Isolation, Chemical Characterization, and Immunohistochemical Localization of a Protein from the Basolateral Plasma Membrane of the Rat Intestinal Absorptive Cell

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The protein pattern of the basolateral membrane (BLM) of the rat small intestinal absorptive cell shows about 20 major and a multitude of minor bands. A simple and efficient method is described for isolation and purification of a major protein in the 17 kDa molecular weight (MW)-range called Prot 17. The isolated BLM of intestinal epithelial cells was dissolved in buffer 1 (Tris/HCl, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, pH 6.8) and subsequently dialyzed for 4 h against buffer 2 (Tris/glycine, pH 8.3) and then for 12 h against buffer 2 containing 25% methanol. The resulting precipitate contained Prot 17 and phospholipids in the form of liposomes. All other BLM proteins remained dissolved in the supernatant. Chemical characterization of Prot 17 suggested that it is an integral membrane protein amounting to about 5% of the total BLM protein. Amino acid analysis revealed a MW of 17.6 kDa. The Prot 17 molecule did not contain any PAS-positive carbohydrates. In its isolated form, and apparently also in the BLM, Prot 17 occurred as a polymerized structure with a MW of about 90 kDa. By dissolution in buffer 1 and heating to 100 °C for 1 min the complex was split into its 17 kDa subunits. By oxidation with performic acid it was also broken down into its subunits. A specific antiserum against Prot 17 was obtained from immunized Balb/c mice. Immunofluorescence labelling of rat small intestinal sections with this serum showed that Prot 17 was not a BLM-specific protein. It occurred in both plasma membrane domains of the intestinal absorptive cell.

Introduction

The plasma membrane (PM) of the absorptive cell in the rat intestine consists of two morphologically [1, 2], chemically [3, 4], and functionally [5–7] different domains. The brush border membrane (BBM) covers the luminal microvilli and extends laterally to the zonula occludens. It contains many specific proteins involved in digestion [8] and resorption [5, 6] which have been well characterized. The basolateral membrane (BLM) covers the lateral and basal part of the cell. It differs morphologically [1, 2, 8, 9] and also in its protein composition [4, 10] from the BBM. Only a few of the proteins of the BLM have so far been studied in detail (examples are: Na^+-K^+-ATPase [11, 12], ectogalactosyltransferase [13], uvomorulin [14], GZ1-antigen [15, 16]). However, for most of the major proteins chemical or functional data are lacking. There is also no information which of these bands are specific for the BLM and which may occur in both domains of the absorptive cell PM.

In the course of our investigations of the BLM proteins we isolated a major protein with a apparent molecular weight (MW) of 17 kDa; thus it is here called Prot 17. It differed somewhat from the other strong BLM protein bands in that it appeared in varying relative intensity in various charges of the (according to Weiser *et al.* [17]) isolated BLM fractions. In reports by other investigators (Quaroni *et al.* [4], Harms *et al.* [12]), one of whom (Harms) used other methods to isolate the BLM, this band was present only weakly or not at all. Therefore the question arose, whether the Prot 17 band is actually a contaminant appearing in variable amounts dependent on different isolation techniques.

This paper first describes a method for isolating Prot 17 and its partial chemical characterization. It then addresses the cause of the variable relative intensity of the Prot 17 band. Finally, it describes the production and characterization of an antiserum against Prot 17 used for its immunohistochemical detection.

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Materials and Methods

All buffers used to isolate Prot17 contained 0.5 mM PMSF and 0.5 mg/ml aprotinin (Trasylol®, Bayer) for protease inhibition. All isolation steps were performed at 0 °C if not indicated otherwise.

Isolation of Prot17

Four Wistar rats (200 g body weight) were used per experiment. The small intestine was removed from the animals and its content flushed out with isotonic saline + 1 mM dithiothreitol. The epithelial cells were then isolated from the small intestine [18]. The cell fractions were pooled and their BLM isolated by the technique described by Weiser *et al.* [17]. This entailed the homogenization of the cells. The homogenate was subjected to differential centrifugation to isolate the membranes, which were then further separated by discontinuous sucrose gradient centrifugation. The membrane fractions between 30% and 40% sucrose and the one in 40% sucrose, in which the BLM were enriched, were pooled. The BLM was then dialyzed against PBS and pelleted by centrifugation for 1 h at $100{,}000 \times g$. The resulting BLM pellet was used for the isolation of Prot17.

