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Biosynthesis of intestinal microvillar proteins

Processing of aminopeptidase N by microsomal membranes

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The biosynthesis of small-intestinal aminopeptidase N (EC 3.4.11.2) was studied in a cell-free translation system derived from rabbit reticulocytes. When dog pancreatic microsomal fractions were present during translation, most of the aminopeptidase N synthesized was found in a membrane-bound rather than a soluble form, indicating that synthesis of the enzyme takes place on ribosomes attached to the rough endoplasmic reticulum. The microsomal fractions process the M_r-115000 polypeptide, which is the primary translation product of aminopeptidase N, to a polypeptide of M_r 140000. This was found to be sensitive to the action of endo-β-*N*-acetylglucosaminidase H (EC 3.2.1.96), showing that aminopeptidase N undergoes transmembrane glycosylation during synthesis. The position of the signal sequence in aminopeptidase N was determined by a synchronized translation experiment. It was found that microsomal fractions should be added before about 25% of the polypeptide was synthesized to ensure processing to the high-mannose glycosylated form. This suggests that the signal sequence is situated in the *N*-terminal part of the aminopeptidase N. The size of the cell-free translation product in the absence of microsomal fractions was found to be similar to that of one of the forms of the enzyme obtained from tunicamycin-treated organ-cultured intestinal explants.

Studies on the early stages in the life cycle of small-intestinal aminopeptidase N (EC 3.4.11.2) have shown that this enzyme occurs in three different molecular forms during its biogenesis and that these forms represent aminopeptidase N in variously processed intracellular states (Sjöström *et al.*, 1983). The primary translation product of the enzyme is of M_r 115000, corresponding to the size of the non-glycosylated A-subunit of mature aminopeptidase N (Danielsen *et al.*, 1982*b*). A transient high-mannose glycosylated polypeptide of M_r 140000 has been found as the earliest detectable form of the enzyme in pulse-labelled organ-cultured intestinal explants (Danielsen, 1982). The conversion into the mature form of M_r 166000 takes place before the enzyme reaches the microvillar membrane. The finding that these glycosylated forms were membrane-bound suggests that membrane insertion is an early event in the biogenesis of aminopeptidase N. However, it is not known whether this process occurs co- or post-translationally, i.e. whether synthesis takes place on free polyribosomes or on ribosomes attached to the rough endoplasmic reticulum. Neither is it known where the signal sequence responsible for membrane insertion is located on the aminopeptidase-N polypeptide, or if the signal is cleaved during synthesis.

In the present work, cell-free translation of aminopeptidase N was performed in the presence of dog pancreatic microsomal fractions in order to study the early events in the biosynthesis of aminopeptidase N.

Materials and methods

Materials

Chemicals and equipment for performing organ culture of intestinal explants were obtained as previously described (Danielsen *et al.*, 1982*a*). A cell-free translation and processing kit, consisting of nuclease-treated rabbit reticulocyte lysate, 'translation cocktail', dog pancreatic microsomal fractions and [^{35}S]methionine (specific radioactivity > 1000 Ci/mmol), was obtained from NEN Chemicals, Dreieich, W. Germany. Tunicamycin and 7-methylguanosine 5'-monophosphate were products of Sigma Chemical Co., St. Louis, MO, U.S.A.

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

Labelling of explants in organ culture

Organ culture of pig small-intestinal explants (Browning & Trier, 1969) was performed as previously described (Danielsen *et al.*, 1982*a*). In experiments with tunicamycin, explants were cultured for 4h in the presence of tunicamycin (20μg/ml); 1h before culture was stopped, [^{35}S]-methionine (200μCi/ml) was added to the medium. After culture, a Triton X-100 extract of total explant was prepared as described by Danielsen (1982).

Cell-free synthesis of aminopeptidase N

Crude RNA was isolated from whole pig small intestine and translated in a cell-free system as previously described (Danielsen *et al.*, 1982*b*). When translation was performed in the presence of dog pancreatic microsomal fractions (Jackson & Blobel, 1977), these were present at a concentration of 10 A_{260} units/ml. Synchronized translation experiments were performed at 22°C, and synchrony was accomplished by the addition of 7-methylguanosine 5'-monophosphate (3mM) 5min after the start of the incubation (Rothman & Lodish, 1977). After incubation, translation mixtures containing microsomes were centrifuged at 133000*g* for 30min (30° A-100 rotor, Beckman Airfuge). The supernatant (soluble fraction) and pellet (membrane fraction) were collected. The membrane fraction was solubilized in 200μl of 25mM-Tris/HCl, pH7.3, containing 5% (w/v) Triton X-100, before immunopurification of aminopeptidase N.

