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Characterization of an integral membrane glycoprotein associated with the microfilaments of pig intestinal microvilli

Evelyne Coudrier, Hubert Reggio* and Daniel Louvard

European Molecular Biology Laboratory, Postfach 102209, D-6900 Heidelberg, FRG

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An integral membrane glycoprotein of pig intestinal microvilli which exists in two polypeptide forms [mol. wt. 140 K and 200 K as measured by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)] was purified to homogeneity and characterized. The 200-K form is probably a precursor of the 140-K species. We have localized the glycoprotein by electron microscope immunochemistry using specific antibodies and determined its topological organization with respect to the membrane bilayer. Triton X-100 treatments which solubilize most other microvillar membrane glycoproteins from purified, closed, right-side out vesicles do not efficiently extract this protein. The protein can be partially solubilized from the detergent-insoluble residue, either by treatment with proteases (trypsin or papain) or by exposure to low ionic strength buffer in the presence of chelating agents and detergents. Once solubilized by papain or trypsin, the protein co-migrates on SDS-PAGE with the protein obtained by low ionic strength extraction. However, the form of the protein released by papain does not bind detergents and exhibits hydrophilic properties. Our observations are consistent with the 140-K protein having a small hydrophobic domain that anchors it to the microvillar membrane. The 140-K glycoprotein binds *in vitro* to a 110-K protein of the core cytoskeleton residue. These observations suggest that the 140-K glycoprotein may be a transmembrane protein which may *in vivo* provide attachment sites for direct or indirect association with polypeptides of the microvillus cytoskeleton.
Key words: microvilli/microfilaments/membrane proteins, membrane-cytoskeleton interactions/immunocytochemistry

Introduction

The brush border of intestinal cells is highly organized and provides a useful model for studying membrane-cytoskeleton interactions in nucleated cells for the following reasons. (1) Each microvillus contains a bundle of axial microfilaments anchored at its tip. In addition, short filaments connect each bundle to the membrane perpendicular to the long axis of the bundle (Mooseker and Tilney, 1975). (2) Fractionation procedures are available to prepare large amounts of intact microvilli (Bretscher and Weber, 1978a; Howe *et al.*. 1980) or small sealed vesicles oriented right-side out (Louvard *et al.*, 1973). Both preparations are composed of microvillar membranes still associated with microfilaments. (3) Insoluble material obtained by extraction of microvilli with non-ionic detergents has a relatively simple polypeptide composition. Five major polypeptides have been identified, but none of the actin-binding proteins associated with microfilaments in non-muscle cells have been found in intestinal microvilli (Bretscher and Weber, 1978b; Geiger *et al.*, 1979).

Two major proteins are associated with the axial actin bundles: (a) villin (95 K), an actin-binding protein of intestinal and renal microvilli (proximal tubule) (Bretscher *et al.*, 1981) and regulated by Ca^{2+} ions (Bretscher and Weber, 1980a); and (b) fimbrin (68 K), found in several cell types in culture (Bretscher and Weber, 1980b). Two other major proteins are associated with the lateral bridges connecting the axial actin bundles to the membrane: (a) a 110-K protein whose association with the actin bundles may be controlled *in vitro* by ATP (Matsudaira and Burgess, 1979), and (b) calmodulin (Glenney and Weber, 1980).

We have reported the presence of another polypeptide (140 K) which is associated with the core residue of intestinal microvillar vesicles after extraction with Triton X-100 and is presumably glycosylated since it binds to Concanavalin A-ultrogel (Coudrier *et al.*, 1981, 1982). Here we report the further characterization of this 140-K polypeptide, its organisation with respect to the membrane bilayer and its binding to detergent micelles. This protein also binds to a 110-K polypeptide *in vitro* indicating that it may be a trans-membrane glycoprotein and thus may provide *in vivo* an attachment site for the microvillar microfilaments to their surrounding membranes.

Results

The core residue prepared from intestinal microvillar vesicles contains a glycosylated polypeptide of high mol. wt. (140 K)

Vesicles derived from pig intestinal microvilli, obtained in the presence of calcium ions, were treated with Triton X-100. A supernatant and an insoluble residue were obtained by centrifugation, and analysed on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 1). The core residue (P_1) contains known cytoskeletal polypeptides and, in addition, a 140-K species. Aminopeptidase and disaccharidases associated with intestinal microvilli are found in the same mol. wt. range, but they are recovered quantitatively in the first detergent extract (S_1). The core residue has $<1\%$ of these intestinal enzymes, as well as of alkaline phosphatase and γ-glutamyl transferase (Louvard *et al.*, 1975).

Only $50-70\%$ of the 140-K polypeptide was extracted by Triton X-100. Since it co-migrates with aminopeptidase and disaccharidases its presence in the first supernatant (S_1) could only be assessed using a quantitative immunological technique (Howe and Hershey, 1981) with specific antibodies (see below). This protein exists in two forms co-migrating on SDS-PAGE and represents $7-10\%$ of the proteins in our vesicle preparation. One form is extractable by Triton X-100, as are the membrane hydrolases, while the other remains associated with the core residue. Further extraction of the latter with various ionic or non-ionic detergents, and chaotropic or denaturing agents, proved to be inefficient for quantitative recovery of this polypeptide in a non-sedimentable form (100 000 *g*, 45 min).