For this purpose the BLM pellet was dissolved in buffer 1 (62 mM Tris/HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol). Undissolved particles were removed by centrifugation for 30 min at $10{,}000 \times g$. The resulting BLM solution was subsequently dialyzed for 4–5 h against buffer 2 (25 mM Tris/192 mM glycine, pH 8.3, 0.01% SDS). Methanol was then added to the BLM solution to a final concentration of 25% (v/v). The mixture was then dialyzed for 12 h against buffer 2 + 25% (v/v) methanol, resulting in a precipitate. This precipitate contained Prot17 and was pelleted by centrifugation at $50{,}000 \times g$ for 30 min. After two washings with the buffer 2–methanol combination, a pellet resulted containing Prot17 as its sole protein. This Prot17 pellet was used for further studies.

To evaluate whether Prot17 could be also extracted from the phospholipid (PL)-free BLM, the BLM pellet was lyophilized and the PL extracted from the lyophilizate with chloroform : methanol (2:1). The PL-free BLM was pelleted and the extraction medium completely removed from the pellet. The dry pellet was dissolved in buffer 1 and Prot17 precipitated as described above.

Chemical characterization of Prot17

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [19] with 2.7 mm thick gels (8% acrylamide) was used to test the purity and determine the MW of the BLM proteins and the isolated protein fractions. The samples were either heated to 100 °C for 1 min before application or applied without heating. The low-MW standard of Bio-Rad served as a MW reference. The gels were stained with Coomassie blue R 250. PAS-staining of Prot17 in the gel was done according to Fairbanks *et al.* [20].

For the proteolytic cleavage of Prot17 with pronase or trypsin, part of the Prot17 pellet equivalent to about 100 μg of protein was lyophilized, suspended in 0.25 ml of the enzyme solution, and incubated at 37 °C for 1 h. Pronase E (Serva) was used at a concentration of 250 μg/ml buffer (17 mM Tris/HCl, pH 7.6, 130 mM NaCl, 3.6 mM KCl, 1.6 mM $CaCl_2$, 1.2 mM $MgSO_4$) [21], trypsin (Sigma) at a concentration of 100 μg/ml buffer (12 mM Tris/HCl, pH 8.1, 130 mM NaCl, 10 mM $CaCl_2$) [22].

For performic acid oxidation of Prot17, a lyophilized Prot17 pellet was suspended in 1 ml formic acid. After 30 min at 0 °C, 1 ml performic acid (0 °C) was added and the mixture was allowed to react for 2 h at 0 °C [23]. The performic acid was then removed by renewed lyophilization.

Amino acid analysis of Prot17 was done after 24 h HCl hydrolysis (6 N HCl at 110 °C) of the lyophilized Prot17 pellet in an amino acid analyzer (Beckman) [24].

Protein was determined according to Lowry *et al.* [25], PL according to Bartlett [26].

For freeze etch studies, small drops of a Prot17 pellet were shock-frozen in propane at the temperature of liquid nitrogen. Their surface was then cut and etched in a freeze etching device (Balzers BAF 400 D). A replica was prepared by shadowing with platinum at angels of 30° and 15°, respectively, followed by reinforcement with a carbon film. The specimens were viewed with a Siemens Elmiskop 102.

Production and specification of the anti-Prot17 serum

Balb/c mice were injected with Prot17 (a protein content of 100 μg), suspended in a mixture of 0.3 ml PBS and 0.3 ml complete Freund's adjuvant. Half of the suspension was injected s.c., the other half i.p.

Two further antigen injections, which were suspended in incomplete Freund's adjuvant, were administered at 3-week intervals in the same manner. Blood was drawn and serum obtained 5 days after the third injection. The sera of 3 immunized mice were pooled and stored at −80 °C.

To evaluate the specificity of the serum for Prot 17, 1 ml of serum diluted 1:50 with PBS was added to a Prot 17 pellet and the mixture stirred for 2 h at 37 °C. Then the mixture was centrifuged for 30 min at $15{,}000 \times g$ and the resulting pellet washed 3 times with 10 ml PBS to extract any remaining non-bound antibody. The washed pellet was dissolved in 0.2 ml Laemmli buffer [19], and heated for 1 min to 100 °C. Then antibody binding was studied by SDS-PAGE (see above). A control test was done in parallel using non-immun mouse serum instead of *anti*-Prot 17 serum. A further similar incubation test used a Prot 17 pellet from which the PL had been extracted.

