Functional diversity and interactions between the repeat domains of rat intestinal lactase

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Lactase–phlorizin hydrolase (LPH), a major digestive enzyme in the small intestine of newborns, is synthesized as a high-molecular-mass precursor comprising four tandemly repeated domains. Proteolytic cleavage of the precursor liberates the pro segment (LPHα) corresponding to domains I and II and devoid of known enzymic function. The mature enzyme (LPHβ) comprises domains III and IV and is anchored in the brush border membrane via a C-terminal hydrophobic segment. To analyse the roles of the different domains of LPHα and LPHβ, and the interactions between them, we have engineered a series of modified derivatives of the rat LPH precursor. These were expressed in cultured cells under the control of a cytomegalovirus promoter. The results show that recombinant LPHβ harbouring both domains III and IV produces lactase activity. Neither domain III nor IV is alone sufficient to generate active enzyme, although the corresponding proteins are transport-competent. Tandem duplication of domains III or IV did not restore lactase activity, demonstrating the separate roles of both domains within LPHβ. Further, the development of lactase activity did not require LPHα; however, LPHα potentiated the production of active LPHβ but the individual LPHα subdomains I and II were unable to do so. Lactase activity and targeting required the C-terminal transmembrane anchor of LPH; this requirement was not satisfied by the signal/anchor region of another digestive enzyme: sucrase–isomaltase. On the basis of this study we suggest that multiple levels of intramolecular interactions occur within the LPH precursor to produce the mature enzyme, and that the repeat domains of the precursor have distinct and specific functions in protein processing, substrate recognition and catalysis. We propose a functional model of LPHβ in which substrate is channelled from an entry point located within domain III to the active site located in domain IV.

INTRODUCTION

Internal tandem duplication has been described in a large number of genes. The evolutionary advantage of this arrangement is unknown, although multimerization could plausibly increase overall protein activity or, alternatively, permit the different repeated domains to adopt specialized functions. Here we examine the separate roles of the repeat domains comprised within rat intestinal lactase–phlorizin hydrolase (LPH; EC 3.2.1.62/108), a major digestive enzyme responsible for the metabolism of dietary lactose in mammalian newborns [1]. LPH is synthesized as a single-chain 215–245 kDa precursor comprising four tandemly repeated domains [2–8]. These show sequence similarity with the type 1 family of glycosyl hydrolases (T1GH family), a family of proteins that includes single-domain enzymes present in micro-organisms (see [9,10] and references therein] and plants [11–13], and in the liver of mammals [14]. The T1GH family also comprises multi-domain proteins expressed in the intestine of non-mammalian vertebrates and in invertebrates, but their sequences have not been fully determined [15].

In the LPH precursor, the four T1GH domains are flanked by an N-terminal signal sequence and, at the C-terminus, a hydrophobic extension thought to tether the enzyme in the brush border membrane in the N_{out}–C_{in} orientation [16–18]. Maturation of the precursor involves N- and O-glycosylation, proteolytic cleavage and dimerization [4,19–25]. Cleavage between domains II and III generates mature active LPH, or LPHβ, comprising domains III and IV together with the C-terminal transmembrane anchor. The pro segment of the precursor, designated LPHα, comprises domains I and II; no enzyme activity has been attributed so far to LPHα [26] and it has a shorter half-life than LPHβ [27,28]. In rat and rabbit, in contrast with human, internal cleavage by a membrane protease of the furin family occurs within LPHα [4,29,30]. Studies on the human precursor suggested an obligate requirement of LPHα for the production of enzymically active and transport-competent LPHβ [28,31].

The natural substrates of LPH are lactose and aryl or alkyl β-glycosides contained in milk [32,33], but the enzyme also hydrolyses laminaribiose, a β-glucoside derived from the plant cell wall [34]. The activity of LPHβ towards different substrates suggested the existence of two distinct active sites [32,35,36], corroborated by labelling experiments with conduritol β-epoxide, a covalent inhibitor that binds to a conserved glutamic residue present in both domain III and domain IV [16]. The same amino acid was identified as the nucleophile of the active site in other members of the T1GH family [37,38]. The kinetics of inhibition of lactase and phlorizin hydrolase activities, compared with the rate of labelling of the two Glu residues of LPHβ, suggested that the Glu residue within domain III constitutes part of the lactase active site [16]. In contrast, site-directed mutagenesis of this residue did not affect lactase activity [39]. Instead, replacement of the equivalent residue in domain IV by another amino acid abolished lactase activity and most phlorizin hydrolase activity, arguing that LPHβ contains a single major active site located in domain IV [39].

The structural organization of the LPH precursor prompted us to address in detail the functional role of the different domains. For this purpose we have studied the properties of LPH derivatives in which the different domains were expressed separately or in combination.

Abbreviations used: LPH, lactase–phlorizin hydrolase; mAb, monoclonal antibody; T1GH, type 1 glycosyl hydrolase; SI, sucrase–isomaltase; X-Fuc, 5-bromo-4-chloroindol-3-yl β-D-fucopyranoside; X-Gal, 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside.

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MATERIALS AND METHODS

Amplification of DNA fragments by PCR

To construct recombinant cDNA species encoding combinations of the LPH domains, several PCR fragments were generated from the oligonucleotides listed below (see also Figure 1A). Oligonucleotides were from Eurogentec (Seraing, Belgium):
LPH-S5, 5′-dAAACTAGTTCCTTCACCATGGAGCTCCCT-TGG-3′;
LPH-S3, 5′-dTTGCATGCATAAGCTTAAATTTCTGTCGG-ATTCCCAGTC-3′;
LPH-13, 5′-dTTTTAAGCTTGCCCAAACTCTCTG-3′;
LPH-25, 5′-dAAAAAAGCTTTGCTAACCAGTCTA-3′;
LPH-23, 5′-dTTTTAAGCTTACTTTCTTGGCTGAA-3′;
LPH-24A, 5′-dTTGGCTGGGAATGGCAT-3′;
LPH-24B, 5′-dTTTTTTGCATGCACACTGATTTTAGCCT-3′;
LPH-T5, 5′-dAAGCATGCCCGACCCTGCACAAGGACCT-CATCC-3′;
LPH-T3, 5′-dTTGATCAGAAGGAGGAAATTGGACTCA-3′;
SI-ST5, 5′-dAAACTAGTCTGGTACAACATA-3′;
SI-ST3, 5′-dTTTTAAGCTTTCATCAACAGCAGGT-3′.

