BIOSYNTHESIS OF INTESTINAL MICROVILLAR PROTEINS

Nature of precursor forms of microvillar enzymes from Ca^{2+}-precipitated enterocyte membranes

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1. Introduction

In the synthesis of membrane glycoproteins, the existence of a common asparagine-linked oligosaccharide intermediate, characterized by possessing a large number of mannose residues (high mannose form) has been proposed [1]. This intermediate is in turn modified by partial reglycosylation, yielding the final complex oligosaccharides, characterized by terminal sialic acid or fucose residues.

In [2] we found new molecular forms of pig intestinal aminopeptidase (EC 3.4.11.2) and sucrase-isomaltase (EC 3.2.1.48–10) obtained from a Ca^{2+}-precipitated membrane fraction of the enterocyte. Due to their likely intracellular and/or basolateral location, these new molecular forms of the two microvillar enzymes were proposed to represent different precursor stages of the final enzymes. Here, putative precursor forms of two other microvillar enzymes, maltase-glucoamylase (EC 3.2.1.20) and lactase-phlorizin hydrolase (EC 3.2.1.23–62), have been purified from the Ca^{2+}-precipitated membrane fraction and the susceptibility of the Ca^{2+}-pellet forms of the microvillar enzymes to digestion by endo H and neuraminidase has been studied.

2. Experimental

2.1. *Materials*

Endo-β-acetylglucosaminidase H (from *Streptomyces griseus*, free of exoglycosidase and protease activities, 30 U/mg) was purchased from Seikagaku Kogyo Co., Tokyo and neuraminidase (from *Clostridium perfringens*, 0.6 U/mg) from Boehringer, Mannheim. The sources of all other chemicals were those in [3,4].

Antibodies against denatured maltase-glucoamylase were prepared as follows: The pure enzyme [5] was boiled for 5 min in the presence of 1% SDS. Excess of SDS was removed by precipitation with 0.05 M KCl (final conc.). After mixing with an equal volume of incomplete Freunds adjuvant, the denatured enzyme was injected into rabbits (50 μg/injection) at 2 week intervals. A week after the fourth injection, the rabbits were bled for 40 ml.

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

2.2. *Preparation of Ca^{2+}-precipitated membrane fraction*

The Ca^{2+}-precipitated membrane fraction was isolated as in [2], using 150–300 g portions of frozen, everted and washed small intestinal pieces as starting material. In the purification of maltase-glucoamylase and lactase-phlorizin hydrolase, the Ca^{2+}-pellet was washed and resuspended in 50 mM potassium phosphate (pH 7.4) before solubilization with Triton X-100.

2.3. *Immunoadsorbent purification of the enzymes*

This was performed on small (2–3 ml) immunoadsorbent columns with specificity directed against the respective pig enzymes using the procedures referenced: aminopeptidase [3]; sucrase-isomaltase [4]; maltase-glucoamylase [5]; and lactase-phlorizin hydrolase [6]. Enzymically active eluate fractions were pooled, concentrated by ultrafiltration (Amicon PM 10 filter) to ~200 μg/ml and stored at −20°C until use.

2.4. *Polyacrylamide gel electrophoresis*

Gel electrophoresis in SDS–10% polyacrylamide

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gels was performed as in [7]. Prior to electrophoresis, samples were denatured by boiling for 5 min in the presence of 1% SDS and 2.5% 2-mercaptoethanol. The following M_r indicators were used: pro-sucrase-isomaltase (M_r 260 000) [4]; subunits A (M_r 162 000) and B (M_r 123 000) of aminopeptidase [3]; polypeptides (M_r 240 000; M_r 135 000 and M_r 125 000) of maltase-glucoamylase [5]; lactase-phlorizin hydrolase (M_r 160 000) [6]; albumin (M_r 67 000); and ovalbumin (M_r 45 000). After electrophoresis protein bands were stained for protein with Coomassie brilliant blue R 250.

2.5. *Immunoelectrophoresis*

First-dimensional polyacrylamide gel electrophoresis [7] followed by second-dimensional immunoelectrophoresis against anti-denatured microvillar maltase-glucoamylase was done as in [2].

2.6. *Treatment with glycosidases*

Samples, 50 μl (10 μg of protein) of purified protein in 0.1 M sodium citrate (pH 5.2) containing 0.1% Triton X-100, were incubated with endo H (4 mU) or neuraminidase (6 mU) at 37°C for 24 h. After incubation, the samples were either directly used in electrophoresis or frozen at −20°C until use. Control samples without addition of glycosidases were incubated in parallel.

2.7. *Other methods*

Protein was determined as in [8], using the Bio-Rad protein assay (microassay procedure, Bio-Rad Labs., Munich) using bovine serum albumin as standard. Enzyme activities were determined as in [3].