Dissociation procedures used to release the 140-K species in a non-sedimentable form

Extraction of the core residue in low ionic strength buffer containing chelating agents (2.5 mM EDTA) and 0.1% Triton X-100 solubilized up to 30% of the 140-K form

*To whom reprint requests should be sent.

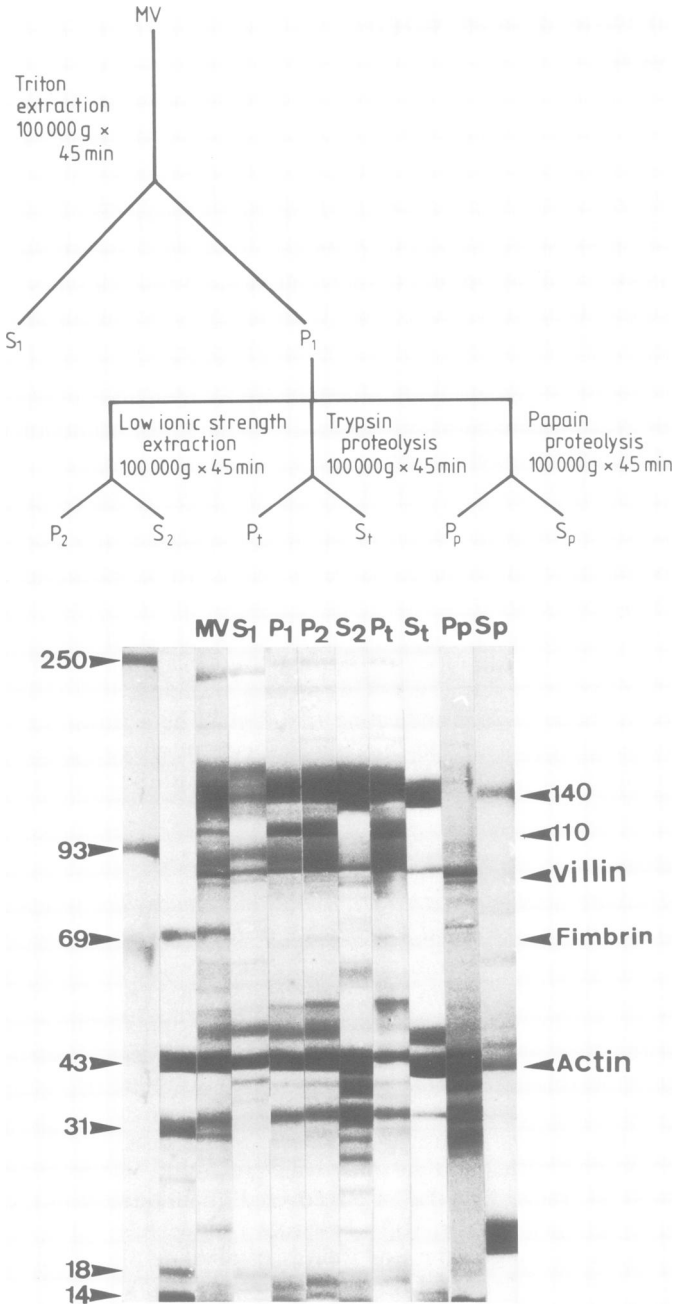

Fig. 1. Protein composition of isolated vesicles derived from pig intestinal microvilli and fractions containing the various forms of the 140-K protein analysed on SDS-PAGE. MV: total microvillar protein from right-side out vesicles (Louvard *et al.*, 1973); S_1: Triton X-100 extract of the vesicles preparation; P_1: core residue obtained after Triton X-100 extraction of the vesicles; P_2: pellet obtained after extraction of the core residue with a low ionic strength buffer; S_2: supernatant obtained after extraction of the core residue with a low ionic strength buffer; P_t: pellet obtained after trypsin treatment of the core residue; S_t: supernatant obtained after trypsin treatment of the core residue; P_p: pellet obtained after papain treatment of the core residue; S_p: supernatant obtained after papain treatment of the core residue. Mol. wt. markers: filamin (250 K), phosphorylase b (93 K), BSA (69 K), actin (43 K), pancreatic DNase (31 K), soybean trypsin inhibitor (18 K) and lysozyme (14 K). The positions of the major identified microvillar proteins are also indicated; Cal = calmodulin.

