

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 M_r 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-

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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*-acetylglucosaminidase F (Endo F)¹ from New England Nuclear, Boston, MA; and Freund's adjuvant from Difco Laboratories Inc., Detroit, MI. Lactoperoxidase, neuraminidase (clostridium perfringens), endo- α -acetylgalactosaminidase ("O-glycanase", diplococcus pneumoniae), and α -L-fucosidase (beef heart) all came from Boehringer Mannheim GmbH, Mannheim, FRG. Triton X-100 came from Serva, Heidelberg, FRG, Tissue Tek from Miles Laboratories Ltd., Slough, England, and [¹²⁵I]Na (carrier free) from Eidgenössisches Institut für Reaktor-forschung (Würenlingen, Switzerland). Sucrose and chloramin T were from E. Merck, Darmstadt, FRG. All of the following reagents were from Sigma Chemical GmbH, Munich, FRG: Rivanol (6,9-diamino-2-ethoxyacridine lactate), BSA (essentially globulin free), horseradish peroxidase, glucose oxidase, ovomucoid, Triton X-114, NP-40, *O*-phenanthroline, phenylmethylsulfonyl fluoride (PMSF), pepstatin, benzamide, and aprotinin.

Purification of Membranes

Microvillar membranes (FI fraction) were purified from isolated rat small-intestinal enterocytes or colonocytes as described previously (Stieger et al., 1986). Proximal colonocytes originated from the proximal half of the colon while distal colonocytes originated from the distal half of the colon. Brush-border membranes from human small-intestinal mucosa (P₂ fraction) were isolated according to Schmitz et al. (1973). Total membrane fractions of kidney or liver were prepared as follows. One rat kidney or one rat liver lobe was homogenized in 10 ml 2 mM Tris-50 mM mannitol, pH 7.1, for 2 min with a homogenizer (Polytron Kinematica GmbH, Kriens, Switzerland) (15,000 rpm). The homogenate was centrifuged at 2,800 g for 10 min and the resulting supernatant was centrifuged at 105,000 g for 1 h. The pellet designated "total membrane fraction" was immediately used for immunoprecipitation experiments.

mAb Technique

Mice were injected intraperitoneally with 50 μ g colonic brush-border membrane protein in 200 μ l PBS mixed with 200 μ l complete Freund's adjuvant. A booster injection with the same amount of antigen in PBS, mixed with incomplete Freund's adjuvant, was administered 4 wk later. 7 (distal colonocytes) or 9 wk (proximal colonocytes) after the first immunization, a final booster with 100 μ g membrane protein without adjuvant was given intraperitoneally and 3 d later cell fusion was carried out according to established techniques (Fazekas de St. Groth and Scheidegger, 1980; Galfré et al., 1977), as described by Hauri et al. (1985b) using PA1 myeloma cells (Stocker et al., 1982). Screening of antibodies was performed by a solid-phase radioimmunoassay (Hauri et al., 1985b) with colonocyte microvillar membranes (20 μ g/ml) adsorbed to soft polyvinylchloride multiwell plates. Supernatants displaying nonspecific binding to BSA or to the glycoprotein ovomucoid were discarded. Positive cultures were expanded and frozen when approaching a cell number of $\sim 3-5 \times 10^6$ cells. The culture supernatants were concentrated 10-fold by ammonium sulphate precipitation and tested for brush-border specificity using cryosections of rat colon in conjunction with the immunofluorescence technique (see below). Hybridoma-producing antibodies that specifically bound to the luminal membrane of colonocytes were subcloned by limiting dilution. mAbs were either used as 10-fold concentrated culture supernatants or in ascites form. In every instance, the antibodies in the ascites form had the identical specificity to the corresponding culture supernatant. For some experiments the mAbs were purified by DEAE 52 chromatography and coupled to horseradish peroxidase (Engvall, 1980). The antibody classes were determined by Ouchterlony double-diffusion assay using subclass-specific rabbit anti-mouse immunoglobulins.

Immunofluorescence

For the secondary screening of mAbs and for the developmental study, 2% paraformaldehyde-fixed rat colon tissue was included in Tissue Tek and 5- μ m-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- μ m-thick sections were cut with an ultramicrotome with cryoattachment (Reichert Jung S.A., Paris, France; Tokuyasu, 1978). The indirect immunofluorescence technique was used to

1. *Abbreviation used in this paper:* Endo F, endo- β -*N*-acetylglucosaminidase F.

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.

Immunoelectron 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 preincubated 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 CD1/62 (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.

Iodination

Triton X-100-solubilized small- or large-intestinal microvillar membranes were labeled with [¹²⁵I]Na using the lactoperoxidase-glucose oxidase method as described (Hauri et al., 1985b). Protein A- or DEAE 52-purified mAbs were labeled with [¹²⁵I]Na using the chloramine T procedure (Hunter and Greenwood, 1962).

Radiometric Assay for Quantification of Antigen

This assay is based on a recent study by Rothwell et al. (1985) and was performed as follows. Cell fractions to be assayed were solubilized with Triton X-100 in 100 mM sodium phosphate, 1 mM EDTA (pH 7.4, detergent/protein ratio [wt/vol] = 3) for 1 h on ice. To 100 μ l of twofold serially diluted sample (1% BSA in PBS, pH 7.4) 150 μ l of [¹²⁵I]-labeled mAbs (containing 60,000 cpm) were added. After overnight incubation at room temperature, the immunocomplexes were precipitated with Rivanol, the supernatant was discarded, and the radioactivity of the pellet was determined in a γ -counter. The data were analyzed by computer using program I developed by Ritchie et al. (1983) and the formula $Y = (a-d)/[1+(X/c)^b]+d$, where Y is the response; X , the arithmetic concentration; a , the response when $X = 0$; d , the response for infinite concentration; c , the ED₅₀, i.e., the concentration resulting from a response halfway between a and d with b as the slope factor that determines the steepness of the curve. The enrichment factor of a given brush-border membrane fraction relative to the corresponding homogenate was determined by comparing ED₅₀ values of the two curves that were fixed to the same d value.

