A Novel Marker Glycoprotein for the Microvillus Membrane of Surface Colonocytes of Rat Large Intestine and Its Presence in Small-intestinal Crypt Cells

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Abstract. Murine mAbs were produced against purified microvillus membranes of rat colonocytes in order to establish a marker protein for this membrane. The majority of antibodies binding to the colonic microvillus membrane recognized a single protein with a mean apparent Mr of 120 kD in both proximal and distal colon samples. The antigen is membrane bound as probed by phase-partitioning studies using Triton X-114 and by the sodium carbonate extraction procedure and is extensively glycosylated as assessed by endoglycosidase F digestion. Localization studies in adult rats by light and electron microscopy revealed the microvillus membrane of surface colonocytes as the principal site of the immunoreaction. The antigen was not detectable in kidney or liver by immunoprecipitation but was present in the small intestine, where it was predominantly confined to the apical membrane of crypt cells and much less to the microvillus membrane of differentiated enterocytes. During fetal development, the antigen appears first in the colon at day 15 and 1–2 d later in the small intestine. In both segments, it initially covers the whole luminal surface but an adult-like localization pattern develops soon after birth. The antibodies were also used to develop a radiometric assay for the quantification of the antigen in subcellular fractions of colonocytes in order to assess the validity of a previously developed method for the purification of colonic brush-border membranes (Stieger, B., A. Marxer, and H. P. Hauri. 1986. J. Membr. Biol. 91:19–31.). The results suggest that we have identified a valuable marker glycoprotein for the colonic microvillus membrane, which in adult rats may also serve as a marker for early differentiation of enterocyte progenitor cells in small-intestinal crypt cells.

The main function of the mammalian colon is absorption of water, sodium, and other minerals (Schultz, 1984; Smith and McCabe, 1984; Binder and Sandle, 1986), as well as short-chain fatty acids (Engelhardt and Rechkemmer, 1983; Luciano et al., 1984). It is likely that the microvillus "brush-border" membrane of colonocytes at the surface of the large-intestinal mucosa mediates some of these absorption processes (Schultz, 1981). However, knowledge of the functions and the molecular composition of the large-intestinal microvillus membrane is limited due to the difficulties encountered with the purification of this surface domain (Hauri et al., 1986; Gustin and Goodman, 1981, 1984). A key problem for the isolation of pure large-intestinal microvillar membranes is the lack of a valid marker protein. The usefulness of alkaline phosphatase, a proposed marker enzyme for this membrane in the rat (Vengesa and Hopfer, 1979; Brasitus and Keresztes, 1984), is controversial and has recently been questioned (Stieger et al., 1986).

As a first step in studying the properties and functions of the colon brush-border membrane, we have recently developed a procedure based on morphological criteria for the isolation of this surface membrane (Stieger et al., 1986) that proved to be useful for the characterization of sodium–proton exchange (Binder et al., 1986). As a second step, we have in the present study used the mAb approach to identify and characterize a marker membrane protein for the microvillus membrane of rat colon. The marker protein established is heavily N-glycosylated and is also expressed in the luminal surface of small-intestinal crypt cells.

Materials and Methods

Chemicals and Reagents

Protein A was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden; rabbit anti-mouse immunoglobulin (affinity purified) from Sera Lab, Sus-
sex, England; rhodamine-conjugated rabbit anti-mouse IgG from Nordic Immunology, Tilburg, The Netherlands; fluorescein-labeled sheep anti-mouse IgG from the Institut Pasteur, Paris, France; endo-β-N-acetylgalactosaminidase F (Engvall, 1980). The antibody classes were determined by Ouchterlony double-diffusion assay using subclass-specific rabbit anti-mouse immune sera. Rat IgG from the Institut Pasteur, Paris, France; endo-13-N-acetylglucosaminidase ("O-glycanase"); diplococcus pneumoniae, and α-L-fucosidase (beef heart) all came from Boehringer Mannheim GmbH, Mannheim, FRG. The immunofluorescence technique was used to visualize the antigens with mAbs (ascites fluid 1:100 or 10-fold concentrated culture supernatants, undiluted). The second antibody was a rhodamine-conjugated goat anti-mouse IgG or a fluorescein-conjugated sheep anti-mouse IgG.

