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Tyrosine sulfation, a post-translational modification of microvillar enzymes in the small intestinal enterocyte

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Protein sulfation in small intestinal epithelial cells was studied by labelling of organ cultured mucosal explants with [^{35}S]-sulfate. Six bands in SDS–PAGE became selectively labelled; four, of 250, 200, 166 and 130 kd, were membrane-bound and two, of 75 and 60 kd, were soluble. The sulfated membrane-bound components were all enriched in the microvillar fraction but either absent or barely detectable in intracellular or basolateral membranes. Immunopurification of sucrase-isomaltase, maltase-glucoamylase, aminopeptidase N and aminopeptidase A showed that these microvillar enzymes become sulfated. Most if not all the sulfate was bound to tyrosine residues rather than to the carbohydrate of the microvillar enzymes, showing that this type of modification can occur on plasma membrane proteins as well as on secretory proteins.

Key words: biosynthesis/microvillar enzymes/protein sulfation/small intestine

Introduction

Sulfation of proteins, i.e. a post-translational modification entailing covalent attachment of sulfate, is a fairly common type of processing of newly synthesized proteins that originate from the RER and are destined for either the plasma membrane or cellular export (Huttner, 1982, 1984). The principal sites for sulfation are either tyrosine residues in the polypeptide backbone or different sugars in the carbohydrate moiety (Kornfeld and Kornfeld, 1985). Contrary to the latter, tyrosine sulfation has been considered a specific type of modification for secretory proteins (Hille *et al.*, 1984), and it has been suggested that tyrosine sulfation may be involved in the sorting of proteins destined for cellular export (Rosa *et al.*, 1985). This notion has gained support not only from the many examples of tyrosine sulfated secretory proteins but also from an observation that the secreted but not the cellular form of dopamine β-hydroxylase in rat pheochromocytoma cells undergoes tyrosine sulfation (McHugh *et al.*, 1985).

The biosynthesis of intestinal and kidney microvillar enzymes has been studied intensively over the past years (Danielsen *et al.*, 1984; Norén *et al.*, 1986; Semenza, 1986). Without exception, stalked, integral membrane proteins of this type undergo cotranslational high mannose glycosylation followed by trimming and attachment of complex sugars during passage through the Golgi complex. We have studied the relevance of this processing for microvillar expression of newly synthesized enzymes (Danielsen *et al.*, 1983b; Danielsen and Cowell, 1984, 1986) and have found that cotranslational attachment of N-linked oligosaccharides is essential for molecular survival and subsequent transport to the microvillar membrane. Cotranslational trimming is likewise important for acquisition of molecular stability but is not required *per se* for microvillar-directed transport. Finally, the Golgi-associated carbohydrate processing is of only small significance for both stability and surface expression, but for at least one