Possible reactivities of the *anti*-Prot 17 serum against other BLM proteins were evaluated with immunoblotting. The proteins of the isolated BLM were separated by SDS-PAGE and subsequently transblotted by electrophoresis to nitrocellulose sheets (NCS). The immunodetection was done according to Towbin *et al.* [27]. The first antibody was the *anti*-Prot 17 serum diluted to 1:250 (or 1:300 to 1:500) with PBS + 1% BSA. The second antibody layer consisted of HRP-conjugated *anti*-mouse Ig diluted 1:500. HRP was demonstrated with *o*-dianisidine and H_2O_2 [28]. In the controls, the incubation with the first antibody was omitted.

Immunohistological localization of Prot 17

Histologic sections of rat small intestine were tested with a indirect fluorescent labelling method. The specimens were first fixed in 4% formaldehyde + 0.1% glutaraldehyde, dissolved in PBS, pH 7.2, for 30 min at 0 °C. The specimens were washed briefly in PBS and infiltrated with 2 M sucrose (in PBS) for 8 h [29]. Then frozen sections were made. For labelling the sections were incubated for 30 min with the first antibody (*anti*-Prot 17 serum, diluted 1:500 in PBS + 1% BSA), washed 3 times with PBS, and incubated for 30 min with the second antibody (FITC-conjugated *anti*-mouse Ig, dissolved in PBS + 1% BSA). After further washing with PBS, the sections were sealed in Moviol and studied with a fluorescence microscope (Zeiss Axiophot). Incubation in which the first antibody had been omitted served as controls.

Results

The freeze etch electron microscopic study of the Prot 17 pellet showed both vesicles of very uniform size with a completely smooth surface and amorphous material that clearly appeared only at a shadowing angel of 17°. No other structures were found.

Chemical analysis of the Prot 17 pellet revealed PL and protein. Measuring the Prot 17 yield with the Lowry method showed that a Prot 17 pellet containing about 250 µg of protein could be obtained from a BLM pellet containing 5 mg protein.

The characterization of the proteins in the Prot 17 pellet by SDS-PAGE revealed the following results. When the Prot 17 pellet was dissolved in buffer 1 and heated to 100 °C for 1 min before electrophoresis, a single sharply demarcated band appeared in the 17–18 kDa MW-range (Fig. 1c). This band corresponded exactly to the Prot 17 band of the BLM (Fig. 1a). Contamination by other BLM proteins was not seen with the Coomassie stain. In the band pattern of the BLM supernatant remaining after precipitation of Prot 17 (Fig. 1b), the 17 kDa band was completely absent. This was evident in a comparison with the protein pattern of the unchanged BLM (Fig. 1a). A 90 kDa band was also missing in this supernatant (Fig. 1b, arrow). When the Prot 17 pellet dissolved in buffer 1 was subjected to electrophoresis without prior heating, a single 90 kDa band appeared (Fig. 1e). This band corresponded to the missing 90 kDa in the supernatant of the Prot 17 pellet (Fig. 1b, arrow). Fig. 1d shows that the 17 kDa band reappeared after 1 min of boiling the same sample before SDS-PAGE. The same electrophoretic behavior of the Prot 17 band was observed, when the Prot 17 pellet for the SDS-PAGE analysis was dissolved in buffer 1 without (the disulfide bond reducing) β-mercaptoethanol.

An evaluation of this behavior of Prot 17 in the unchanged BLM showed clearly that the 17 kDa band and the 90 kDa band were complementary here too, depending on whether the sample was heated to 100 °C (Fig. 1g) or not (Fig. 1f). This finding indicates that the protein component of the Prot 17 pellet consists of a single protein of about 17 kDa MW, *i.e.* Prot 17. But in the BLM it apparently occurs as a stable 90 kDa pentamer.