Other methods

Immunopurification of aminopeptidase N from cell-free translation mixtures and from organ-cultured intestinal explants, treatment with endo-β-*N*-acetylglucosaminidase H, sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and fluorography of gels were performed as described elsewhere (Danielsen *et al.*, 1982*a*,*b*; Danielsen, 1982).

Results and discussion

Aminopeptidase N is translocated across microsomal membranes and glycosylated

Fig. 1 shows aminopeptidase N, translated in the cell-free system, isolated from the soluble and membrane fractions of a translation mixture containing dog pancreatic microsomal fractions (aminopeptidase N labelled in organ-cultured intestinal explants is shown for comparison). Only a minor portion of the aminopeptidase N synthesized occurred in the soluble fraction and was found in the form of a M_r-115000 polypeptide, the translation product of the enzyme in the absence of microsomal fractions (Danielsen *et al.*, 1982*b*). In addition to this polypeptide, aminopeptidase N from the membrane fraction was found to contain another component, of M_r 140000, which is equal in size to a polypeptide of the enzyme isolated from organ-cultured explants. This form of aminopeptidase N was found to be the earliest detectable after a 10min pulse of [^{35}S]methionine and to be intracellularly located (Danielsen, 1982). In addition, it was found to be susceptible to treatment with endo-β-*N*-acetylglucosaminidase H, in contrast with the M_r-166000 polypeptide of the enzyme, which represents aminopeptidase N in its mature 'complex' state of glycosylation. Similarly, the M_r-140000 polypeptide of aminopeptidase N, synthesized in the cell-free system, was sensitive to the action of endo-β-*N*-acetylglucosaminidase H; on treatment, only the polypeptide of M_r 115000 could be seen (Fig. 2).

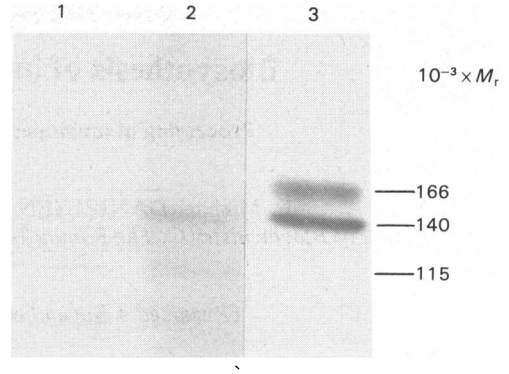

Fig. 1. *Cell-free translation of aminopeptidase N in the presence of dog pancreatic microsomal fractions*
Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of aminopeptidase N, isolated from the supernatant (lane 1) and pellet (lane 2) fractions of a cell-free translation mixture performed as described in the Materials and methods section. Lane 3 shows aminopeptidase N, isolated from intestinal explants organ cultured for 90min in the presence of [^{35}S]methionine (100μCi/ml). Exposure time: 7 days. Apparent M_r values ($\times 10^{-3}$) are shown.

When added to cell-free translation systems, dog pancreatic microsomal fractions are able to perform some of their characteristic functions *in vivo* in the processing of nascent polypeptide chains, most notably cleavage of the *N*-terminal signal from the polypeptide and the addition of high-mannose or core oligosaccharides to asparagine residues (Jackson & Blobel, 1977). The results of the present work directly show that high-mannose glycosylation is an early event in the biogenesis of aminopeptidase N, as has previously been suggested (Danielsen *et al.*,

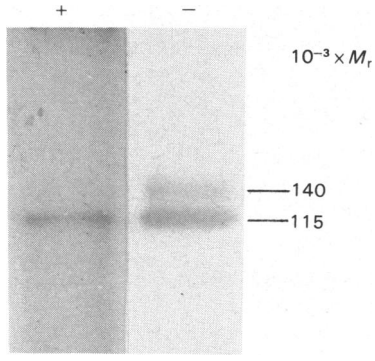

Fig. 2. *Sensitivity of aminopeptidase N to endo-β-N-acetylglucosaminidase H*

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of aminopeptidase N, isolated from the pellet fraction of a cell-free translation mixture. The purified enzyme was incubated for 20h at 37°C with endo-β-*N*-acetylglucosaminidase H as described in the Materials and methods section (+). A control sample without the addition of the glucosidase was incubated in parallel (−). Exposure time: 30 days. Apparent M_r values ($\times 10^{-3}$) are shown.

