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The pro sequence of lactase-phlorizin hydrolase is required for the enzyme to reach the plasma membrane

An intramolecular chaperone?

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Various cDNAs coding for part or all of human pre-pro lactase-phlorizin hydrolase (pre-proLPH) were transfected into COS cells and the subcellular location of the lactase-related proteins assessed. Only the complete proLPH reached the plasma membrane. LPH without the pro sequence, and a construct containing the pro sequence and the lactase domain of mature LPH, accumulated intracellularly; the pro sequence with no mature domain was secreted. We conclude that the pro sequence is important for LPH to be transported to the cell surface.

Sorting; Targeting; Pro sequence; Lactase-phlorizin hydrolase; Plasma membrane; Proteolytic processing

1. INTRODUCTION

The primary translation products of most secretory, lysosomal and some plasma membrane proteins undergo, in addition to the action of signal protease (if the case), more or less extensive proteolytic processing. The biological function of the pro sequences thereby split away is often not known.

The case of the lactase (LPH, lactase-phlorizin hydrolase, EC 3.2.1. 23–26) of the intestinal brush border membrane is rather special. In the sequence of its primary translation product (pre-proLPH, 1,927 amino acid residues long in human [1]), one finds at the NH_2-terminus a typical signal sequence, which, as predicted [1], is split after pos. 19 [2]; the COOH-terminal end encompasses the hydrophobic transmembrane anchor and a hydrophilic cytosolic segment. In between, four homologous regions (I to IV) can be recognized; only the two distal ones appear in mature brush border LPH and have lactase and phlorizin hydrolase activity, respectively [3]. The amino acid sequence between pos. 20 and 868 (the NH_2-terminus of mature brush border LPH is pos. 869) does not appear in the brush borders (save for the proLPH escaping intracellular proteolytic processing; see below). Mature LPH therefore comprises only some 60% of the sequence of its primary translation product. Lactase is perhaps the only (or one of the very few) intrinsic membrane protein which undergoes such extensive proteolytic processing. Also, it is rare, if not unique, that the 'discarded' pro sequence should be homologous to the mature protein – as mentioned above, only regions III and IV appear in mature LPH; the homologous regions I and II belong to the pro sequence, and do not reach the plasma membrane.

Does this long pro sequence have any function? An obvious possibility would be that region I and/or II has an enzymatic activity similar to that of the homologous regions III and IV in mature LPH. However, unprocessed proLPH expressed in COS cells has the same molar specific lactase and phlorizin hydrolase activity as mature LPH [4]; also, a key amino acid residue of the active sites of lactase and phlorizin hydrolase (a Glu) is missing in the corresponding positions of regions I and II [3]. It is therefore most unlikely that regions I and II, i.e. the pro sequence, have glycosidase activity.

In the present investigation we have explored the possibility that the pro sequence of proLPH may be necessary for LPH to reach the plasma membrane. We have expressed constructs encompassing various parts of human pre-proLPH in COS cells, and found that the pro sequence is in fact required for the protein to reach the plasma membrane.

2. MATERIALS AND METHODS

2.1. *Construction of plasmids*

Various segments of LPH cDNA, derived from plasmids pHlac61 and pHlac5 [1] were cloned into the vector pSCT-Gal-X556 [5] downstream of the cytomegalovirus promoter, between *Hin*dIII and *Pvu*II sites: (i) For pSCT-pre-proLPH (Fig. 1a) *Hin*dIII/*Sac*I (0/697) from pHlac61 and *Sac*I/*Eco* RI blunted (697/6286) from pHlac5 were used.

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Dedicated to Prof. S. Auricchio on the occasion of his 60th birthday.