Immunoprecipitation, SDS-PAGE, Autoradiography, and Immunoblotting

Antigens were immunoprecipitated 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 gels 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-114

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. $6\text{--}10 \times 10^6$ cpm were used for phase partitioning in 100 mM sodium phosphate containing 2% (wt/vol) precluded 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 CPI/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_2CO_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 immunisolated, ^{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 $\mu\text{g}/\text{ml}$ pepstatin, 17.5 $\mu\text{g}/\text{ml}$ benzamide, 10 $\mu\text{g}/\text{ml}$ 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 mU of the enzyme in the citrate-maleate buffer as for neuraminidase. α -L-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_r 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 ^{125}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

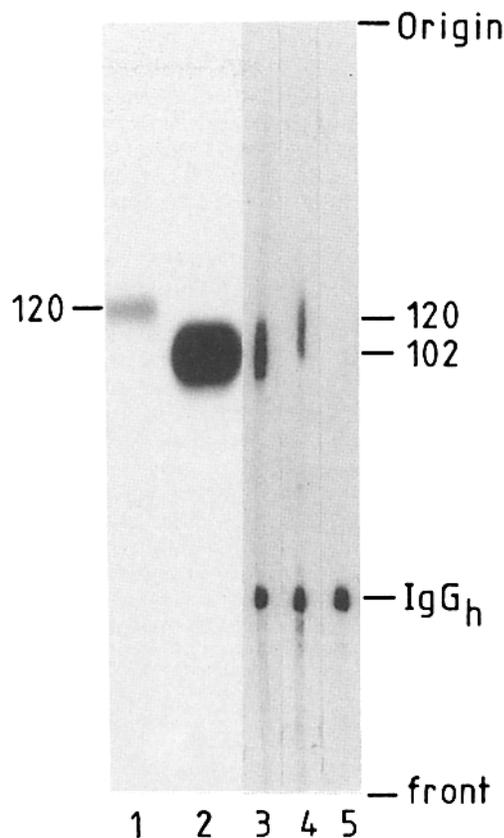


Figure 1. Identification of a novel rat brush-border membrane glycoprotein, cmv 120, in large-intestinal colonocytes (lanes 1 and 4) and small-intestinal enterocytes (lanes 2 and 3) by means of mAbs. Purified brush-border membranes (FI fraction) of isolated colonocytes or enterocytes were solubilized with Triton X-100 and labeled with ^{125}I Na by the lactoperoxidase-glucose oxidase method. 6×10^5 cpm (lane 1) or 5.5×10^6 cpm (lane 2) were subjected to immunoprecipitation with mAb CPI/126 and the immunoprecipitates were separated by SDS-PAGE followed by autoradiography. In lanes 3–5, the antigens were first immunoprecipitated from unlabeled colonic FI brush-border membrane fraction (100 μg protein per lane) by means of antibody CPI/126 and the immunoprecipitates were subjected to SDS-PAGE followed by Western blotting. The blots were incubated with antibody CDI/62 (lanes 3 and 4) or with a mAb, HBB2/614/88, against human sucrase-isomaltase as a negative control (lane 5). The immunoreaction was visualized by using a second rabbit anti-mouse antibody followed by ^{125}I protein A. The immunoreaction in the 50-kD region is due to the heavy chains of the mouse mAbs (IgG_h).

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).

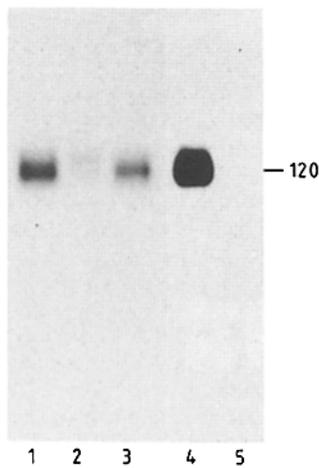


Figure 2. Membrane association of cmv 120 assessed by phase partitioning in Triton X-114 or sodium carbonate treatment. Phase partitioning of cmv 120 in Triton X-114 (lanes 1-3). Purified brush-border membranes (FI fraction) of isolated rat colonocytes were solubilized with Triton X-100 and labeled with [125 I]Na. 10^{-7} cpm were subjected to phase separation in Triton X-114 as described in Materials and Methods. The detergent phase (lane 3), the aqueous phase (lane 2), or an untreated sample (10^7 cpm, lane 1) were immunoprecipitated with antibody CPI/126. The sodium carbonate treatment (see Material and Methods for details) was carried out with 125 I-labeled brush-border membranes. The antigen was immunoprecipitated from the membrane fraction (lane 4) or the supernatant (lane 5). The immunoprecipitates were separated by SDS-PAGE and the gel was processed for autoradiography.

tated with antibody CPI/126. The sodium carbonate treatment (see Material and Methods for details) was carried out with 125 I-labeled brush-border membranes. The antigen was immunoprecipitated from the membrane fraction (lane 4) or the supernatant (lane 5). The immunoprecipitates were separated by SDS-PAGE and the gel was processed for autoradiography.