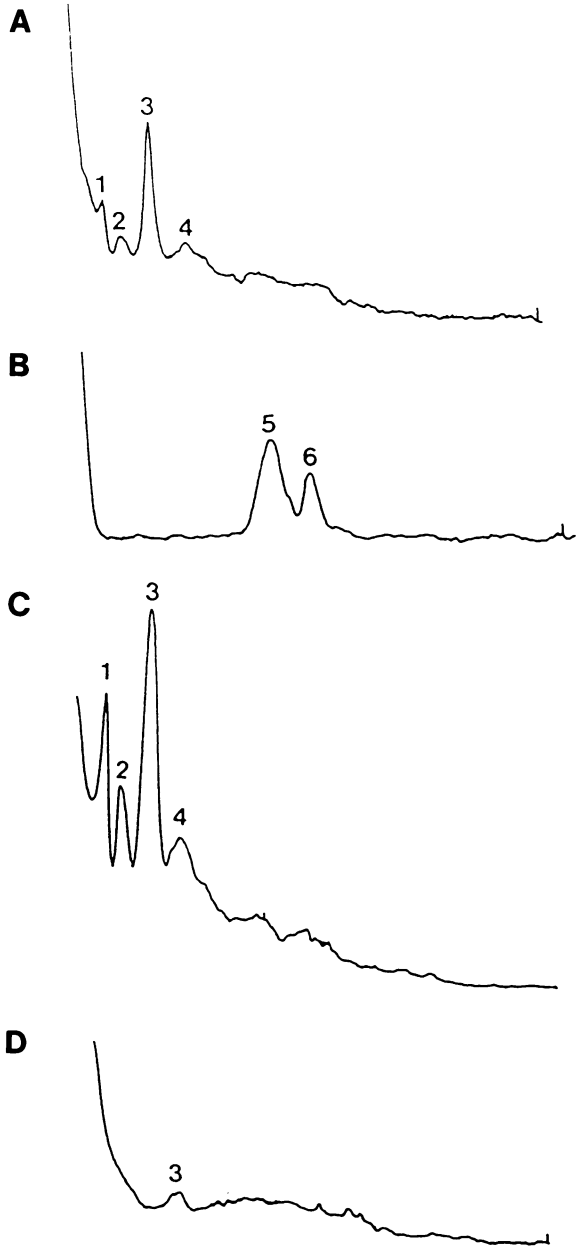

Fig. 1. Sulfation of mucosal explant proteins. Densitometric scanning of SDS–PAGE of subcellular fractions of mucosal explants, labelled with [^{35}S]sulfate. **A**, total fraction of membrane-bound proteins (pellet after centrifugation at 100 000 *g*, 1 h of a homogenate); **B**, soluble proteins (supernatant after centrifugation at 100 000 *g*, 1 h of a homogenate); **C**, microvillar fraction; **D**, Mg^{2+}-precipitated fraction (intracellular and basolateral membranes).

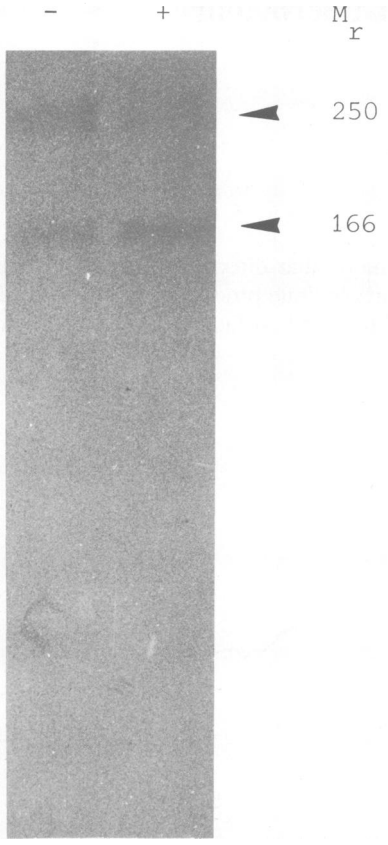

Fig. 2. Alkaline treatment of sulfated proteins. A total fraction of membrane-bound proteins (pellet after centrifugation at 100 000 *g*, 1 h of a homogenate) from [^{35}S]sulfate-labelled mucosal explants was subjected to alkaline treatment (Cowell and Danielsen, 1984). After pH neutralization, the alkaline-treated membranes were solubilized by Triton X-100 (5%) and microvillar enzymes immunoprecipitated as described in Materials and methods. The immunoprecipitates of alkaline-treated (+) and controls (−) were subjected to SDS−PAGE, followed by fluorography. M_r values for the sulfated bands are indicated.

enzyme, sucrase-isomaltase, the biological activity depends upon it (Sjöström *et al.*, 1985).

So far, no reports have been concerned with sulfation of microvillar enzymes. The present paper deals with this aspect of microvillar enzyme biosynthesis.

Results

Labelling of mucosal explants with [^{35}S]sulfate

The total membrane fraction of mucosal explants, cultured for 20 h in the presence of [^{35}S]sulfate, showed four labelled bands of about 250, 200, 166 and 130 kd (designated bands 1−4 respectively, Figure 1). [^{35}S]Sulfate-labelling of the corresponding soluble fraction yielded two broad bands of 75 and 60 kd (designated bands 5 and 6 respectively). In contrast, labelling with [^{35}S]methionine in parallel experiments resulted in a multitude of bands over the entire M_r range of the gel (data not shown). For both membrane-bound and soluble proteins, sulfation is therefore limited to only a few of the components synthesized by the mucosal explants.

The sulfated bands of membrane-bound origin were analyzed further by subcellular fractionation into microvillar- and Mg^{2+}-precipitated (intracellular and basolateral) membranes (Figure 1). Compared with total membranes, all four bands were markedly enriched in the microvillar fraction whereas they were absent from the Mg^{2+}-precipitated fraction (except for minute amounts of band 3). The microvillar origin of bands 1 and 3 was further documented by their immunoprecipitation with an antibody raised against the brush border membrane that reacts specifically with the apical plasma membrane as judged by immunofluorescence and which has been shown to recognize five of the major microvillar enzymes, sucrase-isomaltase, maltase-glycoamylase, lactase-phlorizin hydrolase, aminopeptidase N and aminopeptidase A (Danielsen *et al.*, 1977; Figure 2). A Triton X-100 extract of [^{35}S]sulfate-labelled explants, absorbed with this antibody, was void of bands 1 and 3, showing that these major microvillar enzymes are their only constituents (data not shown).

