Vol. 122, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 18, 1984 Pages 82-90

BIOSYNTHESIS OF INTESTINAL MICROVILLAR PROTEINS
Intracellular processing of lactase-phlorizin hydrolase

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Received May 7, 1984

The biosynthesis of pig small intestinal lactase-phlorizin hydrolase (EC 3.2.1.23-62) was studied by labelling of organ cultured mucosal explants with [^{35}S] methionine. The earliest detactable form of the enzyme was an intracellular, membrane-bound polypeptide of M_r 225 000, sensitive to endo H as judged by its increased electrophoretic mobility (M_r 210 000 after treatment). The labelling of this form decreased during a chase of 120 min and instead two polypeptides of M_r 245 000 and 160 000 occured, which both barely had their electrophoretic mobility changed by treatment with endo H. The M_r 160 000 polypeptide is of the same size as the mature lactase-phlorizin hydrolase and was the only form expressed in the microvillar membrane. Together, these data are indicative of an intracellular proteolytic cleavage during transport. The presence of leupeptin during labelling prevented the appearance of the M_r 160 000 form but not that of the M_r 245 000 polypeptide, suggesting that the proteolytic cleavage takes place after trimming and complex glycosylation. The proteolytic cleavage was not essential for the transport since the precursor was expressed in the microvillar membrane in the presence of leupeptin.

Lactase-phlorizin hydrolase (EC 3.2.1.23-62) belongs to a group of intestinal microvillar enzymes commonly characterized as 'stalked' integral membrane proteins. The peptidases and glycosidases constituting this group are typically dimeric proteins of high M_r, anchored to the membrane by a small hydrophobic segment near the N-terminus (for a review, see 1). In terms of their structure and biosynthesis, it has been suggested that the microvillar enzymes be divided into two categories (2); one, examplified by aminopeptidase N (EC 3.4.11.2), is composed of identical subunits which are synthesized sepa-

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0006-291X/84 $1.50

rately (3); another, the sucrase-isomaltase (EC 3.2.1.48-10) type has different subunits which carry different enzyme activities and are synthesized as a large single-chain precursor (4). Lactase-phlorizin hydrolase is known to have two catalytic sites, but it is uncertain if they are located on different subunits or on different parts of the same polypeptide (5,6). The existence of high M_r precursors has been demonstrated in the microvillar membrane of pigs which three days prior to death had their pancreatic ducts ligated and dissected (7,8). Lactase- phlorizin hydrolase, purified from similarly pre- treated animals had a similar subunit structure as the enzyme, seen in normal pigs (9), indicating that lactase-phlorizin hydrolase does not undergo extracellular proteolytic cleavage by pancreatic proteinases. However, these experiments left open the possibility of an intracellular proteolytic cleavage, occuring during biosynthesis.

In the present work, organ culture of intestinal explants, a technique previously used in biosynthesis studies on microvillar enzymes (10,11), was used to study the biosynthesis of lactase-phlorizin hydrolase.

MATERIALS AND METHODS

Equipment for performing organ culture was obtained as described in (12). Endo-β-N-acetylglucosaminidase H (endo H) was purchased from Miles Biochemicals, Slough, England. All other chemicals used were of an analytical grade.

Pig small intestines were kindly given by the Department of Experimental Pathology, Rigshospitalet, Copenhagen, Denmark.

Organ culture of intestinal mucosal explants (13) was performed as in (12). In pulse-chase labelling experiments, explants were labelled for 10 min with [^{35}S]methionine (500 μCi/ml) and chased with non-radioactive medium containing an increased concentration of methionine (2.5 mM) for various periods of time (10). In some experiments, leupeptin (5 μg/ml) was added to the culture medium 1 h before continuous labelling for 2-3 h with [^{35}S]methionine (100 μCi/ml).

Fractionation of labelled explants by the Ca^{2+}-precipitation technique (14,15) immunopurification of microvillar enzymes, SDS/polyacrylamide gel electrophoresis (16) and fluorography of gels (17) were performed as in (10,18). In some experiments, leupeptin (5 μg/ml) was added to all buffers used

in the subcellular fractionation. The following [^{35}S] methionine labelled M_r-indicators were used: Polypeptides of M_r 240 000 and 265 000 of sucrase-isomaltase and polypeptides of M_r 140 000 and 166 000 of aminopeptidase N.