Tissue Distribution of cmv 120

To determine whether the colon antigen was also found in epithelial cells of other rat tissues, brush-border membranes of jejunum and total-membrane fractions of liver and kidney were prepared, detergent-solubilized, and individually immunoprecipitated with antibody CPI/126. Fig. 3 shows a silver-stained SDS gel on which such immunoprecipitates were separated. No antigen was detectable in immunoprecipitates of kidney (Fig. 3, lane 6) and liver (not shown) while a 102-kD protein was precipitable from the small-intestinal fraction (Fig. 3, lane 4; see also Fig. 1, lane 2). Since this antigen was also recognized by CD1/62 (Fig. 1, lane 3) the results suggest that this protein is indeed related to cmv 120 despite its somewhat faster mobility on SDS gels. The small-intestinal antigen was predominantly expressed in crypt cells (see below). We therefore wondered whether cmv 120 was also present in the established crypt cell lines IEC 6 (Quaroni et al., 1979), IEC 17, and IEC 18 (Quaroni and Isselbacher, 1981) of rat small intestine. For this purpose confluent monolayers of these cells were metabolically labeled with [35 S]methionine for 3 h, solubilized with Triton X-100, and immunoprecipitated with the CPI/126 antibody. No cmv 120 was detectable on fluorograms of a gel run with such immunoprecipitates (not shown). Thus cmv 120 does not seem to be expressed at detectable levels in these three cell lines.

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 M_r 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 sialic acid. Further treatment with O-glycanase only minimally lowered the apparent M_r 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 M_r of the small-intestinal but not the large-intestinal antigen.

Immunolocalization of cmv 120 in the Rat Intestine

Light microscopy using 1- μ m 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 CPI/126 (Fig. 5, a and b). An identical pattern was obtained with antibody CD1/62 (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 lumi-

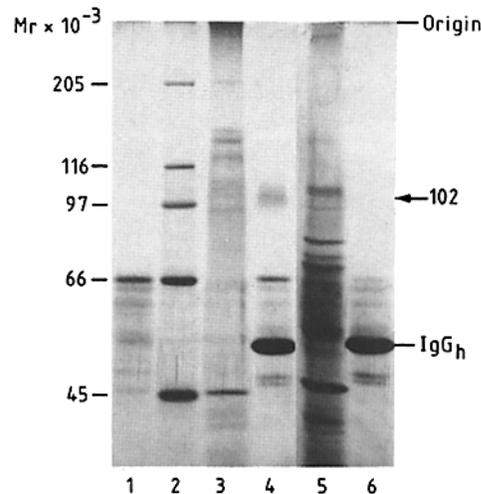


Figure 3. cmv 120 is not detectable in the rat kidney. A total membrane fraction was prepared from one rat kidney and subjected to immunoprecipitation with antibody CPI/126. The immunoprecipitate was separated by SDS-PAGE and the proteins on the gel were stained with silver. For comparative purposes brush-border membranes from rat small intestine were subjected to the same procedure. (Lane 1) Control (no antibody); (lane 2) M_r markers (myosin = 205 kD; β -galactosidase = 116 kD; phosphorylase = 97 kD; BSA = 66 kD; ovalbumin = 45 kD); (lane 3) brush-border membranes (10 μ g protein); (lane 4) immunoprecipitate from 250 μ g brush-border membranes; (lane 5) total kidney membranes (10 μ g protein); (lane 6) immunoprecipitate from 250 μ g total kidney membrane protein. IgG_h indicates the position of the heavy chain of the mAb in lanes 4 and 6. The arrowhead points to the position of the cmv 120 antigen which has an M_r of 102 kD in the small intestine. Bands appearing in the 66-kD region and below in lanes 1, 4, and 6 are due to albumin (and its breakdown products) used during the immunoprecipitation.

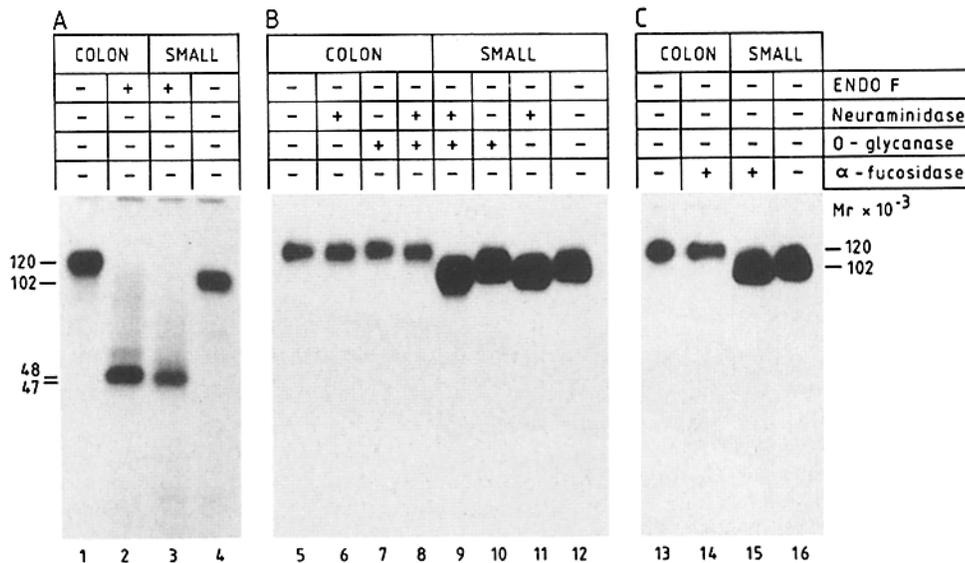


Figure 4. Glycosylation of ^{125}I -labeled cmv 120 in the colon and the small intestine as determined by sensitivity to endo F, neuraminidase, *O*-glycanase, and α -fucosidase. Isolated brush-border membranes were detergent solubilized and enzymatically labeled with ^{125}I Na. Aliquots containing 5×10^6 cpm were immunoprecipitated with antibody CPI/126 and the immunoprecipitates were subjected to digestion with the indicated glycosidases as described in Material and Methods. Shown are autoradiograms of SDS-polyacrylamide gels. COLON, FI fraction of colonocytes; SMALL, FI fraction of small-intestinal enterocytes.