### Immunelectron Microscopy

Small- or large-intestinal tissue fragments were fixed in a mixture of 2% paraformaldehyde and 0.1% glutaraldehyde for 1 h at room temperature, and then stored in 2% paraformaldehyde at -4°C until further processing. Low temperature embedding in Lowicryl K4M was performed according to techniques described previously (Roth et al., 1981; Armbruster et al., 1982; Tokuyasu, 1978; Fransen et al., 1985). In short, fixed tissue fragments were dehydrated in a graded ethanol series, during which the temperature was gradually lowered to -35°C. Infiltration into the resin and embedding took place at -35°C. Polymerization by UV light was performed for 48 h at -40°C, followed by 48 h at room temperature. Sections were then incubated for 5 min at room temperature on drops of 1% BSA dissolved in PBS, pH 7.4. Sections were incubated at room temperature successively with mAb CDI/62 (10-fold concentrated culture supernatant) diluted 1:2 with PBS/BSA for 1 h, with rabbit anti-mouse IgG in PBS/BSA for 1 h, and finally with protein A complexed to 10-nm colloidal gold particles (Slot and Geuze, 1985) in PBS/BSA for 1 h. The sections were washed thoroughly with PBS/BSA after each incubation. In control incubations the first antibody was omitted, which resulted in little to no background labeling. After washing with distilled water, the sections were stained with a saturated aqueous solution of uranyl acetate and lead citrate, and observed in an electron microscope (model EM201, Philips Electronic Instruments, Inc., Mahwah, NJ) operating at 80 kV.

### Isolation of Antibodies

For the secondary screening of mAbs and for the developmental study, 2% paraformaldehyde-fixed rat colon tissue was included in Tissue Tek and 5-μm-thick cryosections were cut with a cryostat. For fine localization studies small- or large-intestinal tissue fragments were fixed with 2% paraformaldehyde-0.1% glutaraldehyde for 2 h, infused with 60% sucrose, and frozen in liquid nitrogen. 0.5-1-μm-thick sections were cut with an ultra-microtome with cryoattachment (Reichert Jung S.A., Paris, France; Tokuyasu, 1978). The indirect immunofluorescence technique was used to visualize the antigens with mAbs (ascites fluid 1:100 or 10-fold concentrated culture supernatants, undiluted). The second antibody was a rhodamine-conjugated goat anti-mouse IgG or a fluorescein-conjugated sheep anti-mouse IgG.

### Immunoprecipitation, SDS-PAGE, Autoradiography, and Immunoblotting

Antigens were immunoisolated from Triton X-100-solubilized membranes by using protein A-Sepharose at pH 8.0 (Hauri et al., 1985b; Ey et al., 1978). The antigen-antibody complexes were separated by SDS-PAGE according to Laemmli (1970). 125I-labeled proteins in the dried gel were visualized by autoradiography using Kodak X-Omat AR or S films. Unlabeled proteins were stained with silver (Merill et al., 1984) or Coomassie Blue. The method of Towbin et al. (1979) was used to transfer proteins from SDS-PAGE to nitrocellulose sheets (Schleicher & Schuell, Inc., Keene, NH). The immunoreaction on nitrocellulose strips was visualized by a second antibody (rabbit anti-mouse) followed by 125I-labeled protein A in the presence of 1% defatted milk (Hauri and Bucher, 1986).

### Phase Separation in Triton X-I14

This procedure was carried out according to Bordier (1981). Detergent-
solubilized small- or large-intestinal brush-border membranes were labeled with $^{125}$I as described above. 6–10 x $10^6$ cpm were used for phase partitioning in 100 mM sodium phosphate containing 2% (wt/vol) preloaded Triton X-114. The final detergent phase contained 70–80% and the aqueous phase 20–30% of the counts. Both phases were separately immunoprecipitated with antibody CP1/126 and subjected to SDS-PAGE and autoradiography.

**Sodium Carbonate Extraction**

1 mg purified brush-border membranes of rat small intestine were radioiodinated by the chloramine T procedure. Free iodine was removed by centrifugation (100,000 g, 1 h). The membrane pellet was resuspended in 0.1 M Na$_2$CO$_3$, pH 11.5, and left on ice for 30 min (Fujiki et al., 1982), after which time the membranes were recovered by centrifugation (100,000 g, 1 h). After solubilization with Triton X-100, the membrane and the supernatant fractions were separately immunoprecipitated.