RESULTS

Pulse-chase labelling

Fig. 1,A shows the appearance in the Ca^{2+}-precipitated membrane fraction of labelled lactase-phlorizin hydrolase. Two polypeptides of M_r 225 000 and 205 000 were visible immediately after the 10 min pulse; the latter only when leupeptin was omitted during subcellular fractionation and enzyme purification. After about 60 min of chase, the intensity of these polypeptides decreased and a polypeptide of M_r 160 000 appeared with increasing intensity.

The appearance in the microvillar fraction of lactase-phlorizin hydrolase (from the same experiment) is shown in fig. 1,B. After 60 min of chase, the polypeptide of M_r 160 000 became visible. No other molecular forms of the enzyme were detected in the microvillar fraction during the chase period, regardless whether leupeptin was present during subcellular fractionation and enzyme purification.

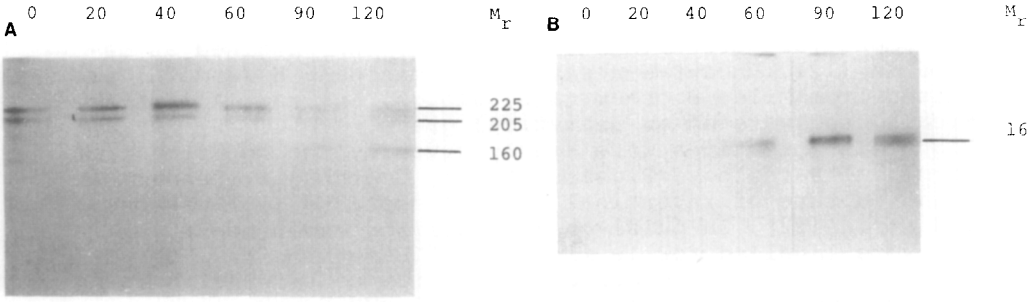

Fig.1. Pulse-chase labelling of lactase-phlorizin hydrolase
After 10 min of labelling, explants were chased with non-radioactive methionine for the indicated periods of time (min). Lactase-phlorizin hydrolase, immunopurified from the Ca^{2+}-precipitated (A) and microvillar (B) membrane fractions was subjected to SDS/polyacrylamide gel electrophoresis. After electrophoresis, the gels were prepared for fluorography. Exposure time: 30-45 days. Apparent M_r values (x 10^{-3}) are shown.

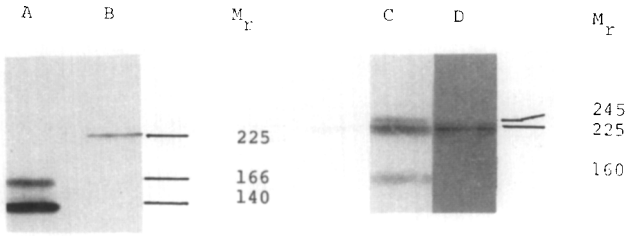

Fig.2. High M_r precursors of lactase-phlorizin hydrolase
Explants were labelled for 40 min (A,B) or 2 h (C,D) in the absence (A-C) or presence (D) of leupeptin. Lactase-phlorizin hydrolase (B-D) and aminopeptidase N (A, shown for comparison) were immunopurified from a Triton X-100 extract of the total explants as in (6) in the presence of leupeptin and subjected to SDS/polyacrylamide gel electrophoresis. After electrophoresis, the gels were prepared for fluorography. Exposure time: 4-40 days. Apparent M_r values (x 10^{-3}) are shown.

When lactase-phlorizin hydrolase was purified in the presence of leupeptin from explants, labelled for 40 min, only the polypeptide of M_r 225 000 was seen (fig. 2, lane B). Upon longer labelling (2 h), polypeptides of M_r 245 000 and 160 000 could be seen as well (fig. 2, lane C). When the labelling for 2 h was performed in the presence of leupeptin, only the polypeptides of M_r 225 000 and 245 000 could be detected (fig. 2, lane D). (In this as well as the following figures shown, the M_r 225 000 polypeptide repeatedly appeared as a band with a blurred front, extending in the direction of migration, or even as a doublet. We interpret this appearance as an insufficent inhibition of proteolysis by leupeptin).

When leupeptin was present during labelling for 3 h, both polypeptides of M_r 225 000 and 245 000 were transported to the microvillar membrane (fig. 3). None of the molecular forms of lactase-phlorizin hydrolase found in the membrane fractions could be detected in the soluble fraction (data not shown).