3. Results and discussion

In [2] we found putative precursor forms of microvillar aminopeptidase and sucrase-isomaltase present in the Ca^{2+}-precipitated membrane fraction of enterocytes. Here, these studies have been extended to two other microvillar enzymes, lactase-phlorizin hydrolase and maltase-glucoamylase which have both been isolated from the Ca^{2+}-precipitated membrane fraction. Though the amount of each of the enzymes in this fraction was not quantitated, they were estimated to be present in only very minute amounts as was the case with aminopeptidase.

Fig.1 shows the polypeptide composition of maltase-glucoamylase, obtained from the Ca^{2+}-precipitated membrane fraction, compared to that of the corresponding microvillar enzyme. Ca^{2+}-pellet maltase-glucoamylase consists of polypeptides of M_r 245 000 and 225 000; the microvillar enzyme of polypeptides of M_r 240 000, 135 000 and 125 000 [5]. The two polypeptides of the Ca^{2+}-pellet maltase-glucoamylase produced fusing precipitates in the second-dimensional immunoelectrophoresis against antibodies to the denatured microvillar enzyme, indicating a close structural resemblance with the latter.

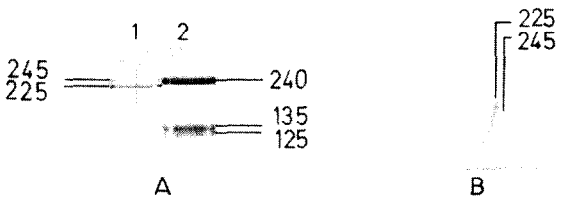

Fig.1. (A) SDS–Polyacrylamide gel electrophoresis of Ca^{2+}-pellet maltase-glucoamylase (1) and the corresponding microvillar enzyme (2); 10 μg and 25 μg of protein, respectively, were applied to the gel. (B) First-dimensional SDS–polyacrylamide gel electrophoresis followed by second dimensional immunoelectrophoresis of Ca^{2+}-pellet maltase-glucoamylase against anti-denatured microvillar maltase-glucoamylase; ~1 μg protein was applied to the gel and after electrophoresis, precipitates were stained with Coomassie brilliant blue R 250.

In [9], lactase-phlorizin hydrolase, obtained from the Ca^{2+}-precipitated membrane fraction was found to consist of two polypeptides of M_r 160 000 and 150 000, compared to the polypeptide of M_r 160 000 of the microvillar enzyme.

The result of digestion with endo H of aminopeptidase, sucrase-isomaltase, maltase-glucoamylase and lactase-phlorizin hydrolase, obtained from the Ca^{2+}-precipitated membrane fraction, is shown in fig.2. As it is evident in the case of all 4 enzymes, the polypeptide with lowest M_r was found to be sensitive to treatment with endo H whereas the polypeptide of higher M_r appeared to be endo H resistant.

The effect of neuraminidase treatment on the Ca^{2+}-pellet enzymes is shown in fig.3. Contrary to endo H, neuraminidase was found to increase only the mobility of the polypeptide with the higher M_r of each of the enzymes. (For sucrase-isomaltase, the shift in mobility was very small but it consistently appeared in several experiments.) In none of the cases was the polypeptide of lower M_r affected by incubation with neuraminidase.

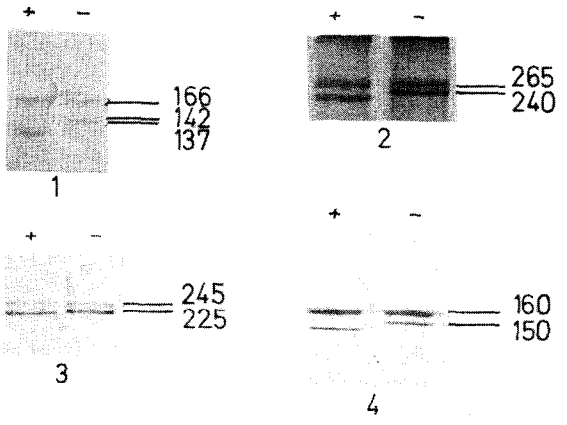

Fig.2. Effect of endo H on Ca^{2+}-pellet enzymes. Each of the enzymes was treated with endo H as in section 2. Endo H-treated samples (+) and control samples (−) were then electrophoresed in SDS–polyacrylamide gels in parallel lanes: (1) aminopeptidase; (2) sucrase-isomaltase; (3) maltase-glucoamylase; (4) lactase-phlorizin hydrolase.

