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Expression and Intracellular Transport of Microvillus Membrane Hydrolases in Human Intestinal Epithelial Cells

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ABSTRACT A panel of monoclonal antibodies was produced against purified microvillus membranes of human small intestinal enterocytes. By means of these probes three disaccharidases (sucrase-isomaltase, lactase-phlorizin hydrolase, and maltase-glucoamylase) and four peptidases (aminopeptidase N, dipeptidylpeptidase IV, angiotension I-converting enzyme, and *p*-aminobenzoic acid peptide hydrolase) were successfully identified as individual entities by SDS PAGE and localized in the microvillus border of the enterocytes by immunofluorescence microscopy. The antibodies were used to study the expression of small intestinal hydrolases in the colonic adenocarcinoma cell line Caco 2. This cell line was found to express sucrase-isomaltase, lactase-phlorizin hydrolase, aminopeptidase N, and dipeptidylpeptidase IV, but not the other three enzymes. Pulse-chase studies with [^{35}S]methionine and analysis by subunit-specific monoclonal antibodies revealed that sucrase-isomaltase was synthesized and persisted as a single-chain protein comprising both subunits. Similarly, lactase-phlorizin hydrolase was synthesized as a large precursor about twice the size of the lactase subunits found in the human intestine. Aminopeptidase N and dipeptidylpeptidase IV, known to be dimeric enzymes in most mammals, were synthesized as monomers. Transport from the rough endoplasmic reticulum to the *trans*-Golgi apparatus was considerably faster for the peptidases than for the disaccharidases, as probed by endoglycosidase H sensitivity. These results suggest that the major disaccharidases share a common biosynthetic mechanism that differs from that for peptidases. Furthermore, the data indicate that the transport of microvillus membrane proteins to and through the Golgi apparatus is a selective process that may be mediated by transport receptors.

The absorptive columnar cell of the mammalian small intestine is a highly polarized epithelial cell that exhibits a regular array of microvilli on its apical pole. The microvillus membrane is endowed with a number of hydrolytic enzymes that are involved in the digestion of dietary carbohydrates and peptides (see references 1 and 2 for reviews). Besides disaccharidases and peptidases, which are believed to be major membrane constituents, the human microvillus membrane has a currently unknown number of poorly characterized, less abundant, intrinsic membrane proteins, including alkaline phosphatase (3) and various receptors as well as carriers for amino acid and sugar transport (4). Despite the many studies dealing with intestinal microvillar hydrolases, only a few of these enzymes have been purified to homogeneity from the human intestine (3, 5–7). Current thinking assumes that sucrase-isomaltase, maltase-glucoamylase, lactase-phlorizin hydrolase, and trehalase are the principal, if not the only, hydrolases responsible for the digestion of dietary carbohydrates on the intestinal surface. However, there is evidence for additional enzymes (8). For two reasons the situation with human microvillar peptidases is even more complicated. First, none of these enzymes has been purified to homogeneity, and second, enterocytes contain a number of intracellular soluble peptidases with similar substrate specificities (2).

To understand more completely the various functions of the human intestinal microvillus membrane it is necessary to identify and characterize all of its constituents. The most promising method to achieve such an enormous task is the

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monoclonal antibody technique. Successful application of this approach in membrane research has been demonstrated in numerous studies in which specific monoclonal antibodies to individual membrane proteins have been obtained starting with highly complex antigen mixtures such as whole membranes or even whole cells. We previously used this approach to produce monoclonal antibodies against rat sucrase-isomaltase. One of these antibodies proved to be an excellent tool with which to study the structure and biosynthesis of this enzyme (9, 10).

With the ultimate goal of elucidating the number and types of intestinal hydrolases, as well as the mechanism of their biosynthesis and polarized surface expression, we have now produced monoclonal antibodies to highly purified human jejunal microvillus membranes. In this article we report the identification of three disaccharidases and four peptidases by means of these antibodies. As an application we have used these immunological tools to study the expression of microvillar enzymes in the differentiated adenocarcinoma cell line Caco 2. These cells were found to synthesize the microvillar enzymes sucrase-isomaltase, lactase-phlorizin hydrolase, aminopeptidase N, and dipeptidylpeptidase IV and therefore represent a promising in vitro model for studying molecular mechanisms that underlie the intracellular membrane traffic and the biogenesis of surface membrane polarity in epithelial cells.

MATERIALS AND METHODS

Production of Monoclonal Antibodies: Highly purified intestinal microvillus membranes were used as an antigen to produce monoclonal antibodies in mice. The membranes were purified according to Sterchi and Woodley (11) from normal adult human jejunal mucosa obtained from kidney donors. The purified membranes (F II fraction) had a specific activity for sucrase of 2.16 ± 0.19 U/mg protein (mean ± SD, $n = 5$), representing a 23- to 27-fold purification as compared with the homogenate. Mice were injected intraperitoneally with 200 μg membrane protein in 200 μl phosphate-buffered saline (PBS) mixed with 200 μl complete Freund's adjuvant. A booster injection with the same amount of antigen in PBS, but mixed with incomplete adjuvant, was administered 3 wk later by the same route. At 4 wk the animals were bled from the tail, and antibody titers were measured in the sera using the solid-phase radioimmunoassay described below. 6, 8, or 47 wk after the first immunization a final booster of 200 μg membrane protein without adjuvant was given intraperitoneally and 4 d later cell fusion was performed according to established techniques (12). 2×10^8 spleen cells were fused with 10^8 FO myeloma cells (13), and the fusion mixture was resuspended in Iscove's modified Dulbecco's medium containing hypoxanthine, aminopterine, and thymidine and distributed into ten 96-well plates (Costar, Cambridge, MA). 1 d before fusion, irradiated (1,000 rad) mouse peritoneal macrophages (13) were added to the wells as feeder cells. Hybrids were selected in hypoxanthine, aminopterin, thymidine medium (14). 9 to 11 d after fusion, culture supernatants were screened for antibody production.

