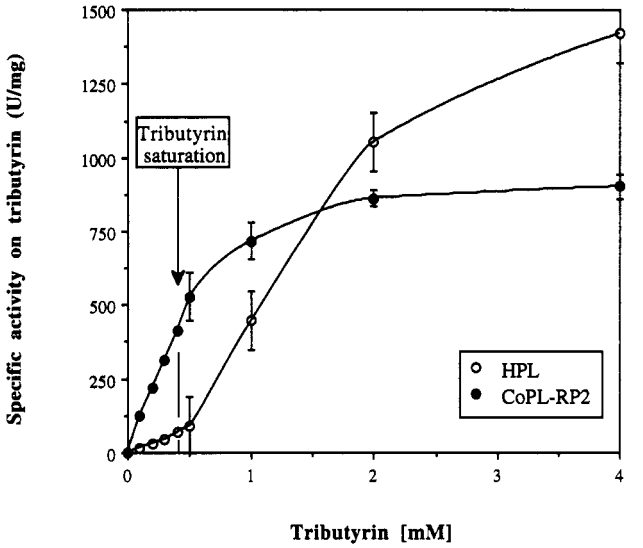


FIGURE 3: Interfacial activation. The influence of tributyrin concentration on CoPL-PR2 and HPL was measured by the pH-stat technique. As indicated by the arrow, tributyrin saturation under the present experimental condition is found at 0.4 mM. The assay (37 °C, pH 8.0) contained between 2 and 4 μg of CoPL-RP2 in 15 mL of NaCl solution (150 mM) and various concentrations of tributyrin. HPL data are from Thirstrup *et al.* (1993). One unit (U) = 1 μmol of fatty acid released/min. Values are expressed as means ± standard deviation (n = 3).

4 mM $CaCl_2$, filtered through a cheese cloth, adjusted to pH 8.0) in 10 mL of 20 mM deoxycholate. Phosphatidylcholine is the main phospholipid found in egg yolk. One phospholipase unit = 1 μmol of fatty acid released/min.

Phospholipase activity was also measured by the monolayer technique (Ransac *et al.*, 1991) using monomolecular films of various phospholipids as substrate (1,2-didodecanoylphosphatidylcholine; 1,2-didodecanoylphosphatidylethanolamine; 1,2-didodecanoylphosphatidylglycerol; Fluka, Paris, France). The lipid monolayer was spread over 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 21 mM $CaCl_2$, and 1 mM EDTA, contained in a zero-order trough. In all cases lipases were injected into the reaction compartment (volume, 51 mL; surface area, 31 cm^2). For each phospholipid substrate, the enzymatic activity was measured at different surface pressures.

Extraction of Coypu Pancreatic Enzymes and Gel Filtration. Sixty grams of coypu pancreas were cut into small pieces and crushed with Ultra-turax: three times in 100 mL of acetone, three times in 200 mL of chloroform–butyl alcohol (9:1), two times in 200 mL of chloroform–butyl alcohol (4:1), and finally in 100 mL of diethyl ether. All solvents were kept at 4 °C before use. After each extraction step, the mixture was filtrated through a Büchner to remove the solvent, and the material retained on the paper filter was submitted to the next extraction step. The final step provided 8.4 g of powder after all diethyl ether had been evaporated. Two grams (sample 1) of the previous powder was soaked in 16 mL of 20 mM Tris-HCl, 150 mM NaCl, pH 7.5 during 15 min at 4 °C, with gentle magnetic stirring. The mixture was submitted to centrifugation (10 000 rpm, 15 min, 4 °C), and the pelled was removed. Lipase and phospholipase activities were assayed immediately in the supernatant (around 12 mL). Sample 1 was then incubated at 37 °C during several hours in order to follow the evolution of lipolytic activity with time. A similar extraction procedure was carried out (sample 2) in the presence of 2 mM benzamidine in order to avoid proteolysis. Sample 2 was further submitted to gel filtration using fast protein liquid chromatography. One milliliter of extract was injected onto a Hiload 16/60 column (Pharmacia) equilibrated in 20 mM Tris-HCl, 150 mM NaCl, 2 mM benzamidine, pH 7.5. The flow rate was 0.8 mL/min, and protein elution was recorded spectrometrically at 280 nm. Lipase and phospholipase activities were measured in all collected fractions. Gel filtration chromatography was also performed with recombinant CoPL-RP2 and porcine pancreatic phospholipase A2 (generous gift from Lars Thim, Novo Nordisk) as molecular weight standards.