PCR reactions (50 μl) contained 5 ng of plasmid, 25 pmol of primer, each dNTP at 0.4 mM, 50 mM Tris/HCl, pH 9.2, 16 mM $(NH_4)_2SO_4$, 1.75 mM $MgCl_2$ and 2.5 units of Expand Long Template PCR Enzyme (Boehringer-Mannheim, Mannheim, Germany). Conditions were: 93 °C for 20 s; 55 °C for 30 s; 68 °C for 30 s to 6 min. PCR used a Thermojet apparatus (Eurogentec) for 10 cycles.

The PCR fragments were inserted into cloning vectors and sequenced on both strands by the dideoxy method, with appropriate primers and the T7 Sequencing Kit (Pharmacia, Orsay, France). Nucleotide sequences were also verified at the junctions between the restriction fragments that were ligated to construct the different recombinants cDNA species.

For reverse transcriptase–PCR analysis, RNA was extracted with Trizol Reagent (Gibco/BRL, Cergy Pontoise, France). Oligo(dT)-primed cDNA synthesis was for 1 h at 42 °C in 50 μl of solution containing 4 μg of RNA, 100 pmol of oligo(dT), 15 units of AMV reverse transcriptase (Promega), each dNTP at 0.4 mM, 50 mM Tris/HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol and 0.5 mM spermidine. PCR was performed for 30 cycles with one-tenth of the cDNA mixture, using oligo(dT) and LPH-S5 as amplification primers.

Starting plasmids and site-directed mutagenesis

Constructs were derived from pRLU61 (see Figure 1A) containing the nearly full-length 6.2 kb rat LPH cDNA [8,40]; from pRL5B comprising the rat genomic *Bam*HI fragment overlapping the first exon of the rat LPH gene [41]; and from pSI2 containing the 5′ end of the human sucrase–isomaltase (SI) cDNA [42]. Plasmid pRLE118EI (see Figure 1A) was constructed by inserting the 1.6 kb *Eco*RI fragment of pRL5B into the *Eco*RI site of Bluescript pSK$^-$ (Stratagene, La Jolla, CA, U.S.A.). DNA fragments overlapping the sequence encoding the signal peptide and the transmembrane anchor of the LPH precursor were respectively produced by PCR with plasmid pRLE118EI and the primers LPH-S5/-S3 (121 bp), or using plasmid pRLU61 and the primers LPH-T5/-T3 (274 bp). These fragments were inserted into pGEM (Promega, Madison, WI, U.S.A.) to give pGEM-Sg and pGEM-Tm; in pGEM-Sg and pGEM-Tm they are flanked by restriction sites allowing in-frame ligation with different segments of the LPH cDNA.

To incorporate a *Hin*dIII site at a presumed lactase proenzyme cleavage site (KKVKR/NPL, located between domains II and III of the precursor), the *Bam*HI–*Sal*I fragment of pRLU61 ([8,40], coordinates 2157–3706, accession number X56747) was subcloned into phage M13 and subjected to site-directed mutagenesis by standard procedures [43] with a 30-mer oligonucleotide. The oligonucleotide and polypeptide sequences at the modified site were 5′-dAAGAAAGTAAGCTTAAACCCA-3′ and N-KKVSLNPL-C respectively. The resulting mutated *Bam*HI–*Sal*I fragment was ligated with the downstream *Sal*I–*Eco*RI fragment of lactase cDNA and inserted into pUC19 (Pharmacia, Uppsala, Sweden) to give pLacMut (see Figure 1A).

To introduce a consensus start site for translation initiation at the presumed proenzyme cleavage site, a double-stranded oligonucleotide (5′-dAGCTTCTCGAGGCCACCATGGGC-3′; 5′-dAGCTGCCCATGGTGGCCTCGAGA-3′) was inserted at the unique *Hin*dIII site of pLacMut. In the resulting plasmid, pLacMutOligo, an *Xho*I site is present upstream of the translation initiation consensus (CCACCATGR [44,45]).

Assembly of modified LPH coding sequences and transfer to the eukaryotic expression vector pCR3

pS34T. The *Sal*I–*Hin*dIII fragment of pGEM-Sg was inserted into Bluescript pSK$^-$ to give pSK-Sg; the *Hin*dIII–*Nhe*I fragment of pLacMut was subcloned between the *Hin*dIII and *Xba*I sites of pSK-Sg, giving pSK-S34T. The cDNA fragment was cut with *Not*I, filled in with Klenow DNA polymerase and inserted into the *Eco*RV site of pCR3 (Invitrogen, Leek, The Netherlands) to give pS34T.

pS1234T. The *Sca*I fragment overlapping the 5′ region of the nearly full-length LPH cDNA of pRLU61 was replaced by the *Sca*I fragment of pSK-S34T that comprises the signal-coding sequence of the precursor, to reconstitute the full-length LPH cDNA (pSK-S1234T). The cDNA fragment was cut with *Kpn*I plus *Sma*I and inserted between the *Kpn*I and *Eco*RV sites of pCR3 to give pS1234T.

pS134T. The *Hin*dIII–*Nhe*I fragment of pLacMut was inserted between the *Hin*dIII and *Spe*I sites of Bluescript pSK$^-$, giving pSK-34T. A PCR fragment from pSK-S1234T (primers LPH-S5 and LPH-13) was cut with *Nsi*I and the resulting 148 bp fragment was inserted between the *Eco*RV and *Nsi*I sites of pGEM, generating pGEM-1E. pSK-34T was cut with *Hin*dIII plus *Sph*I, and the fragment was subcloned between the equivalent sites of pGEM-1E. This was then excised with *Nsi*I plus *Bst*BI and transferred to pSK-S1234T to give pSK-S134T. The resulting cDNA fragment was cut with *Spe*I plus *Eco*RI, filled in with Klenow DNA polymerase and inserted into the *Eco*RV site of pCR3 to give pS134T.

pS234T. The PCR product of pRLU61 (primers LPH-25 and LPH-23) was cut with *Hin*dIII and the resulting 1461 bp fragment was inserted into the *Hin*dIII site of pSK-S34T, giving pSK-S234T. The cDNA fragment was excised with *Not*I, filled in with Klenow DNA polymerase and inserted into the *Eco*RV site of pCR3 to give pS234T.

pS123T. The pGEM-Tm *Sph*I–*Sac*I fragment was inserted between the equivalent sites of pSK-34T, giving pSK-3T. The *Xho*I–*Bst*BI fragment of pSK-S1234T was transferred to pSK-3T, generating pSK-S123T. The resulting cDNA fragment was excised with *Not*I, filled in with Klenow DNA polymerase and inserted into the *Eco*RV site of pCR3 to give pS123T.