nal surface of crypt cells (Fig. 5, *c* and *d*). The labeling was found to be present down to the base of the crypts (not shown). 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-f*). 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 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 side of the microvillus membrane of colonocytes or small-intestinal crypt cells (Fig. 6, *b* and *c*). This suggests that the epitope recognized by antibody CD1/62 is exposed on the luminal side of the apical surface membrane. The cells in the crypt of the colon showed a weaker labeling compared to surface colonocytes (Fig. 6 *a*). The labeling of enterocytes in the villus decreased from the base to the tip. Enterocytes of the tip showed a very weak labeling (Fig. 6 *d*). Apart from the brush-border membrane, labeling of intracellular apical vesicles and tubules was also observed in particular with the large-intestinal surface colonocytes (Fig. 6 *b*). This labeling may be due to newly synthesized cmv 120 that is en route to the plasma membrane or due to endocytosed antigen.

Development of cmv 120 Antigen during Ontogeny

The development of cmv 120 was studied with the immunofluorescence technique using 5- μm cryosections. First appearance of the antigen was detectable in the colon at day 15 of gestation and in the small intestine at day 16-17 (Fig. 7). Until birth, the antigen was evenly distributed at the luminal cell surfaces of the epithelial cells lining the intestinal lumen in both segments. Surprisingly, during crypt formation (1-3 d after birth) the antigen disappeared temporarily from the villi in the small intestine and from surface colonocytes concomitant with its reappearance in the crypts. At day 6 af-

ter birth the cellular distribution of cmv 120 was already virtually identical to that described for the adult intestine. Immunoblotting experiments carried out with antibody CD1/62 revealed that the fetal colonic and fetal small-intestinal antigens had the same mean apparent M_r 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 M_r by 15-20 kD. It is possible that this change in M_r 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 CPI/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 ^{125}I -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.