RESULTS

A full-length CoPL cDNA was isolated and sequenced, and the corresponding polypeptide was found to have the highest degree of homology to the RP2 family as shown in Figures 1 and 2. Within this subfamily, CoPL-RP2 is closely related to GPL-RP2 (73% identities). However, the lid region shortened in GPL-RP2 is full-length in CoPL-RP2 (Figure 2). We have previously reported that GPL-RP2 possesses new enzymatic properties, namely, phospholipase A1 activity, apparent insensitivity to bile salts on tributyrin, and absence of interfacial activation (Hjorth *et al.*, 1993). These kinetic characteristics were proposed as being consequences of the shortened lid domain in GPL-RP2. CoPL-RP2 was expressed using the baculovirus system and purified in one step using cationic exchange chromatography (data not shown) as already described for recombinant HPL (Thirstrup *et al.*, 1993). Surprisingly, characterization of CoPL-RP2 revealed kinetic

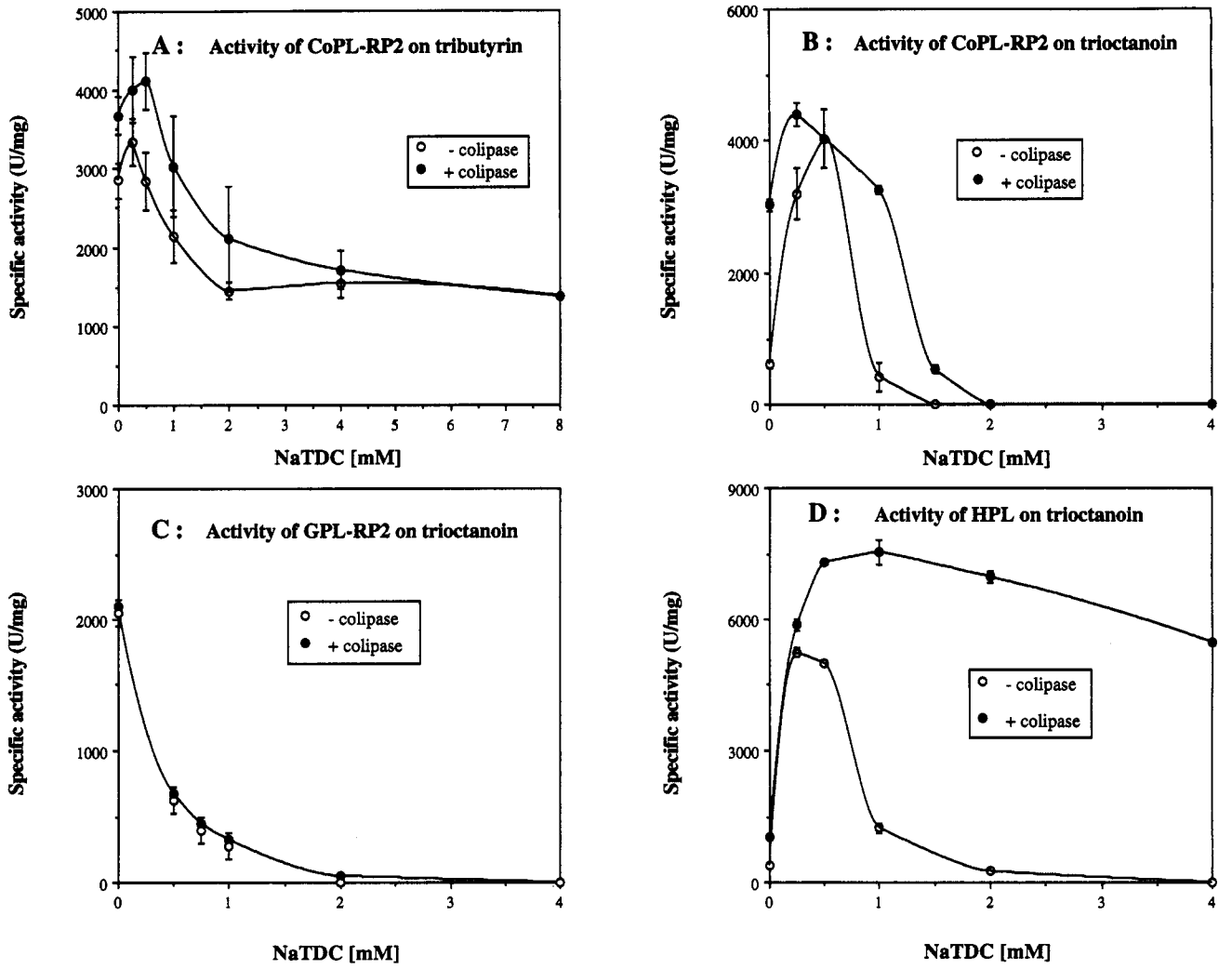


FIGURE 4: Bile salts and colipase effects. (A) Tributyrin as substrate; sensitivity to bile salts and dependence on colipase of CoPL-RP2. The lipase specific activity was measured with the pH-stat technique at pH 7.5, 37 °C. The final assay volume consisted of 15 mL, containing 0.5 mL of tributyrin, 14.5 mL of 0.28 mM Tris, 150 mM NaCl, 1.4 mM $CaCl_2$, and various concentrations of sodium taurodeoxycholate (NaTDC). Two micrograms of CoPL-RP2 was used for each measurement, and when required, colipase was added at molar excess above 2. (B–D) Trioctanoin as substrate; sensitivity to bile salts and dependence on colipase of CoPL-RP2, GPL-RP2, and HPL, respectively. Experimental procedures are the same as above except that highly pure trioctanoin is replacing tributyrin (impure trioctanoin may contain lipolysis products soluble in water such as C8-monoglycerides and C8-diglycerides, and the effect of bile salt does not appear). The large drop in CoPL-RP2 activity (B) when using trioctanoin as substrate in absence of bile salts and colipase is due to the instability of CoPL-RP2 in the presence of the substrate alone. This behavior is also observed with HPL (D). In the presence of colipase, CoPL-RP2 seems to be protected from an irreversible interfacial inactivation (B). One unit (U) = 1 μmol of fatty acid released/min. Values are expressed as means ± standard deviation (n = 3).

properties similar to those of GPL-RP2.

Interfacial Activation. The main property of classical pancreatic lipases is their ability to be activated by a water/lipid interface as shown in Figure 3 by the variation of HPL specific activity as a function of tributyrin concentration. Tributyrin is a short-chain triglyceride possessing low solubility in water. Below the saturation point, HPL activity is very low; but once saturation is exceeded and a lipid phase appears, there is a sharp increase in HPL activity.

CoPL-RP2 was found to display no interfacial activation in contrast to the classical HPL (Figure 3). This indicates that CoPL-RP2 like GPL-RP2 (Hjorth *et al.*, 1993) behaves more as an esterase according to Sarda and Desnuelle (1958); *i.e*, it is fully active on substrate monomers present in solution.

