Tyrosine sulphation is not required for microvillar expression of intestinal aminopeptidase N

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The effect of 2,6-dichloro-4-nitrophenol (DCNP), an inhibitor of phenol sulphotransferases (EC 2.8.2.-), on the biosynthesis of aminopeptidase N (EC 3.4.11.2) was studied in organ-cultured pig intestinal mucosal explants. At 50 μ M DCNP did not affect protein synthesis but it decreased incorporation of [35 S]sulphate into aminopeptidase N and other major microvillar hydrolases by 70–85 % compared with controls, indicating an inhibition of their post-translational tyrosine sulphation. In labelling experiments with [35 S]methionine from 0.5 to 5 h, DCNP was tested for its possible influence on synthesis, processing and microvillar expression of aminopeptidase N, but no effect on any of these parameters could be detected. It can therefore be concluded that tyrosine sulphation is not required (for instance as a sorting signal) for the targeting of newly synthesized enzymes to the microvillar membrane.

INTRODUCTION

Sulphation of tyrosine residues is considered to be a common type of post-translational modification of secretory proteins (Huttner, 1982, 1984), but in spite of this little is known about its biological significance. Owing to the fact that only proteins destined for cellular export appear to undergo tyrosine sulphation, it has been speculated that it might act as a sorting signal for secretory proteins (Hille *et al.*, 1984; Rosa *et al.*, 1985).

Intestinal microvillar proteins are useful as model proteins for studies on plasma-membrane protein synthesis in polarized cells (Danielsen *et al.*, 1984; Norén *et al.*, 1986; Semenza, 1986). It has been observed that several microvillar enzymes become sulphated during biosynthesis, and that most, if not all, of the incorporated sulphate is attached to tyrosine residues rather than to carbohydrate, showing that tyrosine sulphation is more ubiquitous than previously thought. Interestingly, sulphation of other types of membrane proteins in the enterocyte could not be detected, raising the possibility that tyrosine sulphation in polarized cells might be relevant for the targeting of newly synthesized proteins to the apical cell surface (Danielsen, 1987).

A way to test the relevance of tyrosine sulphation for targeting is to inhibit this type of processing specifically and to study the consequences for the subcellular distribution of newly made proteins. 2,6-Dichloro-4-nitrophenol (DCNP) has been reported to inhibit phenol sulphotransferases both *in vivo* and *in vitro* (Mulder & Scholtens, 1977). In the present paper, DCNP was used to affect tyrosine sulphation in organ-cultured mucosal explants as a means to investigate the importance of this type of processing in the biosynthesis of the microvillar enzymes of the enterocyte.

EXPERIMENTAL

Materials

Equipment for performing organ culture, including Trowell's T-8 medium, fetal-calf serum, plastic dishes

with grids and [35 S]methionine, were obtained as previously described (Danielsen *et al.*, 1982a). Na₂³⁵SO₄ was purchased from Amersham International, Amersham, Bucks., U.K. DCNP was a product of Fluka AG, Buchs, Switzerland.

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

Organ culture and processing of mucosal explants

Small-intestinal mucosal explants were labelled for periods of up to 5 h with either [35 S]methionine (100 μ Ci/ml) or [35 S]sulphate (200 μ Ci/ml) as previously described (Danielsen *et al.*, 1982a). When DCNP was included in the medium, the drug was added 1 h before the radioactive labelling compound. After culture, the mucosal explants were further processed immediately or else frozen at -80°C .

The mucosal explants were fractionated by the bivalent-cation technique (Schmitz *et al.*, 1973; Booth & Kenny, 1974) as previously described (Danielsen, 1982).

Immunological methods

Immunopurification of individual microvillar enzymes by line immunoelectrophoresis was performed as indicated in Danielsen & Cowell (1983). Immunoprecipitation of major microvillar enzymes with an antibody directed against the microvillar membrane was carried out as previously described (Danielsen, 1987).

Electrophoresis

SDS/PAGE was performed as described by Laemmli (1970), and fluorography of the gels in accordance with Bonner & Laskey (1974). Exposed X-ray films were scanned in a Kipp und Zonen (Delft, The Netherlands) DD2 densitometer.

