Disposition of the carboxy-terminus tail of rabbit lactase-phlorizin hydrolase elucidated by phosphorylation with protein kinase A in vitro and in tissue culture

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Abstract The intracellular disposition of the carboxy-terminus tail of rabbit lactase-phlorizin hydrolase (LPH) is demonstrated, using a specific phosphorylation of Ser^{1916} by protein kinase A (PKA). This phosphorylation is shown to occur not only in vitro (with pure LPH and pure catalytic subunit of PKA), but also in an organ culture of the small intestine. Cholera toxin, which is known to act in vivo on the membranes of the small intestine, with severe clinical consequences, and to elevate the intracellular cyclic AMP of enterocytes, is shown to enhance significantly the phosphorylation of LPH in intact cells grown as an organ culture. These findings establish the cytosolic orientation of the carboxy-terminus tail of LPH in situ, and raise the possibility that the tail itself and its phosphorylation by PKA may have a physiological or physiopathological significance.

Key words: Lactase-phlorizin hydrolase; Cyclic AMP-dependent protein kinase; Cholera toxin; Protein phosphorylation; Small intestine

1. Introduction

Lactase-phlorizin hydrolase (LPH, EC 3.2.1.23–62) from the small intestine is a single polypeptide chain with an apparent molecular weight of 130–160 kDa, depending on the species from which it is isolated. It can be solubilized from brush border membranes either by a proteolytic cleavage [1–3], or by a detergent treatment [4–6]. The sequence of the LPH precursor [7,8] has revealed (i) a signal sequence (predicted by Mantei et al. [7]) which was shown to be split off by cleavage at Gly^{19}-Ser^{20} during maturation [9]; (ii) a hydrophilic stretch of about 1850 amino acids, composed of four homologous regions, two of which are still present in mature LPH; (iii) a hydrophobic stretch in the carboxy-terminus moiety of the molecule, which serves as a membrane anchor [10]; and (iv) a short hydrophilic tail of about 25 amino acids.

Being hydrophilic, this tail could be either cytosolic or extra cellular, endowing LPH with either an N_{out}–C_{in} or an N_{out}–C_{out} positioning in the membrane. Using a specific phosphorylation with protein kinase A (PKA) and working with pure enzymes (in vitro) and with organ cultures (in situ), we provide here direct evidence demonstrating the intracellular disposition of this carboxy-terminus tail in intact cells, and set the stage for the elucidation of its possible physiological assignment.

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Abbreviations: LPH, lactase-phlorizin hydrolase; PKA, cyclic AMP-dependent protein kinase; C, catalytic subunit of PKA; CT, cholera toxin; PKI, the Walsh-Krebs specific PKA inhibitor; PKI(5–24), a synthetic peptide with a sequence corresponding to positions 5–24 in PKI; IBMX, 3-isobutyl-1-methylxanthine.

2. Materials and methods

2.1. Materials

The specific PKA inhibitor PKI(5–24) was from Peninsula Laboratories (Belmont, Ca, USA). [γ-^{32}P]ATP and $^{32}P_i$ were from Amersham, CT and IBMX from Sigma, phosphate-free RPMI-1640 medium from Gibco, and protein A-Sepharose CL4B from Pharmacia. All other reagents were of the highest available purity and were obtained from Fluka, Buchs, Switzerland.

2.2. Purification of LPH

Rabbit LPH (from the small intestine of adult New Zealand White rabbits) and human LPH (from a healthy organ donor) were prepared by immunoaffinity chromatography, as described previously [10]. For the human LPH, the purification was carried out with the monoclonal antibody HBB/1/909/34/74 described by Hauri et al. [11]. The homogeneity of the preparations was ascertained by SDS-PAGE (see below), where both enzymes yielded a single band of expected size.

2.3. SDS-PAGE

Electrophoresis was performed using linear (5%), or gradient (4–10%) polyacrylamide gels [12].

2.4. In vitro phosphorylation of LPH

Phosphorylation was carried out at 30°C in a mixture containing final concentrations of 50 mM HEPES (pH 7.5), 400 μM magnesium acetate, 0.05% Triton X-100, 50 μg/ml LPH, 50 U/ml of pure catalytic subunit (C) of PKA (prepared according to Reimann and Beham, [13]) and 10 μM ATP. Where indicated, PKI was added at a final concentration of 20 μg/ml. Radiolabeling was carried out with [γ-^{32}P]ATP (final concentration 50 μCi/ml).