pS124T. A fragment of pRLU61 was PCR amplified with primers LPH-24A and LPH-24B, cut with *Sph*I and the resulting 387 bp fragment was inserted into the *Sph*I site of pSK-S1234T, giving pSK-S124T. The cDNA fragment was cut with *Kpn*I plus

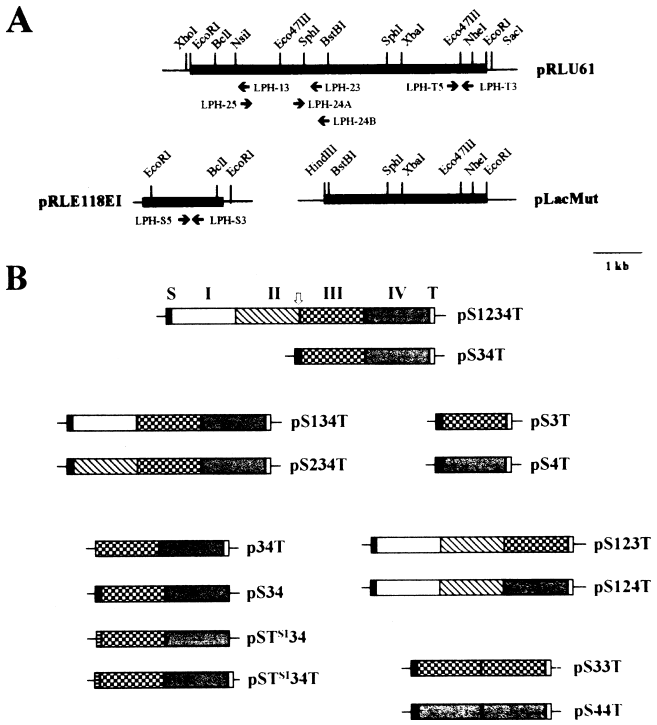

Figure 1 Recombinant LPH plasmids

(**A**) Restriction map of plasmids pRLU61, pRLE118EI and pLacMut. The position and orientation of the synthetic oligonucleotides used for PCR are indicated by horizontal arrows. (**B**) Plasmids prefixed by S incorporate the authentic LPH signal sequence (black, at the left); those suffixed by T include the C-terminal transmembrane domain (white, at the right). Intervening numerals indicate the domains included in the construct (domain I, white; domain II, diagonal hatching; domain III, chequered; domain IV, grey). pS1234T is thus the full-length LPH cDNA (vertical arrow indicates the presumed cleavage site between LPHα and LPHβ). In pST^{SI}34 and pST^{SI}34T the signal sequence of the LPH precursor has been replaced by that from sucrase isomaltase (horizontal hatching, at the left).

*Sma*I and inserted between the *Kpn*I and *Eco*RV sites of pCR3 to give pS124T.

pS3T. The *Sph*I–*Eco*47III fragment of pGEM-Tm was inserted between the equivalent sites of pSK-S34T, giving pSK-S3T. The cDNA fragment was excised with *Not*I, filled in with Klenow DNA polymerase and inserted into the *Eco*RV site of pCR3 to give pS3T.

pS4T. A PCR product from pSK-S34T with primers LPH-S5 and LPH-24B was digested with *Spe*I plus *Sph*I and the resulting 206 bp fragment was inserted between the equivalent sites of pSK-S34T, giving pSK-S4T. The cDNA fragment was excised with *Not*I, filled in with Klenow DNA polymerase and inserted into the *Eco*RV site of pCR3 to give pS4T.

pS33T. The *Spe*I–*Eco*47III fragment of pSK-S3T was inserted between the *Xba*I and *Hin*cII sites of pUC19. The resulting plasmid was cut with *Hin*dIII and the cDNA fragment was inserted in the corresponding site of pSK-S3T to give pSK-S33T. The cDNA fragment was excised with *Not*I, filled in with Klenow DNA polymerase and inserted into the *Eco*RV site of pCR3 to give pS33T.

pS44T. The *Sal*I–*Eco*47III fragment of pSK-S4T was inserted between the *Sal*I and *Pvu*II sites of ppolyIII-I [46]. The *Hin*dIII fragment of the resulting plasmid was inserted into pSK-S4T to give pSK-S44T. The cDNA fragment was excised with *Not*I, filled in with Klenow DNA polymerase and inserted into the *Eco*RV site of pCR3 to give pS44T.

p34T. The *Xho*I–*Eco*RI fragment of pLacMutOligo was subcloned in Bluescript pSK$^-$, then cut with *Kpn*I plus *Nhe*I and the fragment inserted between the *Kpn*I and *Xba*I sites of pCR3 to give p34T.

pS34. Plasmid pS34T was cut with *Eco*47III plus *Eco*RV and re-ligated.

pST^{SI}34T and *pST^{SI}34*. A PCR fragment from pSI2 (primers SI-ST5 and SI-ST3) was cut with *Spe*I plus *Hin*dIII and the 164 bp fragment was inserted between the same sites of pSK-S34T, giving pSK-ST^{SI}34T. This was cut with *Kpn*I plus *Eco*47III and the fragment was inserted between the equivalent sites of pS34T to give pST^{SI}34T. This was cut with *Eco*47III plus *Eco*RV and re-ligated to give pST^{SI}34.

Cell transfection

COS-7 cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco) containing 10 % (v/v) fetal bovine serum (Gibco), 1 % (w/v) penicillin/streptomycin (Gibco) and 0.1 % gentamicin (Schering-Plough, Segré, France). HT29-G$^-$ cells were grown in glucose-free medium [47]. COS-7 cells (2×10^4 cells/cm^2) were transfected with calcium phosphate/DNA co-precipitates [43] of recombinant plasmids (0.4 μg per 10^4 cells); for some experiments, stably transfected cells were selected for growth in the presence of 0.8 mg/ml G418 (geneticin; Gibco). Identical results were obtained in transient and stably transfected cells. HT29-G$^-$ cells (5×10^4 cells/cm^2) were transfected with DOSPER Liposomal Transfection Reagent (5 μl of reagent/2.5 μg of DNA per 3×10^5 cells), as recommended by the supplier (Boehringer).