Our observation that the molecular forms of Ca^{2+}-pellet aminopeptidase and sucrase-isomaltase are different from those of the corresponding final microvillar enzymes, has been extended here to include two other enzymes of the microvillar membrane. A common pattern of Ca^{2+}-pellet polypeptide components emerges from these studies: For the enzymes so far examined a polypeptide about the size of that of the enzyme in [3–6] is found together with a polypeptide (in case of aminopeptidase: two polypeptides) which is ~10 000–25 000 M_r smaller in size. Only the smaller of the two polypeptides is sensitive to treatment with endo H, indicating that it alone possesses the mannose-rich oligosaccharide sequences which are hydrolyzed by endo H. In contrast, treatment with neuraminidase resulted in a greater mobility of the larger of the two polypeptides, indicating the presence of sialic acid as terminal sugar residue.

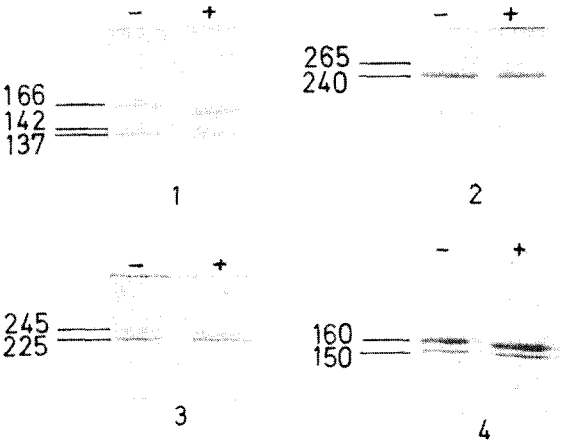

Fig.3. Effect of neuraminidase on Ca^{2+}-pellet enzymes. Each of the enzymes was treated with neuraminidase as in section 2. Neuraminidase treated samples (+) and control samples (−) were then electrophoresed in SDS–polyacrylamide gels in parallel lanes: (1) aminopeptidase; (2) sucrase-isomaltase; (3) maltase-glucoamylase; (4) lactase-phlorizin hydrolase.

For several other plasma membrane and secretory proteins, the cellular location of the high mannose and complex precursor forms have been identified and have provided support for the general membrane flow hypothesis [10]. The presence in the Ca^{2+}-precipitated membrane fraction of the new M_r forms of the microvillar enzymes is at least consistent with this general hypothesis of intracellular transport; the Ca^{2+}-pellet enzymes are firmly attached to the membrane vesicles and need solubilization by detergent, indicating an early, intracellular membrane insertion. The endo H sensitive polypeptides of lower M_r tentatively represent the early stages of the biogenesis of the microvillar enzymes on their way from the rough endoplasmic reticulum to the Golgi apparatus and the polypeptides of higher M_r which resemble the final enzymes with regard to size are the forms reglycosylated at the Golgi apparatus, destined for the plasma membrane.

It seems that the microvillar enzymes included here are all dimeric molecules during at least a part of the time they reside in the microvillar membrane [3–6]. For sucrase-isomaltase, it has been shown that the enzyme is synthesized as a single, large polypeptide which is split post-translationally by pancreatic proteases into its constituent subunits [4,11]. The high M_r of the Ca^{2+}-pellet polypeptides of sucrase-isomaltase agrees well with these findings. We have shown in [2] that artifactual proteolysis of aminopeptidase and sucrase-isomaltase during preparation of the Ca^{2+}-precipitated membrane fraction must be considered highly unlikely. The question arises whether other microvillar enzymes follow a mechanism of synthesis and modification similar to sucrase-isomaltase. These results suggest that only maltase-glucoamylase may resemble sucrase-isomaltase in that respect, being the only other enzyme with high

enough M_r of the Ca^{2+}-pellet polypeptides to contain both subunits. If any of the other enzymes are translated as single, large polypeptides, the cleavage of these must take place during polypeptide synthesis or very shortly after since only polypeptides with M_r comparable to that of the subunits can be detected in the Ca^{2+}-precipitated membrane fraction. Post-translational cleavage of single, large polypeptide precursors to microvillar enzymes by pancreatic proteases therefore cannot be considered as a general modification procedure. With the exception of lactase-phlorizin hydrolase, the polypeptides of higher M_r of the Ca^{2+}-pellet forms of the enzymes were slightly larger than those of the corresponding microvillar enzymes, suggesting that a final modification of these takes place extracellularly. This modification possibly consists of cleavage(s) in the protein or carbohydrate moiety of the enzymes and may be attributable to the action of pancreatic enzymes.

To summarize, the new M_r forms of the microvillar enzymes found in the Ca^{2+}-precipitated membrane fraction by their differing sensitivity to treatment with endo H and neuraminidase suggest the existence in the enterocyte of a mechanism of plasma membrane protein biogenesis compatible with the general membrane flow hypothesis. However, further studies including pulse-labelling and subcellular fractionation are necessary to gain more information about the timing and subcellular location of the individual steps in the biogenesis of the microvillar enzymes. Such work, using an organ culture technique, is currently in progress in our laboratory.

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