2.5. Determination of the phosphorylation stoichiometry

Rabbit LPH was phosphorylated as described above. At different times, aliquots were taken and the reaction was stopped by adding 5× Laemmli sample buffer and boiling for 2 min. Samples were analyzed by SDS-PAGE (4–10% gradient gels), quantitative protein staining and autoradiography. To determine the specific radioactivity of the [γ-^{32}P]ATP, an aliquot of the radiolabeled nucleotide solution was spotted onto filter paper and counted the same way. In our calculations we used a molecular weight of 135 kDa for LPH, and assumed (on the basis of our own results) that LPH is phosphorylated only at one site.

2.6. Phosphorylation of LPH in rabbit small intestinal explants

Explants from the rabbit small intestine (6-months-old male New Zealand White rabbits) were cultured as described by Browning and Trier [14] with the modifications introduced by Naim et al. [15] and Lottaz et al. [16]. Specifically, after 1 h in a phosphate-free RPMI-1640 medium, explants were labeled continuously for 2 h with 150 μCi $^{32}P_i$ in 1 ml medium. Then 1 μg CT and IBMX (1 mM, final concentration) were added for 3 h. No CT and IBMX were added in the control samples. After washing, explants were solubilized by homogenization

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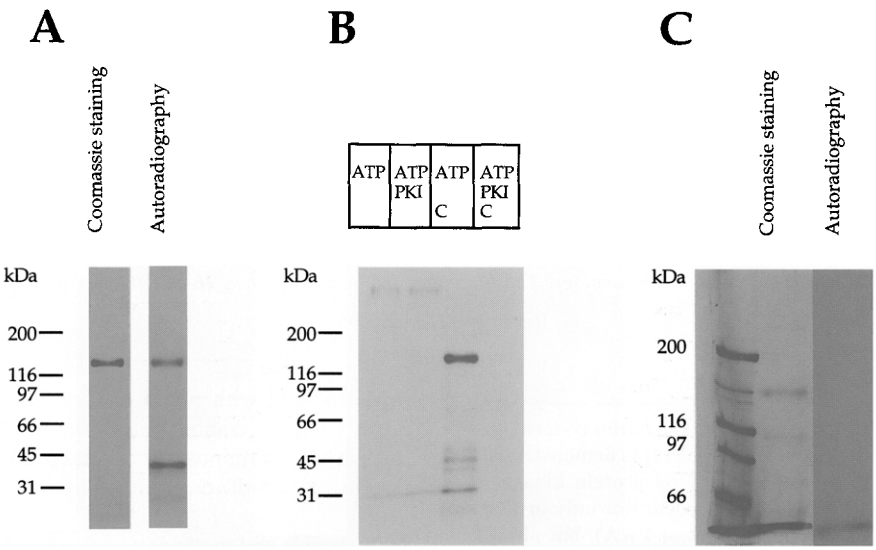


Fig. 1. Rabbit LPH, but not human LPH, can be phosphorylated in vitro by the catalytic subunit of PKA. (A) A sample (2.5 μg) of rabbit LPH was phosphorylated for 2 h at 30°C and then analyzed by SDS-PAGE using a 4–10% gradient gel. The radioactive band at approx. 40 kDa results from autophosphorylation of C. (B) Rabbit LPH was phosphorylated for 75 min at 30°C in the presence of ATP. C and PKI were added as indicated. (C) A sample (1.5 μg) of human LPH was phosphorylated for 2 h at 30°C and then analyzed by SDS-PAGE using a 5% gel. Molecular weight of the markers are indicated on the left.

in 1% Nonidet P-40, 1% deoxycholate in the presence of both protease inhibitors as described earlier [17], and a mixture of phosphatase inhibitors (5 mM EDTA, 50 mM NaF, 5 mM NaH_2PO_4). Insoluble particles were removed by centrifugation at 100,000 × g. The LPH from explants was immunoprecipitated in the presence of phosphatase inhibitors, using guinea pig anti-rabbit LPH bound to protein A-Sepharose [10,16]. Impurities, which unspecifically bind to protein A-Sepharose were first removed by a pre-adsorption on protein A-Sepharose. The resulting LPH samples were analyzed by SDS-PAGE in 5% polyacrylamide gels. Digital images of the gels produced with a PhosphorImager scanner were quantitated using Imagequant 3.2 software (both Molecular Dynamics, Sunnyvale, Ca, USA).