Lactase activity

Transfected cells grown for 4 days on coverslips were fixed for 15 min with 1 % (w/v) paraformaldehyde, incubated in a humid chamber (2–7 h at 37 °C) with 1 mM 5-bromo-4-chloroindol-3-yl β-D-fucopyranoside (X-Fuc; Sigma, St. Quentin Fallavier, France) or 1 mM 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside (X-Gal; Sigma) in 0.1 M maleate buffer, pH 6.5 or pH 4, containing 0.05 M potassium ferricyanide, 0.05 M potassium ferrocyanide and 0.5 mM *p*-chloromercuribenzenesulphonate (Sigma). Slides were mounted in glycerol/PBS/phenylenediamine for microscopy (Axiophot; Zeiss). The enzymic detection method of lactase was validated on cryosections of rat and mouse intestinal mucosa, confirming that X-Fuc is the optimal substrate of lactase, whereas X-Gal is poorly hydrolysed by this enzyme ([48], and B. Jost and J.-N. Freund, unpublished work). Lactase activity in intact cells was determined as above after fixation [1 % (w/v) glutaraldehyde for 15 min] by measuring the production of glucose from lactose. Lactose (20 mg/ml) was added to cells in 0.1 M maleate buffer, pH 6.5 or pH 4, containing 1 mM *p*-chloromercuribenzenesulphonate. Glucose release was measured by the glucose oxidase method [49].

Immunocytochemistry and confocal microscopy

Transfected cells grown for 4 days on coverslips were fixed [1 % (w/v) paraformaldehyde for 15 min] and in some cases permeabilized [0.25 % (w/v) Triton X-100 in PBS for 10 min]. Indirect immunodetection of rat lactase was performed with monoclonal antibody (mAb) FBB3421 [50] or a rabbit antiserum raised against mature rat lactase purified to homogeneity from intestinal brush border membranes [51]. Primary antibodies were used at a 1:100 dilution as described previously [1,52]. Secondary anti-mouse and anti-rabbit antibodies were labelled with Texas Red (dilution 1:250; Amersham, Les Ullis, France). Slides were

mounted in glycerol/PBS/phenylenediamine for fluorescence microscopy (Axiophot, Zeiss) or confocal microscopy (Leica). For simultaneous detection of lactase activity and antigen, the detection of enzyme activity was performed first *in situ*, followed by immunocytochemistry.

Protein extraction and immunoblotting

Integral membrane proteins were extracted with Triton X-114 [53], separated by SDS/PAGE and electroblotted on nitrocellulose filters [43]. To detect lactase proteins, filters were incubated with the rabbit polyclonal antiserum raised against rat lactase ([51], dilution 1:1500) and then with anti-(rabbit IgG) coupled to horseradish peroxidase (dilution 1:5000; Amersham). Chemiluminescent detection used the Western View kit (Transduction Laboratories, Lexington, KY, U.S.A.).

RESULTS

Expression of full-length lactase precursor; detection by staining with X-Fuc

Plasmid pS1234T encodes the complete rat LPH precursor (Figure 1) under the control of a cytomegalovirus promoter. When introduced into COS-7 cells, this construct generated active lactase enzyme, as previously reported [39]. Surface staining of non-permeabilized cells with anti-LPH mAb confirmed that the enzyme was anchored in the outer plasma membrane (Figure 2). Staining with a usual chromogenic substrate for β-galactosidases such as X-Gal gave substantial background (Figure 2) owing to the activity of endogenous acidic

Table 1 Lactase activity and immunostaining in transfected COS-7 cells

Lactase activity was determined with the chromogenic substrate X-Fuc, whereas immunodetection used mAb FBB3421 or polyclonal antiserum raised against rat lactase. Transfected COS-7 cells were not permeabilized before enzymic and immunological detection. Symbols: +, positive signal; −, absence of enzyme activity or cross-reacting material. Abbreviaiton: n.d., not determined. Enzyme detection and immunostaining were performed at day 4 after transfection. Results were reproduced in six to eight independent transfections.

	Staining with		
Plasmid	X-Fuc	mAb FBB3421	Polyclonal anti-LPH
pS1234T	+	+	+
pS134T	+	+	n.d.
pS234T	+	+	n.d.
pS34T	+	+	+
pS3T	−	+	+
pS4T	−	−	+
pS3T + pS4T	−	+	n.d.
pS33T	−	+	+
pS44T	−	−	+
pS123T	−	+	+
pS124T	−	−	+
pS34	−	−	n.d.
p34T	−	−	n.d.
$pST^{SI}34$	−	−	n.d.
$pST^{SI}34T$	+	+	+