**Glycosidase Digestion**

Digestion with Endo F was performed as follows. The immunoprecipitated, $^{125}$I-labeled antigens were heated to 100°C for 3 min in 50 µl Endo F buffer (100 mM Na phosphate, pH 6.1, containing 50 mM EDTA, 1% NP-40, 0.1% SDS, 1% mercaptoethanol, 1 µg/ml pepstatin, 17.5 µg/ml benzamidine, 10 µg/ml aprotinin, 1 mM PMSF, and 2 mM O-phenanthroline). The samples were then digested with 2 µl endoglycosidase F (500 U/ml) for 22 h at 37°C, after which time they were subjected to SDS-PAGE. Neuraminidase was dissolved in and dialyzed against 20 mM sodium citrate, 20 mM maleate/Tris, pH 6.0. The immunoprecipitate was washed in 10 mM chloride-free sodium phosphate, pH 7.0, heat denatured, and digested for 22 h at 37°C with 0.09 U neuraminidase in a final volume of 80 µl of citrate–maleate buffer containing protease inhibitors. O-glycanase digestion was done with 2 µM of the enzyme in the citrate–maleate buffer as for neuraminidase. α-1-Fucosidase digestion (100 µg) was carried out with heat-denatured immunoprecipitates in a final volume of 100 µl 0.2 M Na acetate, pH 4.5, at 37°C for 22 h.

**Other Methods**

Protein was determined according to Bradford (1976). Sucrase was assayed according to Dahlqvist (1968).

**Results**

**Identification of an M. 120-kD Protein Recognized by Antimicrovillar Antibodies**

To identify the microvillar antigens recognized by the various hybridoma antibodies, proximal and distal colon brush-border membranes were solubilized with Triton X-100 and the 100,000 g supernatant was labeled with $^{131}$I followed by immunoprecipitation. None of the antibodies precipitated an antigen that was exclusively present in either the proximal or distal colon sample. Most supernatants precipitated a broad radioactivity band with a mean $M_r$ of 120,000. This protein was designated colon microvillus membrane protein cmv 120. Two hybridoma lines producing antibodies against this antigen were selected and subcloned to yield mAbs CPI/126 (originating from a mouse immunized with proximal colon brush borders) and CDI/62 (originating from a mouse immunized with distal colon brush borders). CPI/126 is an IgG2a and appears to recognize a discontinuous epitope of cmv 120 since it can immunoprecipitate the native Triton X-100–solubilized antigen (Fig. 1, lane 1), but fails to give an immunoreaction on Western blots. CDI/62 is an IgG1 and recognizes both the native, detergent-solubilized antigen in the immunoprecipitation and the denatured antigen on Western blots, and it was useful for antigen localization in Lowicryl-embedded tissue. CPI/126 and CDI/62 bind to the same protein (Fig. 1, lane 4) in a competitive manner as assessed by competitive ELISA (not shown). This indicates that the epitopes for these two antibodies might be in proximity on the protein.

The cmv 120 was judged to be an internal membrane protein since it partitioned predominantly into the detergent phase when $^{125}$I-labeled brush-border proteins were phase separated by the Triton X-114 procedure (Bordier, 1981). This is shown in Fig. 2, where 72% of the counts were immunoprecipitable from the detergent phase (lane 3) while only 28% were recovered from the aqueous phase (lane 2). Membrane association of cmv 120 was confirmed by the sodium carbonate extraction procedure (Fujiki et al., 1982). When colon microvillar membrane vesicles were subjected to this treatment, cmv 120 was quantitatively immunoprecipitable from the membrane fraction (Fig. 2, lanes 4 and 5).
The Size Difference of Colonic and Small-intestinal cmv 120 is Primarily Due to N-glycosylation

The fairly broad band of cmv 120 on SDS gels pointed to the possibility that this protein may be a glycoprotein. Digestions with Endo F were performed to determine if the difference in electrophoretic mobility between the colonic and the small-intestinal antigen was due to glycosylation. Endo F, which removes both complex and high-mannose N-linked oligosaccharides, greatly reduced the molecular mass of cmv 120 in both intestinal segments and almost completely abolished the difference in electrophoretic mobility (Fig. 4). The Endo F-treated antigens had an apparent Mr of 48 kD in the colon and 47 kD in the small intestine. This suggests that the size difference of the colonic and the small-intestinal glycoprotein is essentially due to N-glycosylation. Both glycoproteins are, at least to a low level, sialylated as indicated by their slight mobility shift after treatment with neuraminidase (Fig. 4). However, the size difference was not abolished and hence not attributable to steric acid. Further treatment with O-glycosidase only minimally lowered the apparent Mr, further. This finding suggests that cmv 120 is predominantly, if not exclusively, N-glycosylated. α-Fucosidase produced a very small but reproducible reduction in the apparent Mr of the small-intestinal but not the large-intestinal antigen.

