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Cloning of the classical guinea pig pancreatic lipase and comparison with the lipase related protein 2

Frédéric Carrière*, Kenneth Thirstrup, Siv Hjorth, Esper Boel

Novo Nordisk, Novo allé, DK-2880 Bagsværd, Denmark

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Abstract

Starting from total pancreatic mRNAs, the classical guinea pig pancreatic lipase was cloned using rapid amplification of 3′ and 5′ cDNA ends. Internal oligonucleotide primers were designed from a partial cDNA clone including the region coding for the lid domain. Using this strategy, we did not amplify the cDNA corresponding to the pancreatic lipase related protein 2 in which the lid domain is deleted. Amino acid sequences of the classical guinea pig pancreatic lipase and the related protein 2 were compared based on the primary and tertiary structures of the classical human pancreatic lipase. Their distinct physiological roles are discussed in the light of functional amino acid differences.

Key words: Colipase, Polymerase chain reaction; Phospholipase; Phospholipid; Triglyceride

1. Introduction

Over the last four years, a better understanding of the pancreatic lipase (PL) structure/function relationships has been reached with the resolution of several three-dimensional structures [1–3]. Activation of the lipase at a lipid/water interface and binding of colipase can now be interpreted at the molecular level. At the same time, new pancreatic lipases have been cloned from several species [4–7], and it appeared that the PL family could be divided into three subgroups according to primary structures: (1) classical pancreatic lipases, including the well known human and porcine PLs, (2) pancreatic lipase related proteins 1, PLRP1, and (3) pancreatic lipase related proteins 2, PLRP2 [5]. Whereas the kinetic properties and the physiological role of PLRP1 remain to be investigated, PLRP2 might act as a phospholipase under physiological conditions [8].

The phospholipase activity of PLRP2 has been demonstrated in the guinea pig [6], a species in which the classical pancreatic phospholipase A2 is missing [9]. The guinea pig PLRP2 (GPLRP2) is characterized by a large deletion in the so-called lid domain that regulates access to the active site of the classical PL [6], and by the fact that it does not bind colipase [8]. Using trioctanoin as substrate, it has been shown in vitro that GPLRP2 lipase activity is inhibited by increasing bile salt concentration, and addition of colipase does not restore the activity [8]. Accordingly, GPLRP2 cannot hydrolyze insoluble triglycerides in the small intestine where colipase normally counteracts the inhibitory effect of bile salts by anchoring the lipase at the water/triglyceride interface [10]. On the other hand, GPLRP2 hydrolyzes phospholipids associated with bile salts in mixed micelles [6,11–12].

The digestion of dietary triglycerides is carried out by a classical PL which is also found in the guinea pig pancreas [12]. In the present article, we report on the cloning of this classical guinea pig pancreatic lipase (GPL) cDNA and the comparison of GPL amino acid sequence with GPLRP2 and human pancreatic lipase (HPL).

2. Materials and methods

2.1. Amplification of 3′ and 5′ GPL cDNA ends

Total mRNAs were isolated from guinea pig pancreas using the guanidine thiocyanate extraction procedure [13]. GPL cDNA was obtained from total mRNAs by the procedure of Frohman [14] for rapid bamplification of 3′ and 5′ cDNA ends (RACE, see Fig. 1).

First strand cDNAs were prepared using heat-denaturated (5 min at 65°C) total mRNAs (3 μg) as the template, Superscript reverse transcriptase (Life Technologies/Gibco BRL, Copenhagen, Denmark), and primer #1 (100 pmol, see Table 1). Reverse transcription was carried out in a total reaction volume of 20 μl for 10 min at room temperature and 45 min at 42°C, in presence of RNAsin (20 units, Promega Corp., Madison, WI). The cDNA/mRNA heteroduplex was then denaturated at 95°C for 10 min and cooled on ice.

*Corresponding author.

Present address: Laboratoire de Lipolyse Enzymatique du CNRS, 31 Chemin Joseph Aiguier, F-13402 Marseille cedex 09, France. Fax: (33) 91 71 58 57.