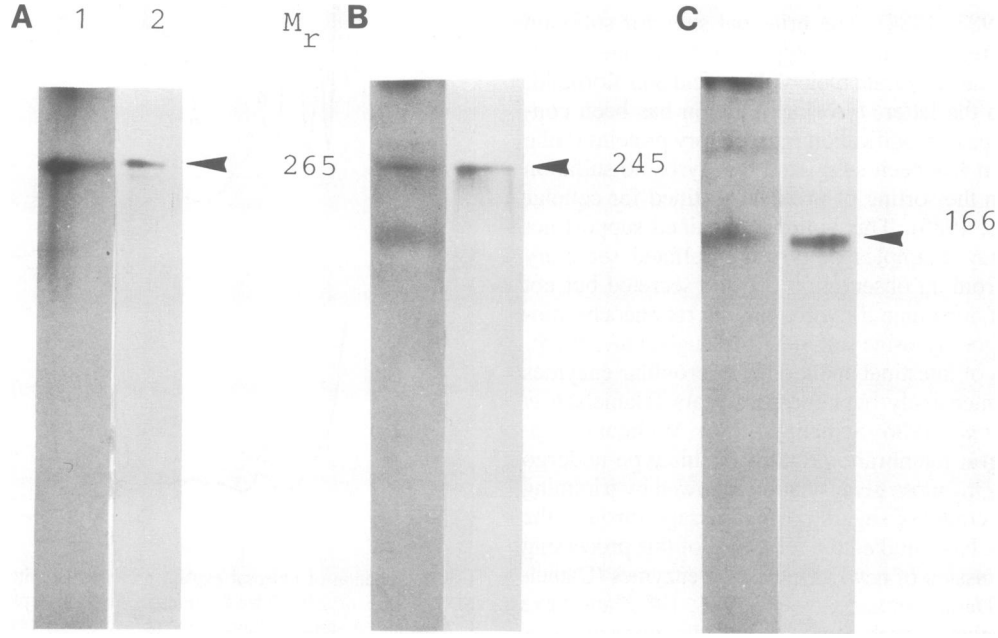

Fig. 3. Sulfation of microvillar enzymes. SDS−PAGE of microvillar enzymes, immunopurified from explants labelled with [^{35}S]sulfate (1) or [^{35}S]methionine (2). **A**, sucrase-isomaltase; **B**, maltase-glucoamylase; **C**, aminopeptidase N. M_r values (kd) are indicated.

The sulfated band 1 and band 3 were unaffected by an alkaline treatment of the membranes prior to the immunoprecipitation, showing them to be integral membrane proteins (Figure 2). This treatment has previously been shown to release cytoskeletal components such as actin, villin and the 110 kd protein from microvilli whereas the microvillar enzymes remain in the membrane (Cowell and Danielsen, 1984).

Direct evidence that bands 1 and 3 represent microvillar enzymes is shown in Figure 3 where sucrase-isomaltase, maltase-glucoamylase and aminopeptidase N were individually immunopurified from explants, labelled with either [^{35}S]sulfate or [^{35}S]-methionine. Similarly, the less abundant aminopeptidase A (Figure 4) and dipeptidyl peptidase IV (data not shown) were found to become sulfated. It thus seems fair to conclude that band 1 is constituted by sucrase-isomaltase and maltase-glucoamylase and band 3 by aminopeptidases N and A.

