Biosynthesis of intestinal microvillar proteins

The effect of swainsonine on post-translational processing of aminopeptidase N

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The post-translational processing of pig small-intestinal aminopeptidase N (EC 3.4.11.2) was studied in organ-cultured mucosal explants. Exposure of the explants to swainsonine, an inhibitor of Golgi mannosidase II, resulted in the formation of a M_r -160 000 polypeptide, still sensitive to endo- β -*N*-acetylglucosaminidase H. Swainsonine caused only a moderate inhibition of transport of the enzyme through the Golgi complex and the subsequent expression in the microvillar membrane. This may imply that the trimming of the high-mannose core and complex glycosylation of *N*-linked oligosaccharides is not essential for the transport of aminopeptidase N to its final destination. A different type of processing was observed to take place in the presence of swainsonine, resulting in a considerable increase in apparent M_r (from 140 000 to 160 000). This processing could not be ascribed to *N*-linked glycosylation, since treatment of the M_r -160 000 polypeptide with endo- β -*N*-acetylglucosaminidase H only decreased its apparent M_r by 15 000. The susceptibility of the mature M_r -166 000 polypeptide, but not the M_r -140 000 polypeptide, to mild alkaline hydrolysis suggests that aminopeptidase N becomes glycosylated with *O*-linked oligosaccharides during its passage through the Golgi complex. Aminopeptidase N was not labelled by [3 H]palmitic acid, indicating that the processing of the enzyme does not include acylation.

In the biosynthesis of membrane and secretory proteins, glycosylation of asparagine residues with high-mannose oligosaccharides is a frequently occurring modification that takes place in the rough endoplasmic reticulum during or shortly after polypeptide synthesis. During the subsequent intracellular transport, the carbohydrate moiety undergoes a series of modifications, resulting in the mature complex glycosylation (Hubbard & Ivatt, 1981). *O*-Linked glycosylation and acylation are two other types of modification that have been described for membrane proteins (Olden *et al.*, 1982; Magee & Schlesinger, 1982).

It has been speculated that carbohydrate structures might act as sorting signals, directing proteins bound for different subcellular compartments to the correct destination (Olden *et al.*, 1982). According to this view, the diversity of various subcellular addresses might be specified by different terminal monosaccharides, the number of branches on the oligosaccharides and the number of oligosac-

charides attached to the polypeptide chain. The best example of a unique carbohydrate found on glycoproteins with a specific subcellular location is mannose 6-phosphate on lysosomal acid hydrolases. The hypothesis has also gained some support by the finding that the blocking of *N*-linked glycosylation (by tunicamycin) in some cases interferes with processing and transport (Olden *et al.*, 1982).

Several intestinal microvillar enzymes, including aminopeptidase N (EC 3.4.11.2), have been shown to undergo high-mannose glycosylation during biosynthesis (Danielsen, 1982; Danielsen *et al.*, 1983*a*). The trimming and complex glycosylation of the *N*-linked oligosaccharides of these enzymes was found to take place in the Golgi complex (Danielsen *et al.*, 1983*b*), but the significance of this processing with regard to the final transport to the microvillar membrane is not known.

Swainsonine is an indolizidine alkaloid, isolated from *Swainsona canescens* (Colegate *et al.*, 1979), that blocks the trimming of *N*-linked oligo-

saccharides and thereby inhibits the further processing of these to the complex state (Elbein *et al.*, 1981). In the present work, the effect of swainsonine on the biogenesis of aminopeptidase N was studied in organ-cultured intestinal-mucosal explants. In addition, the possible occurrence of other types of post-translational modification than *N*-linked glycosylation was investigated.

Materials and methods

Materials

Equipment for performing organ culture, including Trowell's T-8 medium, foetal-calf serum, sterile plastic dishes with grids and [³⁵S]methionine (sp. radioactivity > 1000 Ci/mmol), were obtained as previously described (Danielsen *et al.*, 1982a). [9,10-³H(n)]Palmitic acid (sp. radioactivity 15.2 Ci/mmol) was purchased from NEN Chemicals G.m.b.H., Dreieich, W. Germany, and endo- β -*N*-acetylglucosaminidase H was from Miles Biochemicals, Slough, Bucks., U.K. Swainsonine was isolated from *Swainsona canescens* as described by Colegate *et al.* (1979).

