Biosynthesis of Intestinal Microvillar Proteins. Dimerization of Aminopeptidase N and Lactase-Phlorizin Hydrolase†

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ABSTRACT: The pig intestinal brush border enzymes aminopeptidase N (EC 3.4.11.2) and lactase-phlorizin hydrolase (EC 3.2.1.23-62) are present in the microvillar membrane as homodimers. Dimethyl adipimidate was used to cross-link the two [^{35}S]methionine-labeled brush border enzymes from cultured mucosal explants. For aminopeptidase N, dimerization did not begin until 5-10 min after synthesis, and maximal dimerization by cross-linking of the transient form of the enzyme required 1 h, whereas the mature form of aminopeptidase N cross-linked with unchanged efficiency from 45 min to 3 h of labeling. Formation of dimers of this enzyme therefore occurs prior to the Golgi-associated processing, and the slow rate of dimerization may be the rate-limiting step in the transport from the endoplasmic reticulum to the Golgi complex. For lactase-phlorizin hydrolase, the posttranslational processing includes a proteolytic cleavage of its high molecular weight precursor. Since only the mature form and not the precursor of this enzyme could be cross-linked, formation of tightly associated dimers only takes place after transport out of the endoplasmic reticulum. Dimerization of the two brush border enzymes therefore seems to occur in different organelles of the enterocyte.

All known major integral membrane glycoproteins of the intestinal brush border, or microvillar, membrane are hydrolases playing an important digestive role in the uptake of dietary carbohydrate and protein (Norén et al., 1986; Semenza, 1986). Their gross structural properties are well-known (they are commonly characterized as "stalked" integral membrane proteins), and within the last few years, the primary structure of many brush border enzymes has been deduced from cloned cDNA. Olsen et al. (1988) have recently sequenced aminopeptidase N (EC 3.4.11.2) and were able to define at least 4 domains in the 967 amino acid long polypeptide: a short positively charged cytoplasmic tail comprising the N-terminus; a single membrane-spanning stretch of 23 largely hydrophobic amino acids; a "stalk" of about 40 amino acids, rich in serine and threonine residues and probably carrying O-linked carbohydrate; a globular domain, containing the Zn^{2+}-binding active site and constituting >90% of the molecular mass. The quaternary structure of aminopeptidase N has been studied by electron microscopy of the purified enzyme, reconstituted into liposomes (Hussain et al., 1981). Here, aminopeptidase N is seen predominantly as homodimers with the globular domains closely associated (by noncovalent forces) at a distance of 5 nm from the membrane.

Concerning oligomerization, one of the best-studied membrane glycoproteins is influenza virus hemagglutinin which in its mature form is a homotrimer. The trimerization of hemagglutinin takes place in the endoplasmic reticulum with a half-time of 7-10 min following synthesis (Gething et al., 1986; Copeland et al., 1986). Monomers that fail to assemble correctly are not transported to the Golgi complex, but bind instead to a 77-kDa protein (BiP) and undergo proteolytic degradation. This suggests that oligomerization of membrane glycoproteins may be important not only for their biological function but also for the intracellular transport to the cell surface (Copeland et al., 1988).

Nothing is known about the subcellular location or kinetics of dimerization of intestinal brush border enzymes. In the present work, this biosynthetic event was studied in a mucosal explant system.

Experimental Procedures

Materials. Equipment for performing organ culture of mucosal explants, including Trowell's T-8 medium, plastic dishes with grids, and [^{35}S]methionine (specific radioactivity >1000 Ci/mmol), were obtained as previously described (Danielsen et al., 1982). The bifunctional reagents dimethyl adipimidate and dimethyl 3,3′-dithiobis(propionimidate) were purchased from Sigma Chemical Co., St. Louis, MO.

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

Organ Culture of Mucosal Explants. Small intestinal mucosal explants of about 100 mg wet weight were excised and maintained in organ culture as previously described (Danielsen et al., 1982). After culture, the explants were kept frozen at −80 °C until use.

Chemical Cross-Linking. Labeled mucosal explants were thawed and homogenzied in 400 μL of 0.1 M triethanolamine hydrochloride, pH 8.5, containing 5% (w/v) Triton X-100 and 50 μg/mL leupeptin. The homogenate was centrifuged at 25000*g*, 5 min, to obtain a supernatant of total detergent-extracted mucosal protein. Two hundred microliters of dimethyl adipimidate (20 mg/mL) in 0.2 M triethanolamine hydrochloride, pH 8.5, was added to the mucosal extract, and cross-linking was allowed to proceed for 20 h at room temperature. [In some experiments, dimethyl 3,3′-dithiobis(propionimidate) was used as cross-linker at a concentration of 5 mg/mL instead of dimethyl adipimidate.]