(ii) For pSCT-pre-III-IV (Fig. 1b), first *Hin*dIII/*Ava*II (blunted) (0/128) from pHlac61 and *Xmn*I/*Nco*I (2547/3052) were subcloned into *Hin*dIII and *Nco*I-cleaved pSCT-Gal-X556; the *Hin*dIII/*Nco*I fragment isolated from this plasmid and *Nco*I/*Eco*RI blunted (3052/6286) from pHlac5 were then cloned into pSCT-Gal-X556 as before. (iii) pSCT-pre-pro-III (Fig. 1c) was constructed with *Hin*dIII/*Sac*I from pHlac61 and *Sac*I/*Cla*I blunted (697/4441) from pHlac5. (iv) pSCT-pre-pro (Fig. 1d) contains *Hin*dIII/*Sac*I from pHlac61 and *Sac*I/*Pvu*II (697/2885).

2.2. *Transfection of COS cells*

COS-7 cells were cultured in Dulbecco's modified Eagles medium (Gibco) with 10% fetal calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin (= complete medium). For transfection, cells in 6-well plastic dishes at 20–50% confluence were washed twice with 1 ml Opti-MEM (Gibco), and treated with 1 ml Opti-MEM containing 1.5 μg plasmid DNA plus 6 μl Lipofectin (Gibco). After 6 h at 37°C 1 ml complete medium was added and cells were grown for another 48–72 h. Cells were labelled by incubating in methionine-free complete medium (with dialyzed fetal calf serum) for 0.5–1 h followed by 1 ml of the same medium with 35 to 50 μCi [^{35}S]methionine for 3–5 h; in some cases a 'chase' of 18 h was performed in complete medium supplemented with 2.5 mM methionine.

2.2. *Antiserum against the pro region of human proLPH*

A segment of cloned cDNA [1] coding for amino acids 267–361 of human proLPH was amplified by PCR using primers furnished with a *Bam*HI site for the plus strand and an *Eco*RI site for the minus strand, and cloned into the pGEX-2T expression vector (Pharmacia) so that the protein was expressed as a fusion with the glutathione-*S*-transferase of *Schistosoma japonicum*. Fusion protein produced in *E. coli*, purified by SDS-PAGE and electroelution, was used for immunization of rabbits (125 μg for the first immunization, with Freund's complete adjuvant, 63 μg with incomplete adjuvant for each of 2 subsequent injections).

2.3. *Immunoisolation and immunofluorescence*

Detergent extracts were prepared as described previously for mucosal explants [6], except that the protease inhibitor mix contained 5 mM EDTA, 150 μg/ml PMSF, 2 μg/ml pepstatin A, 2 μg/ml aprotinin, 10 μg/ml leupeptin, 40 μg/ml benzamidine, 2 μg/ml bestatin, 3 μg/ml E-64, and 400 μg/ml *o*-phenanthroline. LPH and its derivatives were isolated [6] with the antiserum described above or with a monoclonal antibody against mature LPH [7] and the products were separated on 6% acrylamide gels according to Laemmli [8] and detected by fluorography.

For immunofluorescence, transfected COS-7 cells grown on coverslips were fixed for 30 min with 3% paraformaldehyde, treated for 15 min with 0.1 M glycine in PBS, and permeabilized or not for 5 min with 0.25% Triton X-100 in PBS. LPH or its derivatives were detected with the monoclonal anti-human LPH antibody and FITC-conjugated goat anti-mouse secondary antibody (1:100, Tago AG).

3. RESULTS

3.1. *Production of LPH derivatives in COS cells*

The complete pre-proLPH cDNA was cloned downstream from the cytomegalovirus promoter in the expression vector pSCT-Gal-X556 (Fig. 1A) [5]. When this construct was introduced into COS cells and the cells labelled with [^{35}S]methionine for 3.5 h beginning 60 h after infection, a protein with apparent molecular weight 215,000 could be immunoisolated with either monoclonal antibody against mature LPH (Fig. 2, lane 1) or with antiserum against a segment of the pro region (lane 5). Thus proLPH was not proteolytically processed (at least not completely), as has been previously reported [4]. Similarly, pSCT-pre-pro-III, coding for the pre-pro region plus region III (Fig. 1b), could be detected with both antibodies (Fig. 2, lanes 4 and 6). The observed molecular weight of 180,000 is larger than the 165,000 expected for the polypeptide chain alone, suggesting that the protein is glycosylated and that proteolytic processing is minimal. With plasmid pSCT-pre-III-IV, coding for the signal sequence plus mature LPH (Fig. 1c), a polypeptide with apparent molecular weight of 95,000 was seen (Fig. 2, lane 3), considerably smaller than the 121,000 predicted for the polypeptide chain alone. The pre-pro segment alone (Fig. 1d), without the mature LPH domains, was as expected not bound by the monoclonal antibody against mature LPH (Fig. 2, lane 2), but was readily detectable with the antiserum against the pro region (lane 6). After a 'chase' of 18 h, the product could be recovered from the medium (lane 8), showing that it is secreted. None of the other LPH-related polypeptides was found in the medium (not shown).