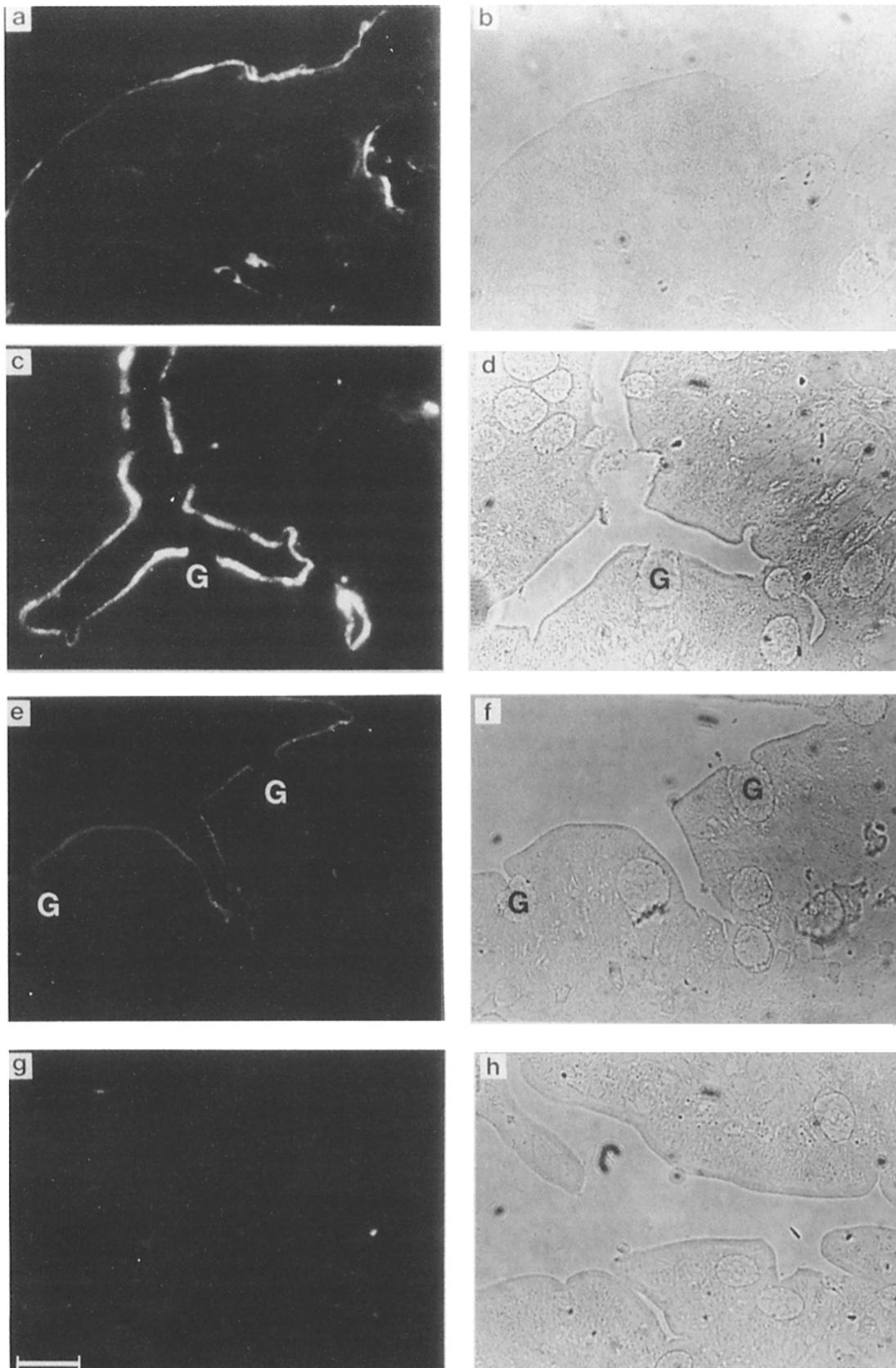


Figure 5. Immunofluorescence staining of cmv 120 in 1- μ m cryosections of rat distal colon (*a*) and rat small intestine (*c*, *e*, and *g*) at the level of crypts (oblique section, *c*), villus base (*e*), and villus tip (*g*). The sections were incubated with antibody CPI/126 (ascites fluid 1:100) 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 μ m.

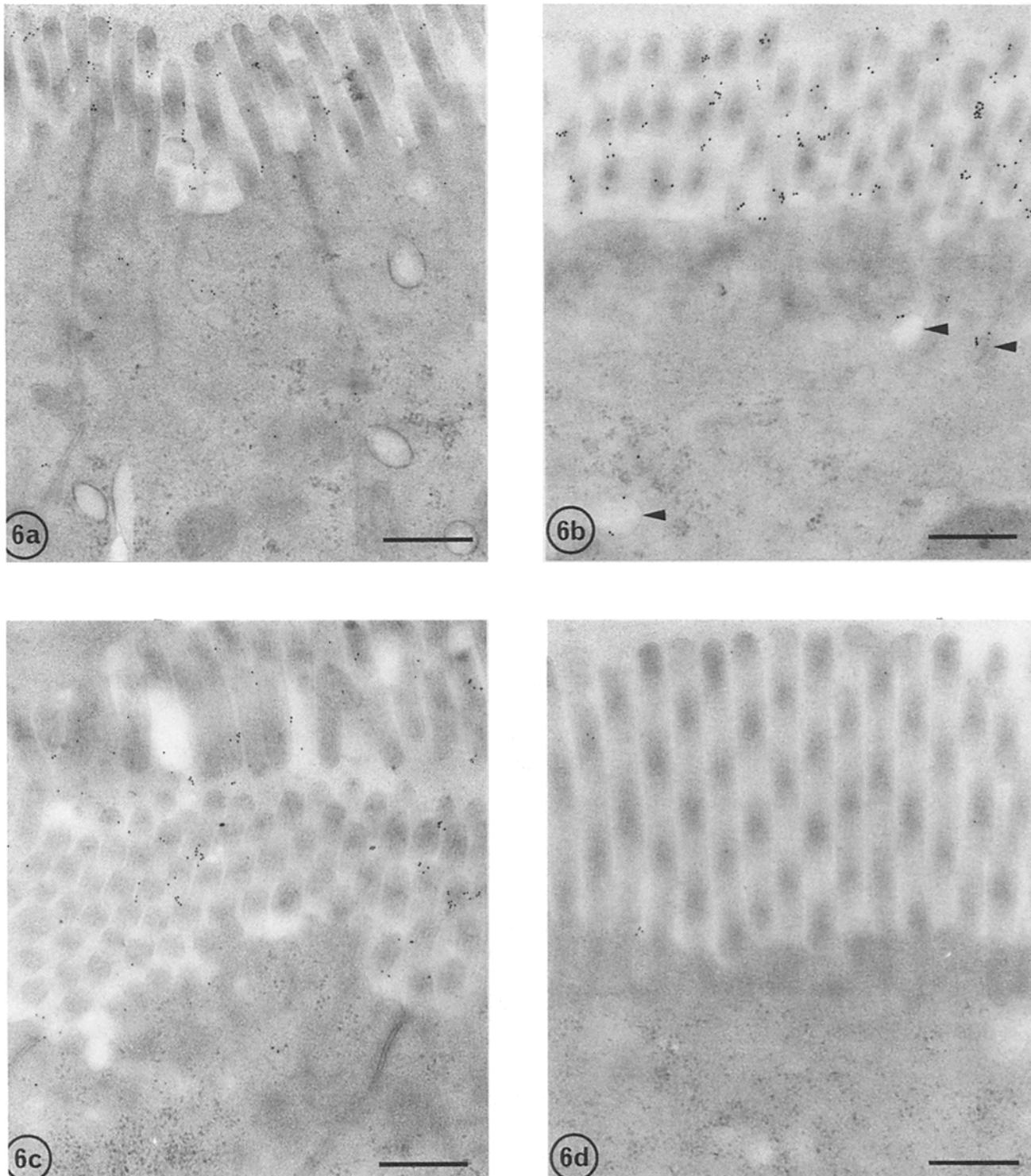


Figure 6. Localization of cmv 120 in ultrathin sections of Lowicryl K4M-embedded rat colonic and small-intestinal mucosa. Sections were incubated with antibody CD1/62 (culture supernatant 1:2), followed by rabbit anti-mouse IgG, and finally with protein A-10 nm colloidal gold. Brush-border labeling is 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 μ m.