Purification of Brush Border Enzymes. Immediately after cross-linking, aminopeptidase N and lactase-phlorizin hydrolase were immunopurified by line-immunoelectrophoresis as previously described (Danielsen & Cowell, 1983).

Electrophoresis. SDS/PAGE in 7% gels was performed according to Laemmli (1970) and fluorography as described by Bonner and Laskey (1974). Gel tracks were scanned in a LKB Ultroscan XL densitometer (LKB, Bromma, Sweden).

Results

We have previously used the intestinal mucosal explant

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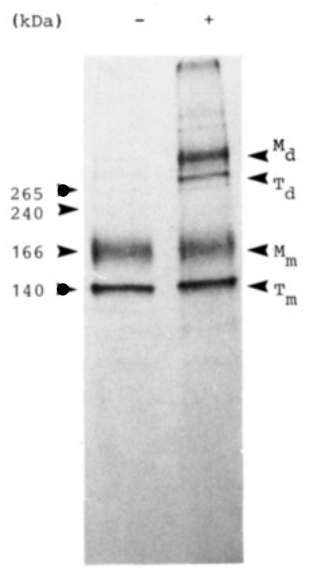

FIGURE 1: Cross-linking of aminopeptidase N. A detergent extract was prepared from mucosal explants, labeled for 1 h with 100 μCi/mL [^{35}S]methionine, and incubated in the presence (+) or absence (−) of dimethyl adipimidate as described under Experimental Procedures. After cross-linking, aminopeptidase N was immunopurified and subjected to SDS/PAGE. The transient (140 and 240 kDa) and mature (166 and 265 kDa) forms of aminopeptidase N and sucrase–isomaltase, respectively, were used as molecular weight indicators. T_m, monomeric transient form; M_m, monomeric mature form; T_d, dimeric transient form; M_d, dimeric mature form.

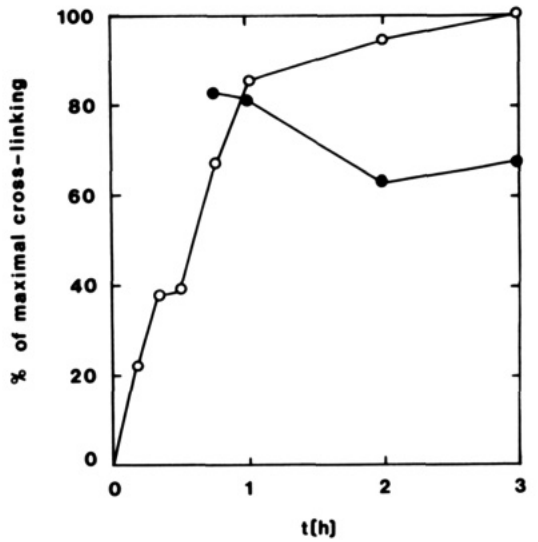

FIGURE 2: Kinetics of dimerization of aminopeptidase N. Mucosal explants, labeled with 100–200 μCi/mL [^{35}S]methionine for the indicated periods of time, were extracted and cross-linked with dimethyl adipimidate. The proportion of cross-linked transient form (○) and mature form (●) was determined by densitometric scanning of the gel tracks. Each value is the mean of two experiments and is expressed as the percent of maximal cross-linking of the transient form (which was obtained after labeling for 3 h). In six series of experiments, each including a 3-h determination, the mean maximal cross-linking of the transient form was 49%.

system to characterize the biosynthetic processing and intracellular transport of brush border enzymes [for a review, see Danielsen et al. (1987)]. They are commonly assembled in the rough endoplasmic reticulum where they also invariably become glycosylated with N-linked, high-mannose oligosaccharides. During passage through the Golgi complex, the N-linked carbohydrate is processed in a way also known for other types of membrane and secretory proteins (Kornfeld & Kornfeld, 1985), and O-linked glycosylation and tyrosine sulfation (Danielsen, 1987) are other types of common post-translational, Golgi-associated modifications of brush border enzymes.