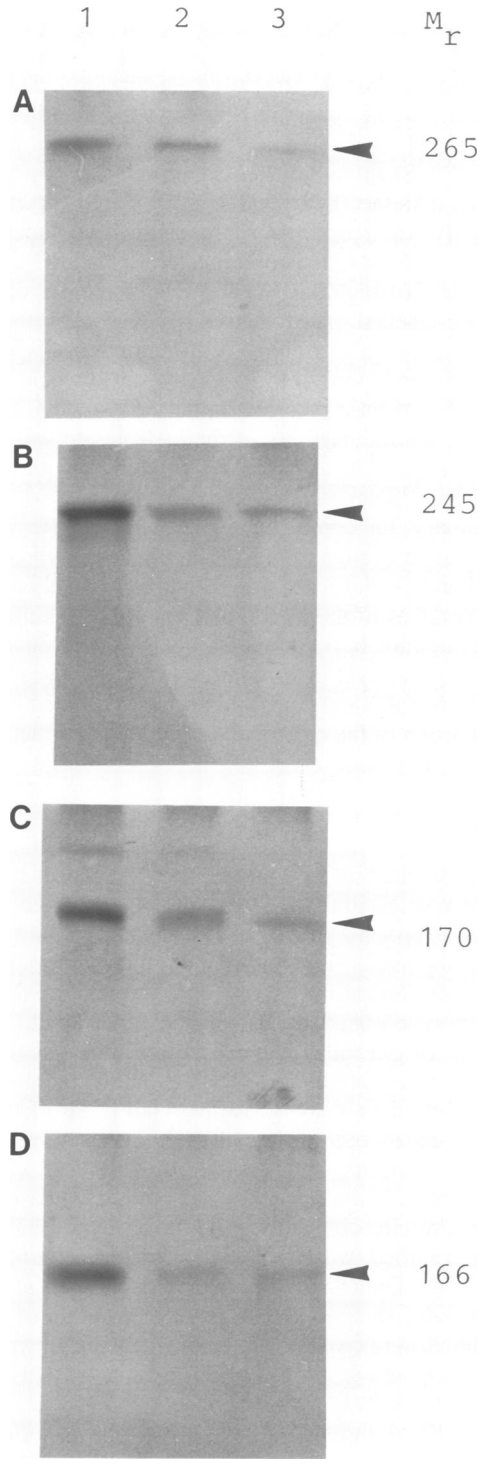

Fig. 4. Effect of swainsonine and castanospermine on sulfation. SDS–PAGE of microvillar enzymes, immunopurified from explants labelled with [^{35}S]sulfate in the absence of inhibitor (1) or in the presence of swainsonine (2) or castanospermine (3). **A**, sucrase-isomaltase; **B**, maltase-glucoamylase. **C**, aminopeptidase A; **D**, aminopeptidase N. M_r values (kd) are indicated.

Effect of swainsonine and castanospermine

Swainsonine and castanospermine are alkaloids that interfere with the processing of N-linked oligosaccharides; swainsonine at a post-translational stage by inhibiting Golgi mannosidase II (Tulsiani *et al.*, 1982), and castanospermine cotranslationally by blocking RER glucosidase I (Saul *et al.*, 1983). In previous studies with aminopeptidase N, both inhibitors gave rise to mature forms of the enzyme of about normal M_r (166 kd) that were sensitive to endo H, indicating a blocked attachment of complex sugars to the N-linked carbohydrate (Danielsen *et al.*, 1983b; Danielsen and Cowell, 1986). As shown in Figure 4, neither swainsonine nor castanospermine prevented labelling of the microvillar enzymes, showing that processing of N-linked oligosaccharides is not required for their sulfation. The decrease in labelling can be fully accounted for by an induced degradation of the newly synthesized microvillar enzymes, caused by the aberrant glycosylation; incorporation of [^{35}S]methionine was reduced to 15–35% of controls within a 3 h labelling period (Danielsen and Cowell, 1986).

Treatment with endo F

Endo F is a glycosidase that cleaves N-linked oligosaccharides of both high mannose and complex types (Elder and Alexander, 1982), and both transient and mature forms of aminopeptidase N and sucrase-isomaltase are susceptible to endo F (Danielsen and Cowell, 1984). In accordance with this, both bands 1 and 3, obtained from [^{35}S]sulfate-labelled explants by immunoprecipitation, had their electrophoretic mobility increased upon treatment with endo F (Figure 5). However, the fact that the labelling persisted the treatment argues against the sulfate being bound to N-linked carbohydrate.

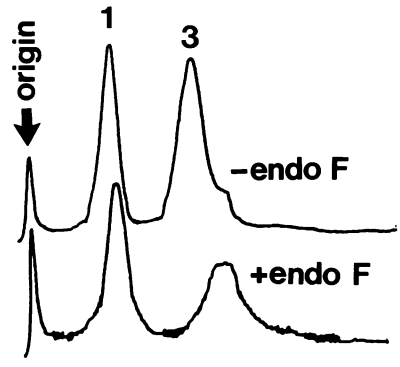

Fig. 5. Endo F analysis of sulfated microvillar enzymes. Densitometric scanning of SDS–PAGE of microvillar enzymes, immunoprecipitated from explants, labelled with [^{35}S]sulfate. Lower track, endo F-treated; upper track, sample incubated in parallel without the glycosidase.