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For 5′ RACE, the single strand cDNAs (from 3 μg of total mRNAs) were poly-dC tailed with terminal deoxynucleotidyl transferase (Pharmacia LKB, Copenhagen, Denmark). Amplification of the specific GPL cDNA 5′ end was carried out by PCR using AmpliTaq polymerase (Perkin-Elmer/AH Diagnosis, Århus, Denmark) and primers #3 and #4 (50 pmol each, see Table 1). For the 3′ RACE, the single strand cDNAs were directly used as template. PCR was carried out with primers #1 and #2 (50 pmol each, see Table 1).

2.2. DNA analysis

PCR products were examined by electrophoresis in 1% agarose gel with ethidium bromide staining and southern blot analysis. After denaturation within the agarose gel, DNA was transferred under vacuum to a nitrocellulose membrane (Schleicher and Schuell) and then immobilized at 80°C for 2 h. Membranes were soaked for 1 h at 65°C in a prehybridization solution of 5 × SSC, 10 × Denhardt's solution, 100 μg/ml salmon sperm DNA, 0.5% SDS, 10 mM EDTA then hybridized overnight at 65°C with ^{32}P-labelled probe (1 × 10^6 cpm per ml of prehybridization solution). Post-hybridization membranes were washed twice for 20 min at 65°C in 6 × SSC. The radioactive probe was generated from a GPL cDNA fragment of 600 bp isolated in a previous work [6]. Labelling was performed with [α-^{32}P]-dCTP using the Klenow polymerase (New England Biolabs/Finnzymes, Espoo, Finland) and a random hexameric primer (Pharmacia LKB, Copenhagen, Denmark). The labelling procedure as well as other molecular biology procedures were taken from [15].

2.3. Cloning and sequencing of GPL cDNA ends

PCR products hybridizing with the GPL specific probe were isolated from 1% agarose gel and purified using the Geneclean kit (BIO 101 Inc., La Jolla, CA). The purified 3′ and 5′ DNA ends were then digested by *Cla*I and *Eco*RI endonucleases (New England Biolabs/Finnzymes, Espoo, Finland) for cloning into the pBluescript SK II vector (Stratagene, La Jolla, CA). ElectroMAX DH10B cells were transformed by electroporation as described by the supplier (Life Technologies, Gaithersburg, MD) and plasmid DNAs were isolated from appropriate clones according to [15]. DNA sequences were determined by the dideoxynucleotide chain-termination method [16] using alkaline-denaturated plasmid DNAs and the Sequenase version 2.0 kit (US Biochemical, Cleveland, OH).

3. Results

Using the RACE protocol (Fig. 1), the 3′ and 5′ ends of GPL cDNA were amplified separately starting from total mRNA of guinea pig pancreas. For this purpose, we synthesized sense and anti-sense oligonucleotides (#2 and # 4, see Table 1) corresponding to an internal region of the GPL coding sequence (see Fig. 2). A partial GPL cDNA clone was isolated previously during the cloning of GPLRP2 cDNA [6]. From sequence alignment with other classical pancreatic lipases, this partial cDNA of 600 bp was found to include the lid domain coding sequence. Our strategy was then to design primers in this region in order to amplify specifically GPL cDNA and not GPLRP2 in which the lid domain is deleted. There was still the possibility that the primers would anneal to a PLRP1 cDNA, but we never observed any clone corresponding to a lipase of the RP1 type.

For cloning purpose, a *Cla*I restriction site was introduced in primers #2 and #4 by substituting A by C in the codon corresponding to Ile-249 (see Fig. 2 and Table 1). Other restriction sites were introduced at the 5′ and 3′ ends by using primers #1 and #3, respectively (Table 1).