In the present work, dimethyl adipimidate (Hartman & Wold, 1967; Tae, 1983) was used as a chemical cross-linker of the brush border enzymes aminopeptidase N and lactase–phlorizin hydrolase, radioactively labeled in the mucosal explant system. Figure 1 shows the result obtained for aminopeptidase N from explants, labeled continuously for 1 h with [^{35}S]methionine. Two major bands resulted from cross-linking; one corresponding to a dimer of the transient, high-mannose glycosylated form of 140 kDa and one corresponding to a dimer of the mature, complex-glycosylated form of 166 kDa. Occasionally, very faint bands migrating with a mobility of trimers of the transient and mature forms could be detected as well. In the absence of cross-linker, only minute amounts of dimers could be seen (<5%). This experiment proves dimethyl adipimidate to be a useful cross-linker for aminopeptidase N and shows that dimerization of the newly synthesized brush border enzyme occurs prior to its acquisition of complex carbohydrate, i.e., before its processing in the Golgi complex. Two other bifunctional reagents, dimethyl suberimidate and dimethyl 3,3′-dithiobis(propionimidate), have previously been used to cross-link aminopeptidase N from microvillar vesicles (Svensson, 1979; Svensson et al., 1982). None of these reagents, however, appeared to be nearly as efficient as dimethyl adipimidate in cross-linking aminopeptidase N; initially, dimethyl 3,3′-dithiobis(propionimidate) was used in the present work, and it, too, cross-linked both the transient and also the mature form of aminopeptidase N, but with an efficiency well below that of dimethyl adipimidate (data not shown).

The kinetics of dimerization of aminopeptidase N as probed by chemical cross-linking are shown in Figure 2. Barely detectable amounts of dimer of the transient form of the enzyme were formed after 5 min of labeling, but from 10 min, the cross-linking efficiency gradually increased to reach a maximum level by 1 h of labeling. The mature form of aminopeptidase N, which starts appearing after about 30 min of labeling, immediately cross-linked with a maximal efficiency (that was somewhat below that of the transient form). This result shows that dimers of aminopeptidase N only begin to form 5–10 min after synthesis in the rough endoplasmic reticulum and that dimerization steadily continues until the Golgi-associated carbohydrate processing begins. One may wonder why the cross-linking efficiency of the transient form of the enzyme continues to increase between 0.5 and 1 h after synthesis, when conversion to the mature form has already begun. However, in earlier experiments with mucosal explants, we observed that the rate of processing from transient to mature form only reaches its maximum after about 1 h of labeling (Danielsen & Cowell, 1985), indicating a slow acquisition of steady-state. This phenomenon may reflect a differential viability of the epithelial cells according to their position along the crypt–villus axis and ascribe the ongoing dimerization of the transient form after 0.5 h to "slow" cells. In any case, it seem fair to conclude that dimerization continues during the entire lifespan of the transient form of aminopeptidase N. Interestingly, the cross-linked dimer appeared initially as a doublet of bands of about equal intensity (even a third band was barely detectable) (Figure 3). After 20 min labeling, only the transient form of aminopeptidase N exists, so the two major and one minor bands resulting from cross-linking represent dimers of the transient form of the enzyme. After 30 min, one of the bands emerges as the major product of the cross-linked transient form, and after 3 h, this band is virtually the only detectable dimer of the transient form of aminopeptidase N (Figure 3). In contrast, the mature form

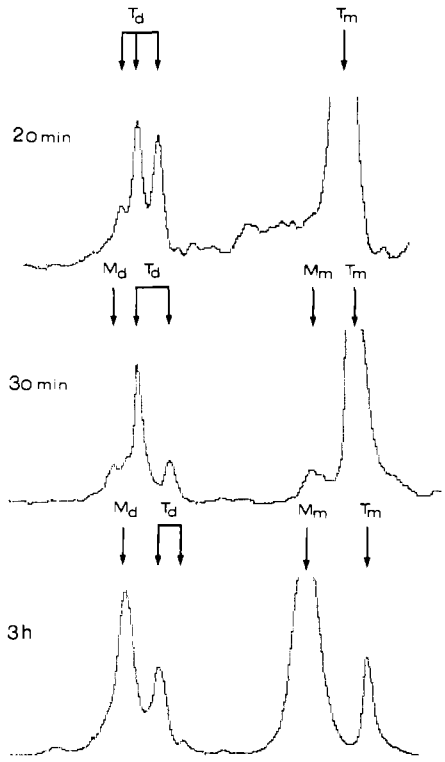

FIGURE 3: Evidence of successive stages in dimerization of aminopeptidase N. Densitometric scanning of SDS/PAGE tracks of aminopeptidase N, immunopurified from cross-linked extracts of mucosal explants, labeled with 100 μCi/mL [^{35}S]methionine for the indicated periods of time. T_m, monomeric transient form; M_m, monomeric mature form; T_d, dimeric transient form; M_d, dimeric mature form.