3. Results and discussion

While carrying out phosphorylation experiments on brush border membranes from the small intestine of rabbits, and searching for optimal PKA phosphorylation sites in LPH, we observed that this large bifunctional enzyme contains only one canonical PKA site (Arg/Lys-Arg/Lys-X-Ser/Thr, [18–21]), residing in its carboxy-terminus tail (Scheme 1). Experiments with pure LPH and pure C showed that in vitro, LPH is indeed a substrate for this kinase (Fig. 1A). This phosphorylation was found to take place only upon addition of C (Fig. 1B), showing that it is not due to a kinase impurity in our LPH preparation. In addition, it was shown to be completely blocked by the specific PKA inhibitor, PKI(5–24), confirming that all the phosphorylation seen is by PKA only. It should be noted that LPH prepared from human tissue did not undergo any phosphorylation whatsoever (Fig. 1C). In view of the high homology between the amino acid sequences of rabbit and of human LPH (83% identity, [7]), the lack of phosphorylation in the human enzyme (Fig. 1C), and the fact that the human LPH is lacking the PKA phosphorylation site around Ser^{1916}, it seems reasonable to conclude that the PKA phosphorylation is targeted to the tail of LPH where the sequence homology is rather low (Scheme 1). Furthermore, upon phosphorylation of rabbit LPH with [γ-^{32}P]ATP and subsequent cleavage of the radiolabeled enzyme at Leu^{1873}-Ser^{1874} (with chymotrypsin, as previously described in [10]), all the radiolabel is detached from the core protein (data not shown), indicating that the phosphorylation occurs at Ser^{1916}, the only Ser residue in that tail which is within any PKA consensus sequence [21].

Attempting to assess the physiological significance of this phosphorylation, we monitored its stoichiometry under a variety of conditions. An example for such a determination is given in the experiment depicted in Fig. 2, where the stoichiometry reached a value of 0.13 mol of phosphate per mol of LPH. While this stoichiometry varied from one experiment to an-

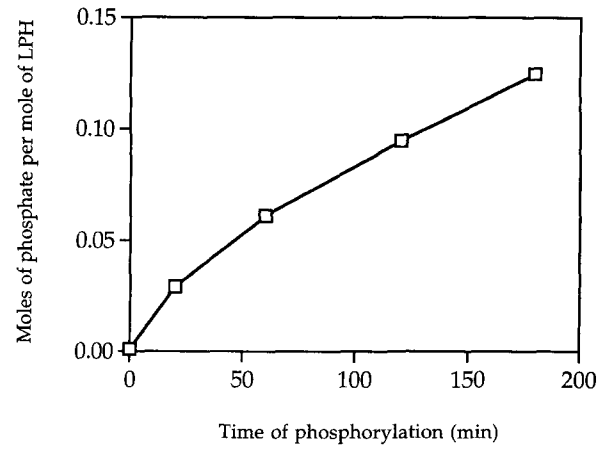
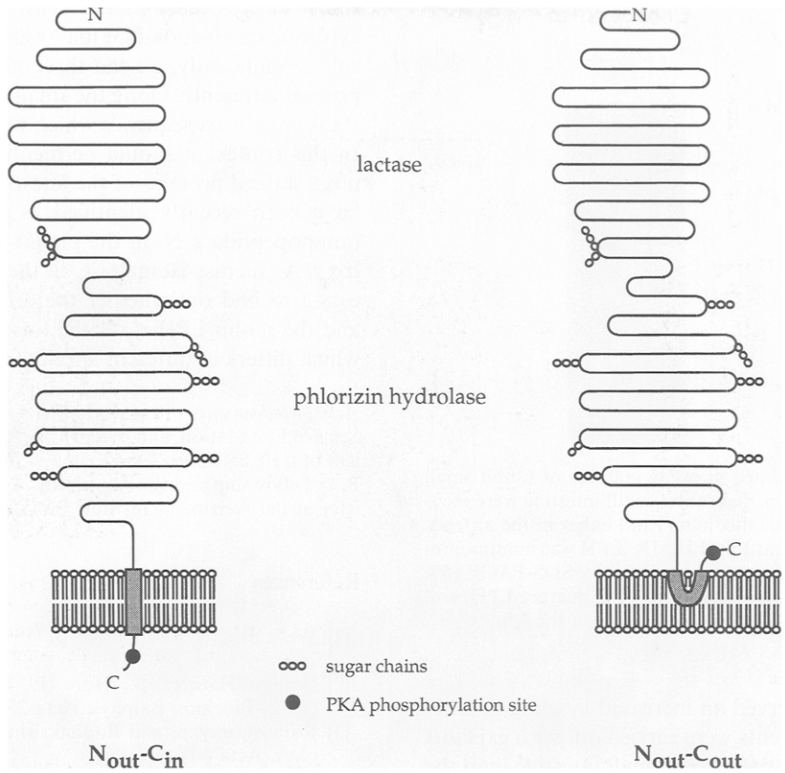
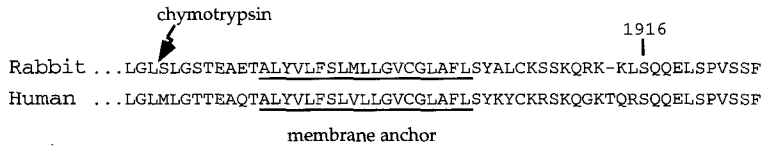


