Biosynthesis of intestinal microvillar proteins

Translational evidence *in vitro* that aminopeptidase N is synthesized as a M_r -115 000 polypeptide

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A crude RNA fraction, prepared from pig small intestine, was found to be more efficient than a fraction enriched in polyadenylated RNA in directing translation of polypeptides with $M_r > 100\,000$ in a rabbit reticulocyte lysate system. Aminopeptidase N (EC 3.4.11.2) synthesized *in vitro* was immunopurified from the translation mixture and analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. It was found to have an apparent M_r of 115 000 regardless of whether the translation was performed in the absence or presence of proteinase inhibitors. This result contradicts the possibility of aminopeptidase N being synthesized as a large single-chain precursor polypeptide.

Studies on the biogenesis of intestinal microvillar enzymes have so far been restricted to using radioactive labelling techniques in systems *in vivo* or in organ culture. These methods have given detailed information about the pathway used by the enterocyte to transport newly synthesized proteins, destined for the microvillar membrane, through the cell, and also some knowledge of the post-translational modifications occurring during this process. Thus the molecular form of several microvillar enzymes during their intracellular transport has been studied (Hauri *et al.*, 1979; E. M. Danielsen, unpublished work). The microvillar enzymes have also been shown to become glycosylated during their biogenesis, first to an endo-H-sensitive 'high mannose' glycosylated form, followed by conversion into a neuraminidase-sensitive 'complex' form (Danielsen *et al.*, 1981; E. M. Danielsen, unpublished work). Because glycosylation is a very early post-translational modification or possibly one that is initiated before the termination of mRNA translation, organ culture is not a suitable system for studying the nature of the unmodified translation product. Cell-free translation, however, is the ideal system for this. Translation systems derived from rabbit reticulocytes (Pelham & Jackson, 1976) have proved capable of translating mRNA molecules coding for polypeptides with M_r larger than 200 000 (Kahn *et al.*, 1981), making it technically feasible to study the unmodified polypeptides of the rather large microvillar enzymes. The present work describes the preparation of translationally active RNA from pig

small intestine and its use in the study of the synthesis *in vitro* of a microvillar enzyme, aminopeptidase N (EC 3.4.11.2).

Materials and methods

Materials

Pig small intestine was kindly given by the Department of Experimental Pathology, Rigshospitalet, Copenhagen, Denmark.

A kit for translation *in vitro*, consisting of nuclease-treated rabbit reticulocyte lysate, 'translation cocktail' and [^{35}S]methionine (sp. radioactivity $> 1000\text{ Ci/mmol}$), was obtained from NEN Chemicals GmbH, Dreieich, W. Germany, poly-(U)-Sephacrose 4B from Pharmacia Fine Chemicals, Uppsala, Sweden, and guanidinium chloride (analytical grade) from Merck, Darmstadt, W. Germany. Technical-grade guanidinium chloride (minimum 98.5% pure, from Süddeutsche Kalkstickstoff-Werke, Trostberg, W. Germany) was kindly given by Mr. E. Danielsen, Afridana A/S, Copenhagen, Denmark.

Specific rabbit antibodies against native or SDS/heat-denatured pig intestinal aminopeptidase N were prepared and purified by affinity chromatography on protein A-Sepharose as described previously (Sjöström *et al.*, 1978; Sjöström & Norén, 1982). Specific rabbit antibodies against glycyl-L-leucine hydrolase (glycyl-leucine dipeptidase, EC 3.4.13.11) and aminoacyl-L-proline hydrolase (proline dipeptidase, EC 3.4.13.9) were as used by Norén *et al.* (1977).

[^{35}S]Methionine-labelled aminopeptidase N and sucrase (EC 3.2.1.48)–isomaltase (EC 3.2.1.10)

Abbreviations used: SDS, sodium dodecyl sulphate; poly(A)⁺ RNA, polyadenylated RNA.

were isolated from organ-cultured intestinal explants (Danielsen *et al.*, 1982).

The sources of other chemicals were as previously described (Danielson *et al.*, 1982).

Preparation of RNA

Unless stated, all procedures were performed at 4°C.

Immediately after slaughter of the pig, the small intestine of the animal was removed from the abdomen, cut into 2–3 cm-long pieces and quickly frozen in liquid nitrogen. Pieces of intestine thus obtained were stored at –80°C until use.