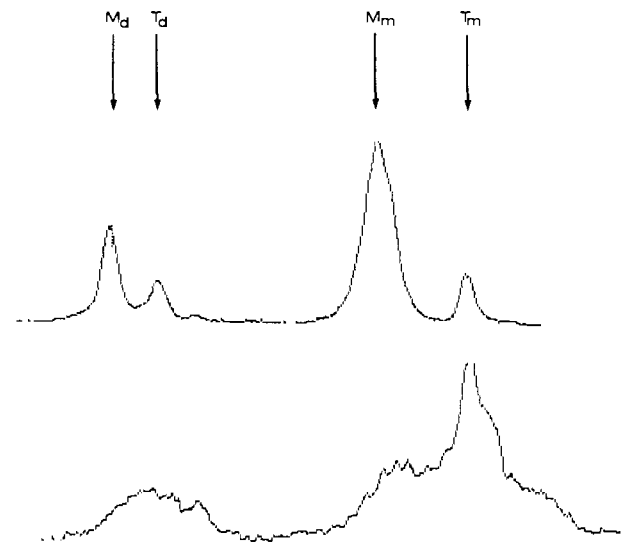

FIGURE 4: Effect of abnormal glycosylation on dimerization of aminopeptidase N. Densitometric scanning of SDS/PAGE tracks of aminopeptidase N, immunopurified from cross-linked extracts of mucosal explants, labeled for 3 h with 100 μCi/mL [^{35}S]methionine in the absence (upper track) or presence (lower track) of 50 mM fructose and 50 μg/mL leupeptin. Fructose resulted in a broad blur of labeled bands, extending above and below the "normal" transient form of aminopeptidase N. Cross-linking resulted in a similar blur of bands the size of the dimer of the transient form. The positions of "normal" monomeric and dimeric transient and mature forms of aminopeptidase N are indicated by arrows.

of the enzyme, appearing after 30 min of labeling, only gives rise to one band of dimers throughout the entire labeling period. The phenomenon is likely to be caused by differences in intra- or interchain cross-linking which is known to produce bands of varying mobility (Tae, 1983). If this is the case, then the transiently occurring dimers of the transient form of aminopeptidase N may represent early stages in the dimerization process. An alternative explanation could be that aminopeptidase N before dimerization transiently, probably during folding, associates with a "chaperone" type of protein.

With dimerization being clearly a posttranslational event in the biosynthesis of aminopeptidase N, it follows that the cotranslational attachment and trimming of N-linked carbohydrate (Kornfeld & Kornfeld, 1985) have occurred before the formation of dimers begin. Since the subunits of aminopeptidase N are held together solely by noncovalent interaction, the carbohydrate (about 10 high-mannose chains per subunit) might be essential for dimerization. To test this hypothesis, mucosal explants were labeled for 3 h in the presence of fructose. It has recently been shown that this monosaccharide induces an abnormal glycosylation of newly made brush border enzymes (Danielsen, 1989), most likely by interfering with the assembly of the high-mannose dolichol phosphate intermediates of glycosylation (Elbein, 1987). In the presence of fructose (and leupeptin, to prevent degradation), aminopeptidase N appears as a broad blur of abnormally glycosylated molecules (Figure 4). Surprisingly, the cross-linking efficiency of the brush border enzyme was only moderately affected by the abnormal glycosylation, as judged by the amount of blurred bands generated with molecular weights of "normal" dimers (dimerization efficiency was 22% compared with 31% for the control). It can therefore be concluded that correct cotranslational glycosylation cannot be essential for the formation of dimers of aminopeptidase N.