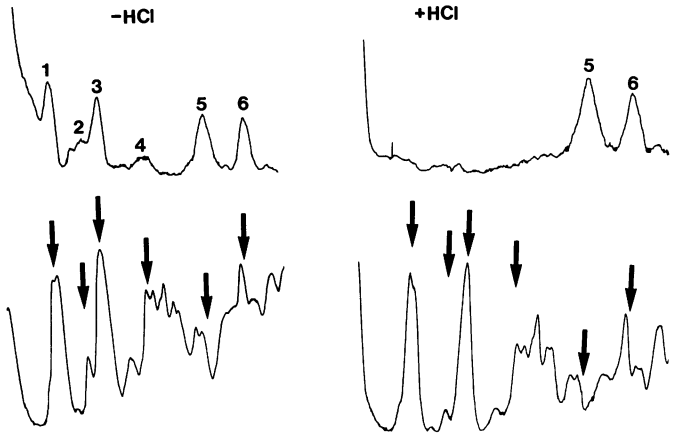

Fig. 6. Acid hydrolysis of sulfated proteins. Triton X-100 extracts of explants, labelled with [^{35}S]sulfate (upper tracks) or [^{35}S]methionine (lower tracks) were subjected to SDS–PAGE. After electrophoresis, the gel tracks were immersed in 1 M HCl at 80°C for 30 min **(right panel)** before fluorography. Neighbouring lanes from the same gel were used as controls **(left panel)**. Arrows indicate the positions of the sulfated bands.

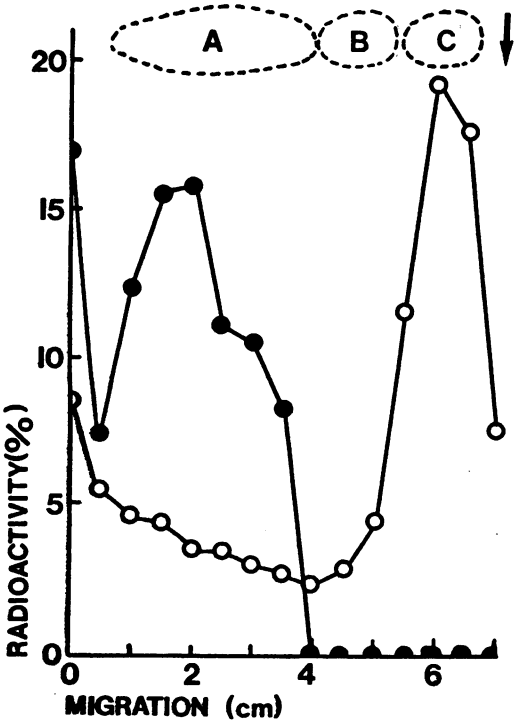

Fig. 7. T.l.c. analysis of alkaline hydrolysates of microvillar enzymes. Alkaline hydrolysates of microvillar enzymes, immunoprecipitated from Triton X-100 extracts of [^{35}S]sulfate-labelled explants, were subjected to t.l.c. as described in Materials and methods. Samples of about 1000 c.p.m. were applied. ○, alkaline hydrolysate; ●, alkaline hydrolysate after acidification (1 M HCl) and boiling for 1 h, followed by pH neutralization. The chromatographic position of free [^{35}S]sulfate (**A**), cysteic acid (**B**) and tyrosine sulfate (**C**) are shown. The solvent front is indicated by the arrow.

Susceptibility to acid and alkaline hydrolysis

Sulfate ester linkages to either tyrosine residues in the polypeptide backbone or to the carbohydrate moiety of glycoproteins can be distinguished by their differing susceptibility to hydrolysis under acid and alkaline conditions. Thus, the tyrosine sulfate ester linkage is labile at acid pH, even to brief exposures; a treatment that does not release sulfate, linked to carbohydrate (Huttner, 1984; Rosa *et al.*, 1985). As shown in Figure 6, bands 1–4 vanished upon the low pH treatment of the gel whereas bands 5 and 6, of soluble origin, persisted despite the acid exposure. The [^{35}S]methionine-labelled gel track, treated in parallel, was unaffected by the treatment.