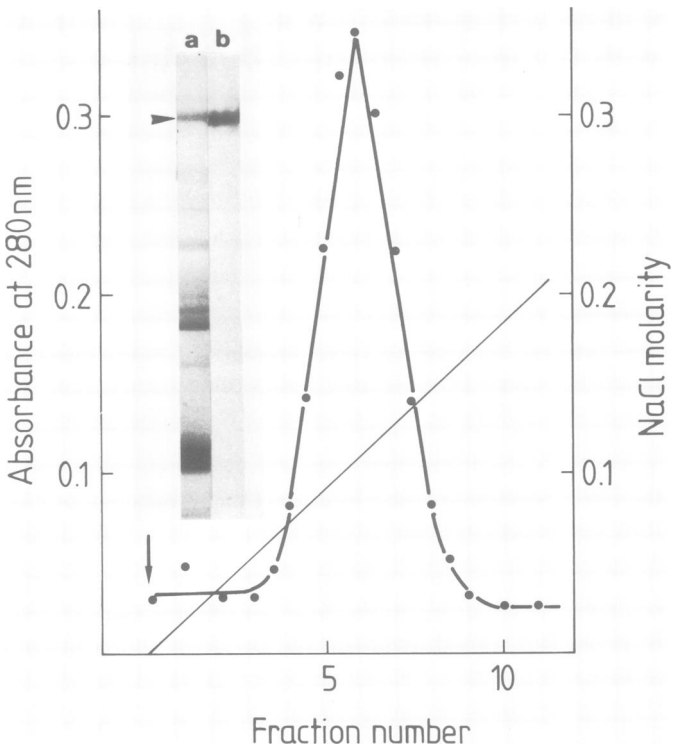

Fig. 2. Elution profile on DEAE-trisacryl resin of the supernatant obtained after papain treatment (S_p). 2 ml of DEAE-trisacryl was packed and equilibrated in 10 mM phosphate buffer adjusted to pH 6.2 containing 1 mM PMSF, 1.5 mg of S_p was then applied to the column. The elution was performed with a linear NaCl gradient and the fractions collected measured for their absorbance at 280 nm. **Inset**: composition of eluted material as analysed by electrophoresis on 6.5–17% SDS-PAGE and stained with Coomassie blue.

(Figure 1, S_2). However, subsequent purification of the 140-K polypeptide from this extract using conventional chromatography procedures proved to be difficult and irreproducible. Possibly, the large amounts of cyoskeletal proteins, particularly villin and actin, aggregate with the extracted 140-K form.

Limited digestion of the core residue by trypsin, or papain solubilized the 140-K protein (Figure 1, S_t, S_p) to yield polypeptides which co-migrate with the intact corresponding protein in the core residue (Figure 1, P_1). Using ion exchange and gel filtration chromatography, the 140-K protein has been purified to homogeneity from the supernatant obtained by trypsin proteolysis (Coudrier *et al.*, 1982). The protein released by papain has been purified in one step by chromatography on DEAE-trisacryl (Figure 2). The solubilized protein extract was fully absorbed on the resin when equilibrated in 10 mM phosphate buffer, pH 6.2. A single symmetric peak of protein was eluted by linearly increasing the ionic strength (0–0.2 M NaCl) (Figure 2). SDS-PAGE shows that this peak contains one major polypeptide, poorly stainable with Coomassie blue, with an apparent mol. wt. of 140 K (Figure 2, inset). The papain form of the 140-K protein lacks a hydrophobic domain still present in the 'trypsin form' and the 'low ionic strength form' (see below). This difference presumably accounts for its easy purification.

Antibodies raised against the purified 140-K polypeptide

The material obtained by mild trypsin digestion (Coudrier *et al.*, 1982), by papain digestion or by electroelution of a preparative SDS-polyacrylamide gel (Coudrier *et al.*, 1981) were each injected into rabbits. The specificity of the antibodies produced was assayed by 'Western blotting' of whole homogenate from pig intestine mucosa (Burnette, 1981).

Antibodies raised against antigens obtained by the three

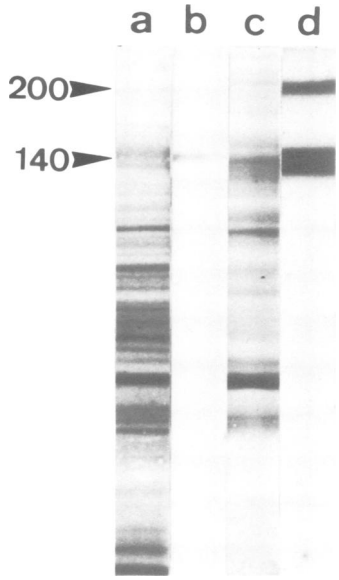

Fig. 3. Characterization of the antibody raised against the 140-K protein. A crude microsomal fraction **(b)** derived from intestinal mucosa or proteins of purified vesicles **(d)** were separated on SDS-PAGE and stained with Coomassie blue **(a,c)** or transferred on nitrocellulose and labelled with specific antiserum against purified 140 K (trypsin form) and [^{125}I]protein (bd).

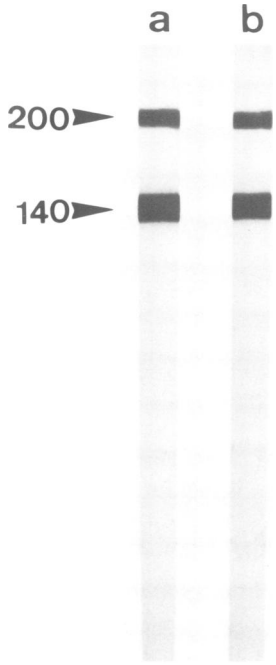

Fig. 4. Evidence for antigenic homology between the 140-K and 200-K polypeptides. Antibodies specific for each polypeptide were affinity purified against either the 140-K or the 200-K polypeptide. A total microvillar vesicle fraction was separated on SDS-PAGE, transferred on to nitrocellulose and incubated; **(a)** With antibody affinity purified on the 140-K protein. **(b)** With antibody affinity purified on the 200-K protein. The antibody-antigen complexes were labelled with the peroxidase antibody. Note that the antibodies affinity purified for each polypeptide still recognize both antigens.

preparative procedures, gave identical results. The sera specifically recognized one polypeptide of mol. wt. 140 K (Figure 3a, b). When the specificity of the antibodies was assayed on purified microvillar proteins, two polypeptides of