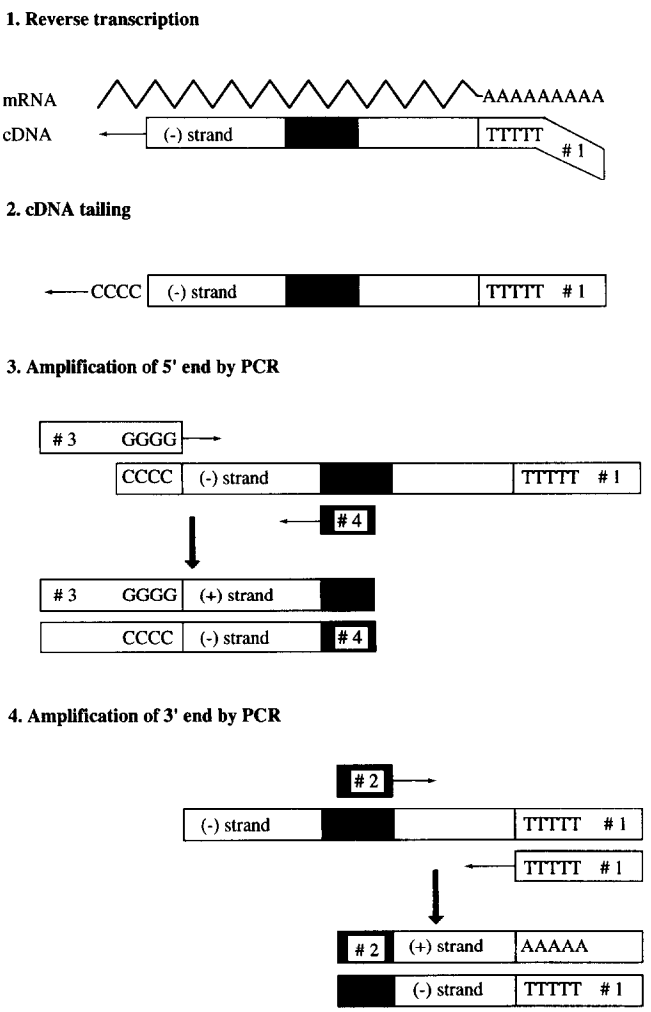

Fig. 1. Schematic diagram for rapid amplification of the 3′ and 5′ cDNA ends (RACE) of GPL. The oligonucleotides primers #1 to #4 are described in Table 1. Dark boxes indicate DNA sequence corresponding to the lipase lid domain.

RACE PCRs were carried out for 40 cycles with denaturation, annealing and extension temperatures of 94°C (1 min), 55°C (2 min) and 72°C (2 min), respectively. The PCR products were separated by electrophoresis and examined by southern blot using the partial GPL cDNA as a probe. A clear hybridization was observed with bands of around 1000 and 750 bp, corresponding to the 5′ and 3′ ends, respectively. These DNAs were cut from a preparative agarose gel stained with ethidium bromide, purified, digested by *Cla*I and *Eco*RI endonucleases, and ligated into the pBluescript SK II vector for transformation of *E. coli* electro-competent cells. Several clones were isolated and characterized.

Concerning the 3′ end, several plasmid DNAs digested with *Cla*I and *Eco*R I were found to contain an insert of 730 bp. One of them was sequenced on both strands and a second on one strand to check that mutations were not introduced by Taq polymerase during the PCR reaction.