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

Organ culture of intestinal explants

Organ culture of intestinal-mucosal explants (Browning & Trier, 1969) was performed as previously described (Danielsen *et al.*, 1982a). In experiments with swainsonine, the explants were organ-cultured for 4.5–6 h in the presence of 20 μ g of swainsonine/ml; after 3 h of organ culture, [³⁵S]methionine (50 μ Ci/ml) was added to the medium. Control explants without the addition of swainsonine were organ-cultured in parallel. For studying acylation, explants were continuously labelled for 20 h with [³H]palmitic acid (500 μ Ci/ml). Explants labelled with [³⁵S]methionine (20 μ Ci/ml) were organ-cultured in parallel. After culture, the explants were collected and frozen at -80°C until further processing.

Fractionation of explants and purification of aminopeptidase N

Radioactively labelled organ-cultured explants were fractionated as described by Danielsen (1982), with the modification that MgCl₂ was substituted for CaCl₂ in the precipitation of intracellular and basolateral membranes.

Aminopeptidase N was immunopurified by line immunoelectrophoresis against specific antibodies as previously described (Danielsen & Cowell, 1983).

Binding to *Ricinus communis* lectin–Sephacrose 4B

The binding of radioactively labelled explant aminopeptidase N to *Ricinus communis* lectin–Sephacrose 4B (Norén *et al.*, 1977) was performed as follows. The explants were suspended in 350 μ l of 50 mM-Tris/HCl, pH 8.0, containing 3% (v/v) Triton X-100. After 30 min of solubilization at 4°C, the suspension was centrifuged for 3 min in a Beckman Microfuge. To the supernatant was then added 200 μ l of *Ricinus communis* lectin–Sephacrose 4B [about 5 mg of lectin/ml of gel, prepared by a CNBr procedure (March *et al.*, 1974)]. After incubation for 2 h at 4°C, the non-adsorbed fraction was collected and the lectin–Sephacrose was washed in 2 $\times$ 1 ml of the above buffer. Finally the lectin–Sephacrose was resuspended in 200 μ l of the above buffer containing 1 M-lactose and incubated for 1 h at 4°C. Aminopeptidase N was immunopurified from both the non-adsorbed and the desorbed fractions of the binding experiment.

Mild alkaline hydrolysis

Immunopurified aminopeptidase N was subjected to mild alkaline hydrolysis. A 100 μ l enzyme sample in 0.1 M-KOH was incubated at room temperature (20°C) for 20 h. After incubation, the reaction mixture was neutralized by addition of equimolar amounts of HCl. A control sample without the addition of KOH was incubated in parallel.

Other methods

Digestion of aminopeptidase N with endo- β -*N*-acetylglucosaminidase H was performed as described by Danielsen (1982). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in 10%-acrylamide slab gels was performed as described by Laemmli (1970), and fluorography of gels as described by Bonner & Laskey (1974).

Results

Effect of swainsonine on processing and intracellular transport of aminopeptidase N

Fig. 1 shows the effect of swainsonine on the forms of aminopeptidase N of different *M_r* after 3 h of continuous labelling with [³⁵S]methionine. Aminopeptidase N from the control explants is seen in two forms, one of *M_r* 140 000, which is the earliest detectable form in pulse-chase labelling experiments and which is high-mannose glycosylated, and one of *M_r* 166 000 that represents the enzyme in its mature complex glycosylated state, which appears after about 40 min of labelling and accumulates during further labelling (Danielsen, 1982). In swainsonine-treated explants, a similar *M_r*-140 000 polypeptide was seen, but the polypeptide of higher *M_r* was repeatedly observed to have a higher mobility

Fig. 1. Effect of swainsonine on glycosylation of aminopeptidase N

Aminopeptidase N was immunopurified from a Triton X-100 extract of whole explants, labelled with [35 S]methionine for 3 h in the presence (+) or absence (–) of swainsonine. The purified enzyme was subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. After electrophoresis, the gel was prepared for fluorography. Apparent M_r values ($\times 10^{-3}$) are shown.

in the electrophoretic system than the M_r -166 000 polypeptide from the control explants. This corresponds to a M_r of 160 000.

The effect of swainsonine on the intracellular transport of aminopeptidase N was studied by continuous labelling for 2 h 50 min in the presence or absence of the inhibitor. In the controls, only 28% of the labelled enzyme was in the Mg^{2+} -precipitated membrane fraction, whereas 72% was in the microvillar fraction. Swainsonine moderately inhibited the intracellular transport of aminopeptidase N, since swainsonine-treated explants had 41% of the labelled enzyme in the Mg^{2+} -precipitated membrane fraction. This effect could not be ascribed to a decreased biosynthesis of aminopeptidase N owing to the presence of swainsonine, since the total amount of radioactivity incorporated into the enzyme was 95% of the amount incorporated into the control explants. When analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, the Mg^{2+} -precipitated membrane fraction of swainsonine-treated explants was found to contain both the M_r -140 000 and -160 000 polypeptides of aminopeptidase N, whereas the microvillar fraction contained only the latter form (results not shown).