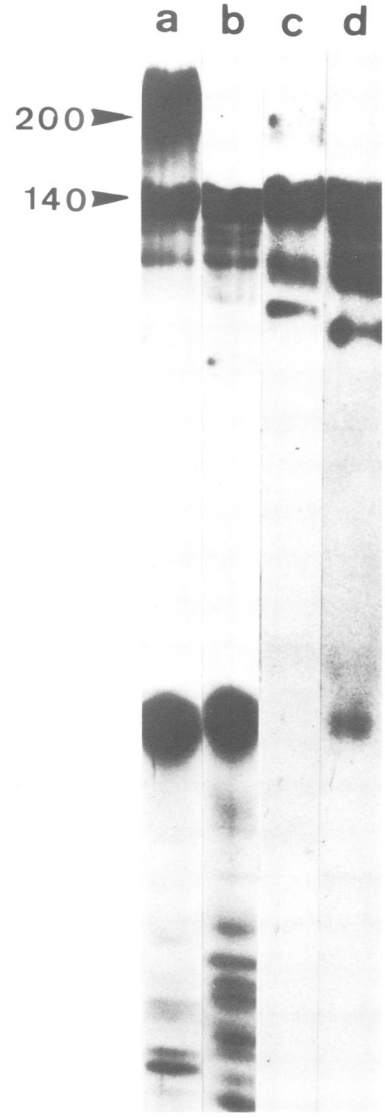

Fig. 5. Peptide mapping in one dimension of the 140-K and 200-K polypeptides analysed on SDS-PAGE. The polypeptides derived from the degradation of the 140-K and 200-K proteins with the *S. aureus* protease V8 were revealed by silver straining **(a,b)** or transferred to nitrocellulose sheets and subsequently incubated with specific sera against 140-K protein and [^{125}I]protein A. As pointed out in Results, the 200-K protein is very poorly revealed using Coomassie blue staining. On the contrary, the silver staining procedure gives a reverse impression of the relative amount of 200-K and 140-K proteins (compare **lane a** with **lane c**). V8 indicates the position of the protease on the gel.

140 K and 200 K were recognized (Figure 3c, d). This 200-K polypeptide was hardly visible on SDS-PAGE stained with Coomassie blue (see Figures 1 and 2). However, quantitative immunoreplicas have shown that the 140-K polypeptide is only twice as abundant as the 200-K polypeptide in our vesicle preparation.

Immunological and structural relationship between the 140-K and 200-K polypeptides

Immunological cross-reactivity. After affinity purification on either the isolated 140-K or 200-K polypeptides, the antibodies still recognized both polypeptides on immunoreplicas of purified microvillar vesicles, thus the two polypeptides share common antigenic determinants (Figure 4).