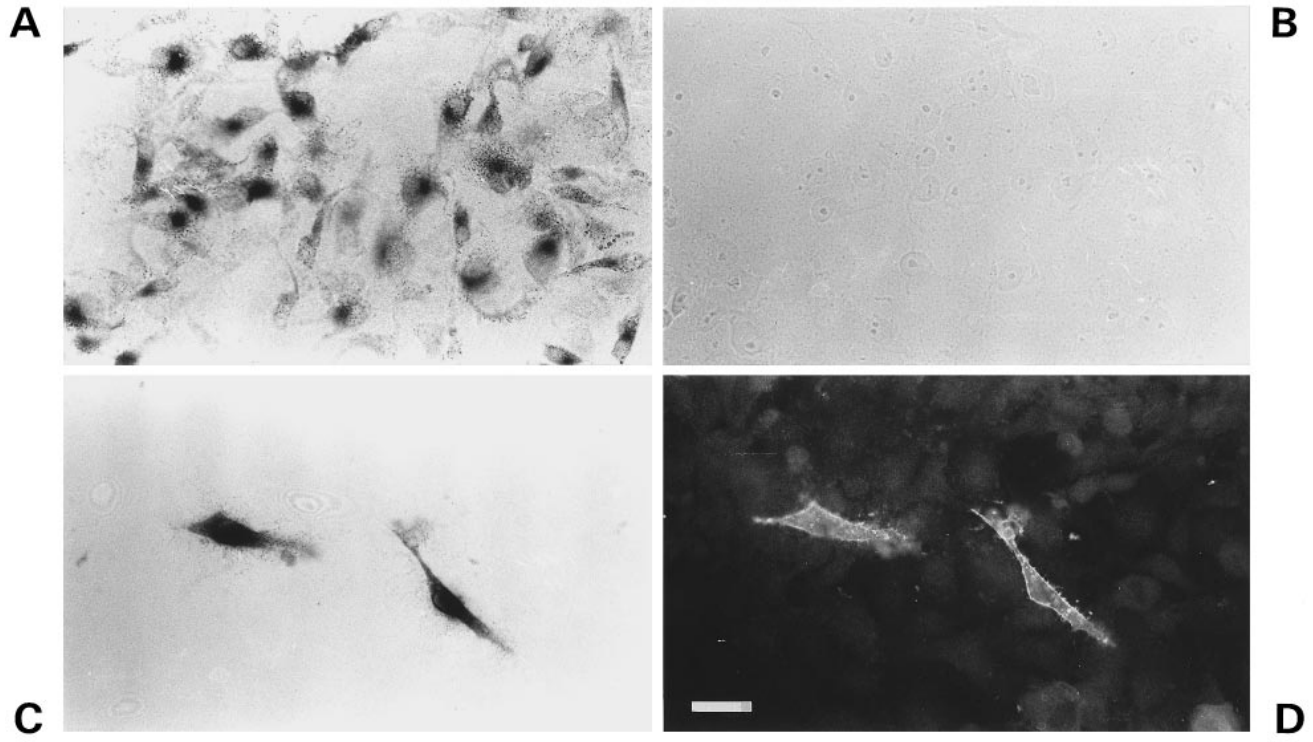

Figure 2 Detection of lactase *in situ* in transfected COS-7 cells

(**A**) Background staining of the lysosomal compartment with the chromogenic substrate X-Gal in control COS-7 cells transfected with pCR3. (**B**) As in (**A**) but stained with the alternative chromogenic substrate X-Fuc. (**C**, **D**) Detection of lactase *in situ* in COS-7 cells transfected with pS1234T, with either X-Fuc as substrate (**C**) or mAb FBB3421 (**D**). Enzymic and protein detection was performed 4 days after transfection. Scale bar, 100 μm.

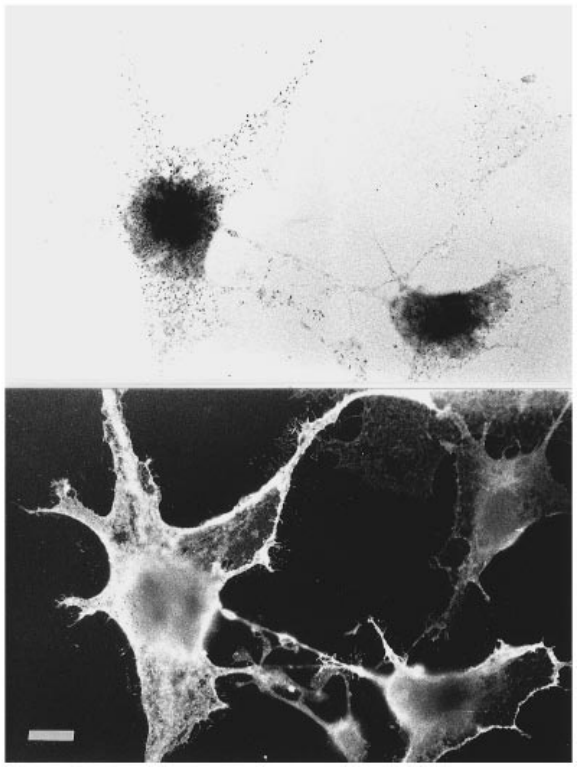

Figure 3 Lactase enzyme in COS-7 cells transfected with pS34T

(**A**) Stained with X-Fuc; (**B**) stained with mAb FBB3421. Enzymic and protein detection was performed 4 days after transfection. Scale bar, 20 μm.

lysosomal β-galactosidase. In contrast, lactase activity was unambiguously revealed by using an alternative chromogenic substrate, X-Fuc, in which the galactose moiety of X-Gal is replaced by fucose. This molecule was not detectably hydrolysed by COS-7 cells transfected with a control plasmid, but was effectively converted by cells transfected with pS1234T (Figure 2).

Rat LPHα is not required for the development of lactase activity

Expression of active LPHβ in human was reported to display an obligate requirement for LPHα [28,31]. To determine whether the rat enzyme has a similar requirement for LPHα, a derivative of the rat precursor was generated (Figure 1) in which the N-terminal signal peptide was inserted in front of LPHβ, whereas domains I and II of LPHα were deleted (plasmid pS34T). Cells transfected with pS34T expressed lactase activity, demonstrated by X-Fuc staining (Table 1; see also Figure 3A). Immunological staining with anti-LPH mAb was observed in non-permeabilized transfected cells, indicating that the recombinant protein was anchored in the outer plasma membrane (Figure 3B). Confocal microscopy of non-permeabilized transfected cells labelled with anti-LPH antibody confirmed the staining of the outer cell membrane (results not shown). In support of membrane association, the 130 kDa LPHβ was also present in Triton X-114 extracts of transfected cells (see Figure 5).

We examined the pH dependence of the recombinant LPHβ through direct activity measurements relying on metabolism of lactose. At pH 6.5, approximately two-thirds of lactase activity expressed by pS34T-transfected cells resulted from recombinant LPHβ. At pH 4, the total lactase activity measured in pS34T-transfected cells was less than at pH 6.5 and was indistinguishable from that obtained in control cells (Table 2). Thus the contribution of recombinant LPHβ to total lactase activity is negligible at acidic pH, in contrast with the situation at neutral pH. Hence isolated LPHβ produced in the transfected cells exhibits typical neutral lactase activity.

Table 2 Lactose hydrolysis by COS-7 cells transfected with pS34T

Glucose release (nmol) from lactose in COS-7 cells transfected with pS34T or with the control plasmid pCR3. Cells grown for 4 days after transfection were fixed and monitored for 2–24 h at pH 6.5, or monitored for 3 h at pH 6.5 or 4. The results are means of three independent experiments.

		Glucose release (nmol)			
Plasmid	Hours of incubation (pH 6.5)...	2	5	8	24
pS34T		14 ± 3	26 ± 4	33 ± 3	39 ± 3
pCR3		4 ± 2	7 ± 2	10 ± 3	11 ± 2
		Glucose release (nmol)			
Plasmid	pH (3 h incubation)...	6.5	4		
pS34T		21 ± 3	15 ± 2		
pCR3		6 ± 2	14 ± 3		

LPHα is also non-essential in an intestinal cell line

Because COS-7 cells are non-polarized, in contrast with intestinal epithelial cells, we were also concerned that a requirement for LPHα might be restricted to specialized secretory cells. Morphological and functional polarization is retained by several colon adenocarcinoma cells in culture, in particular by the HT29-G$^-$ cell line adapted for growth in the absence of glucose [47]; these cells also produce a very low level of endogenous lactase. To determine whether LPHβ is active and correctly transported to the surface of specialized secretory cells, the HT29-G$^-$ cell line was transiently transfected with pS34T and analysed by X-Fuc staining and by immunocytochemistry. Control HT29-G$^-$ cells exhibited a very faint punctate staining with X-Fuc. Transfection with pS34T resulted in groups of cells staining intensely with X-Fuc and labelled with the anti-LPH mAb (Figure 4), indicating that LPHβ, in the absence of LPHα, is transport-competent and enzymically active in polarized intestinal cells cultured *in vitro*.