Treatment with endo H

Fig. 4 shows the effect of endo H on the molecular forms of lactase-phlorizin hydrolase. Whereas the polypeptides of M_r 245

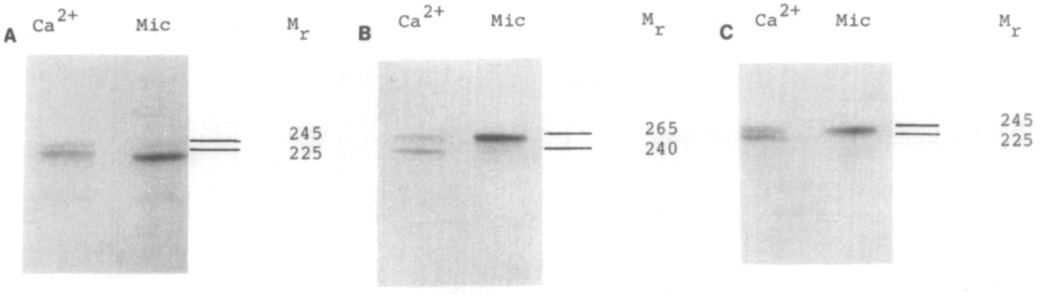

Fig.3. Effect of leupeptin on intracellular transport
Explants were labelled for 3 h in the presence of leupeptin and fractionated into a Ca^{2+}-precipitated (Ca^{2+}) and a microvillar membrane (Mic) fraction in the presence of leupeptin. Lactase-phlorizin hydrolase (A), sucrase-isomaltase (B) and maltase-glucoamylase (C) were immunopurified from both fractions and subjected to SDS/polyacrylamide gel electrophoresis. After electrophoresis, the gel was prepared for fluorography. Exposure time: 7 days. Apparent M_r values (x 10^{-3}) are shown.

000 and 160 000 were barely susceptible, the mobility of the M_r 225 000 polypeptide was markedly increased; corresponding to a M_r of 210 000 after treatment. The presence of leupeptin during labelling did not influence the effect of endo H on the polypeptides of M_r 225 000 and 245 000.

DISCUSSION

High M_r precursors of lactase-phlorizin hydrolase

Like five other microvillar enzymes previously studied by pulse-chase labelling of intestinal explants (10,11), lactase-

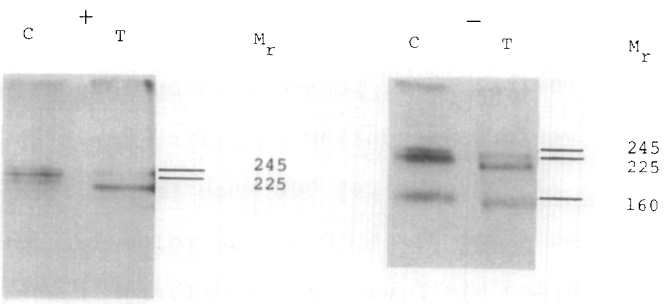

Fig.4. Effect of endo H
Explants were labelled for 2 h in the presence (+) or absence (-) of leupeptin. Lactase-phlorizin hydrolase was immunopurified from a Triton X-100 extract of total explants as in (6) in the presence of leupeptin and treated with endo H as in (7). Endo H treated (T) and control (C) samples were subjected to SDS/polyacrylamide gel electrophoresis. After electrophoresis, the gels were prepared for fluorography. Exposure time: 4-7 days. Apparent M_r values (x 10^{-3}) are shown.

phlorizin hydrolase appeared first in the Ca^{2+}-precipitated membrane fraction. This finding, together with the observation that no labelled enzyme could be detected in soluble form is strongly indicative of a membrane-bound synthesis and intracellular transport. In contrast to the other enzymes studied, lactase-phlorizin hydrolase appeared as two polypeptides (M_r 225 000 and 205 000) after the 10 min pulse. However, since only the M_r 225 000 polypeptide could be detected when purification was performed in the presence of leupeptin, a proteinase inhibitor of bacterial origin (19), the M_r 205 000 polypeptide must be generated by artefactual proteolysis of the M_r 225 000 form during subcellular fractionation and enzyme purification. Since the intensity of the M_r 225 000 polypeptide (and its degration product of M_r 205 000) decreased during the chase period of 120 min, this form is likely to represent a transient precursor state of the mature lactase- phlorizin hydrolase. This interpretation is supported by its marked susceptibility to endo H which indicates the presence of N-linked high mannose glycosylation, a type of processing also seen for other microvillar enzymes (10,11). The apparent M_r after endo H treatment probably corresponds approximately to that of the non-glycosylated form of lactase-phlorizin hydrolase. In contrast, the M_r 245 000 polypeptide, seen after longer periods of labelling when the enzyme was purified in the presence of leupeptin, was barely sensitive to endo H. This molecular form is therefore likely to represent a trimmed and complex glycosylated precursor of lactase-phlorizin hydrolase. The fact that it was totally absent when leupeptin was omitted during preparation further supports the finding that the precursor of lactase-phlorizin hydrolase, unlike those of other microvillar enzymes, is susceptible to an endogeneous proteinase. This sensitivity to