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-

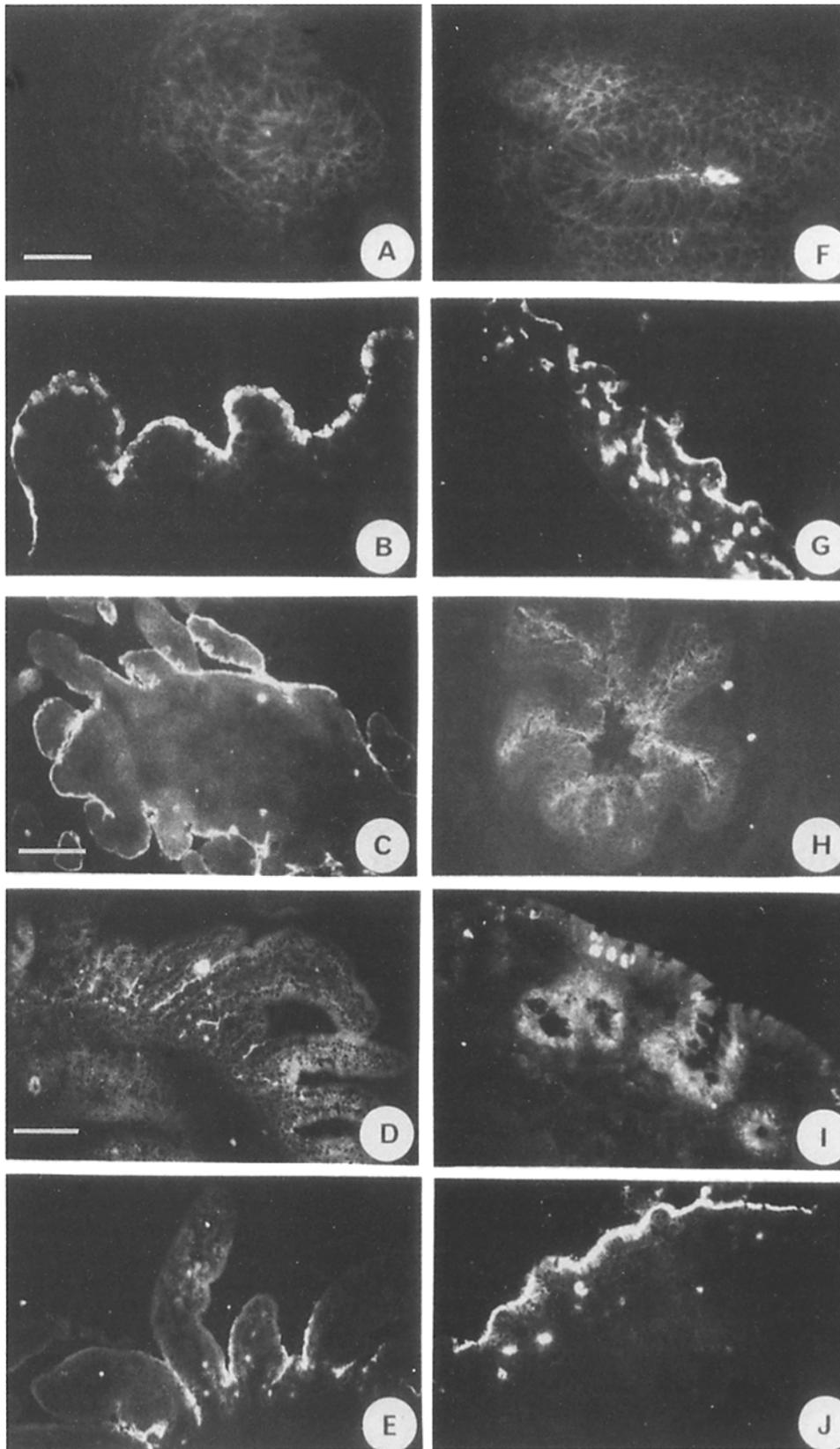


Figure 7. Development of cmv 120 in the small intestine (A-E) and in the colon (F-J) during ontogeny as visualized by immunofluorescence. 5- μ m cryosections of 15- (A and F), 17- (B and G), and 20-d (C and H) fetal intestines or of intestines at 3 (D and I) and 6 d (E and J) after birth were incubated with antibody CPI/126 followed by fluorescein-labeled sheep anti-mouse IgG. Bars: (A, B, F, G, and I) 44 μ m; (C and H) 78 μ m; (D, E, and J) 87 μ m.

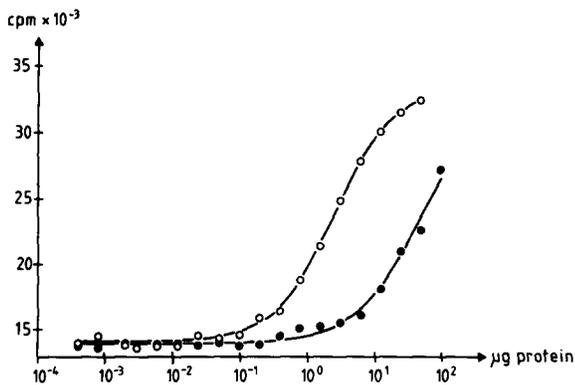


Figure 8. Representative example of the quantification of cmv 120 in the homogenate (●) and the corresponding FI brush-border fraction (○) of isolated rat colonocytes by the Rivanol assay (see Material and Methods for details). The enrichment factor for brush borders was 20.5-fold as determined by the 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.

brane of goblet cells does not appear to contain this protein as assessed by immunofluorescence. According to a postulate of DeDuve (1964), a marker protein or marker enzyme should be associated with a single organelle and distributed homogeneously within its membrane. With the advent of extremely sensitive techniques like high-resolution immunoelectron microscopy, this postulate is more difficult to meet since biosynthetic precursors of the postulated marker may be visualized in various organelles in addition to the principal location. Along this line, we believe that the weak intracellular labeling of apical vesicles and tubules observed at the ultrastructural level with antibody CD1/62 most probably reflects the biosynthetic and/or endocytotic pathway. Apart from its lower absolute amount, cmv 120 favorably compares with sucrase-isomaltase, an accepted marker enzyme of the small-intestinal brush-border membrane, in respect to its subcellular localization at the light and electron microscopic level (Fransen et al., 1985; Lorenzsonn et al., 1987). In the brush-border membrane, the cmv 120 appears to be uniformly distributed with the exception of its absence from 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; Lucocq and Baschong, 1986).