LPHα accelerates the development of enzyme activity of LPHβ

In previous studies on human lactase, enzyme measurements were performed 48 h after transfection [28,31], whereas our analysis was 4 days after the introduction of DNA. To address the question of whether different results could be ascribed to differences in experimental procedure, COS-7 cells were transiently transfected with pS1234T and pS34T, and analysed by X-Fuc staining and lactase immunodetection over 3 days.

Cells transfected with pS1234T exhibited lactase activity (Table 3) and immunostaining as early as 24 h after transfection (results not shown); the number of positive cells subsequently increased, in parallel with the intensity of X-Fuc staining. In contrast, cells transfected with pS34T displayed obvious immunofluorescent staining 24 h after transfection but failed to express significant

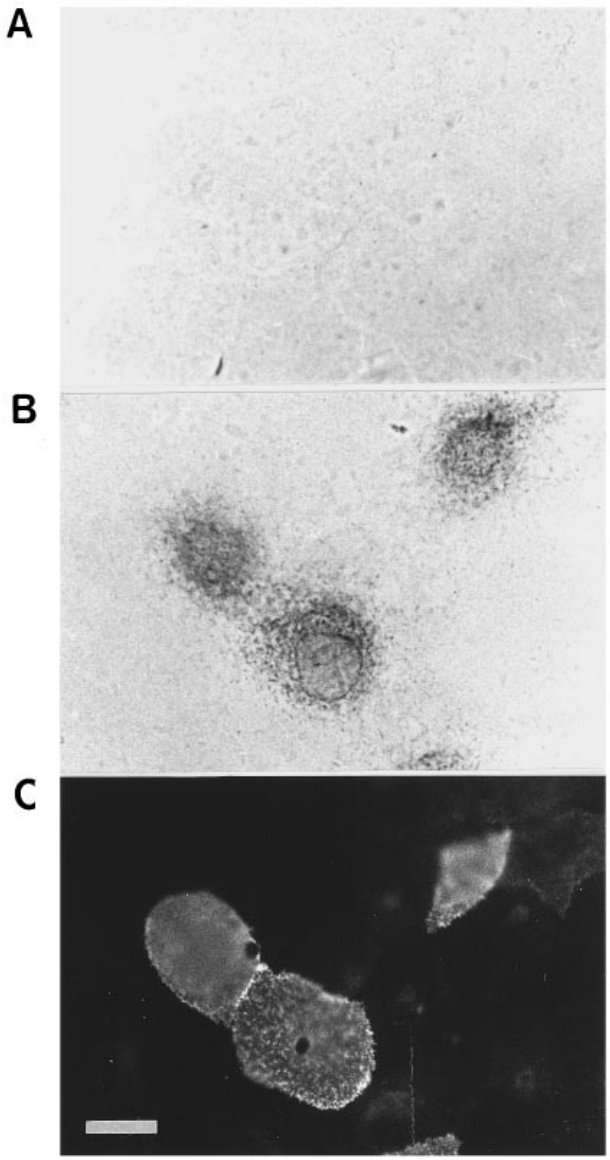

Figure 4 Expression of recombinant LPHβ in intestinal cells

(**A**) Control HT29-G$^-$ cells transfected with pCR3 showed a very faint endogenous staining with X-Fuc, not visible in the photographs. In HT29-G$^-$ cells transfected with pS34T, enzymically active recombinant LPHβ was detected by X-Fuc staining (**B**) and with mAb FBB3421 (**C**). Cells were not permeabilized before immunocytochemistry. Scale bar, 50 μm.

lactase activity until days 2, as determined by X-Fuc staining (Table 3). At 3 days after transfection a majority of pS34T-transfected cells stained with mAB FBB3421 antibody and with X-Fuc, whereas some cells stained intensely with antibody but only weakly with X-Fuc (results not shown). These results suggest that the pro region of the LPH precursor potentiates the expression of mature lactase enzyme at the cell surface.

Domains I and II of LPHα are both required to potentiate LPHβ activity

To address the role of LPHα in the development of rat LPHβ activity, COS-7 cells were transfected with pS134T or pS234T, encoding LPHβ linked to either domain I or domain II of LPHα (Figure 1). In both cases, an enzymically active molecule was

Table 3 Development of lactase activity in COS-7 cells transfected with pS1234T and pS34T

X-Fuc staining (XF) and immunofluorescent staining with mAb FBB3421 (IS) of COS-7 cells transfected with pS1234T or pS34T and analysed 3 days later. The results are expressed as a percentage of the number of labelled cells transfected with pS1234T after 3 days; values are means of three different experiments. Cells were not permeabilized for immunodetection.

	Transfection (%)					
Day after transfection...	1		2		3	
Plasmid	XF	IS	XF	IS	XF	IS
pS1234T	31	30	67	64	100	100
pS34T	0.2	12	0.7	23	35	48

produced (Table 1). The enzyme was located in the outer cell membrane, as assessed by immunostaining of non-permeabilized transfected cells with mAb FBB3421 (Table 1). However, lactase activity only appeared 2-3 days after transfection, as found with cells transfected with pS34T but retarded in comparison with cells that express the full-length LPH precursor (results not shown). This indicates that neither domain I nor II inserted in front of LPHβ can fulfil the function attributed to LPHα in potentiating the expression of mature LPHβ.

Isolated LPHβ subdomains III and IV are functionally inactive

The ability to produce active lactase in COS-7 cells transfected with the plasmid pS34T encoding LPHβ provided an opportunity to investigate the functional substructure of the LPHβ enzyme. Cells were transfected with plasmids pS3T or pS4T, which respectively encode polypeptides representing only domains III or IV of LPHβ, each flanked by the signal sequence and transmembrane region (Figure 1). No lactase activity was detected (Table 1); pS3T and pS4T also failed to produce active lactase in HT29-G$^-$ cells (results not shown).