3.2. *Localization of LPH-related proteins*

COS-7 cells were transfected with pSCT-pre-pro-LPH, pSCT-pre-III-IV, and pSCT-pre-pro-III. After 48 h the cells were fixed and either permeabilized with Triton X-100 (+Tx) or not permeabilized (−Tx) (see Fig. 3), and indirect immunofluorescence was used to assess whether the LPH derivatives reached the cell surface.

The results (Fig. 3) can be summarized as follows: (i) the expression of pre-proLPH leads to the appearance of immunoreactive lactase in the plasma membrane – presumably proLPH [4]; (ii) transfection with pSCT-pre-III-IV, coding for the signal sequence and all of mature LPH but essentially no pro sequence, leads to the *intracellular* expression of a lactase-related polypeptide which is *not* transferred to the plasma membrane. Cotransfection of pSCT-pre-pro with pSCT-pre-III-IV did not 'rescue' transport to the plasma membrane (not shown). (iii) The expression of pre-pro-III (i.e. of a construct encompassing the signal sequence plus regions I, II and III) leads again to the solely *intracellular* expression of lactase-related polypeptide.

4. DISCUSSION

Of the various LPH derivatives tested, only the complete proLPH is transferred to the plasma membrane. The (hydrophilic) pro sequence is secreted into the medium, whereas pro sequence-region III remains in the cells. Whereas the secretion of pro sequence into the medium comes as no surprise, it is not clear why the equally hydrophilic pro sequence-region III is not also secreted. Mature, membrane-anchored LPH, presumably arising from the construct pre-III-IV, does not reach the cell surface.

