Biosynthesis of intestinal microvillar proteins

Further characterization of the intracellular processing and transport

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The effect of tunicamycin on synthesis and intracellular transport of pig small intestinal aminopeptidase N (EC 3.4.11.2), sucrase-isomaltase (EC 3.2.1.48 – 10) and maltase-glucoamylase (EC 3.2.1.20) was studied by labelling of mucosal explants with [^{35}S]methionine. The expression of the microvillar enzymes was greatly reduced by tunicamycin but could be partially restored by leupeptin, suggesting the existence of a mechanism whereby newly synthesized, malprocessed enzymes are recognized and degraded. In the presence of tunicamycin, polypeptides likely to represent non-glycosylated forms of the enzymes persisted in the Mg^{2+}-precipitated membrane fraction, indicating that high mannose glycosylation is essential for transport to the microvillar membrane. Treatment of aminopeptidase N and sucrase-isomaltase with endo F reduced the size of the high mannose forms approximately to those seen in the presence of tunicamycin. The complex forms were also sensitive to endo F but did not coincide with the high mannose forms after treatment, indicating that the size difference cannot alone be ascribed to processing of N-linked carbohydrate.

Microvillar enzyme *Biogenesis* *Processing* *Organ culture* *Tunicamycin* *Endo F*

1. INTRODUCTION

Tunicamycin is an antibiotic that inhibits N-linked high mannose glycosylation of proteins [1]. The drug does not directly interfere with intracellular transport of newly synthesized proteins and is therefore suitable for studying the importance of N-linked glycosylation for the transport to the cell surface [2,3]. From the results obtained so far for several secretory and membrane proteins it seems that this type of processing is of variable importance. One explanation for this could be that glycosylation is necessary to a varying extent for the newly synthesized polypeptides to acquire and maintain a stable conformation [2,3].

During synthesis, the microvillar enzymes, including aminopeptidase N (EC 3.4.11.2), sucrase-isomaltase (EC 3.2.1.48–10) and maltase-glucoamylase (EC 3.2.1.20) receive N-linked high mannose glycosylation [4–6]. However, it is not certain to what extent glycosylation determines the route of transport through the cell. By studying the effect of swainsonine on transport, it was concluded that interference with the trimming and complex glycosylation does not seriously affect the transport to the microvillar membrane [7].

Here, the importance of N-linked glycosylation for the transport of microvillar enzymes was studied in organ-cultured explants of intestinal mucosa by using tunicamycin. In addition, endo F, a glycosidase that cleaves both high mannose and complex N-linked oligosaccharides from glycoproteins [8], was used to characterize the differently glycosylated forms of the microvillar enzymes.

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2. EXPERIMENTAL

2.1. *Materials*

Chemicals and equipment for performing organ culture of intestinal explants were obtained as in [9]. Tunicamycin was a product of Sigma (St. Louis, MO) and leupeptin was purchased from Bachem Feinchemikalien AG (Bubendorf). Endo-β-*N*-acetylglucosaminidase F (endo F) from *Flavobacterium meningosepticum* (manufacturer: NEN Chemicals GmbH, Dreieich) was kindly given by Dr P. Bjerrum, Department of Biophysics, Panum Institute, Copenhagen.

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

2.2. *Labelling of explants in organ culture*

Organ culture of pig small intestinal explants [10] was performed as in [9]. In experiments with tunicamycin and leupeptin, the inhibitors were present in the medium at 20 μg/ml and 2 μg/ml, respectively. After culture, the explants were frozen at -80°C until further processing.

2.3. *Treatment with endo F*

Immunoprecipitated aminopeptidase N and sucrase-isomaltase were resuspended in 100 μl of 100 mM sodium phosphate (pH 6.1), containing 50 mM EDTA, 1% 2-mercaptoethanol, 1% Triton X-100 and 0.1% sodium dodecyl sulphate and denatured by boiling for 2 min prior to the addition of 2.5 μl (1.7 units) of endo F. The samples were incubated at 37°C for 20 h. Similarly, pretreated control samples without the addition of glycosidase were incubated in parallel.

2.4. *Other methods*

Fractionation of the labelled explants into a Mg^{2+}-precipitated membrane fraction, a microvillar fraction and a soluble fraction and immunopurification of the enzymes were carried out as in [4,11]. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis was (SDS–PAGE) performed as in [12] and fluorography of the gels as in [13].