Sensitivity of aminopeptidase N to endo- β -N-acetylglucosaminidase H

Fig. 2 shows the effect of treatment with endo- β -N-acetylglucosaminidase H on the two M_r forms

Fig. 2. Effect of endo- β -N-acetylglucosaminidase H on aminopeptidase N

Aminopeptidase N was immunopurified from a Triton X-100 extract of whole explants, labelled with [35 S]methionine for 90 min in the presence of swainsonine. The purified enzyme was either treated with endo- β -N-acetylglucosaminidase H (+) or incubated in parallel without the addition of the glycosidase (–) and then subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. After electrophoresis, the gel was prepared for fluorography. Apparent M_r values ($\times 10^{-3}$) are shown.

of aminopeptidase N from swainsonine-treated explants. The mobility of the high-mannose-glycosylated M_r -140 000 polypeptide was greatly increased, to correspond to an apparent M_r of 115 000, which is the size of the non-glycosylated primary translation product of aminopeptidase N (Danielsen *et al.*, 1982b). Also, the higher- M_r polypeptide had its mobility significantly increased by the treatment with endo- β -N-acetylglucosaminidase H: the apparent M_r after treatment was about 145 000. In a similarly designed experiment, the M_r -166 000 polypeptide of aminopeptidase N from control explants was found to be insensitive to the action of the glycosidase (Danielsen, 1982).

Sensitivity of aminopeptidase N to mild alkaline hydrolysis

Mild alkaline hydrolysis was observed to affect the gel-electrophoretic pattern of aminopeptidase N (radioactively labelled in the absence of swain-

sonine) (Fig. 3). Upon treatment, a blur of labelled polypeptides was seen, extending between the M_r -140 000 and -166 000 polypeptides, whereas in the control sample these were clearly separated. The blurring did not extend below the M_r -140 000 polypeptide. Alkaline hydrolysis under slightly harsher conditions (37°C instead of 20°C) caused an extensive degradation of both forms of the enzyme (results not shown).

Binding of aminopeptidase N to Ricinus communis lectin-Sepharose

The results of the binding of explant aminopeptidase N to the lectin-Sepharose is shown in Fig. 4(a). Only the M_r -160 000 polypeptide of the enzyme obtained from swainsonine-treated explants bound to the lectin-Sepharose, whereas the M_r -140 000 polypeptide was exclusively found in the

Fig. 3. Effect of mild alkaline hydrolysis on aminopeptidase N

Aminopeptidase N was immunopurified from a Triton X-100 extract of whole explants, labelled with [35 S]methionine for 90 min (in the absence of swainsonine). The purified enzyme was either subjected to mild alkaline hydrolysis (+) or incubated in parallel at neutral pH (–) before sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. After electrophoresis, the gel was prepared for fluorography. Apparent M_r values ($\times 10^{-3}$) are shown.

Fig. 4. Binding of aminopeptidase N to *Ricinus communis* lectin-Sepharose 4B

Aminopeptidase N from explants labelled for 3 h in the presence (a) or absence (b) of swainsonine with [35 S]methionine was incubated with the lectin-Sepharose as described in the Materials and methods section. The enzyme, purified from the non-adsorbed (1) and desorbed (2) fractions, was subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. After electrophoresis, the gel was prepared for fluorography. Apparent M_r values ($\times 10^{-3}$) are shown.

non-adsorbed fraction. An analogous result was seen for the M_r -166 000 and -140 000 polypeptides of control explants (Fig. 4b).

Labelling with [^3H]palmitic acid

A Mg^{2+} -precipitated membrane fraction and a microvillar fraction were obtained from explants labelled with [^3H]palmitic acid. When subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (without prior addition of 2-mercaptoethanol and boiling of the sample), no labelled bands in either of the fractions could be detected even after prolonged exposure of the fluorograph. Similar fractions from explants labelled in parallel with [^{35}S]methionine showed many labelled bands, including one of M_r 166 000, likely to represent aminopeptidase N (results not shown).