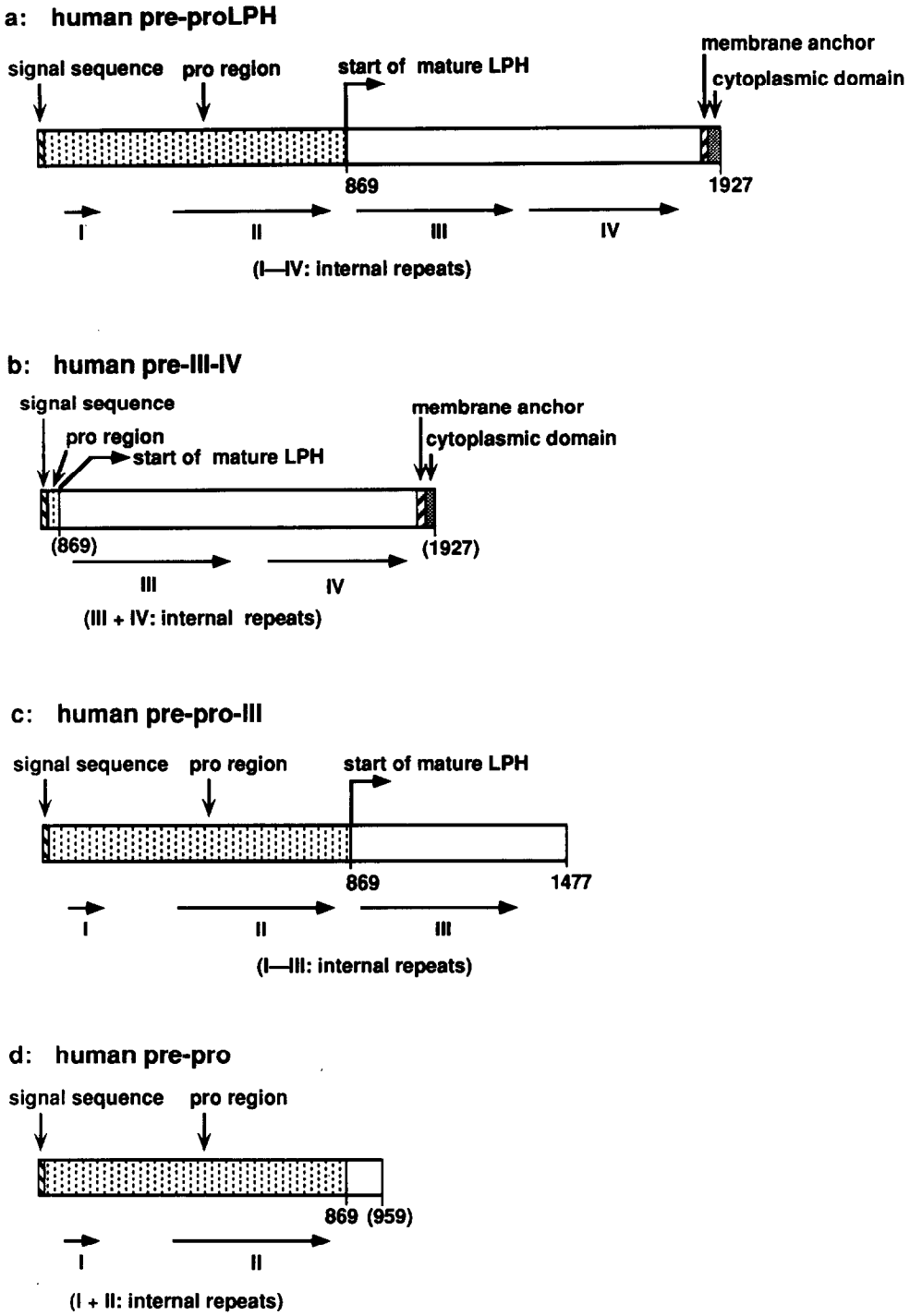

Fig. 1. Human pre-proLPH and derivatives expressed in COS cells. In (a), various domains of the complete polypeptide are labelled, and the horizontal arrows at the bottom indicate four homologous regions within the sequence. In pre-III-IV (b) the pro region is almost entirely deleted. In pre-pro-III (c) the polypeptide is truncated at position 1,477, near the beginning of region IV, and in pre-pro (d) the sequence ends at position 959, deleting almost all of the mature LPH sequence.

Although we do not know in which intracellular compartment or compartments the pre-III-IV or pre-pro-III constructs are retained or transferred to (lysosomes?), it is clear that the pro sequence is needed for LPH to reach the plasma membrane. At least the following mechanisms can be envisaged, alone or in combination:

(i) The pro region in proLPH may carry a target, or allow a target to be exposed, that is required for proLPH to be transferred to the TGN and later compartments (in explants of small-intestine the processing of proLPH to LPH takes place in the TGN [6]).