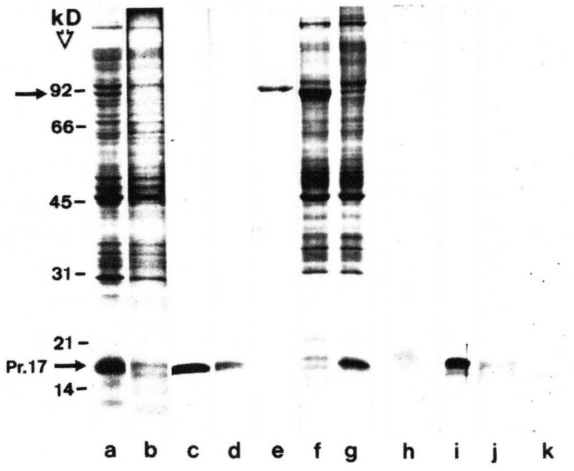

Fig. 1. SDS-PAGE analysis of the isolated Prot17. If not stated otherwise, all samples were heated to 100 °C for 1 min before electrophoresis. The gels were stained with Coomassie blue R 250. (a) Protein pattern of the isolated BLM with the marked (Pr17, arrow) Prot17 band. (b) Protein pattern of the supernatant remaining after the precipitation and pelleting of Prot17: the Prot17 band and the 90 kDa band (arrow) are missing. (c) Purified Prot17 pellet containing the Prot17 as the only protein. The band pattern (d)–(g) shows that both in the isolated Prot17 pellet and in the BLM the Prot17 band emerges from the 90 kDa band only after heating of the sample to 100 °C. (d) Unpurified Prot17 pellet heated and (e) not heated. (f) BLM not heated and (g) heated. Oxidation of the Prot17 pellet with performic acid (h). The Prot17 band becomes diffuse, stains weakly with Coomassie blue, and shifts into the 18–21 kDa MW-range. Omission of heating does not change the position of the band. Treatment of the Prot17 pellet with trypsin (i) shows a partial cleavage of Prot17 into large fragments. Pronase E treatment (j) breaks down the Prot17 molecule completely. The weak bands stem from the enzyme, with the exception of a very weak rest of Prot17, as shown by the blind value with pronase only (k).

Table I shows the results of amino acid analysis for the exact determination of Prot17's MW. MWs of 17.6 kDa and 88.1 kDa were calculated for the Prot17 monomer and pentamer, respectively. They agree well with the values of 17–18 kDa and 90 kDa obtained by SDS-PAGE.

PAS-staining of the Prot17 band obtained by electrophoresis of the Prot17 pellet on polyacrylamide gel was negative. As a control, the separated glycoproteins of human erythrocyte membrane were strongly stained on the same gel.

Oxidation of the Prot17 pellet with performic acid altered significantly the protein pattern. After boiling a band spread diffusely over the 18 kDa to 21 kDa MW-range and staining weakly with Coomassie blue was seen in SDS-PAGE (Fig. 1h). Without boiling, the oxidized Prot17 also moved to the 20 kDa range, whereas no band (or sometimes a very weak one) was seen in the 90 kDa region. Oxidation apparently impairs the ability of Prot17 to form a pentamer.

Table I. Amino acid analysis of Prot17. The Prot17 pellet was hydrolyzed for 24 h in 6 N HCl at 110 °C. The numerical relationship of the amino acids was calculated from the nmol values and are in a range of error of ∓ 3%, with the exception for Thr (+5%) and Phe (+6%). Half-Cys and Trp were not measured.

Asp	12	Gly	10	Ile	9	Lys	9
Thr	6	Ala	8	Leu	10	His	4
Ser	15	Val	9	Tyr	8	Arg	8
Glu	11	Cys	–	Phe	5	Trp	–
Pro	13	Met	0				

Sum of the amino acids: 137; calculated MW of Prot17: 17,611 Da.

Using a PL-free BLM for Prot17 isolation, dialysis against buffer 2 produced a pure protein precipitate that could be pelleted. Electrophoresis revealed that this precipitate had the same characteristics as the Prot17 in the Prot17 pellet.

Examination by SDS-PAGE of the effect of proteases on Prot17 showed that it was completely broken down after incubation with pronase (Fig. 1j). Proteolysis with trypsin produced, apart from intact Prot17, a number of new bands between 15 kDa and 8 kDa (Fig. 1i). The results of pronase cleavage suggests that the enzyme has access to all regions of the Prot17 molecule in the Prot17 pellet.