Since the concentration of monomeric tributyrin cannot exceed saturation, the variation in CoPL-RP2 specific activity with the amount of substrate should not increase significantly above saturation if the enzyme is only active on a monomeric substrate. In fact, CoPL-RP2 specific activity still increases above tributyrin saturation, indicating that CoPL-RP2 is also active toward the aggregated substrate. The curve observed in Figure 3 thus reflects the activity of CoPL-RP2 on two different substrates. Even though CoPL-RP2 activity as a function of substrate concentration seems to display a normal Michaelis–Menten pattern, it is not correct to use this model.

Bile Salts and Colipase Effects. The second main property of classical pancreatic lipases is their inhibition by bile salts and their reactivation by colipase. Using tributyrin as substrate, HPL specific activity decreases with sodium taurodeoxycholate (NaTDC) concentration. Above the critical micellar concentration of NaTDC (1–2 mM), HPL displays no activity and is desorbed from the negatively charged water/lipid interface covered by bile salts. A high activity is restored, however, in the presence of colipase which counteracts the bile salt effect (Thirstrup *et al.*, 1993).

In the absence of colipase and the presence of substrate tributyrin, the specific activity of CoPL-RP2 was found to decrease for bile salt concentrations ranging from 0.5 to 2 mM, but the activity was not inhibited and reached a plateau above 2 mM NaTDC (Figure 4A). Addition of colipase only

Table 1: Comparison between Phospholipase and Lipase Specific Activities of CoPL-RP2, GPL-RP2, Porcine Pancreatic Phospholipase A2 (PLA2), and HPL[a]

enzyme	phospholipase activity on egg yolk (units/mg)	lipase/esterase activity on tributyrin (units/mg)	lipase activity on trioctanoin (units/mg)
CoPL-RP2	180	1700[b]	0
GPL-RP2	500	2000[b]	0
PLA2[c]	700	0	0
HPL	0	8000	6000

[a] Phospholipase and lipase measurements are performed as described in Experimental Procedures. Lipase activity measurements on both tributyrin and trioctanoin were performed in the presence of colipase and 4 mM sodium taurodeoxycholate at pH 7.5. [b] Lipolytic activity also includes esterase activity on tributyrin monomers in the case of CoPL-RP2 and GPL-RP2. [c] From De Haas *et al.* (1968). One unit = 1 μmol of fatty acid released/min.

resulted in a slight effect at low bile salt concentrations. It was, however, impossible to conclude whether CoPL-RP2 was still bound to the interface, since it could also be active on tributyrin monomers in solution. We answered this question by using the completely insoluble medium-chain triglyceride, trioctanoin (Figure 4B). In the absence of colipase, a clear and complete inhibition by bile salts was seen above 2 mM NaTDC. Thus the apparent insensitivity of CoPL-RP2 to high bile salt concentrations as observed previously with tributyrin (Figure 4A) could be attributed to the activity of CoPL-RP2 on tributyrin monomers. The decrease in CoPL-RP2 specific activity for bile salt concentrations ranging from 0.5 to 2 mM might be explained by the inhibition of CoPL-RP2 activity on the aggregated tributyrin (Figure 4A).