Immunolocalization of cmv 120 in the Rat Intestine

Light microscopy using 1-μm intestinal cryosections in conjunction with the indirect fluorescence technique showed that in the colon the brush border of surface colonocytes was the principal site of immunoreaction for antibody CP1/126 (Fig. 5, a and b). An identical pattern was obtained with antibody CDL/62 (not shown). There was no difference in labeling intensity between proximal and distal colon. In the small intestine, the strongest immunoreaction was found with the luminal...
Enterocytes of the villus tip displayed weak reactivity (Fig. 5, g and h), while cells at the base of villi displayed an intermediate signal. The luminal surface of goblet cells was not labeled (Fig. 5, c and d). The labeling was found to be present down to the base of the crypts (not shown). Immunoelectron microscopy using antibody CD1/62 confirmed and extended the results obtained at the light microscope level. Ultrathin sections of Lowicryl K4M-embedded tissue, in conjunction with the protein A-gold labeling technique, revealed CD1/62-immunoreactivity on the extracytoplasmic surface of crypt cells (Fig. 5, c and d). This suggests that in the small intestine cmv 120 is predominantly expressed in crypt cells giving rise to enterocytes and that its amount decreases when the cells undergo differentiation at the base of the villi. Immunoelectron microscopy revealed that the fetal colonic and fetal small-intestinal antigens had the same mean apparent Mr of 100 kD (not shown). Between 21 d of gestation and 1 d after birth, the small-intestinal but not the colonic antigen decreased its apparent Mr by 15–20 kD. It is possible that this change in Mr reflects alterations in glycosylation taking place during crypt formation in the small intestine.

Quantification of cmv 120 in Subcellular Fractions of Colonocytes

In a previous study, the lack of an accepted marker protein rendered it impossible to precisely assess the enrichment factor of a brush-border membrane fraction obtained from large-intestinal epithelial cells by a novel fractionation procedure (Stieger et al., 1986). For this purpose, we adapted a radiometric assay using mAb CP1/126 in conjunction with Rivanol. Rivanol has been shown to precipitate the antibody-antigen complex while leaving the unbound antibody in solution (Rothwell et al., 1985).

To test the validity of the Rivanol assay, it was first applied to sucrase-isomaltase in subcellular fractions of human small intestinal mucosa. For this purpose antibody HBB 2/614/88 against human sucrase-isomaltase (Hauri et al., 1985b) was 125I-labeled and sucrase-isomaltase was quantified in homogenates and brush border membrane fractions. Sucrase activity was measured in parallel. The enrichment of the specific activity of sucrase-isomaltase in the brush-border fraction relative to the homogenate was found to be identical as measured by the two methods (not shown). The Rivanol assay was then used to measure relative enrichment factors of the cmv 120 antigen in brush-border fractions of rat small and large intestine. Representative results of such experiments are given in Fig. 8 for the colon. This method resulted in a relative enrichment of the cmv 120 in the colonic brush-border membrane fraction FI of 15–20-fold.
Figure 5. Immunofluorescence staining of cmv 120 in 1-μm cryosections of rat distal colon (a) and rat small intestine (c, e, and g) at the level of crypts (oblique section, c), villus base (e), and villus tip (g). The sections were incubated with antibody CPI/126 (ascites fluid 1:100) followed by rhodamine-conjugated rabbit anti-mouse IgG. Corresponding phase-contrast pictures are shown in b, d, f, and h. G, goblet cell. The labeling of the brush border in g, although present, was too weak to withstand reproduction. It is important to note that the luminal surface of goblet cells is not labeled. Bar, 20 μm.
Discussion

In the present study, a highly glycosylated protein, cmv 120, was identified by mAbs. This protein is associated with the luminal membrane of rat large-intestinal epithelial cells. A number of observations suggest that in the large intestine, cmv 120 can serve as a marker protein for the brush-border membrane. cmv 120 is a membrane protein as evidenced by phase-partitioning experiments using Triton X-114 and by means of resistance to extraction in carbonate buffer at elevated pH. The protein's principal localization is the brush-border membrane of colonocytes while the luminal mem-