Specificity of the anti-Prot17 serum

SDS-PAGE analysis showed that after incubation of the Prot17 pellet with the *anti*-Prot17 serum the antibody bound specifically to Prot17 (Fig. 2A). Next to the Prot17 band (Pr17) there were the bands of the heavy (H)- and light (L)-chains (with a MW of 55 kDa and 26–28 kDa, respectively), into which the bound antibody was broken down during dissolution in the Laemmli buffer (Fig. 2A, c). In the control test incubation with non-immune mouse serum these bands were missing (Fig. 2A, b). The remaining weak bands are unspecific since they occur simi-

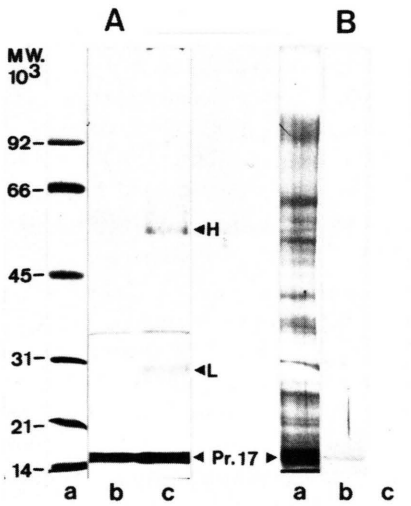

Fig. 2. Specificity of the *anti*-Prot 17 serum. A: SDS-PAGE analysis of Prot 17 pellet after incubation with *anti*-Prot 17 serum (c) and with the mouse non-immunserum (b). In addition to the Prot 17 band (Pr 17) the bands of the heavy (H)- and light (L)-chains of the specifically bound antibody are present in (c) in contrast to the control in (b). (a) MW reference. Staining with Coomassie blue. B: Immunoblotting of BLM proteins on nitrocellulose sheets and immunodetection with *anti*-Prot 17 serum of the specifically reacting bands. (a) protein pattern of the BLM (Coomassie staining). (b) Immunodetection with the 1:250 diluted *anti*-Prot 17 serum: clear labelling of the Prot 17 band (Pr 17); a 30 kDa band shows a weak labelling, which disappears after dilution of the serum to 1:300–1:500. All other BLM-protein bands are unmarked. (c) Control test.

larly in a and b. The same results were obtained using a PL-extracted Prot 17 pellet for incubation.

The results of immunodetection showed that the *anti*-Prot 17 serum recognized the Prot 17 band on the NCS to a dilution of 1:250 (Fig. 2B, b). From the other BLM proteins (Fig. 2B, a) reacted weakly only a band in the 30 kDa MW-range. At dilutions exceeding 1:250, the 30 kDa band disappeared and only the Prot 17 band was clearly labelled. The controls were negative (Fig. 2B, c). An absence of BLM protein bands on the NCS, on which the immunodetection was done, could be ruled out since tests showed that all BLM proteins were transferred from the gel to the NCS during blotting (the only exception was a weak 300 kDa band).

Localization of Prot 17 in the intestinal absorptive cell

Immunofluorescence microscopy of the small intestinal frozen sections with 1:500 diluted *anti*-Prot 17 serum labelled the epithelium exclusively. Studies of the epithelial cells at higher magnifications shows that both the basolateral surface (Fig. 3a and b) and the BBM (Fig. 3b) were significantly stained. The epithelium was not labelled in the control assay (Fig. 3c). The fluorescences at other sites in the microvillous core occurred also in the control test and was thus regarded as non-specific.

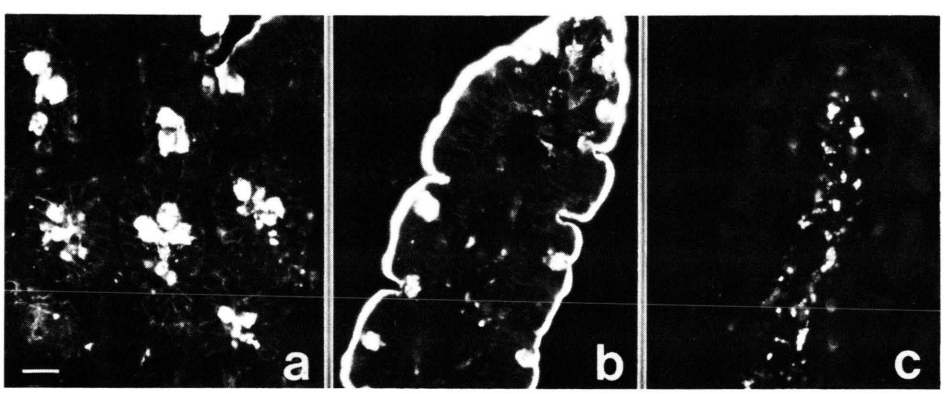

Fig. 3. Fluorescence labelling (FITC test) of the small intestinal epithelial cells with the *anti*-Prot 17 serum (diluted 1:500) on fixed freeze sections of rat small intestine. (a) Crypts: the BLM of the absorptive cells and the mucous part of the goblet cells are specifically labelled. (b) Villus: the BLM and the BBM of the absorptive cells are specifically labelled. The fluorescing sites in the villus core are unspecific, because they can be seen also in the control section of the villus (c). Bar = 20 μm.