On the basis of the previous results, we reinvestigated the effect of bile salts on GPL-RP2 activity using trioctanoin as substrate. GPL-RP2 was also inhibited by high bile salt concentrations in the absence of colipase (Figure 4C) whereas it was shown to be apparently insensitive to bile salts using tributyrin as substrate (Hjorth *et al.*, 1993).

But the main information we obtained from these experiments with trioctanoin as substrate was that colipase did not reactivate either CoPL-RP2 or GPL-RP2 at high bile salt concentrations (Figure 4B,C). Even in the presence of a large excess of colipase, the lipolytic activities of CoPL-RP2 and GPL-RP2 were not expressed above 2 mM NaTDC, whereas the classical HPL recovered a high activity (Figure 4D). In the case of CoPL-RP2, we noted, however, that colipase binding to lipase was not completely abolished since a clear effect of colipase was observed at 1 mM NaTDC using trioctanoin as substrate (Figure 4B).

We used porcine colipase in the preliminary experiments since it is known to reactivate similarly all the known classical pancreatic lipases (Sternby & Borgström, 1981). After we observed a different behavior with CoPL-RP2 in the presence of bile salts and porcine colipase, coypu colipase was also used in order to check any species specificity of the lipase/colipase interactions. No significant change in lipolytic activity was observed.

Phospholipase Activity. The phospholipase activity of CoLP-RP2 was first measured using an emulsion of egg yolk phospholipids as substrate. The CoPL-RP2 showed a high specific activity of 180 units/mg comparable to the activity found for GPL-RP2 (Table 1). In contrast HPL showed no measurable activity on this substrate as also found for other members of this subfamily (Verger, 1984). The phospholipase activity of CoPL-RP2 was also assayed on various phospholipids using the monolayer technique. As shown in Figure 5, CoPL-RP2 displayed its highest activity toward both phosphatidylethanolamine and phosphatidylglycerol, for surface pressure values ranging from 10 to 20 mN·m^{-1}. The activity on phosphatidylcholine was found to be much lower with an optimal surface pressure of 25 mN·m^{-1}.

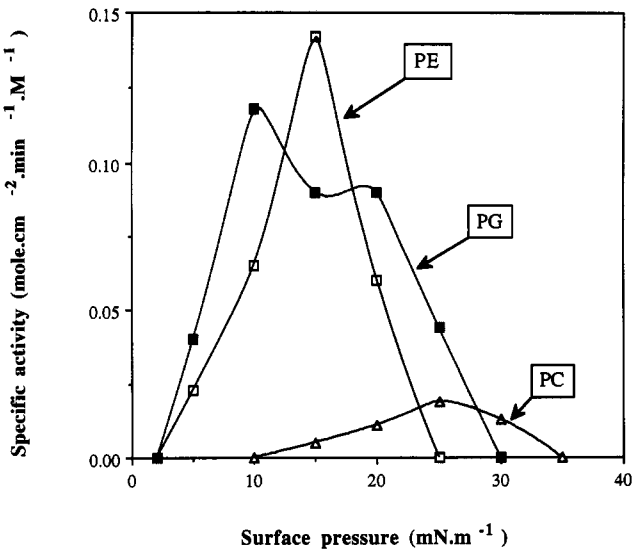


FIGURE 5: Variation in CoPL-RP2 phospholipase activity with surface pressure. The phospholipase activity was measured using the monolayer technique (see Experimental Procedures) and various phospholipids as substrate: 1,2-didodecanoylphosphatidylcholine (△); 1,2-didodecanoylphosphatidylethanolamine (□); 1,2-didodecanoylphosphatidylglycerol (■) monolayer. Hydrolysis was catalyzed by 1–2 μg of recombinant CoPL-RP2 injected under the phospholipid film.

Investigation of Phospholipase Activity in Coypu Pancreas. We found no evidence for a classical phospholipase A2 (PLA2) in the coypu pancreas. Using the egg yolk assay, phospholipase activity was directly measured after extraction of pancreatic enzymes whereas classical PLA2 has to be activated by trypsin (Verheij & de Haas, 1991). In the coypu pancreatic extract, both phospholipase and lipase activities decreased with time, demonstrating the absence of PLA2 zymogen activation (Figure 6). The addition of exogenous trypsin did not increase the phospholipase activity (data not shown). Moreover, the phospholipase activity from coypu pancreas was not dependent on calcium, in contrast to classical PLA2, and addition of EDTA during the egg yolk assay did not modify the activity (data not shown).