Contrary to its stability at acid pH, exhaustive hydrolysis in 0.2 N $Ba(OH)_2$ releases carbohydrate-linked sulfate which can be recovered as $BaSO_4$ after pH neutralization. Sulfate bound to tyrosine, on the other hand, resists the alkaline hydrolysis (Huttner, 1984; Hohmann *et al.*, 1985). When an immunoprecipitate of [^{35}S]sulfate-labelled microvillar enzymes was subjected to alkaline hydrolysis, 89% (568 c.p.m.) of the radioactivity was recovered in the neutralized supernatant and only 11% (68 c.p.m.) precipitated as $BaSO_4$. Alkalkine hydrolysates (using 0.2 N NaOH) of immunoprecipitated [^{35}S]sulfate-labelled microvillar enzymes were further analyzed by t.l.c. As shown in Figure 7, a predominant peak of radioactivity migrated close to the solvent front, coinciding with the position of authentic tyrosine sulfate. Following a short acid hydrolysis, the radioactivity of the hydrolysate was now found in a broad zone of lower mobility, coinciding with the distribution of free [^{35}S]sulfate.

In summary, the combined susceptibility to brief exposure to low pH and resistance to exhaustive alkaline hydrolysis of the sulfated bands 1 and 3 are the hallmarks of a sulfate linkage to tyrosine residues of the microvillar enzymes. If sulfation of the carbohydrate moiety occurs at all it can only account for a very small part of the total incorporation of [^{35}S]sulfate. In contrast, the soluble bands 5 and 6 probably become sulfated in the carbohydrate portion of the molecule, as judged from their resistance to the acid treatment.

Discussion

Labelling of mucosal explants with [^{35}S]sulfate led to a selective incorporation of radioactivity into only six bands in SDS–PAGE. We have no clue as to the identity of the two sulfated soluble components; it is possible that they represent secretory proteins from endocrine cells of the intestinal epithelium. The four membrane-bound bands, on the other hand, were all of microvillar origin rather than belonging to intracellular membranes or the basolateral portion of the plasma membrane. Bands 1 and 3, those most heavily labelled with [^{35}S]sulfate, comprise the most abundant of the microvillar enzymes such as sucrase-isomaltase (265 kd) and maltase-glucoamylase (245 kd) (band 1), and aminopeptidase N (166 kd) and aminopeptidaseA (170 kd) (band 3). Proteins constituting the minor, broad bands 2 and 4 were not identified, but given the uniform character of the biosynthetic events of microvillar enzymes (Danielsen *et al.*, 1984), it is reasonable to assume that they are made up of a multitude of less abundant microvillar enzymes which are all typically composed of polypeptides in the high M_r range. Likely candidates are the angiotensin I-converting enzyme (184 kd; Hauri *et al.*, 1985), a 140 kd antigen (Gorvel *et al.*, 1986), dipeptidyl peptidase IV (137 kd; Danielsen *et al.*, 1983a), aminopeptidase W (130 kd; Gee and Kenny, 1985), PABA peptide hydrolase (100 kd; Hauri *et al.*, 1985) and endopeptidase 24.11 (93 kd; Stewart and Kenny, 1984), to name a few.

Contrary to the processing of their N-linked carbohydrate, sulfation of the four microvillar enzymes studied was not sensitive to either swainsonine or castanospermine, showing that complex sugars are unlikely sites for sulfation. It also demon-

strates that sulfation, which is a post-translational modification taking place in the Golgi complex (Lee and Huttner, 1985), can proceed independently of carbohydrate processing. The experiment with endo F further corroborated the view that sulfation is unrelated to the N-linked carbohydrate moiety of the microvillar enzymes.

More conclusive evidence in favour of tyrosine sulfation was provided by the demonstration that the sulfate, bound to microvillar enzymes, is acid labile but alkaline resistant, a typical property of the tyrosine sulfate ester linkage and, not least, by t.l.c. analysis. There can thus be little doubt that the major part, if not all, of the sulfate bound to microvillar enzymes is linked to tyrosine. This is an unexpected finding since tyrosine sulfation until now has been regarded as a specific type of processing for secretory proteins (Hille *et al.*, 1984). However, as shown by their resistance to alkaline extraction, the sulfated microvillar enzymes are clearly integral membrane proteins and, recently, tyrosine sulfation was reported for another plasma membrane protein, P61 from A431 cells (Liu and Baenziger, 1986). This type of modification therefore seems to be more ubiquitous than currently thought and our notion concerning its occurrence thus needs to be revised accordingly.