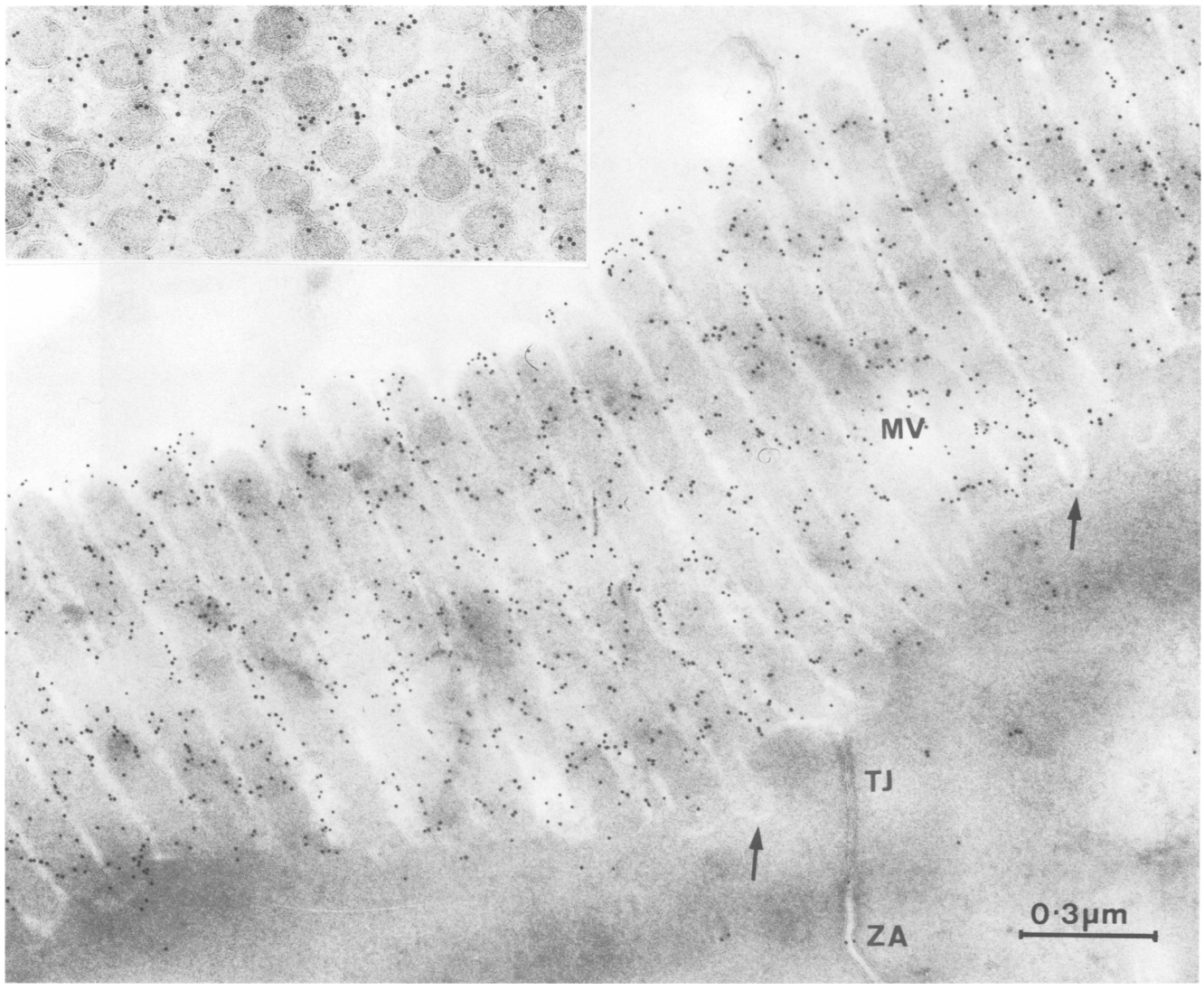

Fig. 6. Electron microscopic immunolocalisation of the 140-K protein in rat intestine epithelial cells. Thin frozen sections were prepared according to the technique of Tokuyasu and stained with antibodies to the 140-K protein and gold-protein A. The labelling was restricted to microvilli. The arrow indicates invagination of the plasma membrane at the base of the microvilli. Tj; tight junction, ZA; zonula adherens. The inset is a cross section through the microvilli.

Peptide map of isolated 140-K and 200-K polypeptides. The structural relationship of the 140-K and 200-K polypeptides was further tested by limited proteolysis (Cleveland *et al.*, 1977) with *Staphylococcus aureus* protease V8 (Figure 5). Both silver staining and immunolabelling of the peptides showed strikingly similar cleavage patterns. The conversion of the 200-K form into a 140-K peptide is particularly noteworthy (Figure 5).

Oligomeric structure of the 140-K species

The material eluted from the DEAE-column was separated into three distinct peaks by filtration in a non-denaturing buffer on an ultragel AC22 column (not shown). About 13% of the applied material was eluted with an apparent mol. wt. of 480 K; ~74% of the eluate had an apparent mol. wt. of 220 K and ~13% of the protein, eluted between the protein markers rabbit immunoglobulin and bovine serum albumin (BSA). The two high mol. wt. peaks have a similar polypeptide composition (a mixture of 140 K and 200 K) as shown by SDS-PAGE. The third peak contained the 140-K protein and a polypeptide migrating with an apparent mol. wt. of 90 K, which is presumably a degradation product; it can also be seen in the Cleveland digest (Figure 5). These results suggest that the native protein is oligomeric. Further work is required to establish the stoichiometric ratios of the 140-K and 200-K polypeptides within the oligomer.

Immunolocalization in intestinal mucosa

Figure 6 shows frozen sections (Tokuyasu, 1973) of the apical portion of an intestinal cell, labelled with affinity-purified antibodies against the 140-K polypeptide. In longitudinal section the staining was restricted to the microvilli. It seemed reduced or absent from the invagination of the plasma membrane, at the base of the microvilli, where coated pits are usually observed with plastic embedded material and conventional staining. The lateral membranes of the cell, the terminal well and the junctional complex were generally free of labelling. In cross section a preferential labelling at the periphery of the microvilli indicates that most antigenic sites are probably exposed to the outside of the plasma membrane.

Topological organisation in the microvillar membrane

External accessibility. Papain quantitatively released the 140-K and 200-K proteins from right-side out vesicles derived

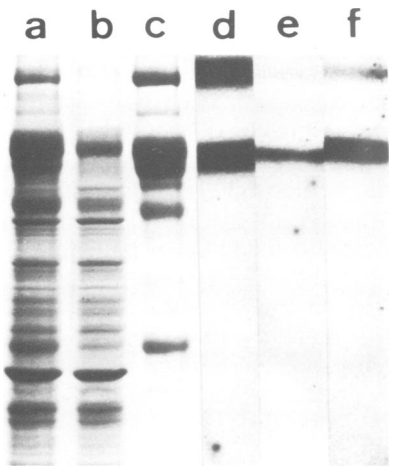

Fig. 7. Proteolytic cleavage and solubilization of the 140-K and 200-K proteins from sealed right-side out vesicles. Proteins of each fraction were separated on SDS-gels, stained with Coomassie blue (**a,b,c**) or transferred to nitrocellulose paper and reacted with specific antisera plus [^{125}I]protein A (**d,e,f**). **a,d**: total microvillar protein, **b,e**: pellet separated after treatment of microvillar vesicles with papain (0.5 mg/ml, 10 min, 37°C). Notice the absence of villin and actin in the protein fraction released by papain (**c**) but their presence in the corresponding pellet (**b**). **c,f**: corresponding papain supernatant.