Gel filtration experiments were carried out using a crude extract of coypu pancreatic enzymes (Figure 7A) and both CoPL-RP2 and porcine pancreatic PLA2 as standards (Figure 7B). No phospholipase activity was detected in fractions corresponding to the molecular weight range of pancreatic PLA2 (14 kDa). On the contrary, phospholipase activity was coeluted with lipase activity in fractions corresponding to 50-kDa proteins as CoPL-RP2.

A classical pancreatic lipase is also found in the coypu (Thirstrup *et al.*, manuscript in preparation). From 1 g of pancreatic powder, it was possible to recover 63 150 ± 420 lipase units (tributyrin as substrate, 4 mM NaTDC, + colipase, pH 7.5) and 1120 ± 100 phospholipase units (egg yolk phospholipids as substrate, pH 8.0). Since, under the previous conditions, the lipase and phospholipase specific activities of CoPL-RP2 were 2000 and 180 units/mg, respectively, and the specific activity of the classical pancreatic lipase could be estimated at around 8000 units/mg (tributyrin as substrate, 4 mM NaTDC, + colipase, pH 7.5), we estimated that 1 g of pancreatic powder (7 g of fresh pancreas) contained 6.2 and 6.3 mg of CoPL-RP2 and classical pancreatic lipase, respectively, i.e., a 1:1 ratio. Some caution is necessary in the

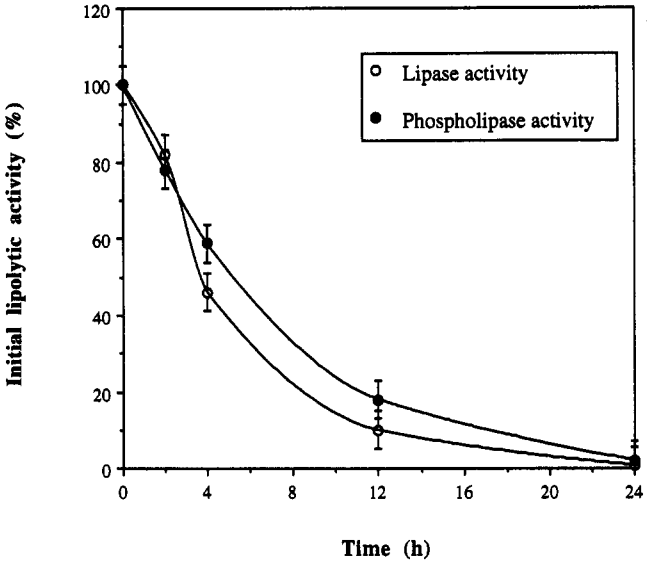


FIGURE 6: Variation vs time in the lipolytic activities extracted from coypu pancreas. The phospholipase activity from coypu pancreas can be directly measured after extraction without trypsin activation. Both lipase and phospholipase activities decrease with time due to proteolytic degradation. This is not the classical behavior of the pancreatic phospholipase A2 (PLA2) which is produced as a proenzyme and requires a tryptic cleavage to be activated. PLA2 activity is not directly measurable after extraction of pancreatic enzymes. This activity is only revealed and starts to increase when trypsin starts to digest the PLA2 propeptide. The lipase and phospholipase activities were measured by the pH-stat technique using tributyrin and egg yolk phospholipids as substrate, respectively. Values are expressed as means ● standard deviation ($n = 3$).