Materials and methods

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.*, 1982). Sodium [^{35}S]sulfate was purchased from Amersham, Bucks, UK. Swainsonine was kindly given by Dr Peter Dorling, Murdoch University, Western Australia and (non-radioactive) tyrosine sulfate by Dr Thue W.Schwartz, Rigshospitalet, Copenhagen, Denmark. Endo-β-*N*-acetylglucosaminidase F (endo F) from *Flavobacterium meningosepticum* was obtained from NEN Chemicals GmbH, Dreieich, FRG and castanospermine from Boehringer, Mannheim, FRG.

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

Organ culture and subcellular fractionation of mucosal explants

Small intestinal mucosal explants were labelled continuously for 20 h with either [^{35}S]methionine (50–100 μCi/ml) or [^{35}S]sulfate (200–300 μCi/ml) as previously described (Danielsen *et al.*, 1982). When labelling was carried out in the presence of swainsonine or castanospermine, the inhibitor was added to the medium (0.02 mg/ml and 0.1 mg/ml respectively) 1 h before the radioactive isotope. After culture, labelled explants were either processed further immediately or else frozen at −80°C.

Mucosal explants were fractionated by the divalent cation precipitation technique (Schmitz *et al.*, 1973; Booth and Kenny, 1974) as previously described (Danielsen, 1982).

Immunological methods

Immunopurification of individual microvillar enzymes by line immunoelectrophoresis was performed as in Danielsen and Cowell (1983). Immunoprecipitation of microvillar enzymes was performed as follows: labelled explants were thawed in 0.5 ml 50 mM Tris–HCl, pH 7.3, containing 2.8 μg/ml aprotinin and solubilized by the addition of Triton X-100 (5%) and vortexing. The detergent extract was cleared by centrifugation for 5 min at 18 000 r.p.m. in a microcentrifuge, and 2–3 vol. of an antibody solution (~10 mg IgG/ml), recognizing the major microvillar enzymes (Danielsen *et al.*, 1977) were added. After 30 min at 20°C, the immunoprecipitate was collected by centrifugation for 5 min in the microcentrifuge and washed twice in 1 ml of the above buffer.

Treatment with endo F

Immunoprecipitates of microvillar enzymes were treated with endo F, using the procedure previously described (Danielsen and Cowell, 1984).

Acid hydrolysis

Acid treatment of sulfated polypeptides after separation in SDS–PAGE was performed according to Huttner (1984). The samples applied to the gel were Triton X-100 extracts of labelled explants.

Alkaline hydrolysis

For alkaline hydrolysis, immunoprecipitates of [^{35}S]sulfate-labelled microvillar enzymes were dissolved in 1 ml 0.2 N $Ba(OH)_2$, sealed in a polyallomer tube and hydrolyzed by boiling for 24 h. The alkaline hydrolysates were next neutralized by the addition of H_2SO_4 and the resulting precipitate of $BaSO_4$ pelleted by centrifugation for 5 min in a microcentrifuge. The supernatant was collected and the precipitate washed three times in 1 ml 50 mM Tris–HCl, pH 7.3. The proportion of alkaline-stable and -labile radioactivity was determined by liquid scintillation counting of the supernatant and pellet, respectively.

For analysis by t.l.c., the immunoprecipitates were instead dissolved in 200 μl 0.2 N NaOH and hydrolyzed by boiling in a sealed tube for 24 h, followed by neutralization with HCl and concentration by evaporation to a vol. of about 20 μl.

T.l.c. analysis

Hydrolyzed immunoprecipitates of [^{35}S]sulfate-labelled microvillar enzymes were subjected to ascending t.l.c. on polyamide sheets using 1-butanol/formic acid/isopropanol/H_2O (3:1:1:1) as solvent. Authentic tyrosine sulfate and free [^{35}S]sulfate were used as markers. After chromatography, radioactivity was detected by scintillation counting of 0.5 cm segments of the chromatogram and tyrosine sulfate was visualized by staining with ninhydrin (0.5% in acetone).

Other methods

Alkaline treatment (100 mM Na_2CO_3, pH 11.0) was carried out as in Cowell and Danielsen (1984). SDS–PAGE was performed as described by Laemmli (1970) and fluorography according to Bonner and Laskey (1974). Exposed fluorographs were scanned in a Kipp & Zonen DD2 densitometer (Delft, Holland).