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 FI brush-border fraction.

Differences relating to ultrastructure (Engelhardt and Reckemmer, 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 M_r by ~72 kD in the colon. Assuming a contribution of a single N-linked oligosaccharide side chain of ~3 kD this reduction in apparent M_r would reflect removal of 24 side chains per polypeptide. The difference in M_r 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 M_r 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 M_r difference of the colonic and the small-intestinal cmv 120 was 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, Quaroni (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 having a similar M_r 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 M_r 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 immunoelectron microscopy. While in the colon, cmv 120 immunoreactivity is low at the bottom of the crypt cells and increases to a maximal level when the cells reach the intestinal surface. This protein is maximally expressed in small-intestinal crypt cells and gradually disappears while the cells migrate from the base to the tip of the villus.

The cmv 120 protein was found to be already expressed during the late fetal stage before the development of crypt cells. Its transient disappearance from the cell surface after birth suggests a block of synthesis of this marker in surface cells during crypt formation.

In conclusion, the present study suggests that cmv 120 is

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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References

- Armbruster, B. L., E. Carlemalm, R. Chiovetti, R. M. Garavito, J. A. Habot, E. Kellenberger, and W. Villiger. 1982. Specimen preparation for electron microscopy using low temperature embedding resins. *J. Microsc.* 126: 77-85.
- Binder, H. J., and G. I. Sandle. 1986. Electrolyte absorption and secretion in the mammalian colon. In *Physiology of the Gastrointestinal Tract*. Vol. 2. L. R. Johnson, editor. Raven Press, New York. 1389-1418.
- Binder, H. J., G. Stange, H. Murer, B. Stieger, and H. P. Hauri. 1986. Sodium-proton exchange in colon brush border membranes. *Am. J. Physiol.* 251: G382-G390.
- Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* 256:1604-1607.
- Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
- Brasitus, T. A., and R. S. Keresztes. 1984. Protein-lipid interactions in antipodal plasma membranes of rat colonocytes. *Biochim. Biophys. Acta.* 773: 290-300.
- Dahlqvist, A. 1968. Assay of intestinal disaccharidases. *Anal. Biochem.* 22: 99-107.
- DeDuve, C. 1964. Principles of tissue fractionation. *J. Theor. Biol.* 6:33-59.
- Engelhardt, W., and G. Rechkemmer. 1983. Absorption of inorganic ions and short-chain fatty acids in the colon of mammals. In *Intestinal Transport*. M. Gilles-Baillien and R. Gilles, editors. Springer-Verlag GmbH, Heidelberg, FRG. 26-45.
- Engvall, E. 1980. Enzyme immunoassay ELISA and EMIT. *Methods Enzymol.* 70:419-439.
- Ey, P. L., S. J. Prowse, and C. R. Jenkin. 1978. Isolation of pure IgG₁, IgG_{2a}, and IgG_{2b} immunoglobulins from mouse serum using protein A-Sepharose. *Immunochemistry.* 15:429-436.
- Fazekas de St. Groth, S., and D. Scheidegger. 1980. Production of monoclonal antibodies: strategy and tactics. *J. Immunol. Methods.* 35:1-21.
- Foster, E. S., J. P. Hayslett, and H. J. Binder. 1984. Mechanism of active potassium absorption and secretion in the rat colon. *Am. J. Physiol.* 246: G611-G617.
- Fransen, J. A. M., L. A. Ginsel, H. P. Hauri, E. E. Sterchi, and J. Blok. 1985. Immunoelectron microscopic localization of a microvillus membrane disaccharidase in the human small-intestinal epithelium with monoclonal antibodies. *Eur. J. Cell Biol.* 38:6-15.
- Fujiki, Y., A. Hubbard, S. Fowler, and P. B. Lazarow. 1982. Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. *J. Cell Biol.* 93:97-102.
- Galfré, G. S., C. Howe, C. Milstein, G. W. Butcher, and J. D. Howard. 1977. Antibodies to major histocompatibility antigens produced by hybrid cell lines. *Nature (Lond.)*. 266:550-552.
- Gorvel, J. P., A. Rigal, A. Bernadac, and S. Maroux. 1986a. Further characterization of an early expressed glycoprotein of the rabbit small intestinal brush border: its interaction with some hydrolases. *Eur. J. Biochem.* 159:499-505.
- Gorvel, J. P., A. Rigal, D. Olive, C. Mawas, and S. Maroux. 1986b. Identification of an early expressed marker of the luminal membrane of rabbit small intestinal columnar cells. Presence of a homologous antigen in kidney proximal tubules and glomeruli. *Biol. Cell.* 56:121-126.
- Gustin, M. C., and D. B. P. Goodman. 1981. Isolation of brush-border membrane from the rabbit descending colon epithelium. Partial characterization of a unique K⁺-activated ATPase. *J. Biol. Chem.* 256:10651-10656.