(ii) The pro region may protect the mature LPH do-

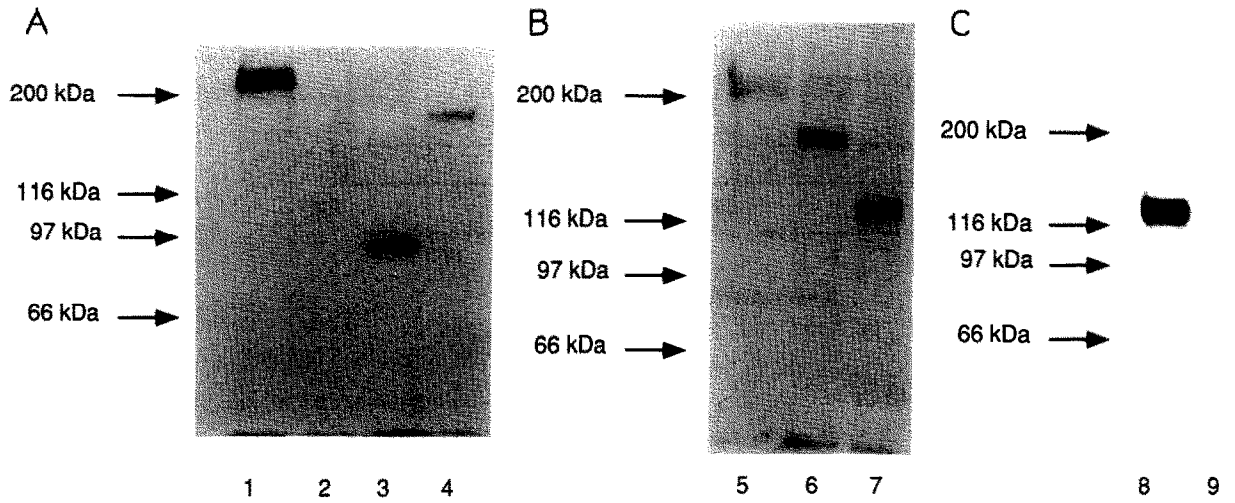

Fig. 2. Immunoisolation of LPH-related polypeptides produced in transiently transfected COS cells. Plasmids coding for the polypeptides shown in Fig. 1 were introduced into COS cells and the cultures labelled with [^{35}S]methionine beginning at 60 h after transfection. LPH derivatives were immunoisolated either from cell homogenates prepared immediately after labelling (A and B) or from the culture medium after an 18 h 'chase' (C). Either monoclonal antibody against mature LPH (A) or antiserum against the pro region (B,C) was used. Lanes 1 and 5, pre-proLPH; lanes 2, 7, and 8, pre-pro; lane 3, pre-III-IV; lanes 4 and 6, pre-pro-III; lane 9, untransfected COS cells.

main from proteolytic degradation. The two constructs that do not reach the cell surface are those occurring as polypeptides with incomplete mature LPH domains – pre-pro-III by design, and pre-III-IV through degradation. If degradation of pre-III-IV is the primary event, and not simply a consequence of the polypeptide not being transported properly, then an important function of the pro sequence may be to stabilize the mature LPH against protease action. This could occur, for example, by hindering the access of protease to a sensitive bond or by inducing a glycosylation event with a similar effect. The pro sequence might be required only transiently for initiation of proper folding and stabilization of mature LPH. Such a 'chaperone-like' role has been suggested for the pro sequence of subtilisin [9,10] and of Kex2p (D. Germain, D.Y. Thomas, and G. Boileau, in preparation).

(iii) The pro sequence in proLPH may be needed for it to proceed past the ER, e.g. by forming an intramolecular 'quasi homodimer'. It is a well established notion that most proteins synthesized in the ER have to form homo- or heterooligomers in order to leave this compartment [11,12]. Proteins easily form homooligomers, and theoretical considerations suggest particularly homodimers may be favored [13,14] (Repeated experiments in our Department reproducibly show that a right hand can shake another right hand more easily than a left hand). Clearly, the *intra*molecular formation of 'quasi-(homo)oligomers' (i.e. the interaction between two or more similar or identical domains belonging to a single polypeptide chain) is kinetically more favorable than the formation of complexes of separate polypeptide chains. We suggest that these *intra*molecular 'quasi-(homo)oligomers' do not have to further interact with like or unlike polypeptides to proceed past the ER. It is thus possible that this is the biological raison d'être of the pro region in proLPH, and explains why many of the brush border hydrolases are synthesized as double, or even four-fold, proteins. These include, in addition to proLPH, sucrase-isomaltase [15,16], angiotensin converting enzyme [17,18], the AdRab-B esterase/phospholipase [19], and probably glucoamylase [15,20,21].