3. RESULTS AND DISCUSSION

In the presence of tunicamycin, a polypeptide of M_r 115 000 of aminopeptidase N, not seen in the control explants, could be detected (fig.1). This is of similar size to the primary translation product of the enzyme and thus represents a non-glycosylated form of aminopeptidase N [14]. However, tunicamycin severely reduced the expression of aminopeptidase N (and other microvillar enzymes), both during a 20-min pulse (fig.1) and during continuous labelling for 20 h (not shown). Leupeptin, a proteinase inhibitor of bacterial origin [15], partially restored the expression of the microvillar enzymes, enabling the detection of the labelled enzymes in the various subcellular fractions and thus making it possible to study the importance of N-linked glycosylation for intracellular transport. Fig.2 shows the labelling of aminopeptidase N, sucrase-isomaltase and maltase-glucoamylase from the Mg^{2+}-precipitated membrane fraction and microvillar fraction of controls and tunicamycin-exposed explants. For all

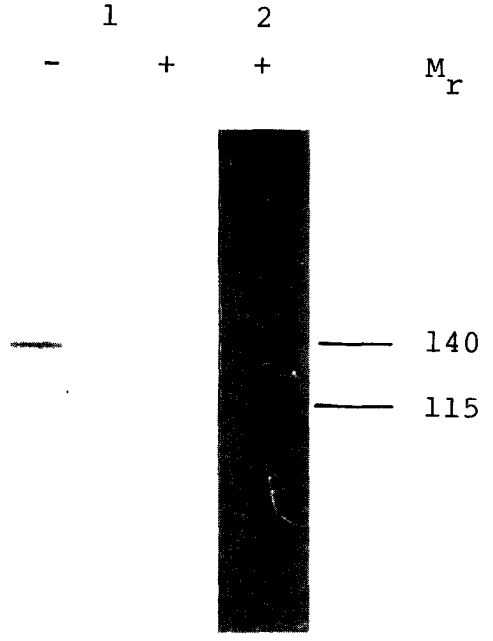

Fig.1. Explants, preincubated for 3 h in the absence (−) or presence (+) of tunicamycin were labelled for 20 min by adding [^{35}S]methionine (220 μCi/ml) to the culture medium. Aminopeptidase N was immunopurified from Triton X-100-solubilized extracts of the explants [4] and subjected to SDS–PAGE. After electrophoresis, the gel was prepared for fluorography. Exposure time: 5 days (1), 30 days (2). Apparent M_r values ($\times 10^{-3}$) are shown.

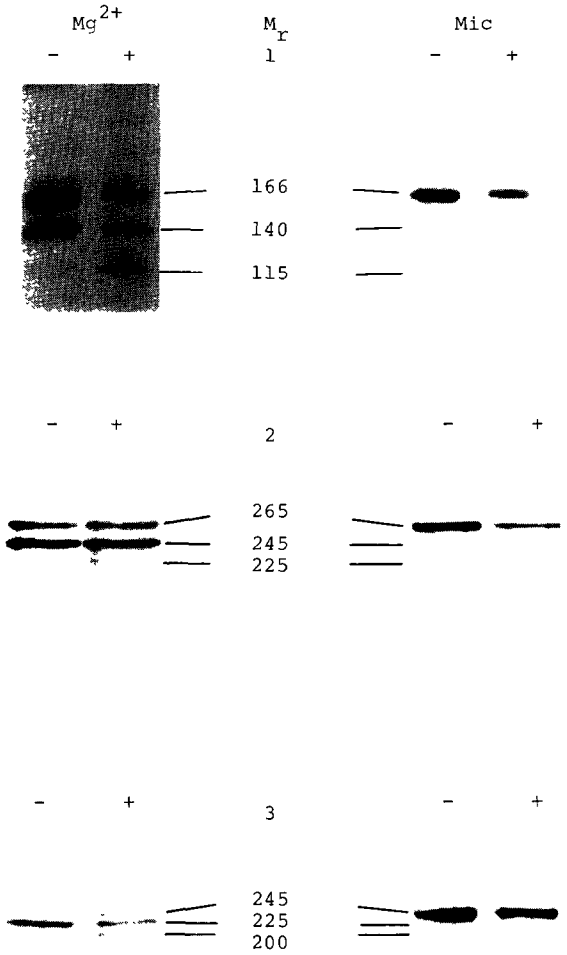

Fig.2. Explants, preincubated for 2 h in the presence of leupeptin and in the absence (−) or presence (+) of tunicamycin, were labelled for 2 h by adding [^{35}S]methionine (100 μCi/ml) to the culture medium. Aminopeptidase N (1), sucrase-isomaltase (2) and maltase-glucoamylase (3) were immunopurified from the Mg^{2+}-precipitated membrane fraction (Mg^{2+}) and the microvillar fraction (Mic) and subjected to SDS-PAGE. After electrophoresis, the gels were prepared for fluorography. Exposure time: 3–7 days. Apparent M_r values ($\times 10^{-3}$) are shown.