To rule out the possibility that lack of enzyme activity might have resulted from a defect in transcription, RNA from transfected cells was subjected to reverse transcriptase–PCR analysis with LPH-S5 and oligo-dT as amplification primers. PCR fragments of the expected size were obtained in each case, indicating that the plasmids pS3T and pS4T were transcribed (results not shown).

To exclude the possibility that the absence of lactase activity might be due to a failure of transport to the cell surface, transfected cells were examined for LPH antigen (Table 1). Molecules reacting with the polyclonal anti-LPH antiserum were detected at the surface of cells transfected with pS3T and pS4T, indicating that the recombinant proteins corresponding to isolated domain III and domain IV are synthesized and transported to the cell membrane. When immunodetection was performed with mAb FBB3421, only the pS3T-transfected cells were labelled, suggesting that the epitope is localized exclusively in domain III. Membrane proteins from COS-7 cells transfected with pS3T, pS4T or pS34T were also extracted with Triton X-114 and subjected to Western blotting with the polyclonal anti-LPH antiserum (Figure 5). This revealed LPH antigen at approx. 70 kDa in cells transfected with either pS3T or pS4T, and a protein band at 130 kDa in pS34T-transfected cells. No specific labelling was observed in the cytosol. These molecular masses are slightly higher than predicted from the cDNA species (respectively 120, 64 and 65 kDa), consistent with glycosylation.

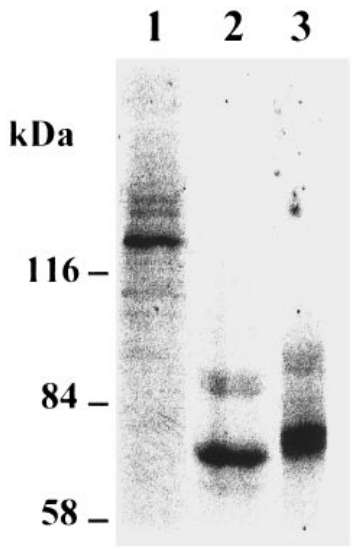

Figure 5 Western blot analysis of membrane protein extracts of COS-7 cells transfected with pS3T, pS4T and pS34T

COS-7 cells transfected with plasmids pS34T (lane 1), pS3T (lane 2) or pS4T (lane 3) were extracted with Triton X-114. Proteins were resolved by SDS/PAGE and immunodetected with rabbit polyclonal anti-(rat LPH) antiserum.

To determine whether lactase activity could be reconstituted from independently translated polypeptide chains representing domains III and IV, COS-7 cells were co-transfected with a mixture of pS3T and pS4T. No lactase activity was detected (Table 1).

Tandem duplication of domain III or IV does not restore lactase activity

Plasmids pS33T and pS44T, which encode proteins comprising tandem repeats of either domain III or IV, were constructed to test whether lactase activity was dependent on the juxtaposition of two T1GH-like domains (Figure 1). As shown in Table 1, no staining was observed with X-Fuc in pS33T-transfected and pS44T-transfected COS-7 cells, demonstrating that these plasmids did not produce active lactase. Immunostaining was indistinguishable from that obtained with pS3T and pS4T, indicating that recombinant proteins corresponding to tandem duplications of domain III or domain IV are synthesized and transported to the cell membrane, but in inactive form.

LPHα does not rescue the deficit of isolated LPHβ domains III and IV

We then tested whether domains III or IV, co-translated with LPHα, might generate active lactase. For this purpose COS-7 cells were transfected with plasmids pS123T or pS124T (Figure 1). No lactase activity was detected by X-Fuc staining, although immunoreaction with the monoclonal and/or polyclonal antibodies demonstrated the presence of recombinant proteins at the cell surface (Table 1).

Both a signal sequence and the C-terminal transmembrane domain are required to produce transport-competent LPHβ

COS-7 cells were transfected with plasmids p34T and pS34, in which the sequences coding respectively for the transmembrane anchor and for the signal peptide had been deleted. Neither lactase activity nor LPH antigen was detectable at the surface of these cells (Table 1).

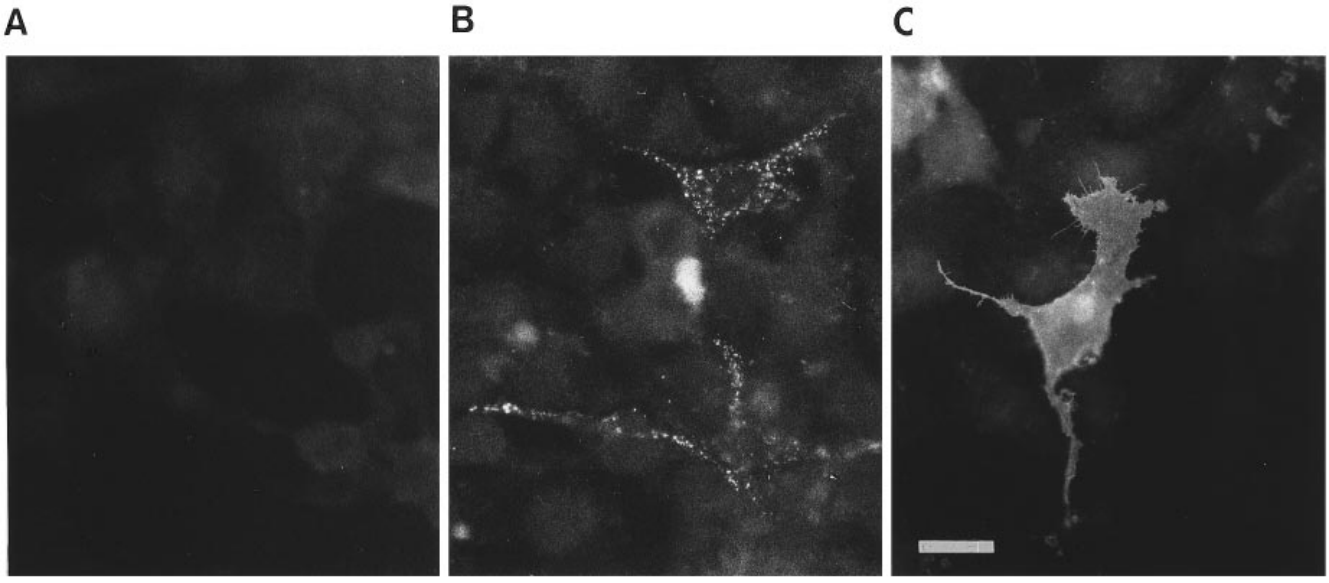

Figure 6 Lactase antigen immunodetection in COS-7 cells transfected with pST^{SI}34 and pST^{SI}34T

COS-7 cells transfected with pST^{SI}34 were not permeabilized (**A**) or permeabilized (**B**) with Triton X-100 before immunodetection with mAb FBB3421; (**C**) non-permeabilized COS-7 cells transfected with pST^{SI}34T showed surface staining with mAb FBB3421. Scale bar, 50 μm.

