Biosynthesis of intestinal microvillar proteins

Role of the Golgi complex and microtubules

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The effect of monensin and colchicine on the biogenesis of aminopeptidase N (EC 3.4.11.2), aminopeptidase A (EC 3.4.11.7), dipeptidyl peptidase IV (EC 3.4.14.5), sucrase (EC 3.2.1.48)–isomaltase (EC 3.2.1.10) and maltase–glucoamylase (EC 3.2.1.20) was studied in organ-cultured pig small-intestinal explants. On the ultrastructural level, monensin (1 μ M) caused an increasingly extensive dilation and vacuolization of the Golgi complex during 4 h exposure of the explants. On the molecular level, the effect of monensin was twofold. (1) The processing from the initial high-mannose-glycosylated form to the mature complex-glycosylated form was arrested. For some of the enzymes studied, intermediate stages between the high-mannose and complex forms could be seen, probably corresponding to 'trimmed' or partially complex-glycosylated polypeptides. (2) Labelled microvillar enzymes failed to reach their final destination. These findings suggest the involvement of the Golgi complex in the post-translational processing and transport of microvillar enzymes. The presence in the growth medium of colchicine (50 μ g/ml) caused a significant inhibition of the appearance of newly synthesized enzymes in the microvillar membrane during a 3 h labelling period. Since synthesis and post-translational modification of the microvillar enzymes were largely unaffected by colchicine, the results obtained suggest that microtubules play a role in the final transport of the enzymes from the Golgi complex to the microvillar membrane.

Several microvillar enzymes, namely aminopeptidase N (EC 3.4.11.2), aminopeptidase A (EC 3.4.11.7), dipeptidyl peptidase IV (EC 3.4.14.5), 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 transported from their site of synthesis, the rough endoplasmic reticulum, to the microvillar membrane in a membrane-bound state (Hauri *et al.*, 1980; Danielsen, 1982; Danielsen *et al.*, 1983a). During synthesis and intracellular transport, the primary translation products of these enzymes undergo modifications; in the rough endoplasmic reticulum they become glycosylated with high-mannose oligosaccharides, which occurs co-translationally or very shortly after synthesis of the polypeptide (Danielsen *et al.*, 1983b). Before reaching the microvillar membrane, the high-mannose oligosaccharides of the enzymes are trimmed and complex-glycosylated to yield the mature form.

However, information about the organelles that participate in the post-translational modification and transport of the microvillar enzymes is scarce. For other types of membrane proteins, the Golgi complex has been found to be active in these processes (for review, see Tartakoff, 1982). One way to obtain information about the possible involvement of the Golgi complex in the biogenesis of the microvillar enzymes is to study the effect of monensin; in low concentrations it impairs the structure and functions of the Golgi complex.

In enterocytes, microtubules have been found in the apical and Golgi areas of the cytoplasm (Ginsel *et al.*, 1975; Reaven & Reaven, 1977), oriented in a fashion that suggests a role in the transport of material between these two parts of the cell. The anti-microtubular agent colchicine (for review, see Dustin, 1978) was found to alter this distribution of microtubules (Ginsel *et al.*, 1975) and to disturb the transport of fucosylated proteins (Quaroni *et al.*, 1979; Blok *et al.*, 1981).

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The present work describes the effect of monensin and colchicine on the biogenesis of microvillar enzymes as studied in organ-cultured pig small-intestinal explants.

Materials and methods

Materials

Equipment for performing organ culture, including Trowell's T-8 medium, foetal-calf serum, sterile plastic dishes with grids and [^{35}S]methionine (sp. radioactivity $>1000\text{ Ci/mmol}$) were obtained as previously described (Danielsen *et al.*, 1982). Monensin (from Calbiochem) was kindly given by Dr. G. Warren, EMBL, Heidelberg, W. Germany. Colchicine was purchased from Merck, Darmstadt, W. Germany.

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

Organ culture of intestinal explants

Organ culture of intestinal explants (Browning & Trier, 1969) was performed as previously described (Danielsen *et al.*, 1982). In experiments with monensin, the explants were organ-cultured for 3 h in the presence of $1\text{ }\mu\text{M}$ -monensin; after 1 h of organ culture, [^{35}S]methionine ($50\text{--}100\text{ }\mu\text{Ci/ml}$) was added to the medium. In experiments with colchicine, the explants were organ-cultured for 5 h in the presence of $50\text{ }\mu\text{g}$ of colchicine/ml; after 2 h of organ culture, [^{35}S]methionine ($50\text{ }\mu\text{Ci/ml}$) was added to the medium. In all experiments, control explants without the addition of either monensin or colchicine were organ-cultured in parallel. Explants used for electron microscopy were organ-cultured in the presence of $1\text{ }\mu\text{M}$ -monensin for 0–4 h.

