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

Patrick Keller*, Giorgio Semenza*, Shmuel Shaltiel**

*Department of Biochemistry, Swiss Federal Institute of Technology, ETH Zentrum, CH-8092 Zurich, Switzerland
**Department of Chemical Immunology, The Weizmann Institute of Science, IL-76100 Rehovot, Israel

Received 31 May 1995

Abstract The intracellular disposition of the carboxy-terminus tail of rabbit lactase-phlorizin hydrolase (LPH) is demonstrated, using a specific phosphorylation of Ser1916 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 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 Gly19-Ser20 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 hydrophilic 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 extracellular, endowing LPH with either an Nout-Cin or an Nout-Cout 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.

2. Materials and methods

2.1. Materials

The specific PKA inhibitor PKI(5–24) was from Peninsula Laboratories (Belmont, Ca, USA). [γ-32P]ATP and 32P 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/1/9093474 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-32P]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 x 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-32P]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 Lottiazz et al. [16]. Specifically, after 1 h in a phosphate-free RPMI-1640 medium, explants were labeled continuously for 2 h with 150 μCi 32P 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.
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 NaH2PO4). Insoluble particles were removed by centrifugation at 100,000 x 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 Phosphor-Imager 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 Ser1916, 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-32P]ATP and subsequent cleavage of the radiolabeled enzyme at Leu1837-Ser1874 (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 Ser1916, the only Ser residue in that tail which is within any PKA consensus sequence [21].

Attemping 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. 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.]

![Fig. 2. Time course and stoichiometry of incorporation of 32P 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.]

[\textcopyright P. Keller et al./FEBS Letters 368 (1995) 563–567]
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, (to radiolabel the intracellular pool of ATP) and subsequently stimulated by

| Sample* and its origin       | $^{32}$P incorporated (counts/ug protein)$^a$ | Ratio of $^{32}$P incorporated $+\text{CT}/-\text{CT}$ |
|----------------------------|-----------------------------------------------|------------------------------------------------------|
| Mature LPH proximal small intestine | 12186                                         | 1.40                                                 |
| Pro LPH proximal small intestine     | 49407                                         | 1.33                                                 |
| Mature LPH medial small intestine     | 10204                                         | 1.49                                                 |
| Pro LPH medial small intestine     | 42522                                         | 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 Phosphor-Imager, 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 ³²P 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.

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 Ser¹⁹¹⁶ 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 Ser¹⁹¹⁶, 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 phosphorylation 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 Ser¹⁹¹⁶.

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.

**Acknowledgements:** This work was supported by the Swiss National Science Foundation, and by the Minerva Foundation. P.K. is the recipient of a FEBS Short-Term Fellowship. S.Sh. is the recipient of a Hans Baer Fellowship and the incumbent of the Kleeman Chair in Biochemistry at the Weizmann Institute of Science.

**References**

[1] Wallenfels, K. and Fischer, J. (1960) Z. Physiol. Chem. 312, 223-245.
[2] Schlegel-Haueuter, S., Hore, P., Perry, K.R. and Semenza, G. (1972) Biochim. Biophys. Acta 258, 506-519.
[3] Ramaswamy, S. and Radhakrishnan, A.N. (1975) Biochim. Biophys. Acta 403, 446-455.
[4] Skovbjerg, H., Sjöström, H. and Norén, O. (1981) Eur. J. Biochem. 114, 653-661.
[5] Skovbjerg, H., Norén, O., Sjöström, H., Danielson, E.M. and Envoldsen, B.S. (1982) Biochim. Biophys. Acta 707, 89-97.
[6] Potter J., Ho, M.-W., Bolton, H., Forth, A.J., Swallow, D.M. and Griffith, B. (1985) Biochem. Genet. 23, 423-439.
[7] Mantei, N., Villa, M., Enzler, T., Wacker, H., Boll, W., James, P., Hunziker, W. and Semenza, G. (1988) EMBO J. 7, 2705-2713.
[8] Duluc, L., Boukamel, R., Mantei, N., Semenza, G., Raul, F. and Freund, J.N. (1991) Gene 103, 273-276.
[9] Dudley, M.A., Hachey, D.L., Quaroni, A., Hutchens, T.W., Nichols, B.L., Rosenberger, J., Parkin, J.S., Cook, G. and Reeds, P.J. (1993) J. Biol. Chem. 268, 13609-13616.
[10] Wacker, H., Keller, P., Falchetto, R., Legler, G. and Semenza, G. (1992) J. Biol. Chem. 267, 18474-18475.
[11] Hauri, H.P., Sterchi, E.E., Bienz, D., Fransen, J.A. and Marxer, A. (1985) J. Cell Biol. 101, 838-851.
[12] Laemmli, U.K. (1970) Nature 227, 680-685.
[13] Reimann, E.M. and Behram, R.A. (1983) Methods Enzymol. 99, 51-55.
[14] Browning, T.H. and Trier, J.S. (1969) J. Clin. Invest. 48, 1423-1432.
[15] Naim, H.Y., Sterchi, E.E. and Lentze, M.J. (1987) Biochem. J. 241, 427-434.
[16] Lottaz, D., Oberholzer, T., Semenza, G., Bähler, P. and Sterchi, E.E. (1992) FEBS Lett. 313, 270-276.
[17] Keller, P., Zwickler, E., Mantei, N. and Semenza, G. (1992) FEBS Lett. 313, 265-269.
[18] Krebs, E.G. (1986) in: The Enzymes: Control by Phosphorylation (Kemp, B.E., Ed.) pp. 171-180.
[19] Taylor, S.S., Buehler, J.A. and Yonemoto, W. (1990) Annu. Rev. Biochem. 59, 971-1005.
[20] Zetterqvist, O.C., Ragnarsson, U. and Engstrom, L. (1990) in: Peptides and Protein Phosphorylation (Kemp, B.E., Ed.) pp. 171-187, CRC Press, Boca Raton, FL.
[21] Kennelly, P.J. and Krebs, E.G. (1991) J. Biol. Chem. 266, 15555-15558.
[22] Moss, J. and Vaughan, M. (1979) Annu. Rev. Biochem. 48, 581-600.
[23] De Jonge, H., Schmeeda, H. and Shaltiel, S. (1987) Eur. J. Biochem. 169, 503-509.
[24] Alhanaty, E. and Shaltiel, S. (1979) Biochem. Biophys. Res. Commun. 89, 323–332.
[25] Alhanaty, E., Patinkin, J., Tauber-Finkelstein, M. and Shaltiel, S. (1981) Proc. Natl. Acad. Sci. USA 78, 3492–3495.
[26] Alhanaty, E., Tauber-Finkelstein, M., Schneeda, H. and Shaltiel, S. (1985) Curr. Topics Cell. Regul. 27, 267–278.
[27] Villa, M., Brunschwiler, D., Gächter, T., Boll, W., Semenza, G. and Mantei, N. (1993) FEBS Lett. 336, 70–74.
[28] Delmas, B., Gelf, J., L’Haridon, R., Vogel, L.K., Sjöström, H., Norén, O. and Laude, H. (1992) Nature 357, 417–420.
[29] Pothoulakis, C., Gao, N., Cladaras, C., Offner, G. and LaMont, J.T. (1994) Gastroenterol. 106, Abstract A833.
[30] Ball, E.H. (1986) Anal. Biochem. 155, 23–27.