proteolytic cleavage probably explains why we in an earlier work found the high mannose glycosylated precursor of the enzyme to be of lower M_r (150 000) than the mature form (9,20).

Intracellular proteolytic cleavage of lactase-phlorizin hydrolase

In the microvillar membrane, lactase-phlorizin hydrolase appeared in the form of the M_r 160 000 polypeptide (fig. 1,B), the only molecular form accumulating during a 20 h labelling period (12). This is also the size of the enzyme, purified from pig small intestine, regardless whether the pancreatic ducts had been ligated (9). It therefore seems reasonable to propose that newly synthesized lactase-phlorizin hydrolase undergoes proteolytic cleavage during its intracellular transport. Although considered unlikely, the results obtained would also be compatible with an extracellular cleavage if this was to act so rapidly that the extracellular pool of non-cleaved precursor would escape detection.

For aminopeptidase N, the primary translation product of M_r 115 000 is glycosylated to yield the mature form of M_r 166 000 (3). Assuming a similar degree of glycosylation of lactase-phlorizin hydrolase, the reduction in M_r (from 210 000 to 2 x 160 000) suggests that the precursor is cleaved into two polypeptides, thereby generating the two subunits of the mature enzyme. Such a mechanism would be analogous to those of sucrase-isomaltase (7,21), maltase-glucoamylase (8) and γ-glutamyl transpeptidase (22-24). An alternative explanation would be that only one subunit is generated per precursor molecule. This would imply that a large fragment is cleaved off; possibly in a way similar to the processing of proforms of lysosomal hydrolase and pro-hormones (25). No such fragments were visible, but since the antibody used in the purification is raised against

the mature enzyme, it possibly would not recognize such fragments. Although such a mechanism must be considered unlikely, it cannot be excluded.

Since the polypeptides of M_r 245 000 and 160 000 were equally resistant to endo H, it is likely that the proteolytic cleavage in vivo takes place after the precursor has undergone trimming and complex glycosylation. It therefore occurs either as a late event during the passage through the Golgi complex or possibly later, at a stage between the Golgi complex and the microvillar membrane. Interestingly, the blocking of the cleavage by the presence of leupeptin during labelling did not inhibit the expression in the microvillar membrane, indicating that this type of processing is not essential for the intracellular transport. It is noteworthy that both the high mannose form of M_r 225 000 as well as the complex form of M_r 245 000 appeared in the microvillar membrane. In contrast, sucrase-isomaltase and maltase- glucoamylase, purified simultaneously from the same fractions, exhibited the "normal" pattern with only the complex form being expressed in the microvillar membrane. A speculative explanation for this could be that inhibition of the proteolytic cleavage disturbs the carbohydrate processing without arresting transport, leading to incomplete maturation of newly synthesized polypeptides.

In summary, the present work shows that pig lactase-phlorizin hydrolase, like sucrase-isomaltase and maltase-glucoamylase, is synthesized as a high M_r precursor. A similar observation was recently reported for the human enzyme (26). Unlike the other microvillar glycosidases, however, lactase-phlorizin hydrolase is cleaved intracellularly during transport by a leupeptin-sensitive proteinase to yield the size of the mature enzyme. A kidney microvillar enzyme, γ-glutamyl transpeptidase also

undergoes intracellular proteolytic cleavage but in this case it occurs before trimming and complex glycosylation, so it may well be different mechanisms that are active for the two enzymes (23).

ACKNOWLEDGMENTS

The work was supported by grants from the Danish Medical Research Council (project 12-3505), the Novo Foundation, J.Madsens og O.Madsens Fond and P.Carl Petersens Fond.

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