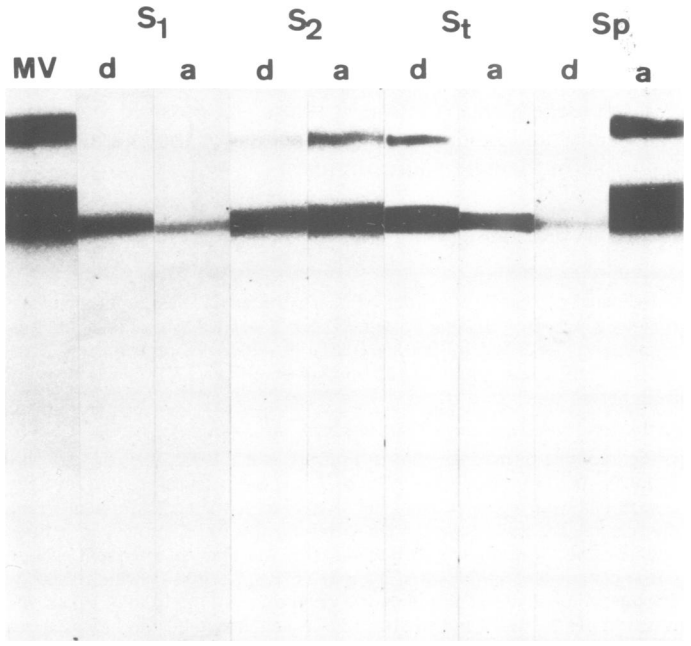

Fig. 8. Binding of the 140-K and 200-K proteins to detergent micelles. Proteins of each fraction were separated on SDS-PAGE transferred to nitrocellulose and reacted with the anti-140 K antisera plus [^{125}I]protein A. The protein fractions shown are: S_1, S_2, S_t (see Figure 1). Each supernatant was processed as described by Bordier (1981) to separate the rich detergent phase (**d**) from the aqueous phase (**a**).

from intestinal microvilli (Figure 7) without significant changes in their mol. wts. Villin and actin were absent from the supernatant. This shows the external accessibility of the two forms of the protein to proteases.

Amphipathic nature of the 140-K polypeptide. To assess the presence of a hydrophobic domain in the various preparations of 140-K protein, we have investigated its binding to

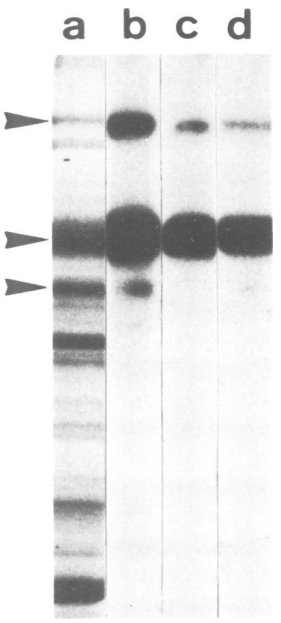

Fig. 9. Binding of the 140-K protein to a 110-K polypeptide of the core residue. (**a**): Polypeptide composition of the core residue (P_2) stained with Coomassie blue. (**b**): Polypeptides of the core residue (P_2) revealed by antisera against the 140-K protein after transfer to nitrocellulose sheets. Prior to immunological staining the blot had been incubated with the detergent-rich phase derived from the low ionic strength extract S_2. (**c**): Same as in (**b**) but the blot had been incubated with the aqueous phase derived from the low ionic strength extract S_2. (**d**): The nitrocellulose paper was processed directly without preincubation for identification of the 140-K and 200-K antigens. Arrows point to 200-K, 140-K and 110-K polypeptides. The load, the conditions of incubation of the nitrocellulose with the reagents solutions as well as the length of exposure for the autoradiograms have been performed in identical conditions for each lane; however, a more intense labelling of the 140-K and 200-K polypeptides is observed in (**b**). We suggest that reformation of the oligomers may have occurred by reassociation of the native protein of the fraction with renatured protein on the nitrocellulose sheet, leading to an overstaining of these bands.

detergent micelles using a simple assay devised by Bordier (1981). The results of such an experiment are shown in Figure 8. The following conclusions can be drawn. The 140-K protein solubilized by papain from sealed right-side out vesicles (Figure 7) or from core residue (Figure 1, S_p) is hydrophilic. The proteins obtained after low ionic strength extraction (Figure 1, S_2) or trypsin cleavage (Figure 1, S_t) still contain hydrophobic domains, presumably responsible for their associations with the detergent micelles. Polypeptides (140 K and 200 K) derived from all the fractions investigated, including those found in the first detergent extract (Figure 8, S_1) have a similar mol. wt. These data suggest that the 140-K species is an amphipathic protein anchored to the membrane bilayer by a short hydrophobic polypeptide region. The hydrophilic bulk of the protein is exposed to the outside, and can be released quantitatively by papain or elastase.

The 140-K and 200-K polypeptides bind to a band co-migrating with the 110-K polypeptide

The 140-K protein behaves like any hydrolases of the microvillus membrane except that a significant part of it (30 – 50%) is recovered in a fraction enriched in cytoskeletal proteins. We assumed that a portion of the 140-K molecule was exposed on the cytoplasmic face of the membrane and provided an anchor for a polypeptide associated with microfilaments. If so, the 140-K protein found in the Triton X-114 detergent phase should contain a binding site for a cyto-

skeletal protein. In contrast, the hydrophilic form of the 140-K protein recovered in the aqueous phase should lack such activity and provide a control. It is necessary to use limited proteolysis in order to purify the protein, but we could use, in the binding assay described below, the fraction solubilized by low salt (S_2). This contains the native 140-K protein as well as some hydrophilic form (see Figure 8) both of which can be followed specifically with the antibodies.