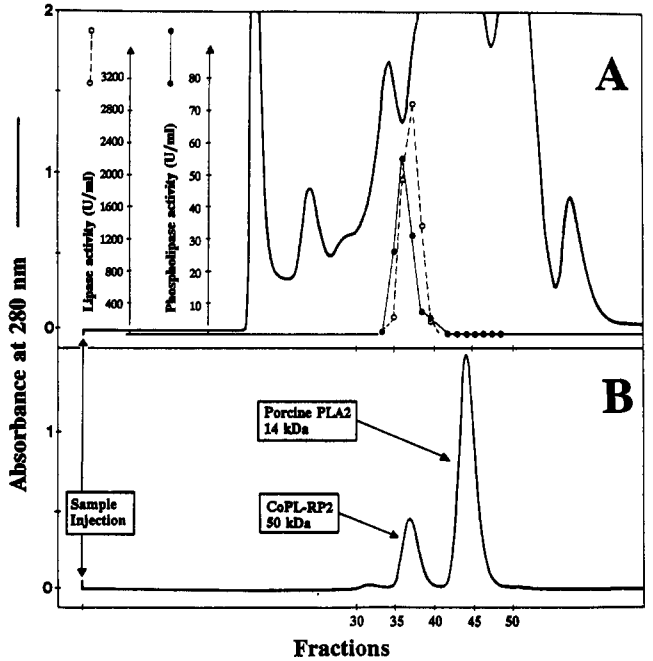


FIGURE 7: Gel filtration chromatography of the proteins extracted from coypu pancreas. (A) Elution profile of all the proteins extracted from an acetonic powder of coypu pancreas and injected on a Hiload 16/60 column (see Experimental Procedures). Lipase and phospholipase activities are coeluted in the fractions corresponding to 50-kDa proteins. (B) Elution profile of CoPL-RP2 and porcine pancreatic PLA2 as standards, under the same experimental conditions as in A. The lipase and phospholipase activities were measured by the pH-stat technique using tributyrin and egg yolk phospholipids as substrate, respectively.

interpretation of our results since pancreatic carboxylic ester hydrolase (CEH) can also hydrolyze tributyrin and can contribute to the total activity measured with this substrate. CEH specific activity on tributyrin is, however, much lower than that of pancreatic lipase, and our estimation cannot be greatly modified.

DISCUSSION

From the previous characterization of GPL-RP2 (Hjorth *et al.*, 1993), it was proposed that the presence of the "mini lid" domain was responsible for its peculiar properties, i.e., high phospholipase activity, absence of interfacial activation, and apparent insensitivity to bile salts using a partially water-soluble triglyceride, tributyrin. The present results show that these kinetic properties are shared by CoPL-RP2 and are not simply associated with the size of the lid domain.

Phospholipase Activity. Whereas a classical pancreatic lipase is found in both the guinea pig and the coypu (Fauvel *et al.*, 1981b; Thirstrup *et al.*, manuscript in preparation), there is no measurable activity of the classical pancreatic phospholipase A2 in both species (Fauvel *et al.*, 1981a; see Results for the coypu). GPL-RP2 and CoPL-RP2 are, however, produced by the pancreas at a high level similar to that of the classical lipase, and their physiological role is probably the digestion of dietary phospholipids, even though a phospholipase A2 of the pancreatic type was recently discovered in the guinea pig stomach (Tojo *et al.*, 1993; Ying *et al.*, 1993). The fact that the lipase activity of RP2 enzymes is inhibited by micellar concentrations of bile salts (as found in the small intestine), in the presence or absence of colipase (Figure 4B,C), indicates that insoluble triglycerides cannot be their physiological substrates and that the classical pancreatic lipase is probably the only pancreatic enzyme degrading dietary triglycerides.

Since both GPL-RP2 and CoPL-RP2 possess phospholipase activity, it is obvious that the lid domain as a whole is not directly responsible for the phospholipase activity of these enzymes. This could explain that the lid domain in GPL-RP2 has been extensively mutated through evolution without major consequences for the phospholipase activity. The reasons for the phospholipase activity of GPL-RP2 and CoPL-RP2 remain, however, unknown.

Concerning the other lipases within the RP2 subfamily, phospholipase activity remains to be investigated but some speculations can be expressed. In humans, HPL-PR2 is described as a lipase marginally dependent on the presence of colipase; *i.e.*, its activity is insensitive to high bile salt concentration and is only slightly increased by addition of colipase (Giller *et al.*, 1992). The activity of HPL-RP2 was assayed using an insoluble long-chain triglyceride and cannot result from hydrolysis of substrate monomers present in solution as in the case of tributyrin as substrate. This suggests that HPL-RP2 could still bind to the triglyceride/water interface in the presence of bile salts and in the absence of colipase. Inspection of the reported experimental conditions for lipase activity measurements reveals, however, the presence of phosphatidylcholine in the assay (Giller *et al.*, 1992) and liberation of fatty acids from the hydrolysis of lecithin cannot be excluded.

MPL-RP2 is a pancreatic lipase related enzyme expressed in mouse cytotoxic T lymphocytes under interleukin-4 stimulation (Grusby *et al.*, 1990). The authors proposed that this enzyme could be involved in cytolysis by degrading lipids of the outer cell membrane. A phospholipase activity of MPL-RP2 might explain the expression of this digestive-like enzyme in T cells.

Finally, GP3 is a pancreatic lipase related enzyme linked to the secretory granule membranes of pancreatic acinar cells

again, a phospholipase activity might be consistent with the postulated biological role.