Fig. 2. Time course and stoichiometry of incorporation of ^{32}P into rabbit LPH. LPH was phosphorylated at 30°C. At the indicated times, samples containing ≈2 μg of protein were removed, and phosphorylation was stopped by adding 10 μl of 5 X Laemmli sample buffer and boiling for 2 min. After SDS-PAGE (4–10% polyacrylamide), the LPH bands were cut out from the dried gel and Cerenkov cpm were determined. Mole of phosphate incorporated per mole of enzyme were calculated as described in section 2.



C-terminus sequences of rabbit and human LPH



Scheme 1. Positioning of LPH in the small intestinal brush border membrane. LPH is oriented either in an N_{out}–C_{in} or an N_{out}–C_{out} orientation in the membrane. The approximate position of the potential phosphorylation site by PKA in rabbit LPH is indicated by a filled circle. The sequence of the C-terminus tail containing the potential phosphorylation site is indicated. Note that only rabbit LPH contains a potential phosphorylation site for PKA. The hydrophobic stretch that serves as the membrane anchor is underlined, and the cleavage site by chymotrypsin in rabbit LPH is indicated. Sequence data were taken from Mantei et al. [7].

other, we were not able to reach values beyond 0.2 mol ^{32}P per mol of protein, as is often the case for phosphorylations carried out in vitro with detergent-solubilized membrane proteins. With the particular membranes used here, this difficulty is somewhat accentuated, in view of the presence of several phosphatases and proteolytic enzymes which are not easy to either inhibit or to remove completely.

We therefore assessed the potential physiological relevance of this phosphorylation by attempting to find out whether it occurs in intact cells under physiologically relevant conditions. In view of the fact that membranes of the small intestine constitute a target for cholera toxin (CT), the clinical importance of this interaction, and the fact that CT is known to elevate intracellular cyclic AMP [22], we attempted to find out whether CT affects the level of phosphorylation of LPH in intact cells. As seen in Fig. 3, when an organ culture of an explant from the medial rabbit small intestine is pre-fed with $^{32}P_i$ (to radiolabel the intracellular pool of ATP) and subsequently stimulated by

Table 1
Phosphorylation of LPH in rabbit small intestinal explants in the presence or absence of cholera toxin (CT) and IBMX

Sample* and its origin	^{32}P incorporated (counts/μg protein)†		Ratio of ^{32}P incorporated
	−CT	+CT	+CT/−CT
Mature LPH proximal small intestine	12186	17094	1.40
Pro LPH proximal small intestine	49407	65554	1.33
Mature LPH medial small intestine	10204	15208	1.49
Pro LPH medial small intestine	42522	62792	1.48

*Data were taken from Fig. 3 (medial small intestine) and from an identical experiment using a sample from proximal small intestine.
†Radioactivity in the LPH bands was determined with the PhosphorImager, and the amount of protein was estimated by elution of Coomassie blue R250 [30] from the LPH bands.