1981; Danielsen, 1982). The fact that high-mannose glycosylation can be performed in a cell-free translation system by using microsomal fractions is indicative that translation of aminopeptidase N *in vivo* takes place on ribosomes attached to the rough endoplasmic reticulum. Furthermore, since the protein glycosylation occurring in microsomal fractions has been found to take place on the luminal side of the membrane, this process is also evidence for a transmembrane insertion of the nascent polypeptide into the membrane (Snider & Robbins, 1982).

The rather large difference in M_r between the non-glycosylated and high-mannose-glycosylated forms of aminopeptidase N (approx. 25000) suggests that about 10 asparagine residues on the enzyme become glycosylated. Since no intermediate bands between the M_r-115000 and M_r-140000 polypeptides can be detected, these individual glycosylation steps must occur in a rapid order. If the glycosylation proceeds co-translationally, this must imply that the asparagine residues that become glycosylated are clustered on the polypeptide. An alternative explanation is that glycosylation of the asparagine residues simultaneously takes place post-translationally.

The processing of the M_r-115000 polypeptide to that of M_r 140000 never occurred completely, even with increased concentrations of microsomal fractions present during translation. In pulse-labelled organ-cultured explants, the non-glycosylated polypeptide of M_r 115000 is never visible unless tunicamycin is present. The result obtained in the cell-free translation system is therefore most probably explained by a limited effectiveness of this system.

The signal in aminopeptidase N is located in the N-terminal part of the polypeptide

Synchronized translation was performed at 22°C rather than at 37°C to obtain a good time resolution of the events. At 22°C, the reticulocyte lysate system requires about 75min to complete the synthesis of the aminopeptidase-N polypeptide (results not shown). Assuming a polypeptide length of 1000 residues, this corresponds to an elongation rate of about 13 amino acids/min. Under the same conditions, a similar value was observed for the synthesis of the erythrocyte band-3 protein and the vesicular-stomatitis-virus G protein (Braell & Lodish, 1982*a*).

Fig. 3. shows the result of the synchronized translation experiment. After a 5min 'window' of initiation, synchrony was accomplished by the addition of 7-methylguanosine 5′-monophosphate. In addition, translation was terminated after 75min to ensure that initiations that may have occurred after the first 5min would not be completed. As Fig. 3 shows, only when microsomal fractions were added pre- or co-translationally was synthesized aminopeptidase N in the membrane fraction; when they were added post-translationally, aminopeptidase N was exclusively found in the soluble fraction, and only in the form of the M_r-115000 polypeptide. When microsomal fractions were added co-translationally, only those present within 10min after initiation were capable of yielding the M_r-140000 polypeptide in amounts comparable with those obtained by pre-translational addition of microsomal fractions. When microsomal fractions were added 20min after initiation, only the M_r-115000 polypeptide was significantly present in the membrane fraction, and after 40min even this polypeptide was scarcely present. We interpret these findings as follows: since only the appearance of the glycosylated M_r-140000 polypeptide is indicative of membrane translocation, microsomal fractions need be present not later than 10–20min after initiation for this process to occur. This means that the signal for membrane recognition must be situated within the first 25% of the molecule, i.e. clearly in the *N*-terminal part of the polypeptide. In a study on the location of the signal in ovalbumin (385 amino acids), Braell & Lodish (1982*b*), by placing the signal before residue 150, concluded that ovalbumin utilizes an *N*-terminal signal. In contrast, in a similarly designed experiment on the erythrocyte band-3 protein, which has a M_r (100000) comparable with that of aminopeptidase N, Braell &

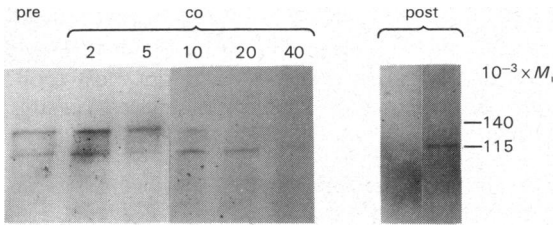

Fig. 3. *Synchronized cell-free translation of aminopeptidase N*

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of aminopeptidase N isolated from the pellet fractions of a cell-free translation mixture with dog pancreatic microsomes added pre- (pre), co- (co, after the indicated time in min) or post-translationally (post). (For experimental details, see the Materials and methods section.) The lane furthest to the right shows aminopeptidase N isolated from the supernatant fraction when microsomal fractions were added post-translationally. Exposure time: 30 days. Apparent M_r values ($\times 10^{-3}$) are shown.