A crude RNA fraction was isolated from the small intestine by the guanidinium chloride method (Cox, 1968; Deeley *et al.*, 1977) as follows (buffers containing technically pure guanidinium chloride were filtered before use): 50 g of frozen whole intestine was homogenized at –10°C in 500 ml of 20 mM-sodium acetate (pH 5.0)/7 M-guanidinium chloride/1 mM-dithiothreitol by a Kenwood blender operated at full speed for 60 s. The homogenate was centrifuged at –10°C (8000 g, 10 min), and to the supernatant was added 0.5 vol. of pre-chilled (–20°C) ethanol. The RNA was allowed to precipitate for 1 h at –20°C and was then collected by centrifugation at –10°C (2500 g, 10 min). The pellet was quickly redissolved at room temperature in a minimum volume of 20 mM-sodium acetate (pH 7.0)/7 M-guanidinium chloride/1 mM-dithiothreitol, and re-precipitated by the addition of 0.5 vol. of ethanol as described above. The procedure of RNA solubilization and precipitation was repeated twice. In the subsequent steps of RNA preparation, all glassware and buffers used were autoclaved before use. The RNA pellet was dissolved at room temperature in 25 ml of 20 mM-EDTA, pH 7.0. Insoluble material was then removed by centrifugation at 2500 g for 10 min, and the supernatant extracted with an equal volume of chloroform/butan-1-ol (4:1, v/v). The aqueous phase was collected and 2 vol. of pre-chilled (–20°C) ethanol was added. After precipitation overnight at –20°C, the RNA was pelleted by centrifugation at –10°C (2500 g, 10 min). The RNA was briefly washed in pre-chilled (–20°C) 70% (v/v) ethanol, pelleted again as described above and dried by a jet of nitrogen. It was finally dissolved in a small volume of water at a concentration of 300 A_{260} units/ml, pipetted into small samples and stored at –20°C until use.

Poly(A)⁺ RNA was affinity purified on poly(U)–Sephrose 4B as follows. Crude RNA was dissolved in 50 mM-Tris/HCl (pH 7.5)/0.7 M-NaCl/10 mM-EDTA, at a concentration of about 30 A_{260} units/ml. Before chromatography, RNA was disaggregated by heating the solution to 55°C for 5

min, followed by a quick cooling on ice. The RNA solution was then mixed with approx. 3 ml of poly(U)–Sephrose 4B, preswollen in the same buffer, in a Büchner funnel at room temperature for 1 h with frequent stirring. After washing of the poly(U)–Sephrose in the above buffer, poly(A)⁺ RNA was eluted at room temperature with 10 mM-potassium phosphate (pH 7.5)/10 mM-EDTA/90% (v/v) formamide. It was finally precipitated with 2 vol. of ethanol and re-dissolved in water as described for the preparation of crude RNA.

Translation of RNA in vitro

Crude RNA and poly(A)⁺ RNA were translated in a nuclease-treated cell-free system derived from rabbit reticulocytes (Pelham & Jackson, 1976). A 200 μ l translation mixture contained 100 μ l of lysate, 55 μ l of 'translation cocktail', 0.65 mM-magnesium acetate, 80 mM-potassium acetate, 150 μ Ci of [³⁵S]-methionine, 1.80 A_{260} units of crude RNA or 0.33 A_{260} unit of poly(A)⁺ RNA. When proteinase inhibitors were present, aprotinin (25 μ g/ml), phenylmethanesulphonyl fluoride (0.1 mM) and *o*-phenanthroline (1 mM) were added to the translation mixture before incubation.

Incubation was performed at 37°C for 90 min and then terminated by quick cooling of the translation mixture on ice. Incorporated radioactivity was determined in 2 μ l samples of translation mixture, spotted on small pieces of filter paper which were placed in boiling 10% (w/v) trichloroacetic acid for 10 min. The filters were rinsed in ethanol, ethanol/acetone (1:1, v/v) and acetone, before the addition of 3 ml of Aqualuma Plus scintillant, and radioactivity counting.

Immunopurification of enzymes

Enzymes synthesized *in vitro* were purified by line immunoelectrophoresis (Axelsen *et al.*, 1973) against specific antibodies to the respective enzymes as described by Danielsen *et al.* (1982).

Before immunopurification of aminopeptidase N, 1 μ l of Triton X-100-solubilized microvillar proteins (10 mg of protein/ml), prepared as described by Sjöström *et al.* (1978), was added to the translation mixture to ensure a detectable precipitation of the translation product. Similarly, about 1 μ g of purified glycyl-leucine dipeptidase (Norén *et al.*, 1973) or proline dipeptidase (Sjöström *et al.*, 1973) was added in the purification of these enzymes.

In one experiment, an antibody preparation raised against SDS/heat-denatured aminopeptidase N was used; in this case, the non-radioactive solubilized microvillar fraction was denatured before addition to the translation mixture by boiling for 3 min in the presence of 0.1% (w/v) SDS, and the translation mixture made 0.1% in SDS before immunopurification.