Lactase–phlorizin hydrolase is another major brush border enzyme that, unlike aminopeptidase N, is synthesized as a precursor which during intracellular transport, probably in the Golgi complex, is proteolytically cleaved to yield the mature form of the enzyme (Skovbjerg et al., 1984; Danielsen et al., 1984; Naim et al., 1987). Contrary to aminopeptidase N, lactase–phlorizin hydrolase is anchored to the membrane by a segment near its C-terminal end (Mantei et al., 1988). Its quaternary structure has not been rigorously established by membrane reconstitution experiments, but gel filtration analysis of native, detergent-solubilized lactase–phlorizin hydrolase indicates this brush border enzyme to be a dimer (Skovbjerg et al., 1981). This conclusion is supported by the cross-linking experiment shown in Figure 5. Here, the mature form of 160 kDa of lactase–phlorizin hydrolase, seen after 1 h of labeling followed by 3 h of chase, gave rise to a band with a mobility identified with that of a dimer, whereas no bands in the molecular weight range of trimers or tetramers could be detected. In a similar experiment with omission of the 3-h chase period, the precursor of M_r 225K/205K was the prevailing molecular form of lactase–phlorizin hydrolase. Following reaction with dimethyl adipimidate, however, it was not possible to detect bands in SDS/PAGE migrating in a position similar to a cross-linked precursor dimer. From this, it must be concluded that newly made lactase–phlorizin hydrolase only begins to associate into cross-linkable dimers after proteolytic cleavage of the precursor, i.e., at the earliest time in the Golgi complex. Kinetically, this corresponds to a lag period preceding dimerization of about 1 h (Danielsen et al., 1984). We have previously shown that the cleavage of the lactase–phlorizin hydrolase precursor can be inhibited by leupeptin, an observation confirmed by Naim et al. (1987), and that the precursor in the presence of leupeptin is transported in its normal way to the apical cell surface. From this and the result of the present work, it seems that dimerization cannot be an important intracellular event for the targeting of this brush border enzyme. Contrary to aminopeptidase N, however, the high molecular weight precursor of lactase–phlorizin hydrolase can itself be considered a dimer which, with

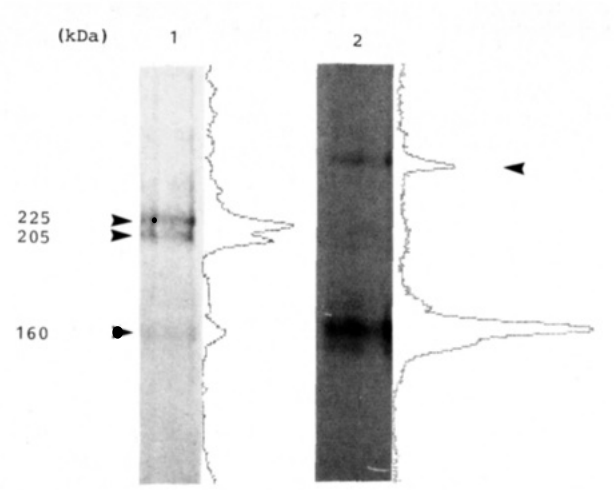

FIGURE 5: Cross-linking of lactase–phlorizin hydrolase. Mucosal explants, labeled for 1 h with 100 μCi/mL [^{35}S]methionine and chased for 0 h (track 1) or 3 h (track 2), were extracted and cross-linked with dimethyl adipimidate. Lactase–phlorizin hydrolase was immunopurified and subjected to SDS/PAGE. Molecular weights of the precursor form (225K/205K) and mature form (160k) of the enzyme are indicated. The arrow to the right indicates the cross-linked dimer of the mature form of lactase–phlorizin hydrolase.

its repeated structure, comprises the mature enzyme and a polypeptide of similar molecular weight, possibly possessing a catalytic activity related to lactase–phlorizin hydrolase (Mantei et al., 1988).

Discussion

In previous studies on the oligomeric assembly of membrane glycoproteins, this event invariably occurs posttranslationally after attachment of carbohydrate, either in the endoplasmic reticulum or in the Golgi complex. As far as endoplasmic reticulum confined assembly is concerned, it seems to be requried for, and possibly even controls, the transport to the Golgi complex, as has been reported for the histocompatibility antigens (Kvist et al., 1982), Ig heavy chains (Bole et al., 1986), and hemagglutinin (Gething et al., 1986; Copeland et al., 1986, 1988). Brush border enzymes are generally transported out of the endoplasmic reticulum at a slow rate, as judged by the time required for the molecular processing from transient to mature form (0.5–1 h) (Danielsen & Cowell, 1985; Hauri et al., 1985; Gorvel et al., 1986). For aminopeptidase N, the slow rate of processing could be explained by its slow kinetics of dimerization if we assume this event to be the rate-controlling step for the exit of newly made enzyme out of the endoplasmic reticulum. For other types of membrane glycoproteins, their final assembly does not occur until after the molecular processing in the Golgi complex. This is the case for the acetylcholine receptor (Merlie, 1984) and the complex subunit structure of acetylcholinesterase (Rotundo, 1984; Toutant & Massoulié, 1987). For lactase–phlorizin hydrolase, the failure of dimethyl adipimidate to cross-link the precursor does not exclude the possibility of an initial, loose association between pairs of precursors already in the endoplasmic reticulum, but it strongly implies that the formation of tightly assembled dimers awaits the Golgi-associated proteolytic cleavage. The difference in cross-linkability beteen the precursor and mature forms of lactase–phlorizin hydrolase may therefore indicate a major conformational change accompanying the cleavage.

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