3 enzymes, the control pattern was principally similar; the Mg^{2+}-precipitated membrane fraction contained a lower M_r polypeptide, bearing high mannose glycosylation and a polypeptide of higher M_r, representing the mature, complex glycosylated enzyme [4,5]. Only the latter form was present in the microvillar fraction and the soluble fraction did not contain detectable amounts of any of the enzymes (not shown). In the tunicamycin-exposed explants, additional molecular forms of all 3 enzymes were visible in the Mg^{2+}-precipitated membrane fraction. For aminopeptidase N, the polypeptide of M_r 115 000 was seen. For sucrase-isomaltase and maltase-glucoamylase, molecular forms smaller than the high mannose glycosylated forms, were seen, corresponding to polypeptides of M_r 225 000 and 200 000, respectively. In analogy with aminopeptidase N, it seems reasonable to propose that these polypeptides represent newly synthesized, non-glycosylated forms of the two enzymes. This interpretation is supported by the experiment shown in fig.3, where treatment with endo F reduced the size of the high mannose glycosylated forms of aminopeptidase N and sucrase-isomaltase approximately to that of the lower M_r polypeptides, seen in the presence of tunicamycin. Interestingly, the M_r 225 000 form of sucrase-isomaltase appeared as a doublet. It is noteworthy that authors in [16], using cell-free translation, obtained two polypeptides of rabbit sucrase-isomaltase of lower M_r than the mature single chain precursor, probably representing non-

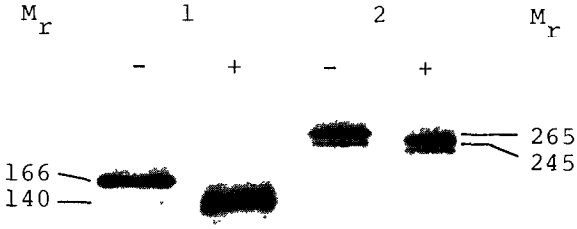

Fig.3. Aminopeptidase N (1) and sucrase-isomaltase (2) were immunopurified from Triton X-100-solubilized extracts of explants [4], labelled for 2 h with [^{35}S]methionine (100 μCi/ml). The purified enzymes were incubated in the presence (+) or absence (−) of endo F as described in section 2 and subjected to SDS-PAGE. After electrophoresis, the gel was prepared for fluorography. Exposure time: 4 days. Apparent M_r values ($\times 10^{-3}$) are shown.

glycosylated forms of the enzyme. Though the doublet seen here most likely reflects a partial cleavage of the hydrophobic anchor, the possibility also exists that it could represent different but closely related gene products.

The additional molecular forms, seen in the presence of tunicamycin, were not detectable in the microvillar or soluble fractions. Even at the high concentration of tunicamycin used, the predominant molecular forms of all 3 enzymes were the high mannose and complex glycosylated polypeptides. This confirms our earlier observation of the inability of tunicamycin to inhibit completely the N-linked glycosylation in the explants [14]. We have no explanation for this phenomenon, but it shows that the normally processed, newly synthesized enzymes are also normally transported to the microvillar membrane. This demonstrates that tunicamycin does not itself directly interfere with the intracellular transport.

Our results indicate that N-linked glycosylation is essential for the transport of newly synthesized enzymes to the microvillar membrane. The ability of leupeptin to partially restore the expression of the enzymes suggests that these are rapidly degraded unless they become high mannose glycosylated during, or shortly after translation; an observation also made for fibronectin and the acetylcholine receptor [3]. The presence of the non-glycosylated forms of the enzymes in the Mg^{2+}-precipitated membrane fraction rather than in soluble form indicates that membrane insertion of the nascent polypeptides takes place even in the absence of glycosylation and that degradation is at least initiated when the polypeptide resides in the membrane. A crinophagic pathway leading to the lysosomal compartment has been proposed to exist in the enterocyte [17], but degradation occurring in the rough endoplasmic reticulum or the Golgi complex would also be compatible with recently proposed models for catabolic regulation of protein expression [18].

The failure of the non-glycosylated enzymes to reach the microvillar membrane might lead to the suggestion that N-linked oligosaccharides act as 'sorting signals', directing the enzymes to their correct destination [3]. However, for several membrane and secretory proteins it has been shown that expression of non-glycosylated forms can take place at the cell surface [3]. Another role for this type of processing would be to ensure that otherwise unstable polypeptides become protected from rapid degradation and therefore survive a first step of 'quality control' in the intracellular transport towards their final destination.

The property of endo F to cleave both high mannose and complex types of N-linked glycosylation [8] makes it useful to examine whether the considerable increase in M_r (about 25 000) from the high mannose to the complex glycosylated form of the enzymes might possibly be attributed to another type of processing. For both aminopeptidase N and sucrase-isomaltase, both the high mannose and the complex glycosylated forms were susceptible to the action of endo F, as expected. However, the fact that the high mannose and complex glycosylated polypeptides for both enzymes exhibited separate bands after endo F treatment, indicates that the M_r difference between these two forms cannot be ascribed solely to structural differences in N-linked carbohydrate; if this had been the case, one should have expected the two bands to coincide after the treatment with endo F. We have previously proposed that O-linked glycosylation also takes place during intracellular transport of the enzymes [7]. Our present results support our earlier observation.

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