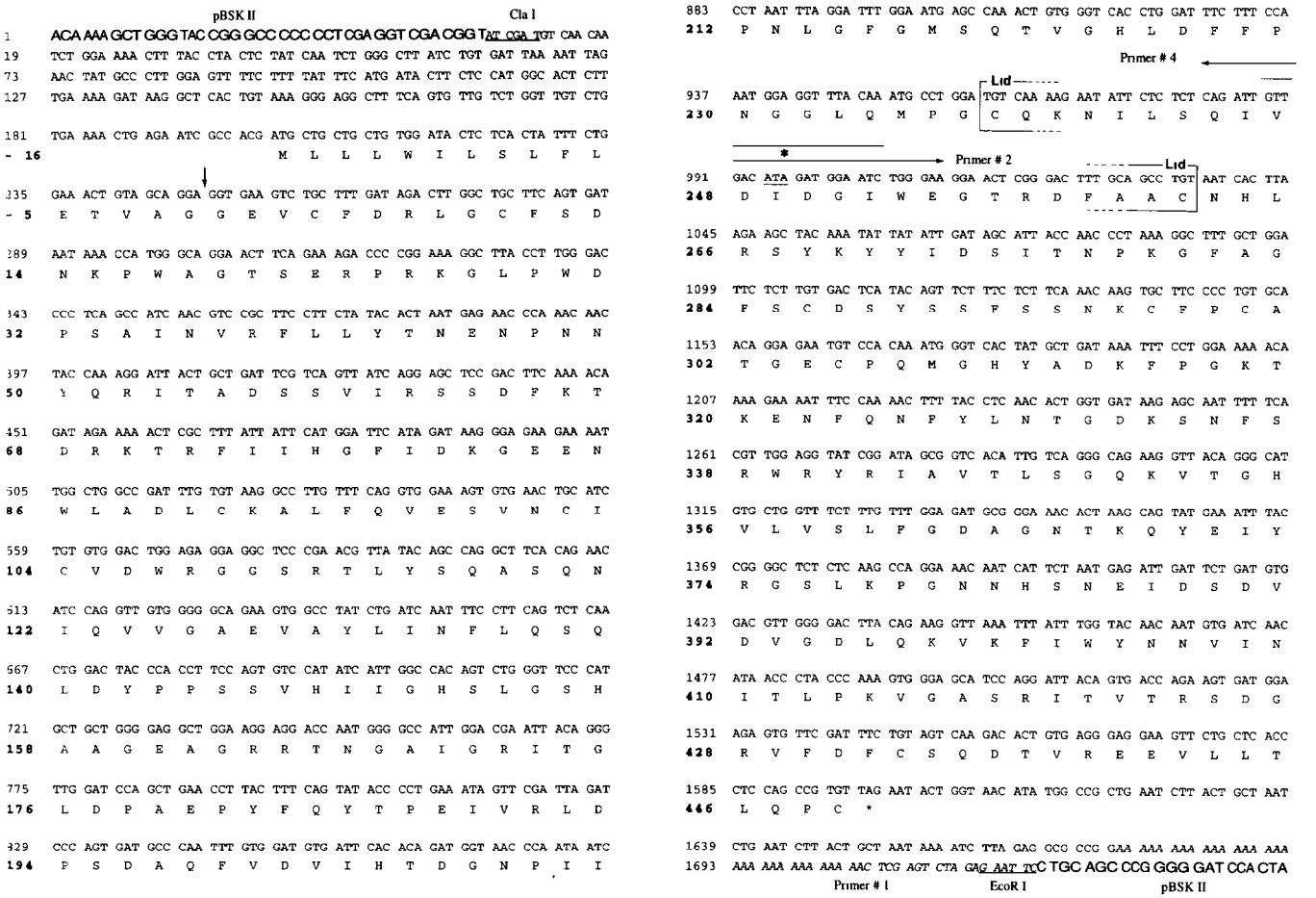

Fig. 2. cDNA and amino acid sequence of the classical guinea pig pancreatic lipase (GPL). Numbering of nucleotides begins and ends at the *Cla*I and *Eco*RI restriction sites, respectively. Numbering of amino acid residues begins at the signal peptide. The signal peptide cleavage site is shown by an arrow. The positions of primers #2 and #4 are shown and the star indicates the point mutation for introducing a *Cla*I restriction site (see Table 1). Letters in italics indicate the nucleotide sequence corresponding to primer #1 and letters in bold indicate the pBluescript SK II vector sequence.

Concerning the 5′ end, it appeared that *Cla*I was also cutting GPL cDNA at around 200 bp upstream of the coding sequence (see Fig. 2), generating a DNA fragment of 990 bp. After cloning in the *Cla*I restriction site of the pBluescript KS II vector, an insert of the same size was found in several clones and two of them were sequenced.

The complete sequence of GPL cDNA was then assembled as shown in Fig. 2.

4. Discussion

From sequence alignment with all the known PLs [4–8,17–22], it is clear that the GPL sequence reported here corresponds to a classical PL (data not shown). GPL possesses 76.2% of amino acid identities with the classical HPL whereas it only shares 60.1% of amino acid identities with GPLRP2 (Fig. 3). When the N- and C-