Discussion

By the method presented Prot 17 can easily be isolated and purified from the BLM of the absorptive cells. The yield is 5% (g/g) of the initial amount of the BLM protein. Since Prot 17 is completely precipitable from the BLM solution, as judged from its absence in the remaining supernatant (Fig. 1b), it can be assumed that Prot 17 makes up 5% of the total BLM protein.

The isolation technique produces a pellet that contains PL in addition to Prot 17. The freeze etch results of the pellet show that the liposomes are completely free of intramembrane particles. This indicates that Prot 17 is not built into the PL vesicle but instead form the amorphous structures also found in the pellet. This leads to the conclusion that the PL vesicles are not necessary for the selective precipitation of Prot 17 from BLM solutions. This is also indicated by the fact that Prot 17 can be precipitated from PL-free BLM solutions. The complete breakdown of Prot 17 by pronase also shows that it occurs in the Prot 17 pellet in a form easily accessible to the enzyme.

Prot 17 is probably a membrane-integrated BLM protein since it cannot be removed from the BLM by either decrease in pH or increase in salt concentration [30]. Also, it can be dissolved in Triton X-100 only at concentrations exceeding 0.5% [31]. Integration of Prot 17 into the liposomes arising during dialysis against buffer 2 was probably prevented by its denaturation during dissolution of the BLM in the SDS-containing buffer 1. During the decrease of SDS concentration caused by dialysis, Prot 17 quickly stabilized by aggregation to large complexes, and by that could not be integrated into the liposomes that developed only at low SDS concentrations. The denaturing effect of the ionic detergent SDS seems an important part of this mechanism, since Prot 17 cannot be selectively precipitated from BLM solutions in buffers with 1% Triton X-100, which cause little denaturation of membrane proteins.

Our results help to explain the variable concentrations of the 17 kDa band in the protein pattern of the isolated BLM as described by other authors (Quaroni *et al.* [4], Harms *et al.* [12]). In its isolated form – and as electrophoresis of the untreated BLM showed, also in the BLM – Prot 17 occurs as a pentamer with a MW of about 90 kDa. The separation into 17 kDa subunits took place only after heating to 100 °C in the SDS-containing buffer 1. After short or without heating of the samples before electrophoresis, no or only a weak 17 kDa band was visible, while the 90 kDa band was correspondingly stronger. Both Quaroni *et al.* [4] and especially Harms *et al.* [12] detected a strong band at 90 kDa instead of a weak or absent 17 kDa band. Thus, Prot 17 occurs regularly in the BLM, but its position in the band pattern can vary from the 17 kDa- to the 90 kDa-MW region.

The type of bonding between the 17 kDa subunits could be a strong hydrophobic interaction since it separates only in extreme antihydrophobic conditions. Other additional bonding modes are likely since oxidation with performic acid, which does not affect the hydrophobic bonds, caused dissociation into subunits even without boiling. Subunit bonding by disulfide bonds does obviously not occur, since separation of the 90 kDa pentamer in the 17 kDa monomers is also found in absence of β-mercaptoethanol.

The specificity tests with the *anti*-Prot 17 serum show that the serum is specifically directed against Prot 17. Incubation of the serum with a Prot 17 pellet, in which no other BLM proteins are demonstrable, results in a specific binding of antibody to the pellet. Since there is only Prot 17 in the pellet, the antibody must have bound this protein specifically. When this Prot 17-bound antibody, which has no specificity for other BLM proteins, is removed with HCl from Prot 17, and, after neutralization, is used for immunohistochemical labelling of small-intestine sections, it stains the same cellular sites as the *anti*-Prot 17 serum. Immunoblotting results confirm serum specificity for Prot 17. The weak reaction with the 30 kDa band occurs only at dilutions up to 1:250. If, as in immunofluorescence microscopy, the serum is diluted over 300×, it can be assumed that the ensuing fluorescence results exclusively from the specific binding to Prot 17.