Discussion

All microvillar enzymes so far studied in the organ-culture system by pulse-chase labelling have been found to become high-mannose glycosylated during or shortly after synthesis (Danielsen, 1982; Danielsen *et al.*, 1983a). During their intracellular transport to the microvillar membrane they gain resistance to endo- β -*N*-acetylglucosaminidase H, indicating a trimming and addition of complex sugars to *N*-linked oligosaccharides. This processing is accompanied by a significant increase in apparent M_r (20 000–25 000), allowing the possibility that the microvillar enzymes undergo another type of modification also. The effect of monensin on post-translational modification and transport of microvillar enzymes has suggested the involvement of the Golgi complex in these processes (Danielsen *et al.*, 1983b). Monensin, however, must be considered a rather indiscriminate destroyer of the processing functions of the Golgi complex, as shown by its severe effect on the ultrastructure of the organelle. Monensin is consequently an unsuitable drug for studying the importance in protein biogenesis of the individual processing functions known to be associated with the Golgi complex.

Swainsonine specifically interferes with the processing of *N*-linked oligosaccharides from the transient endo- β -*N*-acetylglucosaminidase-H-sensitive to the mature, resistant, form (Elbein *et al.*, 1981; Tulsiani *et al.*, 1982). It thus provides an opportunity to study the significance of this event for the intracellular transport and subsequent expression in the microvillar membrane of the enzymes. In the present work, swainsonine at a concentration above that reported to inhibit mannosidase II completely (Tulsiani *et al.*, 1982) did not interfere with synthesis and high-mannose glycosylation of aminopeptidase N. The final form of the enzyme in the presence of swainsonine was a polypeptide of well-defined size,

only marginally smaller than the mature form of aminopeptidase N observed in controls.

The experiment with endo- β -*N*-acetylglucosaminidase H shows that swainsonine did inhibit the processing of the *N*-linked oligosaccharides. In contrast with the M_r -166 000 polypeptide of aminopeptidase N, which is resistant to the glycosidase (Danielsen, 1982), the significant increase in electrophoretic mobility of the M_r -160 000 polypeptide seen in the presence of swainsonine suggests a decrease in apparent M_r of about 15 000. However, in the same experiment, the high-mannose-glycosylated polypeptide of M_r 140 000 had its apparent M_r decreased to 115 000, which is the size of the non-glycosylated translation product of aminopeptidase N (Danielsen *et al.*, 1982b). This confirms our previous finding that the M_r difference between these two forms of the enzyme can be accounted for solely by high-mannose glycosylation, suggesting that about ten high-mannose oligosaccharides become attached to asparagine residues on the polypeptide. The smaller change in M_r of the M_r -160 000 polypeptide in response to treatment with endo- β -*N*-acetylglucosaminidase H is indicative that the processing of aminopeptidase N from M_r 140 000 to M_r 160 000 cannot be ascribed to *N*-linked glycosylation; otherwise, the M_r -160 000 polypeptide should have been expected to increase its electrophoretic mobility to that of the non-glycosylated translation product on treatment. Gross *et al.* (1983), studying the biogenesis of α_1 -antitrypsin in rat hepatocytes, found that swainsonine did not completely block the processing of the *N*-linked oligosaccharides, but gave rise to the formation of hybrid carbohydrate chains that were partially complex-glycosylated but nevertheless sensitive to endo- β -*N*-acetylglucosaminidase H. Such hybrid carbohydrate structures might also be present on the M_r -160 000 polypeptide of aminopeptidase N, seen in the presence of swainsonine. However, the decrease in size of the M_r -160 000 polypeptide in response to endo- β -*N*-acetylglucosaminidase H (about 15 000) is even smaller than that of the high-mannose-glycosylated M_r -140 000 form, suggesting that no further processing of *N*-linked oligosaccharides occurs.

The swainsonine-induced inhibition of complex glycosylation only moderately impaired the passage of aminopeptidase N through the Golgi complex to the microvillar membrane. This implies that the final trimming and subsequent complex glycosylation of *N*-linked oligosaccharides may not be essential steps for the correct transport of the enzyme to the microvillar membrane. A similar observation was made by Gross *et al.* (1983).