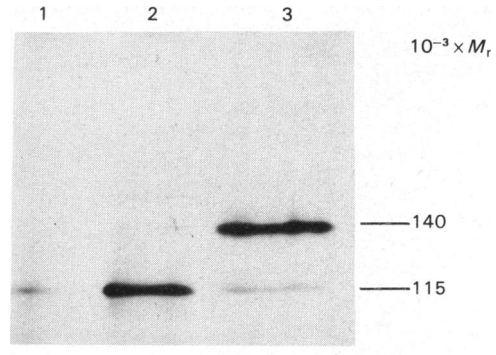

Fig. 4. *M_r comparison of non-glycosylated aminopeptidase N from tunicamycin-exposed organ-cultured explants and from a cell-free translation system*

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of aminopeptidase N isolated from a cell-free translation system (lanes 1 and 2, different loadings) and from tunicamycin-exposed organ-cultured explants (lane 3). (For experimental details, see the Materials and methods section.) Exposure time: 30 days. Apparent M_r values ($\times 10^{-3}$) are shown.

Lodish (1982*a*) found that transmembrane insertion could be accomplished by microsomal fractions added as late as 35–40 min after initiation, some 55–60% of the distance from the *N*-terminal. This led them to suggest the existence of an internal signal in this protein.

The finding that the M_r-115000 polypeptide was significantly associated with the microsomal fractions when these had been added 20 min after initiation must indicate that the nascent polypeptide chain was still in a conformation allowing the signal (or some other part of the molecule) to bind to the microsomal membrane but prohibiting the proper translocation of the polypeptide. This property was markedly decreased when microsomal fractions were added 40 min after initiation and was completely lost when addition occurred post-translationally. An interpretation could be that there are two conformational changes in the translation process for aminopeptidase N; the first (10–20 min after initiation) preventing the normal processing by the microsomes and the second (40 min after initiation) totally obscuring the affinity for the membrane.

Evidence favouring the existence of a non-cleaved signal in aminopeptidase N

Because of glycosylation, a direct comparison of the size of aminopeptidase N synthesized in organ culture and in a cell-free translation system cannot determine whether or not an *N*-terminal signal sequence on the enzyme is cleaved during the membrane insertion of the newly synthesized polypeptide. Tunicamycin is an inhibitor of asparagine-linked high-mannose glycosylation (Gibson *et al.*, 1980), but does not interfere with membrane insertion (Warren, 1981). Tunicamycin was found to inhibit the biosynthesis of aminopeptidase N in organ-cultured intestinal explants (E. M. Danielsen, unpublished work), and a significant amount of the enzyme synthesized in the presence of this inhibitor was of lower M_r, indicating that it lacked asparagine-linked glycosylation.

Fig. 4 shows a M_r comparison of aminopeptidase N synthesized in the cell-free translation system and in organ-cultured intestinal explants in the presence of tunicamycin. No detectable difference in apparent M_r between the polypeptide of lower M_r from the explants and the non-glycosylated one of M_r 115000 was observed. The feasibility of this method evidently depends on the resolution power of the electrophoretic system used. In the apparent M_r range of 100000–130000 of the gel shown, a mobility difference of 1 mm corresponds to a M_r difference of 2500. Thus the method is not accurate enough to recognize M_r differences < 1000, whereas M_r differences > 1500, a value smaller than most cleaved signal sequences reported (Leader, 1979), would be detectable. The absence of a detectable M_r difference between the two forms of aminopeptidase N might therefore indicate that no cleavage of the signal in this enzyme occurs during the insertion into the membrane.

Of proteins synthesized on membrane-bound ribosomes, most of those so far studied have been found to have their signal sequence cleaved during the translocation across the membrane (Sabatini *et al.*, 1982). However, signal cleavage does not always appear to accompany this process; several proteins have been reported to possess a permanent signal. Thus, as mentioned above, the band-3 protein of the erythrocyte membrane was found to have an internal signal (Braell & Lodish, 1982*a*). There are also examples of proteins with permanent *N*-terminal signals, for instance ovalbumin (Braell & Lodish, 1982*b*) and cytochrome *P*-450 (Bar-Nun *et al.*, 1980). Aminopeptidase N, like other microvillar hydrolases, has been shown to be anchored to the membrane by an *N*-terminally located segment of M_r 3500–4500 (Kenny & Maroux, 1982). It remains to be investigated whether this hydrophobic anchor of mature aminopeptidase N is indeed identical with the *N*-terminal of the primary translation product of the enzyme. If this is the case, it suggests an anchoring role for the signal in the mature aminopeptidase N and may indicate by which mechanism the membrane insertion takes place during synthesis.

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