Electron microscopy

After organ culture, explants were divided into small blocks and fixed for 24 h by immersion in 2% (v/v) glutaraldehyde in 0.04 M -cacodylate buffer, pH 7.2 (4°C). The tissues were rinsed in cacodylate buffer for 20 h and post-fixed in 2% OsO_4 in the same buffer for 2 h. They were then rinsed in distilled water, dehydrated through graded concentrations of ethanol and embedded in Epon epoxy resin. Thin sections of golden to silver colour were stained for 20 min in uranyl acetate, followed by 5 min in lead citrate, and were thereafter examined in an electron microscope (model 300; Philips, Eindhoven, The Netherlands).

Other methods

Labelled explants were fractionated (Danielsen, 1982), by using the Ca^{2+} -precipitation technique (Schmitz *et al.*, 1973; Booth & Kenny, 1974) to obtain subcellular fractions containing the enzymes during intracellular transport (Ca^{2+} -precipitated

membrane fraction, containing membranes of intracellular and basolateral origin) and at their final destination (microvillar fraction). The presence of either monensin or colchicine did not markedly influence the yield and quality of the subcellular fractions, as judged by their contents of immunoprecipitable aminopeptidase N, sucrase–isomaltase and maltase–glucoamylase. Immunopurification of microvillar enzymes by line immunoelectrophoresis was performed as described by Danielsen & Cowell (1983). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed as described by Laemmli (1970), with fluorography of gels as described by Bonner & Laskey (1974). Before liquid-scintillation counting, immunopurified microvillar enzymes were dissolved by boiling for 3 min in the presence of 1% sodium dodecyl sulphate and 2.5% 2-mercaptoethanol.

Results

Effect of monensin on ultrastructure

After incubation with monensin, the enterocytes of the surface epithelium developed morphological alterations which progressed in a time-dependent manner (Plate 1). A typical finding after 1 h was dilation of the saccules of the Golgi complex, which in places were surrounded by clear vacuoles. After further incubation the Golgi complex itself was difficult to identify. After 4 h the entire luminal part of the cells was filled with vacuoles, giving the cells a foamy appearance. The fully developed changes could easily be seen also in $1\text{ }\mu\text{m}$ -thick sections at the light-microscopic level. There was considerable quantitative variation in the changes observed in the individual cells in response to monensin. Cells on the villi showed more pronounced changes than did cells in the crypts, where only minor changes were observed, but there was considerable variation also among neighbouring cells.

Effect of monensin on glycosylation

Fig. 1 shows the effect of monensin on the molecular forms of aminopeptidase N, aminopeptidase A, dipeptidyl peptidase IV, sucrase–isomaltase and maltase–glucoamylase after 2 h of continuous labelling. For all five enzymes, the normal pattern without addition of monensin consists of two labelled polypeptides: one of lower M_r , which bears high-mannose glycosylation, and another of higher M_r that represents the enzymes in a mature complex state of glycosylation. In pulse–chase labelling experiments, the high-mannose-glycosylated polypeptides were found to be the earliest labelled form of all five enzymes (Danielsen, 1982; Danielsen *et al.*, 1983a), and they were converted into the mature form after 30–60 min of chase. Within the 2 h labelling period, explants exposed to monensin

EXPLANATION OF PLATE 1

Effect of monensin on the electron-microscopic appearance of enterocytes

(a) Control showing normal enterocytes (magnification $\times 3500$). (b) Supranuclear region of enterocytes after 2 h of exposure to monensin. The Golgi saccules are dilated and clear vacuoles have appeared (magnification $\times 5400$). (c) Larger magnification ($\times 12400$) of the Golgi region of a cell after 2 h of exposure to monensin. (d) Fully developed changes as observed after 4 h of exposure to monensin (magnification $\times 3500$).