Table 1
Oligonucleotides used as primers for cloning GPL cDNA

No	Oligonucleotide	Comment
#1	GAG AGA GAA TTC TCT AGA CTC GAG T_{30}	Anti-sense primer for first strand cDNA synthesis and 3′ RACE. Restriction sites: *Eco*RI, *Xba*I, *Xho*I
#2	GTT GAC ATC GAT GGA ATC TGG GAA	Sense primer for 3′ RACE Restriction site: *Cla*I
#3	CGA GAA TTC GGA TCC G_{17}	Sense primer for 5′ RACE Restriction sites: *Eco*RI, *Bam*HI
#4	GAT TCC ATC GAT GTC AAC AAT CTG	Anti-sense primer for 5′ RACE Restriction site: *Cla*I

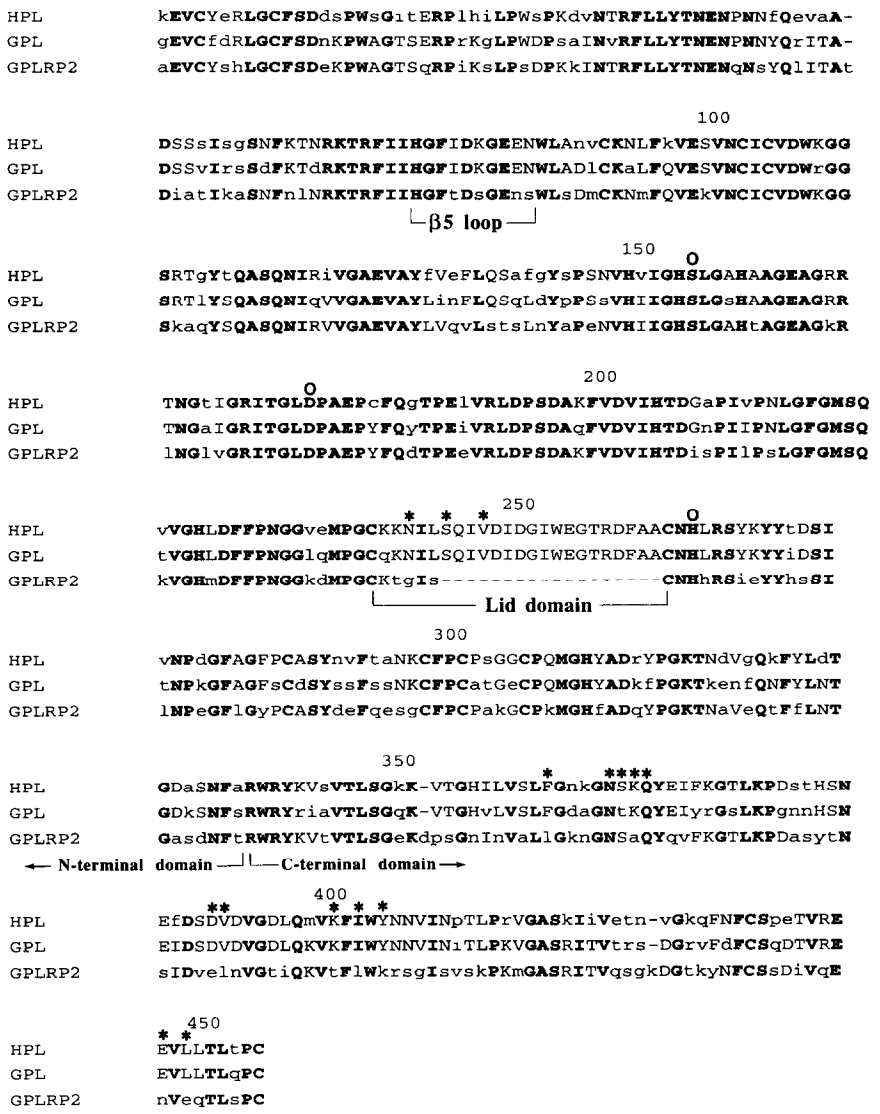

Fig. 3. Amino acid sequence alignment of GPL with HPL and GPLRP2. HPL and GPLRP2 sequences are based on ref. [1,19] and [6], respectively. Active site residues (Ser-152, Asp-176, His-263) are shown by open circles. Stars indicate amino acids involved in colipase binding to HPL [2]. The positions of the b5 loop, the lid domain and the junction between the N- and C-terminal domains are shown. Capital letters in bold indicate amino acid residues conserved within the three lipases.

terminal domains of GPL and HPL are compared independently, it appears that the level of amino acid identities are similar (77.4 and 72.6%, respectively). A similar comparison between GPL and GPLRP2 reveals that the N-terminal domain (with the exception of the lid domain) is much more conserved (68.7%) than the C-terminal domain (48.7%). Globally, the domains involved in the high specificity of classical PLs towards triglycerides through the binding of colipase are well conserved in GPL and are extensively mutated in GPLRP2.