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References

Bonner,W.M. and Laskey,R.A. (1974) *Eur. J. Biochem.*, **46**, 83–88.
Booth,A.G. and Kenny,A.J. (1974) *Biochem. J.*, **142**, 575–581.
Cowell,G.M. and Danielsen,E.M. (1984) *FEBS Lett.*, **172**, 309–314.
Danielsen,E.M. (1982) *Biochem. J.*, **204**, 639–645.
Danielsen,E.M. and Cowell,G.M. (1983) *J. Biochem. Biophys. Methods*, **8**, 41–47.
Danielsen,E.M. and Cowell,G.M. (1984) *FEBS Lett.*, **166**, 28–32.
Danielsen,E.M. and Cowell,G.M. (1986) *Biochem. J.*, **240**, 777–782.
Danielsen,E.M., Sjöström,H., Norén,O. and Dabelsteen,E. (1977) *Biochim. Biophys. Acta*, **494**, 332–342.
Danielsen,E.M., Sjöström,H., Norén,O., Bro,B. and Dabelsteen,E. (1982) *Biochem. J.*, **202**, 647–654.
Danielsen,E.M., Sjöström,H. and Norén,O. (1983a) *Biochem. J.*, **210**, 389–393.
Danielsen,E.M., Cowell,G.M., Norén,O., Sjöström,H. and Dorling,P.R. (1983b) *Biochem. J.*, **216**, 325–331.
Danielsen,E.M., Cowell,G.M., Norén,O. and Sjöström,H. (1984) *Biochem. J.*, **221**, 1–14.
Elder,J.H. and Alexander,S. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 4540–4544.
Gee,N.S. and Kenny,A.J. (1985) *Biochem. J.*, **230**, 753–764.
Gorvel,J.P., Rigal,A., Olive,D., Mawas,C. and Maroux,S. (1986) *Biol. Cell*, **56**, 71–76.
Hauri,H.-P., Sterchi,E.E., Bienz,D., Fransen,J.A.M. and Marxer,A. (1985) *J. Cell Biol.*, **101**, 838–851.
Hille,A., Rosa,P. and Huttner,W.B. (1984) *FEBS Lett.*, **177**, 129–134.
Hohmann,H.-P., Gerisch,G., Lee,R.W.H. and Huttner,W.B. (1985) *J. Biol. Chem.*, **260**, 13869–13878.
Huttner,W.B. (1982) *Nature*, **299**, 273–276.
Huttner,W.B. (1984) *Methods Enzymol.*, **107**, 200–223.
Kornfeld,R. and Kornfeld,S. (1985) *Annu. Rev. Biochem.*, **54**, 631–634.
Laemmli,U.K. (1970) *Nature*, **277**, 680–685.
Lee,R.W.H. and Huttner,W.B. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 6143–6147.
Liu,N. and Baenziger,J.U. (1986) *J. Biol. Chem.*, **261**, 856–861.
McHugh,E.M., McGee,R. and Fleming,P.J. (1985) *J. Biol. Chem.*, **260**, 4409–4417.
Norén,O., Sjöström,H., Danielsen,E.M., Cowell,G.M. and Skovbjerg,H. (1986) In Desnuelle,P., Sjöström,H. and Norén,O. (eds), *Molecular and Cellular Basis of Digestion*. Elsevier, Amsterdam, pp. 335–365.
Rosa,P., Fumagalli,G., Zanini,A. and Huttner,W.B. (1985) *J. Cell Biol.*, **100**, 928–937.
Saul,R., Chambers,J.P., Molyneux,R.J. and Elbein,A.D. (1983) *Arch. Biochem. Biophys.*, **221**, 593–597.
Schmitz,J., Preiser,H., Maestracci,D., Ghosh,B.K., Cerda,J.J. and Crane,R.K. (1973) *Biochim. Biophys. Acta*, **323**, 98–112.
Semenza,G. (1986) *Annu. Rev. Cell Biol.*, **2**, 255–313.

Sjöström,H., Norén,O. and Danielsen,E.M. (1985) *J. Pediatr. Gastroenterol. Nutr.*, **4**, 980–983.
Stewart,J.R. and Kenny,A.J. (1984) *Biochem. J.*, **224**, 549–558.
Tulsiani,D.R.P., Harris,T.M. and Touster,O. (1982) *J. Biol. Chem.*, **257**, 7936–7939.

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