Following the approach of Glenney and Weber (1980), polypeptides from the core residue were separated on SDS-PAGE and transferred on to nitrocellulose sheets. Blots were incubated with protein extracts containing either the 'native', i.e., amphiphathic, or the hydrophilic form of the 140-K polypeptide. The 140-K protein was then visualized by its antibody and [^{125}I]protein A. The nitrocellulose blots showed ^{125}I-labelled bands at 200 K and 140 K as expected. However, the blot incubated with an extract containing the amphipathic form of the 140-K protein, showed an additional band co-migrating with the 110-K polypeptide (Figure 9). This band was never observed when a protein extract containing equivalent amounts of the hydrophilic form of 140 K was used. The same experiment performed using a protein extract containing the amphipathic form of aminopeptidase (another integral membrane protein), which was detected with its specific antibody, showed no binding to the 110-K polypeptide. In this figure the load and the processing of the gels (Figure 9, lanes b, c and d) were absolutely identical. Yet a more intense labelling of the 140-K and 200-K bands was observed in lane b. Presumably, native proteins also bind to the 140-K and the 200-K polypeptides renatured on the nitrocellulose sheet and reform their oligomeric structure.

Discussion

We have characterized, in microvillar vesicle preparations, two polypeptides (200 K and 140 K) that are structurally and immunologically related. The conversion, *in vitro* under controlled proteolysis, of the 200-K into the 140-K polypeptide indicates a sensitive peptide bond which may be located at a hinge between two domains of this molecule. We think that the 200-K polypeptide is a precursor form of the 140-K form reminiscent of the case of sucrase isomaltase (Hauri *et al.*, 1979). The characterization by immunoreplicas of a unique polypeptide, with a mol. wt. of 200 K, in microvillar vesicles purified from kidney cortex, further favors this possibility (unpublished observations).

Like most of the intestine hydrolase, the 140-K protein in our preparation of right-side out vesicles is easily accessible to proteases such as papain or elastase (Maroux and Louvard, 1976; Louvard *et al.*, 1976). A large hydrophilic fragment representing almost the entire length of the molecule is then released. The ability of the uncleaved 140-K protein to bind detergent micelles suggests the existence of a small hydrophobic domain but direct proof of this requires the isolation of this portion of the molecule.

A unique property of the 140-K glycoprotein is its tight association with proteins forming the microvillar cytoskeleton. This association with the insoluble core residue can be destroyed using trypsin or papain. Their site of action is, however, different. Papain released a similar form whether or not it reacted with the intact vesicles or the core residue. In both cases the peptide solubilized is no longer extractable by Triton X-114. In contrast, a significant amount of the protein released by trypsin is still amphipathic and binds to Triton X-114 detergent micelles. In fact, trypsin may act either directly on the site of the 140-K protein involved in the interaction with the cytoskeleton, or on one of several components of the cytoskeleton itself.

We have observed the binding *in vitro* of the 140-K protein with a 110-K protein of the core cytoskeleton residue. We cannot rule out the possibility that this interaction is indirect *via* another protein, since the fraction used in this kind of experiment (S_2) contained additional polypeptides. The hydrophobic domain of the 140-K protein is important in this interaction since the binding *in vitro* occurred only when the hydrophobic form of the 140-K protein was used. The function of the hydrophilic domain is currently unknown: purified 140-K protein exhibits none of the enzyme activities of intestinal brush borders.

Two antigens immunologically related to the 110-K and the 140-K proteins have been found in striated muscle (Coudrier *et al.*, 1982). The antibodies to the 140-K protein cross-reacted with the Ca^{2+}-ATPase of the sarcoplasmic reticulum. Furthermore, the cross-reactive domain was found in the A_2 polypeptide obtained after trypsin cleavage of the intact molecule (Reggio *et al.*, in preparation). This fragment of the Ca^{2+}-ATPase is known to be exposed on the cytoplasmic face of the sarcoplasmic reticulum. The 140-K protein and the Ca^{2+}-ATPase are expressed in cells of totally different origin and function, they also have a different mol. wt. but they share a common structural domain responsible for their cross-reactivity. This domain could also be responsible for a common function. The 140-K protein does not display Ca^{2+}-ATPase-dependent ATPase activity. A possible common function would be to provide an anchor for the lateral bridging of actin filament to membranes.

Materials and methods

Sources of reagents

Iodinated protein A was from NEN. Peroxidase-labelled anti-rabbit Fab fragments were from Institut Pasteur. Triton X-114 was from Fluka and was processed as described by Bordier (1981). *S. aureus* V8 protease was from Miles, DEAE-trisacryl and AC 22 ultrogel were from LKB and Ponceau S from Serva.