The wild-type LPH precursor and recombinant LPHβ constructed in this study are type I membrane-associated proteins anchored in an N_{out}–C_{in} orientation. Conversely, in another intestinal carbohydrate-degrading enzyme, SI, the N-terminal signal sequence acts both to target the polypeptide to the secretory pathway and to tether the mature polypeptide at the cell surface in a type II (C_{out}–N_{in}) orientation [54]. To determine whether the SI signal/transmembrane region could direct active LPHβ to the cell surface, a plasmid was constructed to encode a chimaeric protein in which domains III and IV of LPHβ were linked downstream of the human SI signal peptide (plasmid $pST^{SI}34$; see Figure 1). No staining with X-Fuc was detected in COS-7 cells transfected with $pST^{SI}34$ (Table 1). As shown in Figure 6, immunodetection with mAb FB3421 indicated that lactase antigen was absent from the outer membrane of non-permeabilized cells. However, a punctate staining was observed in permeabilized cells, suggesting that the chimaeric protein is sequestered intracellularly in an inactive form.

We then constructed plasmid $pST^{SI}34T$, in which the C-terminal region of the LPH precursor, including the transmembrane anchor, was added downstream of the chimaeric recombinant protein (Figure 1). COS-7 cells transfected with $pST^{SI}34T$ exhibited both lactase activity and antigen at the cell surface (Table 1; see also Figure 6), demonstrating that the C-terminal region of the LPH precursor contributes to the production of transport-competent enzyme.

DISCUSSION

The intestinal LPH precursor is composed of four tandem T1GH-like domains, most probably generated by two successive rounds of duplication of a single ancestral domain. To define further the contribution of each domain to overall enzyme function, we have studied the properties of LPH derivatives in which the different domains are expressed separately or in combination.

Role of LPHα

We report that the production and sorting of the mature active rat enzyme (LPHβ) does not display an obligate requirement for the pro segment (LPHα) of the precursor. This contrasts with the reported obligate requirement for LPHα in the maturation of the human enzyme [28,31]. However, the role attributed to the human pro region has recently been re-evaluated [54]. In the present study we show that LPHα accelerates the transport of the mature enzyme to the outer cell membrane, consistent with the chaperone-like function attributed to LPHα [28,31]. Furthermore the tandem association of domains I and II is required for the LPHα function, suggestive of a role in folding or in the acquisition of the quaternary structure of the mature enzyme. In rat and rabbit, unlike human, LPHα is subject to internal cleavage during precursor maturation [4,5,29,30]. The functional role (if any) of this cleavage is unknown, but might be related to the different pattern of lactase expression observed along the villus axis in human compared with rat and rabbit; indeed some enterocytes in hypolactasic humans contain LPH mRNA but no protein, whereas other enterocytes express an inactive protein [55–57]. Similar observations have not been made in rat or rabbit [56].

Role of LPHβ subdomains

In the present study we show that the production of active lactase is critically dependent on a tight association of domains III and IV. Indeed neither domain III or IV alone, nor the duplication of these domains, nor the co-expression of the domains III and IV as separate polypeptides, is sufficient to generate enzyme activity, although the proteins are synthesized and transported to the outer cell membrane. These results suggest that domains III and IV have distinct functions within LPHβ. In the light of inhibition studies conducted with a covalent inhibitor interacting preferentially with a nucleophilic Glu residue located within domain III [16], and of site-directed mutagenesis experiments that designated the Glu residue located with domain IV as part of the active site, instead of that present in domain III [39], we propose a functional model in which both domains III and IV co-operate to generate active LPHβ. This model is based on the concept of substrate channelling, which has already been suggested for several multifunctional enzymic complexes (reviewed in [58]). We suggest that domain III contains the entry point of lactose in the enzymic complex and that lactose is subsequently directed for catalysis to domain IV. It should be mentioned that domain II of LPHα, comprising a typical T1GH-like domain but one in which the nucleophilic Glu residue is replaced by Gly, does not complement domain IV (nor domain III) for the production of enzyme activity. This suggests that sequence differences between domains II and III, in particular at the level of the nucleophilic Glu residue absent from domain II, might be essential for entry/transfer of substrate to domain IV.

Role of signal and transmembrane regions

Mature LPHβ is anchored in the outer cell membrane in the N_{out}–C_{in} orientation via a hydrophobic C-terminal extension [16,18]. With the aim of investigating the production of active recombinant lactase anchored in the reverse orientation in the membrane (N_{in}–C_{out}), we replaced the signal peptide and transmembrane anchor of LPHβ with the N-terminal segment of SI, which has the dual role of both signal and membrane anchor in this disaccharidase [59]. The resulting $ST^{SI}34$ chimaeric protein was sequestered intracellularly in inactive form; however, lactase activity and targeting to the cell membrane were restored when the authentic anchor of LPHβ was added downstream of the chimaeric protein. This indicates that the signal peptide of SI can substitute functionally for the signal segment of the LPH precursor, although it cannot fulfil the role of the C-terminal segment of LPH. Consistent with these results, LPHβ dimerization has been shown to be essential for transport to the cell membrane, whereas the C-terminal region, including the transmembrane anchor, is required for dimerization [60]. Alternatively, the failure of the SI signal/anchor region to direct the chimaeric enzyme to the cell membrane suggests that additional segments, located elsewhere within SI, might be required for sorting and targeting of this particular enzyme. This is supported by recent results on congenital SI deficiency [61].

The present study demonstrates the existence of multiple functional interactions between various regions of the LPH precursor. They also emphasize that the tandem T1GH-like domains, resulting from two cycles of duplication, now exhibit distinct and specific properties involved in the processing of the mature enzyme, substrate channelling and catalysis. It is unclear whether the different roles ascribed to domains I + II, III and IV respectively were present in the ancestral T1GH domain, or whether some activities have evolved afresh since duplication. Comparison of the structural and functional organization of the T1GH-like domains of lactase and other members of the T1GH family [14] will provide further insights into both the mechanism of the reaction catalysed by, and the phylogeny of, this evolutionarily ancient family of enzymes.

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