The lid domain is involved in the interfacial activation of classical PL. In the absence of an aggregated lipid substrate, the lid domain prevents the access to the active site [1]. In the presence of a water/lipid interface, the lid domain as well as another surface loop (the so-called b5 loop) undergo large conformational changes thus opening access to the active site and creating the oxyanion hole [3]. The new conformation of the lid domain is stabilized by interactions with the protein core and the b5 loop (Table 2). The colipase interacts with both the lid and the lipase C-terminal domain (Table 2). All the lipase amino acid residues involved in the previous interactions are well conserved within the classical PL subfamily, and this is also the case for GPL (see Fig. 3 and Table 2).

In GPLRP2, these functional amino acid residues are nearly all mutated. The lid domain of 23 residues in GPL and HPL (between Cys-237 and Cys-261) is replaced by a short loop of 5 residues in GPLRP2 (Fig. 3). The lid interactions as observed in HPL cannot exist and the

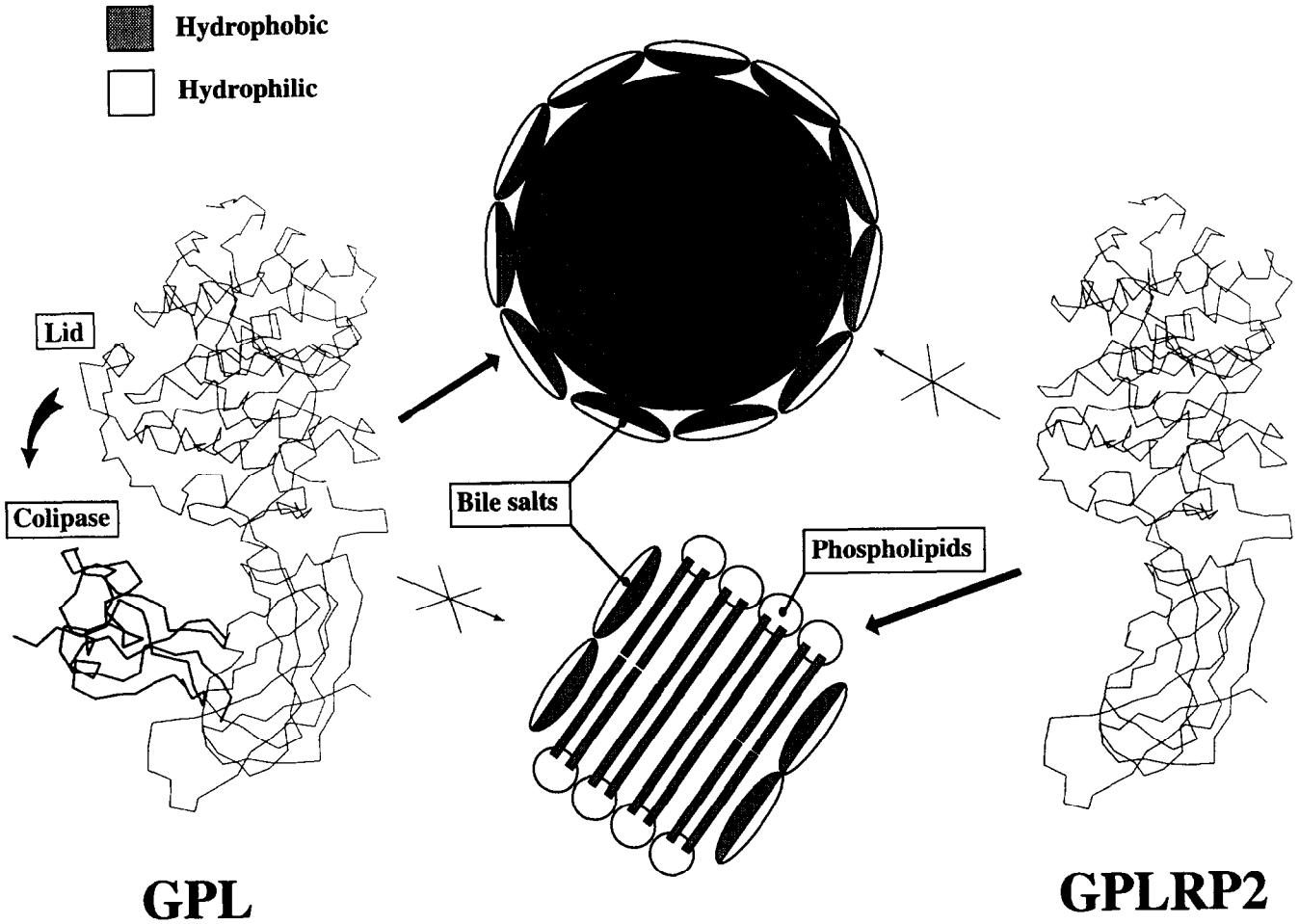

Fig. 4. Schematic diagram representing the distinct biological functions of GPL and GPLRP2. GPL model is based on the Ca tracing of HPL-colipase complex [2]. GPLRP2 model is based on HPL 3D structure [6]. TG is the abbreviation for triglycerides.

Table 2
Conservation of the functional amino acids found in HPL-colipase complex. Numbering is based on ref. [1].

Interactions	HPL	GPL	GPLRP2
Lid/protein core	Arg-256...Tyr-267	Arg/Tyr	-/Ile
	Asp-257...Lys-268	Asp/Lys	-/Glu
Lid/β5 loop	Trp-252...Glu-83	Trp/Glu	-/Asn
	Arg-256...Asp-79	Arg/Asp	-/Asp
Lid/colipase	Asn-240	Asn	-
	Ser-243	Ser	-
	Val-246	Val	-
C-terminal domain/ colipase	Phe-360	Phe	Leu
	Asn-365	Asn	Asn
	Ser-366	Thr	Ser
	Lys-367	Lys	Ala
	Gln-368	Gln	Gln
	Asp-389	Asp	Glu
	Val-390	Val	Leu
	Lys-399	Lys	Thr
	Ile-401	Ile	Leu
	Tyr-403	Tyr	Lys
	Glu-441	Glu	Asn
	Leu-443	Leu	Glu