Interfacial Activation. In solution (absence of interface), the classical HPL was found to exist in a closed conformation (Winkler *et al.*, 1990). The active center is completely inaccessible to solvent, and a residue in the lid region, Trp252, is structurally located directly on top of the active Ser152, with the indole ring packed against Phe77, a residue which belongs to the β5-loop. In the presence of an interface, the lid domain as well as the β5-loop undergoes large conformational changes, opening the active center and creating the oxyanion hole (van Tilbeurgh *et al.*, 1993). This phenomenon was anticipated by Sarda and Desnuelle (1958) and named "interfacial activation". Both GPL-RP2 and CoPL-RP2 display no interfacial activation; *i.e.*, these enzymes are active in solution on monomers of a partially water-soluble triglyceride. In GPL-RP2, the lid domain is shortened and the active center is envisioned to be freely accessible (Hjorth *et al.*, 1993). In CoPL-RP2, the activity in solution is probably due to an open conformation of the lid domain. The lid domain sequence is poorly conserved when CoPL-RP2 and HPL are compared (Figure 2). Particularly the replacement of Trp252 by a Leu in CoPL-RP2 could weaken the interactions of the lid with the β5-loop thus facilitating the spontaneous opening of the active site.

The Absence of a Colipase Effect on CoPL-RP2 and GPL-RP2. Both GPL-RP2 and CoPL-RP2 activities on insoluble triglycerides are inhibited by high bile salt concentration and are not restored by colipase (Figure 4B,C). With CoPL-RP2, however, a minor effect of colipase is observed for bile salt concentrations below 2 mM (Figure 4B). The behavior of GPL-RP2 can be explained as follows: among the 12 residues of the HPL C-terminal domain involved in colipase binding (van Tilbeurgh *et al.*, 1992), nine are different in GPL-RP2, including the important Lys399. Moreover, colipase also interacts with the lid domain of HPL in its open conformation (van Tilbeurgh *et al.*, 1993). These interactions cannot exist in a lipase with a "minilid" such as GPL-RP2.

In CoPL-RP2, the absence of a colipase effect at bile salt concentrations above 2 mM is not easily interpretable. Almost all the residues involved in the interaction with colipase are conserved (both in the C-terminal domain and the lid, Figure 2). One striking exception is, however, residue 403, which is conserved as a tyrosine within all classical lipases. Tyr403 interacts strongly with colipase through several van der Waals contacts in the HPL–colipase complex (van Tilbeurgh *et al.*, 1992). The stacking of Tyr403 of lipase and Arg65 of colipase mainly confers the apolar component of binding energy. The point mutation of residue 403 in CoPL-RP2 could induce a low affinity for colipase, explaining the absence of a colipase effect at high bile salt concentration.

Also the lid/colipase interactions might be suppressed in CoPL-RP2 due to an open conformation of the lid domain different from the one observed in HPL. The open conformation of the HPL lid is stabilized by interactions with the core of the protein, the colipase, and the β5-loop (van Tilbeurgh *et al.*, 1993). The lid domain interacts with the core of the protein through a salt bridge (Asp257···Lys268) and a hydrogen bond (Arg256···Tyr267). In the RP2 subfamily, the corresponding residues are almost all different, whereas they are totally conserved in the two other pancreatic lipase subfamilies (Figure 2). The lid domain also interacts with the β5-loop through a salt bridge (Arg256···Asp79) and a hydrogen bond (Trp252···Glu83). Here again, most of the corresponding residues are different in the RP2 subfamily. All the previous interactions stabilizing the lid domain conformation in HPL cannot exist in a lipase such as CoPL-RP2. It is proposed that the lid domain in CoPL-RP2 possesses a higher degree of freedom and that the interactions with colipase are weakened.

In conclusion, the two enzymes of the RP2 subfamily characterized in our studies exhibit similar kinetic properties, despite the presence of a full-length lid domain in CoPL-RP2 and a "minilid" in GPL-RP2. These properties are (1) a high phospholipase activity, (2) the absence of interfacial activation, and (3) the absence of a colipase effects at high bile salt concentrations. Since both guinea pig and coypu pancreas produce a classical pancreatic lipase and no measurable phospholipase A2 activity, it is suggested that RP2 enzymes act as real phospholipases under physiological conditions. The question remains whether the other enzymes within the RP2 subfamily display similar kinetic behavior, even though it is already clear that they also fulfill new biological functions.

ACKNOWLEDGMENT

We are grateful to Francine Ferrato and Isabelle Douchet for their technical assistance and to Lars Thim and Esper Boel for reading the manuscript and for fruitful discussions.

REFERENCES

Aleman-Gomez, J. A., Colwell, N. S., Sasser, T., & Kumar, V. B. (1992) *Biochem. Biophys. Res. Commun. 188*(3), 964–971.

Aviv, H., & Leder, P. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1408–1412.

Beitema, J. J., & Campagne, R. N. (1987) *Mol. Biol. Evol. 4*, 10–18.