RESULTS AND DISCUSSION

Because of their abundance in epithelial cells, microvillar enzymes are suitable as probes for membrane

Fig. 1. Labelling of microvillar enzymes with [35 S]sulphate and [35 S]methionine

Mucosal explants, labelled either for 5 h with [35 S]sulphate (lanes 1–3) or for 2 h with [35 S]methionine (lanes 4 and 5) were homogenized in 1 ml of 50 mM-Tris/HCl buffer, pH 7.5, containing 5% Triton X-100. The detergent extracts were cleared by centrifugation for 5 min in a Microfuge (18000 rev./min), and an antibody to microvillar membrane was added. After 30 min on ice, the immunoprecipitate was pelleted by centrifugation for 5 min in the Microfuge, washed once in the above buffer, dissolved in reduction buffer, and subjected to SDS/PAGE. Lanes 1 and 4, immunoprecipitate; lane 2, detergent extract, absorbed with antibodies to microvillar membranes; lanes 3 and 5, detergent extract before immunoprecipitation. (Only in the [35 S]methionine-labelled immunoprecipitate are the transient high-mannose glycosylated forms of microvillar enzymes visible, owing to the late post-translational nature of sulphation.)

traffic of endogenous eukaryotic plasma-membrane proteins, and their biosynthesis has been investigated *in vivo* (Feracci *et al.*, 1985), in cell culture (Hauri *et al.*, 1985), in organ culture (Danielsen, 1982; Stewart & Kenny, 1984; Naim *et al.*, 1987) and by cell-free translation (Wacker *et al.*, 1981; Danielsen *et al.*, 1982b). A common theme in their biosynthesis appears to be an extensive *N*-linked co-translational glycosylation, followed by trimming and attachment of complex sugars as well as *O*-linked glycosylation during traversal of the Golgi complex. The post-translational modifications cause a rather large increase in molecular mass (about 25 kDa), which, together with the considerable time required for intracellular transport (about 1 h), makes it feasible to detect induced changes in the molecular processing and kinetics of intracellular transport. I recently observed that four microvillar enzymes undergo sulphation (Danielsen, 1987). The fact that the attached sulphate was resistant to alkaline hydrolysis but labile to a brief acid treatment demonstrated that it is predominantly tyrosine residues rather than the carbohydrate moiety that carries the sulphate. This, hitherto unusual, modification of plasma-membrane proteins prompted the present investigation to determine its possible importance for the intracellular transport and microvillar expression of aminopeptidase N.

Fig. 1 (lanes 1–3) shows the labelling of total explant

Fig. 2. Effect of DCNP on sulphation of microvillar enzymes

Mucosal explants were labelled with [35 S]sulphate for 5 h in the absence (–) or in the presence (+) of 50 μ M-DCNP. Microvillar enzymes were immunoprecipitated from the explants as described in the legend to Fig. 1 and subjected to SDS/PAGE.

protein by [35 S]sulphate during organ culture for 5 h. The most prominent bands are of molecular mass 250, 166, 75 and 60 kDa. The first-mentioned two of these bands could be quantitatively immunoprecipitated from a detergent extract of explants with an antibody directed towards the microvillar membrane (Danielsen *et al.*, 1977). They represent the major microvillar hydrolases sucrase-isomaltase (EC 3.2.1.48–10), maltase-glucosylase (EC 3.2.1.20) (which both contribute to the 250000- M_r band) and aminopeptidase N (EC 3.4.11.2). Supplementing the culture medium with [35 S]methionine instead resulted in a much more complex pattern of labelled bands in the detergent extract, but the microvillar antibody precipitated essentially the same components as with [35 S]sulphate labelling (Fig. 1, lanes 4 and 5). Sulphation is thus restricted to only a few proteins, synthesized by the enterocyte, including its microvillar enzymes. In fact, the sulphated 75 kDa and 60 kDa bands were found to be of soluble origin and their sulphate is probably carbohydrate-bound (Danielsen, 1987).

If tyrosine sulphation is of importance for microvillar-directed transport (for instance as a sorting signal), one should expect that interference of this type of processing would obscure the intracellular transport of newly synthesized microvillar enzymes, either by retarding their apical expression or possibly by causing an intracellular mis-sorting, leading to accumulation of microvillar enzymes in other organelles. DCNP has been described to inhibit selectively the phenol sulphotransferase from rat liver *in vivo* as well as *in vitro* (Mulder & Scholtens, 1977). In the latter case 0.1 μ M caused about 50% inhibition and complete inhibition was achieved at 1 μ M. DCNP thus appears to be a suitable drug for studying

Fig. 3. Effect of DCNP on protein synthesis

Mucosal explants were labelled with [35 S]methionine for 5 h in the absence (–) or in the presence (+) of 50 μ M-DCNP. After culture, the explants were fractionated into Mg^{2+} -precipitated (Mg^{2+}), microvillar (Mic) and soluble (Sol) fractions as described in the Experimental section and subjected to SDS/PAGE.

Fig. 4. [35 S]Methionine labelling of aminopeptidase N in the presence of DCNP

Mucosal explants were labelled with [35 S]methionine for 3 h in the absence (–) or in the presence (+) of 50 μ M-DCNP. After culture, the explants were fractionated into a Mg^{2+} -precipitated (Mg^{2+}) and a microvillar (Mic) fraction. Aminopeptidase N was immunopurified from the subcellular fractions and subjected to SDS/PAGE.