Figure 6. Localization of cmv 120 in ultrathin sections of Lowicryl K4M-embedded rat colonic and small-intestinal mucosa. Sections were incubated with antibody CD1/62 (culture supernatant 1:2), followed by rabbit anti-mouse IgG, and finally with protein A-10 nm colloidal gold. Brush-border labeling is shown in a crypt cell of rat distal colon (a), a surface colonocyte (b), a jejunal crypt cell (c), and a cell at the tip of a small-intestinal villus (d). In surface colonocytes, labeling of apical vesicles is sometimes observed (arrowheads). Bars, 0.5 μm.
Figure 7. Development of cmv 120 in the small intestine (A-E) and in the colon (F-J) during ontogeny as visualized by immunofluorescence. 5-μm cryosections of 15- (A and F), 17- (B and G), and 20-d (C and H) fetal intestines or of intestines at 3 (D and I) and 6 d (E and J) after birth were incubated with antibody CP1/126 followed by fluorescein-labeled sheep anti-mouse IgG. Bars: (A, B, F, G, and I) 44 μm; (C and H) 78 μm; (D, E, and J) 87 μm.
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Figure 8. Representative example of the quantification of cmv 120 in the homogenate (●) and the corresponding F1 brush-border fraction (○) of isolated rat colonocytes by the Rivanol assay (see Material and Methods for details). The enrichment factor for brush borders was 20.5-fold as determined by the comparison of the ED50 values which were 51.2 µg protein for the homogenate and 2.5 µg protein for the brush-border fraction. Each point is the mean of duplicate values.

The cmv 120 antigen was used to assess the validity of a previously established procedure for the isolation of colon brush-border membranes (Stieger et al., 1986). In the absence of an accepted marker, the method had to rely on indirect criteria like morphology and its applicability to the small intestine, for which a number of brush-border enzymes can serve as markers. The present study now confirms the validity of the procedure that results in a 15–20-fold relative enrichment of cmv 120 in the F1 brush-border fraction.

Differences relating to ultrastructure (Engelhardt and Rech-kenmer, 1983) and function (Binder and Sandle, 1986) have been reported for proximal and distal colon. The presence of cmv 120 in both colonic segments, therefore, renders it unlikely that this glycoprotein is involved in segment-specific functions like active K absorption or active K secretion (Foster et al., 1984).

Although we have yet to define the function of cmv 120, some of its features render this protein an interesting subject for studies that go beyond its usefulness as a marker protein for the brush-border membrane of colonocytes. The cmv 120 protein carries a substantial amount of glycans. Endo F digestion decreased its apparent Mr by ~72 kD in the colon. Assuming a contribution of a single N-linked oligosaccharide side chain of ~3 kD this reduction in apparent Mr would reflect removal of 24 side chains per polypeptide. The difference in Mr of the colonic and small-intestinal glycoprotein appears to be essentially due to N-linked side chains since Endo F digestion almost completely abolished the difference in electrophoretic mobility. Glycoproteins that are common to both the small-intestinal and the large-intestinal microvillus membrane have not been analyzed in detail yet. This first example suggests that N-glycosylation of cmv 120 is tissue—rather than protein—dependent. Similar to cmv 120, the β subunit of Na+/K+-ATPase displayed a higher apparent Mr in the colon than in the small intestine (Marxer, A., and H. P. Hauri, unpublished observations). This would be in line with observations by Roth et al. (1985), who found one particular sialyltransferase expressed in rat large intestine but not small intestine. However, it is important to note that the Mr difference of the colonic and the small-intestinal enzyme is not abolished by neuraminidase treatment and therefore is unlikely to be due to sialic acid.

In the small intestine, cmv 120 was found to be expressed on the luminal membrane of most crypt cells and to a lesser extent in the brush border of differentiated villus cells. Most notably, the goblet cells do not express cmv 120. Thus, cmv 120 may also be useful as a marker for progenitor crypt cells giving rise to absorptive epithelial cells. Recently, Quarani (1986) has described a number of mAbs that preferentially bind to the luminal cell surface of rat small-intestinal crypt cells, as shown by immunofluorescence. One of these antibodies (i.e., FBB 1/20) precipitated a protein with a similar Mr to the cmv 120 described here. However, it appears unlikely that FBB 1/20 is identical to cmv 120 since the FBB 1/20 antigen becomes confined to the crypt cells only after weaning. Another similar protein, expressed in the apical membrane of rabbit crypt cells, has been reported by Gorvel et al. (1986a, b). The protein has an apparent Mr of 140 kD but is absent from the rabbit large intestine. Thus, this protein is clearly different from cmv 120.

An interesting observation relates to the inverse gradients of cmv 120 in the adult colon and small intestine as revealed by immunofluorescence and immuno-electron microscopy. While in the colon, cmv 120 immunoreactivity is low at the very tip of the microvilli (the dense plaques). Again this is similar to sucrase–isomaltase, in that it is also excluded from the dense plaques (see Fig. 2 a of Hauri et al., 1985b; Lucoq and Baschong, 1986).

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a valuable marker for the apical membrane of colonocytes. Moreover, the cmv 120 protein may serve as a cell-surface marker for progenitor cells of enterocytes in small-intestinal crypt cells.

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