Borgström, B., & Erlanson, C. (1971) *Biochim. Biophys. Acta 242*, 509–513.

Chirgwin, J. M., Przybila, A. E., Mac Donald, R. J., & Rutter, W. J. (1979) *Biochemistry 18*, 5294–5299.

De Caro, J., Boudouard, M., Bonicel, J., Guidoni, A., Desnuelle, P., & Rovery, M. (1981) *Biochim. Biophys. Acta 671*, 129–138.

De Haas, G. E., Postema, N. M., Nieuwenhuizen, W., & van Deenen, L. L. M. (1968) *Biochim. Biophys. Acta 159*, 103–117.

Fauvel, J., Bonnefis, M. J., Chap, H., Thouvenot, J. P., & Douste-Blazy, L. (1981a) *Biochim. Biophys. Acta 666*, 72–79.

Fauvel, J., Bonnefis, M. J., Sarda, L., Chap, H., Thouvenot, J. P., & Douste-Blazy, L. (1981b) *Biochim. Biophys. Acta 663*, 446–456.

Giller, T., Buchwald, P., Kaelin, D. B., & Hunziker, W. (1992) *J. Biol. Chem. 267*(23), 16509–16516.

Grusby, M. J., Nabavi, N., Wong, H., Dick, R. F., Bluestone, J. A., Schotz, M. C., & Glimcher, L. H. (1990) *Cell 60*, 451–459.

Hide, W. A., Chan, L., & Li, W. H. (1992) *J. Lipid. Res. 33*, 167–178.

Hjorth, A., Carrière, F., Cudrey, C., Wöldike, H., Boel, E., Lawson, D. M., Ferrato, F., Cambillau, C., Dodson, G. G., Thim, L., & Verger, R. (1993) *Biochemistry 32*, 4702–4707.

Houamed, K. M., Kuijper, J. L., Gilbert, T. L., Haldeman, B. A., O'Hara, P. J., Mulvihill, E. R., Almers, W., & Hagen, F. S. (1991) *Science 252*, 1318–1321.

Kerfelec, B., LaForge, K. S., Puigserver, A., & Scheele, G. (1986) *Pancreas 1*, 430–437.

Kerfelec, B., Foglizzo, E., Bonicel, J., Bougis, P. E., & Chapus, C. (1992) *Eur. J. Biochem. 206*, 279–287.

Kirchgessner, T. G., Chuat, J. C., Heinzmann, C., Etienne, J., Guilhot, S., Svenson, K., Ameis, D., Pilon, C., D'Auriol, L., Andalibi, A., Schotz, M. C., Galibert, F., & Lusis, A. J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9647–9651.

Lowe, M. E., Rosemblum, J. L., & Strauss, A. W. (1989) *J. Biol. Chem. 264*, 20042–20048.
Maylié, M. F., Charles, M., Gache, C., & Desnuelle, P. (1971) *Biochim. Biophys. Acta 229*, 286–289.
Ransac, S., Moreau, H., Rivière, C., & Verger, R. (1991) *Methods Enzymol. 197*, 49–65.
Sarda, L., & Desnuelle, P. (1958) *Biochim. Biophys. Acta 30*, 513–521.
Sternby, B., & Borgström, B. (1981) *Comp. Biochem. Physiol. B68*, 15–18.
Thirstrup, K., Carrière, F., Hjorth, S., Rasmussen, P. B., Wöldike, H., Nielsen, P. F., & Thim, L. (1993) *FEBS Lett. 327*, 79–84.
Tojo, H., Ying, Z., & Okamoto, M. (1993) *Eur. J. Biochem. 215*, 81–90.
Van Tilbeurgh, H., Sarda, L., Verger, R., & Cambillau, C. (1992) *Nature 359*, 159–162.
Van Tilbeurgh, H., Egloff, M. P., Martinez, C., Rugani, N., Verger, R., & Cambillau, C. (1993) *Nature 362*, 814–820.
Verger, R. (1984) In *Lipases* (Borgström, B., & Brockman, H. L., Eds.) pp 83–150, Elsevier, London.
Verheij, H. M., & de Haas, H. G. (1991) *Methods Enzymol. 197*, 214–223.
Wicker-Planquard, C., & Puigserver, A. (1992) *FEBS Lett. 296*, 61–66.
Winkler, F. K., D'Arcy, A., & Hunziker, W. (1990) *Nature 343*, 771–774.
Wishart, M. J., Andrews, P. C., Nichols, R., Blevins, G. T., Logsdon, C. D., & Williams, J. A. (1993) *J. Biol. Chem. 268*(14), 10303–10311.
Ying, Z., Tojo, H., Nonaka, Y., & Okamoto, M. (1993) *Eur. J. Biochem. 215*, 91–97.