Fig. 5. Kinetics of post-translational processing and intracellular transport of aminopeptidase N in the presence of DCNP

Mucosal explants were labelled with [35 S]methionine for periods of 0.5–5 h in the absence (●, ■ and ▲) or in the presence (○, □ and △) of 50 μ M-DCNP. Aminopeptidase N was immunopurified from subcellular fractions and subjected to SDS/PAGE as shown in Fig. 4. Gel tracks from exposed X-ray films were scanned and the relative intensities of the transient form (140 kDa), the mature form (166 kDa) in the Mg^{2+} -precipitated fraction and the mature form in the microvillar fraction were determined. ● and ○, Transient form; ■ and □, mature form in the Mg^{2+} -precipitated fraction; ▲ and △, mature form in the microvillar fraction.

the importance of tyrosine sulphation in the biosynthesis of microvillar enzymes. Fig. 2 shows the effect of DCNP (50 μ M) on sulphation of microvillar enzymes. As can be seen, the drug drastically decreased the incorporation of radioactivity in bands of both 250 and 166 kDa. Densitometric scanning of gel tracks from several experiments revealed a 70–85% decrease in labelling compared with the controls. In contrast with its severe effect on sulphation, DCNP (50 μ M) had no general influence on the incorporation of [35 S]methionine into protein from Mg^{2+} -precipitated (intracellular and basolateral membranes), microvillar or soluble fractions of organ-cultured mucosal explants during continuous labelling for 5 h (Fig. 3). Since protein synthesis in cultured mucosal explants is fairly linear for periods of up to 24 h (Danielsen *et al.*, 1982a), this parameter was unaffected by DCNP. DCNP at higher concentrations (500 μ M) did inhibit protein synthesis but was unable to abolish completely the sulphation of microvillar enzymes. The residual sulphation, seen in Fig. 2 in the presence of DCNP (15–30%), may well therefore be ascribed to carbohydrate sulphation of microvillar enzymes, which is not inhibited by the drug.

DCNP thus seems to be an ideal drug for studying the relevance of tyrosine sulphation for intracellular transport and apical expression of newly synthesized microvillar enzymes. Aminopeptidase N was selected as a marker for this; in previous work it has been shown to be synthesized in the rough endoplasmic reticulum as a

140 kDa high-mannose glycosylated polypeptide (Danielsen, 1982). This transient form prevails for about 30 min, whereafter it undergoes processing to the mature form of 166 kDa that is subsequently expressed in the microvillar membrane. As shown in Fig. 4, DCNP caused no detectable changes in the size or subcellular distribution of the transient or mature forms of aminopeptidase N. Its possible effect on the kinetics of intracellular transport and processing was further studied by labelling experiments for 0.5–5 h (Fig. 5). It is evident that these parameters in DCNP-exposed and control explants are very similar. Thus in both cases the half-life of the transient form was about 90 min, indicating an unaffected migration from the site of synthesis to, and through, the Golgi complex. Likewise, the half-time for the appearance in the microvillar membrane was about 105 min, showing that the rate of post-Golgi transport was similarly unaffected. Moreover, DCNP did not cause an accumulation of the mature form of aminopeptidase N in the Mg^{2+} -precipitated fraction, as would have been expected if tyrosine sulphation were to play an essential role as sorting signal for the apical cell surface. Rather, it can be concluded that tyrosine sulphation bears no implications for the targeting of microvillar enzymes to their extracellular site of function.

For several years investigators have tried to elucidate a functional relationship between molecular processing and site-directed intracellular transport, not only for microvillar enzymes but also for membrane proteins in general, as well as for secretory proteins (Sabatini *et al.*, 1982). The quest has no doubt been inspired by the role played by mannose 6-phosphate in the targeting of lysosomal enzymes, and accordingly one should expect to find identical sorting signals only on proteins with a common subcellular destination. Tyrosine sulphation would appear to fulfil this requirement, but clearly the result of the present work is not compatible with this notion. Similarly, it has been reported that tyrosine sulphation is not a prerequisite for the cellular export of thyroglobulin from thyroid follicle cells (Herzog, 1986), suggesting that this type of processing most probably is a result rather than the cause of sorting.

It has recently been argued that transport from the rough endoplasmic reticulum to the plasma membrane can primarily be characterized as a bulk flow of components, lacking a retention signal (Wieland *et al.*, 1987). However attractive, this hypothesis fails to provide an explanation of how polarized cells maintain their two plasma-membrane domains apart. In this context it is interesting that in the enterocyte small amounts of newly made microvillar enzymes have been localized in lysosomes (Fransen *et al.*, 1985; Hansen *et al.*, 1987) as well as in the basolateral membrane (Moktari *et al.*, 1986), suggesting that the process whereby sorting takes place may not be all that accurate. Nevertheless its elucidation is an important task within this field that still needs to be carried out.

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