Extraction procedures carried out to obtain the 140-K polypeptide

Pig intestinal microvillar vesicles (Louvard *et al.*, 1973), were treated with Triton X-100 (Mooseker *et al.*, 1976) or Triton X-114 (Bordier, 1981) and the core residue was separated from the solubilized component. Three different dissociation procedures were used to release the 140-K protein in a non-sedimentable form. (a) The core residue (P_1) was dialyzed overnight against buffer containing 5 mM EDTA, 2.5 mM EGTA, 2.5 mM cysteine, 1 mM β-mercaptoethanol, 0.1% Triton X-100 or Trixon X-114, 10 mM Hepes adjusted to pH 7.3 and a mixture of protease inhibitor (Craig and Lancashire, 1980). The insoluble pellet (P_2) was separated from solubilized components including some of the 140-K protein (S_2) on a sucrose cushion (Coudrier *et al.*, 1981). (b) The core residue (protein concentration 5 mg/ml), was incubated for 1 h at 4°C with trypsin (30 μg/ml). The proteolysis was stopped with soybean trypsin inhibitor (30 μg/ml) and the mixture of protease inhibitors mentioned above. An insoluble pellet (P_t) was separated on a sucrose cushion from solubilized component (S_t) as in (a). (c) The core residue, protein concentration 5 mg/ml, was incubated for 15 min at 37°C with a reactivated papain suspension (0.5 mg/ml) (Louvard *et al.*, 1975). An insoluble pellet (P_p) was separated from a soluble fraction (S_p) as in (a).

Gel electrophoresis

SDS-PAGE were prepared according to the procedure described by Laemmli (1970) in 6.5–17% linear acrylamide gradient slab gels.

Immunoreplica experiments

Electrophoresis transfer of the proteins from the SDS-PAGE to nitrocellulose has been performed as described by Burnette (1981). The nitrocellulose sheet was either stained for 5 min in a solution of 0.2% Ponceau S in 3% TCA and washed with water or immersed 30 min at room

temperature in a solution of phosphate-buffered saline (PBS) pH 7.4 containing 20 mg/ml hemoglobin (buffer I). Under these conditions the Ponceau S staining is completely reversed. The sheet was then incubated for 1 h at room temperature in buffer I containing the appropriate antibodies, and washed four times for 15 min in PBS Triton 0.05% (Burnette, 1981). The nitrocellulose was immersed in a solution of 10^5 c.p.m. [^{125}I]protein A in buffer I for 1 h, washed as for the incubation with the specific antibodies and exposed at −70°C on Kodak X-Omat AR film. In some experiments the antigen-antibody complex was detected with the peroxidase technique. In that case, buffer I was replaced by 10% new born calf serum containing 0.2% Triton X-100 in PBS buffer pH 7.4 (buffer II). Incubation with the first antibody and washing were performed in buffer II as before. The nitrocellulose was then incubated for 1 h at room temperature with sheep anti-rabbit Fab fragment, conjugated to peroxidase, diluted 1/1000 in buffer II. After four washes of 15 min in buffer II, the peroxidase was revealed with diaminobenzidine (Graham and Kanowsky, 1966). Quantitative immunoreplicas were made according to Howe and Hershey (1981).

Antisera and antibody purification

The immunization procedure and the affinity purification of the antibodies were as described by Coudrier *et al.* (1981). Analytical quantities of antibodies were also affinity purified using a modification of the procedure described by Olmsted (1981). Proteins were separated on a preparative SDS-PAGE (13 x 9 x 0.15 cm), transferred to nitrocellulose paper and stained with Ponceau S. Strips of nitrocellulose containing the relevant polypeptide were excised and reacted with specific sera. The antibodies were eluted at 4°C from the nitrocellulose strips with glycine-HCl buffer, pH 2.2, containing 0.2% gelatin, and neutralised immediately with 1 M Tris base solution.

Ion exchange chromatography

Ion exchange chromatography was performed using trisacryl-DEAE (LKB) equilibrated with 10 mM potassium phosphate buffer, pH 6.2 containing 1 mM phenylmethylsulfonyl fluoride (PMSF). 70% of the protein loaded was absorbed on the resin. The elution was achieved with a linear gradient of 0−0.2 M NaCl in the same buffer.

Gel filtration

The protein fraction was iodinated with Bolton Hunter reagent prior to chromatography (Coudrier *et al.*, 1981). The Ac22 ultrogel column (LKB) was 100 x 2.5 cm, equilibrated in 50 mM potassium phosphate pH 7.8 and run at 6 ml/h. The mol. wt. markers were apoferritin (430 K), catalase (250 K), rabbit IgG (150 K) and BSA (69 K).

Peptide map in one dimension

Protein fractions were separated on SDS-PAGE. The relevant polypeptides were cut out and re-run on another gel in the presence of *S. aureus* V8 protease (0.1 mg/ml) (Cleveland, 1977). The peptides were revealed using a silver stain procedure (Ansorge, 1982) or by immunoperoxidase labelling on replicas. In contrast to Coomassie blue staining, the 200 K polypeptide is well revealed with the silver stain procedure.

Separation of hydrophobic proteins from hydrophilic proteins using Triton X-114

The protein fractions were prepared at 0°C in 10 mM Tris pH 7.4, 150 mM NaCl containing 1% Triton X-114. The material was extracted as described by Bordier (1981) Polypeptides recovered in the detergent and in the aqueous phase were subsequently analysed on SDS-PAGE or on immunoreplica.

Electron microscopy

Thin frozen sections (Tokuyasu, 1973) from rat intestine were labelled with anti-140 K followed by protein A-gold (12 nm) and positively stained according to Tokuyasu and Singer (1976) and Tokuyasu (1978). The tissue was fixed with 2% formaldehyde, 0.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4, and infused with 2 M sucrose prior to freezing and sectioning.

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