Polyacrylamide-gel electrophoresis

Gel electrophoresis in 10% slab gels in the presence of SDS was performed as described by Laemmli (1970). Before electrophoresis, samples were denatured by boiling for 5 min in the presence of 1% (w/v) SDS and 2.5% (v/v) 2-mercaptoethanol. The following [35 S]methionine-labelled M_r indicators were used: polypeptides (M_r 265 000 and 240 000) of sucrase-isomaltase, polypeptides (M_r 166 000 and 140 000) of aminopeptidase N (E. M. Danielsen, unpublished work) and actin (M_r 42 000). After electrophoresis, the gels were prepared for fluorography as described by Bonner & Laskey (1974). Kodak X-Omat films were exposed at -80°C .

Results and discussion

Isolation of RNA

The guanidinium chloride extraction method of Cox (1968) was used to prepare a crude fraction of RNA from whole intestine. This method was reported to be superior in obtaining minimally degraded mRNA, coding for vitellogenin (M_r 240 000), to procedures using SDS, chloroform and phenol, or preparation of polyribosomes in the presence of various inhibitors (Deeley *et al.*, 1977). The guanidinium chloride technique was also used by Kahn *et al.* (1981) to prepare RNA, from human muscle, capable of directing synthesis *in vitro* of polypeptides with M_r greater than 200 000. In the present work, approx. 300 A_{260} units of crude RNA were obtained from 50 g of intestine, and the A_{260}/A_{280} ratio of the final preparation was 1.8–1.9, as also reported by Deeley *et al.* (1977) and Kahn *et al.* (1981).

Initially, a poly(A) $^+$ RNA fraction was obtained by affinity chromatography on poly(U)-Sephacrose 4B; the yield of poly(A) $^+$ RNA from 50 g of intestine was about 20 A_{260} units. This purified mRNA fraction gave maximum incorporation of radioactivity into trichloroacetic acid-precipitable protein (about a 6-fold increase in the endogenous activity) at a much lower RNA concentration than for the crude RNA preparation. However, the relative amount of polypeptides with M_r larger than 100 000 synthesized with the purified mRNA fraction was low compared with that obtained with the crude RNA preparation, and for that reason the affinity-purification step was omitted when studying the synthesis of such a high- M_r polypeptide as that of aminopeptidase N. A similar observation was made by Deeley *et al.* (1977) in using poly(U)-Sephacrose in the purification of vitellogenin mRNA from the rooster.

Translation of RNA *in vitro*

Fig. 1(a) shows the pattern of labelled poly-

Fig. 1. Translation *in vitro* of the crude intestinal RNA preparation

A crude RNA fraction was prepared and translated in a rabbit reticulocyte lysate system as described in the Materials and methods section. (a) After incubation, 20 μl of translation mixture was subjected to SDS/polyacrylamide-gel electrophoresis. Exposure time: 1 day (1), 8 days (2). (b), (c), (e) After incubation, proline dipeptidase (b; exposure time 8 days), glycyl-leucine dipeptidase (c; exposure time 1 day) and aminopeptidase N (e; exposure time 3 days) were purified by line immunoelectrophoresis against specific antibodies raised to the respective enzymes from a 200 μl translation mixture. The immunopurified enzymes were electrophoresed in polyacrylamide gels in the presence of SDS. (d) Polyacrylamide-gel electrophoresis in the presence of SDS of [35 S] methionine-labelled aminopeptidase N, isolated from organ-cultured intestinal explants (exposure time 3 days). (a)–(c) and (d)–(e) were run in separate gels. Apparent M_r values ($\times 10^{-3}$) are shown.

peptides synthesized in the presence of crude RNA from small intestine. Distinct polypeptides with M_r above 200 000 are visible, indicating that mRNA degradation during the preparative procedure was not excessive. Actin (M_r 42 000), which is a major protein component of the enterocyte and becomes intensively labelled with [35 S]methionine in organ-cultured intestinal explants (Danielsen *et al.*, 1982), is also a prominent band among the polypeptides synthesized *in vitro*.

Line immunoelectrophoresis was previously found (Danielsen *et al.*, 1982; E. M. Danielsen, unpublished work) to be an excellent purification method for radioactively labelled microvillar enzymes from organ-cultured intestinal explants with high specificity and a low background, and was therefore used in the present work. Two cytosolic peptidases, glycyl-leucine dipeptidase and proline dipeptidase, were immunopurified from the translation mixture

(Figs. 1b and 1c). The physical properties of these proteins have been extensively studied (Sjöström & Norén, 1978) and therefore provide a good standard against which to compare the mRNA-translation products and thus to evaluate authenticity of the polypeptides coded for by the RNA preparation used. Glycyl-leucine dipeptidase and proline dipeptidase translated *in vitro* were polypeptides of M_r 53 000 and 61 000 respectively. These values agree well with the subunit M_r of the proteins, calculated from sedimentation-velocity studies (51 000 and 63 000 for glycyl-leucine dipeptidase and proline dipeptidase respectively; Sjöström & Norén, 1978), and thus indicate the presence of large amounts of intact mRNA in the RNA preparation.