Fig. 1. *Effect of monensin on glycosylation of microvillar enzymes*

Explants were continuously labelled for 2 h in the absence (—) or presence (+) of monensin. After culture, a Triton X-100 extract of total explant protein was prepared (Danielsen, 1982) and the enzymes were immunopurified. The total fractions of purified enzymes were subjected to polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. After electrophoresis, the gels were prepared for fluorography (exposure time 3–7 days). Apparent M_r values ($\times 10^{-3}$) are shown.

synthesized all five microvillar enzymes in about the same amounts as did the control explants, as judged by the approximately similar intensity of the labelled bands. However, the high-mannose-glycosylated form of all five microvillar enzymes remained the predominant molecular form. In contrast, the enzymes obtained from the control explants had largely been processed to the complex-glycosylated form. This latter form could not be detected for any of the enzymes obtained from monensin-treated explants, but a broad blurred band, extending from the high-mannose form towards the complex form, could be seen, most notably with aminopeptidase N and sucrase-isomaltase. For some of the enzymes (most clearly aminopeptidase N, but also sucrase-isomaltase and dipeptidyl peptidase IV) polypeptides were seen to extend below the position of the high-mannose-glycosylated form of the enzymes obtained from the control explants, suggesting intermediate forms with trimmed high-mannose glycosylation.

Effect of monensin on intracellular transport

Fig. 2 compares the distribution between the Ca^{2+} -precipitated membrane fraction, containing intracellular and basolateral membranes, and the microvillar fraction of aminopeptidase N and sucrase-isomaltase, synthesized in control and monensin-treated explants. In pulse-chase labelling

experiments, it was found that it takes about 60–90 min of chase for the enterocyte to transport the microvillar enzymes from their site of synthesis in the rough endoplasmic reticulum to the microvillar membrane (Danielsen, 1982; Danielsen *et al.*, 1983a). Consequently, a significant part of the enzymes from the control explants was seen in the microvillar fraction after the 2 h labelling period. In contrast, the enzymes obtained from monensin-treated explants failed to appear in the microvillar membrane, but instead accumulated in the Ca^{2+} -precipitated membrane fraction.

Effect of colchicine in intracellular transport

Table 1 lists the relative amounts of labelled aminopeptidase N, sucrase-isomaltase and maltase-glucoamylase in the microvillar fraction of control and colchicine-treated explants. Table 1 thus expresses the capability of the explants to transport the newly synthesized microvillar enzymes to their destination. In the control explants, after 3 h of continuous labelling, more than half the amount of labelled enzymes was found in the microvillar fraction. The Ca^{2+} -precipitated membrane fraction contained a significantly larger part of the labelled enzymes in the colchicine-treated explants, where only about one-third of the radioactivity was in the microvillar fraction. The effect of colchicine was not due to a decreased biosynthesis of the enzymes,

Table 1. *Effect of colchicine on transport of microvillar enzymes*

After continuous labelling of the explants for 3 h, aminopeptidase N, sucrase–isomaltase and maltase–glucoamylase were purified from the Ca^{2+} -precipitated membrane fraction and the microvillar fraction, and the amounts of radioactivity incorporated into enzymes were determined. The values listed represent the fraction of radioactivity in the respective enzymes obtained from the microvillar fraction. (Except for aminopeptidase N, two experiments were performed for each enzyme, of which the mean is given.)

Enzyme	Radioactivity of enzyme in microvillar fraction	
	Radioactivity of enzyme in Ca^{2+} -precipitated membrane fraction and microvillar fraction	
Aminopeptidase N	Control	0.56
	+ Colchicine	0.28
Sucrase–isomaltase	Control	0.58
	+ Colchicine	0.35
Maltase–glucoamylase	Control	0.54
	+ Colchicine	0.33

Fig. 2. *Effect of monensin on transport of microvillar enzymes*

Explants were continuously labelled for 2 h in the absence (–) or presence (+) of monensin. After culture, a Ca^{2+} -precipitated membrane fraction (Ca^{2+}) and a microvillar fraction (Mic) were prepared, and the enzymes were immunopurified after solubilization. The total fractions of purified enzymes were subjected to polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. After electrophoresis, the gels were prepared for fluorography (exposure time 3–30 days). Apparent M_r values ($\times 10^{-3}$) are shown.

since the amounts of labelled enzymes found in colchicine-treated explants were approximately the same as in the controls. Thus the total amounts of