corresponding amino acids in the protein core and the b5 loop are also different (Table 2). The lid/colipase interactions are suppressed and among the twelve residues identified as the colipase binding site in the C-terminal domain of HPL, nine are different in GPLRP2 (Fig. 3 and Table 2).

Even though GPL has not been expressed and enzymatically characterized, it is probably this pancreatic enzyme which is responsible for dietary triglyceride hydrolysis in the guinea pig intestine, like HPL in humans. On the contrary, the kinetic properties of GPLRP2 have been studied in vitro [6,8]. This atypical PL is characterized by (1) an absence of interfacial activation, (2) an inhibition by bile salts of the lipase activity towards insoluble triglycerides, (3) an absence of colipase effect, and finally (4) a high phospholipase activity towards phospholipids found in mixed micelles. Whereas the absences of interfacial activation and colipase binding can be easily interpreted from the previous structural analysis, the reasons for GPLRP2 phospholipase activity remain unknown. In the GPLRP2 model based on HPL 3D structure, there is a high degree of conservation of the

amino acid residues found in a 10 Å radius sphere from the active site Ser-152 [6]. The classical PLs are however completely inactive towards phospholipids found in mixed micelles and can only hydrolyze monolayers of negatively charged phospholipids at a very low rate [23].

In conclusion, GPL and GPLRP2 presumably have two distinct physiological roles in the intestine, where the triglyceride droplets are covered by bile salts and the phospholipids are mainly present in mixed micelles: GPL is a triglyceride hydrolase and GPLRP2 is a phosphoglyceride hydrolase (Fig. 4). The role of GPLRP2 as a physiologically important phospholipase is supported by the absence of any measurable phospholipase A2 activity in the guinea pig pancreas [9], even though it has been shown recently that a phospholipase A2 of the pancreatic type is produced by the guinea pig stomach [24,25].