- Gustin, M. C., and D. B. P. Goodman. 1984. Development of an isolation procedure for the brush border membrane of an electrically tight epithelium: rabbit distal colon. *Curr. Top. Membr. Transp.* 20:295-305.
- Hauri, H. P., and K. Bucher. 1986. Immunoblotting with monoclonal antibodies: importance of the blocking solution. *Anal. Biochem.* 159:386-389.
- Hauri, H. P., J. Roth, E. Sterchi, and M. Lentze. 1985a. Transport to cell surface of intestinal sucrase-isomaltase is blocked in the Golgi apparatus in a patient with congenital sucrase-isomaltase deficiency. *Proc. Natl. Acad. Sci. USA.* 82:4423-4427.
- Hauri, H. P., E. E. Sterchi, D. Bienz, J. A. M. Fransen, and A. Marxer. 1985b. Expression and intracellular transport of microvillus membrane hydrolases in human intestinal epithelial cells. *J. Cell Biol.* 101:838-851.
- Hauri, H. P., B. Stieger, and A. Marxer. 1986. The brush border membrane of the rat colonic columnar epithelial cell. In *Molecular and Cellular Biology of Digestion*. P. Desnuelle, H. Sjöström, and O. Noren, editors. Elsevier Biomedical Press, Amsterdam, Holland. 421-431.
- Hunter, W. M., and F. C. Greenwood. 1962. Preparation of iodine-131 labeled human growth hormone of high specific activity. *Nature (Lond.)*. 194: 495-496.
- Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680-685.
- Lorenzsonn, V., H. Korsmo, and W. A. Olsen. 1987. Localization of sucrase-isomaltase in the rat enterocyte. *Gastroenterology.* 92:98-105.
- Luciano, L., E. Reale, G. Rechkemmer, and W. v. Engelhard. 1984. Structure of zonulae occludentes and the permeability of the epithelium to short-chain fatty acids in the proximal and distal colon of guinea pig. *J. Membr. Biol.* 82:145-156.
- Lucocq, J. M., and W. Baschong. 1986. Preparation of protein colloidal gold complexes in the presence of commonly used buffers. *Eur. J. Cell Biol.* 42:332-337.
- Merril, C. R., D. Goldman, and M. L. van Keuren. 1984. Gel protein stains: silverstain. *Methods Enzymol.* 104:441-447.
- Quaroni, A. 1986. Fetal characteristics of small intestinal crypt cells. *Proc. Natl. Acad. Sci. USA.* 83:1723-1727.
- Quaroni, A., and K. J. Isselbacher. 1981. Cytotoxic effects and metabolism of benzo(a)pyrene and 7,12-dimethylbenz(a)anthracene in duodenal and ileal epithelial cell cultures. *J. Natl. Cancer Inst.* 67:1353-1362.
- Quaroni, A., J. Wands, R. L. Trelstad, and K. J. Isselbacher. 1979. Epitheloid cell cultures from rat small intestine. Characterization by morphologic and immunologic criteria. *J. Cell Biol.* 80:248-265.
- Ritchie, D. G., J. M. Nickerson, and G. M. Fuller. 1983. Two simple programs for the analysis of data from enzyme-linked immunosorbent assays (ELISA) on a programmable desk-top calculator. *Methods Enzymol.* 92:577-588.
- Roth, J., M. Bendayan, E. Carlemalm, W. Villiger, and M. Garavito. 1981. Enhancement of structural preservation and immunocytochemical staining in low temperature embedded pancreatic tissue. *J. Histochem. Cytochem.* 29:663-671.
- Roth, J., D. J. Taatjes, J. M. Lucocq, J. Weinstein, and J. C. Pauslon. 1985. Demonstration of an extensive transtubular network continuous with the Golgi apparatus stack that may function in glycosylation. *Cell.* 43:287-295.
- Rothwell, C. E., M. B. Khazaeli, and I. A. Bernstein. 1985. Radiometric assay for direct quantitation of rat liver cytochrome P-450b using monoclonal antibodies. *Anal. Biochem.* 149:197-201.
- Schmitz, J., H. Preiser, D. Maestracci, B. K. Ghosh, J. J. Cerda, and R. K. Crane. 1973. Purification of the human intestinal brush border membrane. *Biochim. Biophys. Acta.* 323:98-112.
- Schultz, S. G. 1981. Ion transport by mammalian large intestine. In *Physiology of the Gastrointestinal Tract*. Vol. 2. L. R. Johnson, editor. Raven Press, New York. 991-1002.
- Schultz, S. G. 1984. A cellular model for active sodium absorption by mammalian colon. *Annu. Rev. Physiol.* 46:435-451.
- Slot, J. W., and H. J. Geuze. 1985. A new method of preparing gold probes for multiple-labeling cytochemistry. *Eur. J. Cell Biol.* 38:87-93.
- Smith, P. L., and R. D. McCabe. 1984. Mechanism and regulation of transcellular potassium transport by the colon. *Am. J. Physiol.* 247:G445-G456.
- Stieger, B., A. Marxer, and H. P. Hauri. 1986. Isolation of brush border membranes from rat and rabbit colonocytes: is alkaline phosphatase a marker enzyme? *J. Membr. Biol.* 91:19-31.
- Stocker, J. W., H. K. Forster, V. Miggiano, C. Staehli, G. Staiger, B. Takacs, and T. Staehelin. 1982. Generation of two new mouse myeloma cell lines "PAI" and "PAI-O" for hybridoma production. *Res. Disclosure.* 217:155-157.
- Tokuyasu, K. T. 1978. A study of positive staining of ultrathin frozen sections. *J. Ultrastruct. Res.* 63:287-307.
- Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.
- Vengesa, P. B., and U. Hopfer. 1979. Cytochemical localization of alkaline phosphatase and Na⁺-pump sites in adult rat colon. *J. Histochem. Cytochem.* 27:1231-1235.