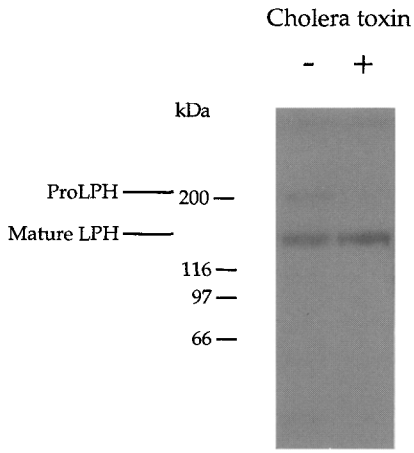


Fig. 3. LPH can be phosphorylated in organ cultures of rabbit small intestine. Pieces of tissue from medial rabbit small intestine were incubated in $^{32}P_i$ containing synthetic medium (1 ml) either in the absence (−) or presence (+) of 1 μg CT and 1 mM IBMX. LPH was immunoprecipitated from the solubilized tissue and analyzed by SDS-PAGE (5% gel) and autoradiography. The position of pro LPH, mature LPH, and the molecular weight of the markers are indicated on the left.

treatment with CT, we observed an increased labeling of LPH (Fig. 3). A series of experiments were carried out with explants from either the proximal or the medial small intestine, and the effect of CT on the labeling of the mature or the pro LPH was measured. As seen in Table 1, there is an increase of 30 to 50% in the specific labeling of the LPH bands in all cases.

Our finding that in intact cells the phosphorylation of Ser^{1916} occurs intracellularly is further supported by our earlier demonstration that the luminal side of similar brush border membranes does not contain an ecto PKA activity [23] and does contain a kinase splitting membranal proteinase (KSMP), which rapidly cleaves and selectively inactivates the catalytic subunit of PKA [24–26]. On the other hand, using right-side-out vesicles from such membranes, we have shown that they contain a PKA activity (inhibited by PKI) with a cytosolic orientation [23], which could be involved in the phosphorylation of the tail of LPH in vivo.

In conclusion, this communication provides evidence to show: (a) that rabbit LPH is phosphorylated in vitro with pure LPH and pure C; (b) that the phosphorylation of the rabbit enzyme is directed to Ser^{1916} which is within a canonical PKA phosphorylation site [21] in the rabbit enzyme (the lack of phosphorylation in the homologous human enzyme supports the selective targeting of this phosphorylation, since the human enzyme lacks a PKA canonical site around the Ser residue which is equivalent to the rabbit Ser^{1916}, Scheme 1); (c) that upon clipping off the carboxy-terminus tail of LPH with chymotrypsin, the radiolabel is removed from the core protein; (d) that CT, which elevates intracellular cyclic AMP, brings about a significant increase in radiolabeling of LPH in intact cells, actually in an organ culture of the small intestine. These findings establish unequivocally the cytosolic orientation of the carboxy-terminus tail of LPH, i.e. its N_{out}–C_{in} orientation (Scheme 1), and that this tail , the sequence of which had been established so far by cDNA sequencing only, is not post-translationally clipped , and is kept intact at least down to Ser^{1916}.

The phosphorylation of the cytosolic tail in rabbit LPH by PKA which occurs in the brush border membranes with a cytosolic disposition [23] may well have an as yet unidentified role. Significantly, all the three genes of LPH which are expressed differently along the small intestine [27] do code for the PKA consensus sequence which we find to be phosphorylated. In this context it should be mentioned that other once-spanning, stalked proteins of the intestinal brush border membrane have been recently identified as receptors of a coronavirus (aminopeptidase N, in the pig [28]) or of Clostridium difficile toxin A (sucrase-isomaltase, in the rabbit [29]). It will be interesting to find out whether the difference between the human and the rabbit LPH is related to some other function of LPH, which differs in different species.

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