There is some experimental evidence that at least some of these double proteins do not form quaternary homo- or heterooligomers in the ER or the Golgi, namely for proLPH [22], for pro-sucrase-isomaltase (in Caco-2 cells, [23]), and angiotensin converting enzyme [18]. In contrast, 'simple' proteins, such as aminopeptidase N (in intestinal explants [22]) or dipeptidyl peptidase IV (in Caco-2 cells [23]) seem to form homodimers at an early stage of their biosynthesis. However, even 'double' proteins such as LPH [22], sucrase-isomaltase [24] and glucoamylase [25] *do* form homodimers at a later stage, shortly before or after they reach the brush border membrane. This may make them less susceptible to degradation by pancreatic proteases.

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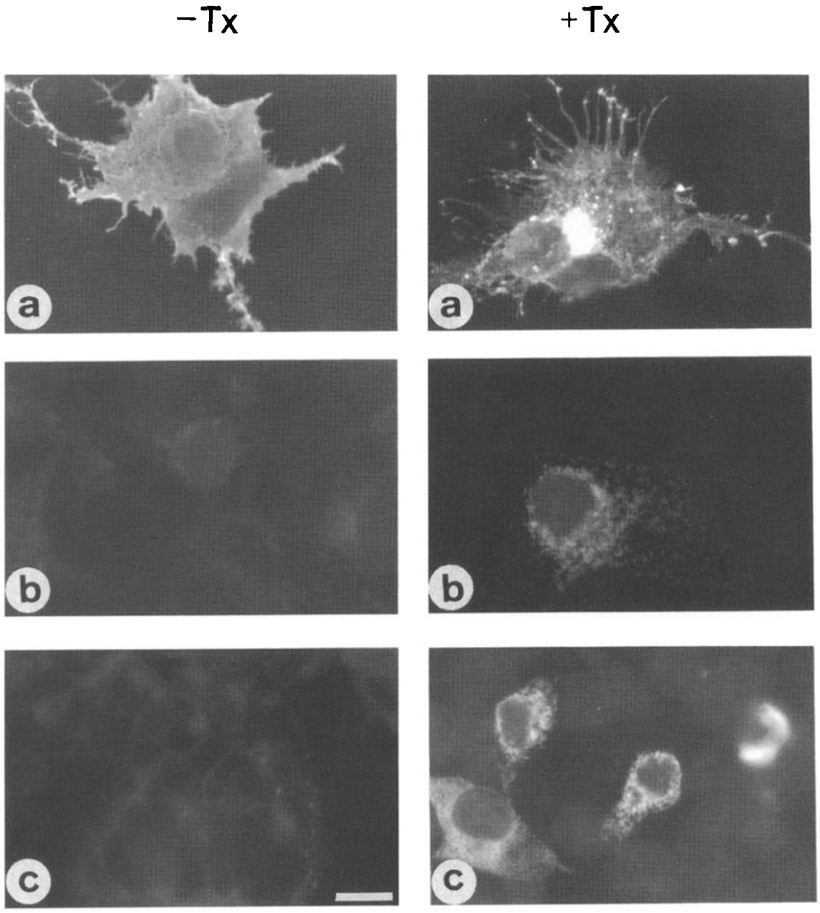

Fig. 3. Surface and intracellular expression of LPH derivatives. Plasmids coding for (a) pre-proLPH, (b) pre-III-IV, or (c) pre-pro-III were introduced into COS-7 cells. At 48 h, cells were fixed and either permeabilized with Triton X-100 (+Tx) or not permeabilized (−Tx). LPH-related polypeptides were detected by indirect immunofluorescence using the monoclonal antibody against mature LPH. Bar = 20 μm.

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NOTE ADDED IN PROOF

Very recently Liang et al. (1993) have shown that a genetically engineered 'double' protein does form intramolecular homodimers substantially faster than identical, but separated, subunits do. (Liang, H., Sandberg, W.S. and Terwilliger, T.C. (1993) Proc. Natl. Acad. Sci. USA 90, 7010–7014.)