The comparison of classical and RP2 pancreatic lipases provides an excellent tool for futher investigation of structure/function relationships including substrate specificity and more generally, lipid/protein interactions.

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References

[1] Winkler, F.K., D'Arcy, A. and Hunziker, W. (1990) Nature 343, 771–774.
[2] Van Tilbeurgh, H., Sarda, L., Verger, R. and Cambillau, C. (1992) Nature 359, 159–162.
[3] Van Tilbeurgh, H., Egloff, M.P., Martinez, C., Rugani, N., Verger, R. and Cambillau, C. (1993) Nature 362, 814–820.
[4] Grusby, M.J., Nabavi, N., Wong, H., Dick, R.F., Bluestone, J.A., Schotz, M.C. and Glimcher, L.H. (1990) Cell 60, 451–459.
[5] Giller, T., Buchwald, P., Kaelin, D.B. and Hunziker, W. (1992) J. Biol. Chem. 267(23), 16509–16516.
[6] Hjorth, A., Carrière, F., Cudrey, C., Wöldike, H., Boel, E., Lawson, D.M., Ferrato, F., Cambillau, C., Dodson, G.G., Thim, L. and Verger, R. (1993) Biochemistry 32, 4702–4707.
[7] Wishart, M.J., Andrews, P.C., Nichols, R., Blevins, G.T., Logsdon, C.D. and Williams, J.A. (1993) J. Biol. Chem. 268, 10303–10311.
[8] Thirstrup, K., Verger, R. and Carrière, F., Biochemistry, in press.
[9] Fauvel, J., Bonnefis, M.J., Chap, H., Thouvenot, J.P. and Douste-Blazy, L. (1981) Biochim. Biophys. Acta 666, 72–79.
[10] Borgström, B. and Erlanson-Albertsson, C. (1984) in: Lipases (Borgström, B. and Brockman, H.L., Eds.) pp. 151–183, Elsevier, London.
[11] Durand, S., Clemente, F., Thouvenot, J.P., Fauvel-Marmouyet, J. and Douste-Blazy, L. (1978) Biochimie 60, 1215–1217.
[12] Fauvel, J., Bonnefis, M.J., Sarda, L., Chap, H., Thouvenot, J.P. and Douste-Blazy, L. (1981) Biochim. Biophys. Acta 663, 446–456.
[13] Chirgwin, J.M., Przybila, A.E., Mac Donald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294–5299.
[14] Frohman, M. (1990) in: PCR Protocols: A Guide to Methods and Applications (Innis, M.A., Gelfand, D.H., Sninsky J.J. and White, T.J., Eds) pp. 28–38.
[15] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular cloning, Vols. 1, 2 and 3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
[16] Sanger, F., Nicklen, S. and Coulson, A. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[17] De Caro, J., Boudouard, M., Bonicel, J., Guidoni, A., Desnuelle, P. and Rovery, M. (1981) Biochim. Biophys. Acta 671, 129–138.
[18] Kerfelec, B., LaForge, K.S., Puigserver, A. and Scheele, G. (1986) Pancreas 1, 430–437.
[19] Lowe, M.E., Rosemblum, J.L. and Strauss, A.W. (1989) J. Biol. Chem. 264, 20042–20048.
[20] Kerfelec, B., Foglizzo, E., Bonicel, J., Bougis, P.E. and Chapus, C. (1992) Eur. J. Biochem. 206, 279–287.
[21] Aleman-Gomez, J.A., Colwell, N.S., Sasser, T. and Kumar, V.B. (1992) Biochem. Biophys. Res. Commun. 188, 964–971.
[22] Wicker-Planquard, C. and Puigserver, A. (1992) FEBS Lett. 296, 61–66.
[23] Verger, R. (1984) in: Lipases (Borgström, B. and Brockman, H.L., Eds.) pp. 83–150, Elsevier, London.
[24] Tojo, H., Ying, Z. and Okamoto, M. (1993) Eur. J. Biochem. 215, 81–90.
[25] Ying, Z., Tojo, H., Nonaka, Y. and Okamoto, M. (1993) Eur. J. Biochem. 215, 91–97.