Immunohistochemistry data indicate that Prot 17 occurs in the entire plasma membrane of the absorptive cell. Its presence in both the BBM and the BLM suggests that it is functionally necessary in both domains. The following steps may also be involved. The target membrane for Prot 17 may be the BBM. But before it is inserted into this membrane, it is first built into the BLM from where it is transported into the BBM. Such a specific protein transport mechanism is assumed for at least some of the proteins of the brush-border cell [4].

[1] T. M. Mukerjee and L. A. Staehelin, J. Cell Sci. **8,** 573 (1971).
[2] M. G. Farquhar and G. E. Palade, J. Cell Biol. **17,** 375 (1963).
[3] T. A. Brasitus and D. Schachter, Biochemistry **19,** 2763 (1980).
[4] A. Quaroni, K. Kirsch, and M. M. Weiser, Biochem. J. **182,** 203 (1979).
[5] U. Hopfer, K. Nelson, J. Perrotto, and K. J. Isselbacher, J. Biol. Chem. **248,** 25 (1973).
[6] H. Murer and U. Hopfer, in: Intestinal Permeation, **Vol. 4** (M. Kramer and F. Lauterbach, eds.), p. 294, Excerpta Medica, Amsterdam, Oxford 1977.
[7] J. L. Madara and J. S. Trier, in: Physiology of the Gastrointestinal Tract, Vol. 2 (L. R. Johnson, J. Christensen, M. J. Jackson, E. D. Jacobson, and J. H. Walsh, eds.), p. 1209, Raven Press 1987.
[8] G. Semenza, in: The Enzymes of Biological Membranes, Vol. 3 (A. Martonosi, ed.), p. 349, Plenum Press, New York 1976.
[9] J. S. Trier, Gastroenterology **56,** 618 (1969).
[10] S. Ito, Philos. Trans. R. Soc. London (Biol.) **1974,** 268.
[11] G. Zampighi, J. Kyte, and W. Freytag, J. Cell Biol. **98,** 1851 (1984).
[12] V. Harms and E. Wright, J. Membr. Biol. **53,** 119 (1980).
[13] J. Roth, M. J. Lentze, and E. G. Berger, J. Cell Biol. **100,** 118 (1985).
[14] K. Boller, D. Vestweber, and R. Kemler, J. Cell Biol. **100,** 327 (1985).
[15] H. Schiechl, G. Dohr, and A. Eherer, J. Histochem. Cytochem. **34,** 1659 (1986).
[16] H. Schiechl and G. Dohr, Histochemistry **87,** 491 (1987).
[17] M. M. Weiser, M. M. Neumeier, A. Quaroni, and K. Kirsch, J. Cell Biol. **77,** 722 (1978).
[18] M. M. Weiser, J. Biol. Chem. **248,** 2536 (1973).
[19] U. K. Laemmli, Nature **227,** 680 (1970).
[20] G. Fairbanks, T. L. Steck, and D. F. H. Wallach, Biochemistry **10,** 2606 (1971).
[21] W. W. Bender, H. Garan, and H. C. Berg, J. Mol. Biol. **58,** 783 (1971).
[22] A. Elgsaeter and D. Branton, J. Cell Biol. **63,** 1018 (1974).
[23] C. H. W. Hirs, Methods Enzymol. **11,** 197 (1967).
[24] S. Moore and W. H. Stein, Methods Enzymol. **6,** 619 (1963).
[25] D. H. Lowry, N. J. Rosenbrough, A. L. Farr, and R. J. Randall, J. Biol. Chem. **193,** 265 (1951).
[26] G. R. Bartlett, J. Biol. Chem. **234,** 466 (1959).
[27] H. Towbin, T. Staehelin, and J. Gordon, Proc. Natl. Acad. Sci. U.S.A. **76,** 4350 (1979).
[28] S. Avrameas and B. Guilbert, Eur. J. Immunol. **1,** 394 (1971).
[29] K. T. Tokuyasu, J. Cell Biol. **57,** 551 (1973).
[30] H. Schiechl, Z. mikrosk.-anat. Forsch. Leipzig **97,** 33 (1983).
[31] H. Schiechl, Z. mikrosk.-anat. Forsch. Leipzig **98,** 746 (1984).