Fig. 1(e) shows the product of translation *in vitro* immunoprecipitated with antibodies to native aminopeptidase N. A narrow band corresponding to a polypeptide of apparent M_r 115 000 is clearly visible. A polypeptide with identical M_r was immunoprecipitated with antibodies to SDS/heat-denatured aminopeptidase N (result not shown). Prolonged exposure of either of the fluorograms did not reveal the presence of any polypeptides with M_r larger than 115 000.

In pulse-chase labelling studies, performed with organ-cultured intestinal explants, the earliest polypeptide of aminopeptidase N detectable by immunoprecipitation is of M_r 140 000 (E. M. Danielsen, unpublished work). This intracellularly located precursor form of the microvillar enzyme has been shown to be glycosylated with the 'high mannose' oligosaccharide sequence (Danielsen *et al.*, 1981; E. M. Danielsen, unpublished work), characteristic for proteins destined for cellular export (Robbins *et al.*, 1977). The lower M_r (115 000) of the polypeptide of aminopeptidase N obtained from the translation system *in vitro* is likely to represent the non-glycosylated polypeptide of the microvillar enzyme. Maroux *et al.* (1973) estimated the carbohydrate content of aminopeptidase N to be 23%. This value is sufficient to explain the discrepancy in apparent M_r between the polypeptide translated *in vitro* and the mature microvillar enzyme. The immunoprecipitability of the 115 000- M_r polypeptide demonstrates that glycosylation is not an essential requisite for the antigenicity of aminopeptidase N. Interestingly, the non-glycosylated enzyme could be immunoprecipitated with antibodies raised to both native and SDS/heat-denatured aminopeptidase N. These two antibodies recognize different antigenic determinants on aminopeptidase N; in its native conformation, the enzyme is not immunoprecipitated by antibodies raised to the SDS/heat-denatured aminopeptidase N (Sjöström & Norén, 1982). To obtain quantitative precipitation of the enzyme in immunoelectrophoresis with the latter

type of antibodies, microvillar aminopeptidase N has to be denatured by boiling in the presence of SDS (E. M. Danielsen, unpublished work). The translation product of M_r 115 000 is therefore likely to be in a conformational state that only partially resembles the final native one. The reason for this may be that the amphiphilic polypeptide of newly synthesized aminopeptidase N needs insertion into a lipid membrane and/or to become glycosylated in order to achieve its final conformation.

All dimeric microvillar enzymes are not synthesized as large single-chain precursors

Two other microvillar enzymes, sucrase (EC 3.2.1.48)—isomaltase (EC 3.2.1.10) and maltase—glucoamylase (EC 3.2.1.20), have been shown to be synthesized as large single-chain precursor polypeptides which are post-translationally split, presumably by pancreatic proteinases, to yield the final mature enzymes (Hauri *et al.*, 1979; Sjöström *et al.*, 1980; Danielsen *et al.*, 1981, 1982; S. Hedeager-Sørensen, personal communication). On the basis of the structural resemblance of all hitherto studied microvillar enzymes (dimeric 'stalked' integral membrane proteins), it has been suggested that aminopeptidase N is synthesized in the same way, i.e. as a large single-chain precursor (Svensson, 1979). The result of the present work, however, argues against the existence of such a general biosynthetic mechanism for the microvillar enzymes. Despite the ability of the RNA preparation to direct synthesis of polypeptides of sufficient size to be candidates for such a large single-chain precursor of aminopeptidase N, a high- M_r polypeptide (M_r about 200 000) could not be detected, even when translation was performed in the presence of proteinase inhibitors. Further evidence is given by the finding of a doublet polypeptide of approx. M_r 220 000, immunopurified from the translation mixture *in vitro* with antibodies to sucrase—isomaltase (E. M. Danielsen, O. Norén & H. Sjöström, unpublished work). Despite a gross structural relationship between the microvillar enzymes and some similarities in their glycosylation pattern during the biogenesis, a satisfactory model describing their biosynthesis will have to be flexible enough to allow some individuality among the enzymes, at least where the early stages of their biogenesis is concerned.

After this paper was submitted, the translation *in vitro* of another microvillar enzyme, sucrase—isomaltase, was reported (Wacker *et al.*, 1981).

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