The considerable increase in apparent M_r (20 000) of aminopeptidase N that occurs in the presence of

swainsonine during passage of the newly synthesized polypeptide through the Golgi complex is strongly suggestive of another type of processing taking place concomitant with the intracellular transport. To determine the nature of this processing, labelled aminopeptidase N was subjected to mild alkaline hydrolysis, a treatment used to distinguish between *N*- and *O*-linked oligosaccharides (Spiro, 1970). The resulting blur of labelled bands between the M_r -166 000 and -140 000 polypeptides most likely represents aminopeptidase N molecules that had lost variable amounts of *O*-linked oligosaccharides. A more complete hydrolysis of *O*-linked oligosaccharides was impossible to monitor, because only slightly harsher conditions than those used caused cleavages of the polypeptide chain. Some *O*-linked oligosaccharides have been found to be resistant to mild alkaline hydrolysis (Swann *et al.*, 1977), whereas some *N*-linked oligosaccharides are liberated under these conditions (Ogata & Lloyd, 1982). The resulting blur of polypeptides in the present experiment, however, is unlikely to be caused by hydrolysis of *N*-linked oligosaccharides from the M_r -166 000 polypeptide. If some *N*-linkages were labile at alkaline pH, one should have expected the blur to extend also below the M_r of the high-mannose-glycosylated polypeptide (140 000) towards the M_r of the non-glycosylated form (115 000). The glycosylation of aminopeptidase N was also studied by a lectin-binding experiment. The *Ricinus communis* lectin recognizes galactose residues (Nicolson & Blaustein, 1972), which are present in complex *N*-linked and *O*-linked oligosaccharides, but are not a constituent of the high-mannose glycosylation (Hubbard & Ivatt, 1981; Olden *et al.*, 1982). As expected, the high-mannose-glycosylated polypeptide of M_r 140 000 of aminopeptidase N did not bind to the lectin-Sepharose. The finding that the M_r -160 000 polypeptide from swainsonine-treated explants bound equally well to the lectin-Sepharose as did the M_r -166 000 polypeptide is strongly indicative that galactose residues are attached to the enzyme during its processing from the M_r -140 000 to the M_r -160 000 polypeptide.

Another type of post-translational modification, acylation, has been shown to take place during intracellular transport (Magee & Schlesinger, 1982). Since this processing has been found to be active not only for viral proteins but also for eukaryotic plasma-membrane proteins (Schlesinger *et al.*, 1980), microvillar enzymes might become acylated during intracellular transport. As fatty acid linkages, like *O*-linked oligosaccharides, are susceptible to alkaline hydrolysis, acylation could partially explain the result shown in Fig. 3. However, the labelling experiment with [3 H]palmitic acid clearly showed that no fatty acid becomes covalently attached to aminopeptidase N during its biogenesis. Even if only

1 molecule of fatty acid per polypeptide chain had been incorporated it would have been detectable under the conditions used. No other microvillar membrane components were labelled except for the lipid. It therefore seems reasonable to propose that at least the major part of the processing of aminopeptidase N, seen in the presence of swainsonine, can be ascribed to *O*-linked glycosylation.

During intracellular transport, *O*-linked glycosylation occurs concomitantly with the trimming and complex glycosylation of *N*-linked oligosaccharides, i.e. during the passage of the newly synthesized polypeptide through the Golgi complex. Similar processing has been described for the E1 glycoprotein of the coronavirus (Niemann & Klenk, 1981). Interestingly, this protein receives only *O*-linked glycosylation during its intracellular transport, whereas its fellow viral glycoprotein, E2, is exclusively modified by *N*-linked glycosylation. Our finding of aminopeptidase N being simultaneously modified by both types of glycosylation must indicate that these are not characteristic types of processing of separate pathways leading through the Golgi complex, but must be understood rather in terms of susceptible residues for either type of glycosylation on the polypeptide chain. Another type of plasma-membrane protein, glycophorin A, also becomes glycosylated with both *N*- and *O*-linked oligosaccharides during biogenesis (Gahmberg *et al.*, 1980).

As mentioned above, several microvillar enzymes have been found to show a significant increase in apparent M_r like aminopeptidase N in the intracellular processing step that simultaneously renders them resistant to endo- β -*N*-acetylglucosaminidase H. It is therefore tempting to assume that other microvillar enzymes during biogenesis receive both *N*- and *O*-linked glycosylation. For dipeptidyl peptidase IV (EC 3.4.14.5) and sucrase (EC 3.2.1.48)-isomaltase (EC 3.2.1.10), we have observed that molecular forms of about the size of the mature enzymes were synthesized in the presence of swainsonine, indicating a processing beyond the high-mannose glycosylation also for these enzymes. For the latter of these enzymes, it has been found that the threonine residue at position 11 near the *N*-terminal end of the isomaltase subunit is probably glycosylated (Frank *et al.*, 1978; Sjöström *et al.*, 1982), supporting this view.

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