Fig. 3. *Effect of colchicine on transport of aminopeptidase N*

Explants were continuously labelled for 2 h in the absence (–) or presence (+) of colchicine. After culture, a Ca^{2+} -precipitated membrane fraction (Ca^{2+}) and a microvillar fraction (Mic) were prepared and aminopeptidase N was immunopurified after solubilization. The total fractions of purified enzymes were subjected to polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. After electrophoresis, the gel was prepared for fluorography (exposure time 2 days). Apparent M_r values ($\times 10^{-3}$) are shown.

radioactivity incorporated into the enzymes from colchicine-treated explants were 94%, 93% and 83% of the corresponding controls for aminopeptidase N, sucrase–isomaltase and maltase–glucoamylase respectively. In Fig. 3, the molecular forms of aminopeptidase N from colchicine-treated explants are compared with the corresponding forms of the enzyme obtained from the controls. Both the high-mannose-glycosylated form and the complex-glycosylated form of the enzyme were seen in colchicine-treated explants, indicating that the post-translational modifications were unaffected. The relatively small amount of aminopeptidase N ap-

pearing in the microvillar membrane in colchicine-treated explants was of the complex-glycosylated form, as in the control.

Discussion

Previous findings of a membrane-bound intracellular route of transport of newly synthesized microvillar enzymes together with the post-translational complex glycosylation urged a closer study of the possible role of the Golgi complex in these processes. The sodium ionophore monensin provides an opportunity to study directly the involvement of this organelle in the biogenesis of the microvillar enzymes. Monensin causes a dilation of the Golgi cisternae (Tartakoff, 1982) and in low concentrations (0.01–1 μM) inhibits the transport of protein through the organelle (Strous & Lodish, 1980).

In the present work, the morphological alterations in the enterocytes caused by monensin were clearly visible in the electron microscope. The vacuolization of the Golgi complex was observed to progress somewhat more slowly than was found by other investigators (Tartakoff *et al.*, 1981), and depended on the state of maturation of the epithelial cells.

Preincubation for 1 h with monensin completely arrested the conversion from the high-mannose into the complex-glycosylated form of all the microvillar enzymes studied. As mentioned above, this process comprises many individual processing steps. The blur of labelled bands extending below and above the high-mannose-glycosylated polypeptide for some of the enzymes (particularly aminopeptidase N) is likely to represent intermediate stages in the processing from high-mannose to complex glycosylation; those of M_r lower than that of the corresponding high-mannose form have been arrested in the processing shortly after the initial trimming of mannose residues, and those extending up towards the M_r of the complex form have had various amounts of terminal complex sugars added before their processing was halted. Under normal conditions, these intermediate stages cannot be detected, presumably because the trimming and complex-glycosylation reactions are occurring so rapidly after one another that the pools of transient forms are extremely small.

Monensin was found to be equally inhibitory of intracellular transport and of complex glycosylation, as judged by the inability of the enzymes from monensin-treated explants to reach the microvillar membrane. The resulting intracellular accumulation of newly synthesized microvillar enzymes (together with other proteins passing through the Golgi complex) may explain the increasingly extensive

vacuolization of the apical cytoplasm seen after prolonged exposure of the explants to monensin.

The significant inhibitory effect of colchicine on transport observed in the present work strongly suggests that microtubules play a role in the intracellular transport of microvillar enzymes. Since not only the high-mannose but also the complex-glycosylated form of the microvillar enzymes could be seen in colchicine-treated explants, the involvement of microtubules must be confined to the final part of the transport process between the Golgi complex and the microvillar membrane. Previously, the effect of colchicine on the transport of radioactively fucose-labelled glycoproteins in intestinal epithelial cells has been studied *in vivo* in the rat (Quaroni *et al.*, 1979) and in organ-cultured human intestinal explants (Blok *et al.*, 1981). In both these studies colchicine was found not to interfere with protein synthesis, but caused an impairment of transport of labelled protein to the microvillar membrane together with an accumulation in other subcellular compartments. Previous pulse-chase labelling studies have shown that the transport of newly synthesized enzymes from the Golgi complex to the microvillar membrane is a rapid process compared with the transport from the site of synthesis to the Golgi complex (Danielsen, 1982; Danielsen *et al.*, 1983a). If microtubules exert their translocating function by the mechanism known as 'treadmilling' (Margolis & Wilson, 1981), their involvement in the final stage of transport would explain its rapidity.

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