Screening of Monoclonal Antibodies: Initial screening was performed by a solid-phase radioimmunoassay using flexible 96-well polyvinylchloride plates as follows. All the buffers contained 0.1% sodium azide. The procedure had five steps: (*1*) antigen coating by the use of 50 μl/well of a suspension of microvillus membranes (20 μg/ml) in PBS overnight at 4°C; (*2*) blocking of remaining binding sites by incubation with 250 μl PBS containing 2% bovine serum albumin (BSA; Sigma Chemical Co., Poole, England) for 1 h at 37°C; (*3*) incubation with culture supernatants, 50 μl, for 1 h at 37°C; (*4*) incubation with second antibody, 50 μl (2.5 μg) rabbit anti-mouse immunoglobulin (IgG fraction, Sera Lab, Sussex, England) in PBS-0.1% BSA, for 1 h at 37°C; and (*5*) incubation with ^{125}I-protein A (New England Nuclear, Boston MA), 50.000 cpm in 50 μl PBS-0.1% BSA, for 1 h at 37°C. After step *1* the antigen was aspirated from the wells and the blocking solution was immediately added. The wells were washed four times each after steps *2*, *3*, *4*, and *5* with 250 μl PBS-0.1% BSA. After the final wash the radioactivity of the individual wells was measured in a γ-counter (Gammamatic, Kontron Elektronik GmbH, Zurich). Every culture supernatant was also tested for nonspecific binding to BSA-coated multiwell plates. As a positive control, a polyclonal rabbit antibody to human microvillus membranes (IgG fraction) was used at a concentration of 10 μg/ml. This antibody reproducibly gave from 20,000 to 22,000 cpm per well, and the negative controls (hypoxanthine, aminopterin, thymidine medium instead of culture supernatants) yielded from 200 to 400 cpm. Cultures with >1,000 cpm were considered positive and were expanded to 100-mm culture dishes. At that stage the cells were frozen.

The culture media were concentrated 10 times by ammonium sulfate precipitation (to 50% saturation) and subsequent dialysis against 100 mM sodium phosphate, pH 8.0. Such concentrated culture supernatants were used to screen for noninhibitory antibodies to various microvillar enzymes using a protein A–Sepharose immunoassay. The assay was performed in 3-ml plastic tubes. To each tube 20 μl of a 30% stock suspension of protein A-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) was added. The beads were washed once with 1 ml 100 mM sodium phosphate-0.1% BSA, pH 8.0 (buffer 1) and incubated with 50 μl concentrated culture supernatants for 2 h at room temperature on a horizontal Lab-shaker (A. Kühner AG, Basel) at 145 rpm. After two washes with 1 ml buffer 1 and one wash with 1 ml 100 mM sodium phosphate buffer containing 1% (wt/vol) Triton X-100, pH 8.0 (buffer 2), the beads were incubated with 50 μl Triton X-100–solubilized microvillus membranes for 90 min at 4°C. Thereafter the beads were washed by three cycles of 1 ml buffer 2 and the washed beads were incubated with the appropriate enzyme substrate at 37°C overnight. On the following day enzyme activities were measured.

Selection of Hybridomas and Cell Cloning: Antibody-producing hybridomas were selected according to (*a*) enzyme specificity assessed by the protein A immunoassay, (*b*) presence of high antibody titers in the concentrated culture supernatant as detected by Ouchterlony double diffusion experiments with class-specific antisera to mouse immunoglobulins (Nordic Immunology, Tilburg, The Netherlands), and (*c*) radioactivity obtained in the solid-phase radioimmunoassay. Through *c*) it was found that monoclonal antibodies against the most abundant protein of the microvillus membrane, sucrase-isomaltase, yielded up to 7,000 cpm. Therefore higher values were indicative of either more than one antibody-producing clone or a clone producing a nonspecific antibody. For these reasons such cultures were not usually considered for further analysis. Selected hybridomas were thawed and cloned from one to three times by the limiting dilution technique using nonirradiated peritoneal macrophages (one mouse/20 ml medium) as feeder cells. Positive cultures were detected by the above protein A–Sepharose immunosorbent assay. Cloned cells were injected into pristane-primed BALB/c mice yielding ascites fluid rich in monoclonal antibody.

Purification of Monoclonal Antibodies: Monoclonal antibodies from culture supernatants or from ascites fluids were purified by ammonium sulfate precipitation (to 50% saturation) followed by DE52 ion exchange chromatography to yield the IgG fraction. Alternatively, monoclonal antibodies were purified by chromatography on protein A-Sepharose (15). Antibodies were stored frozen at −20°C.

Rocket Immunoelectrophoresis: This technique was used to determine antibody titers in ascites fluids. Rocket immunoelectrophoresis was performed in 1% (wt/vol) agarose (Calbiochem-Behring Corp., La Jolla, CA, or Pharmacia Fine Chemicals, Bromma, Sweden) gel slabs on Gelbond (LKB Instruments, Inc.). The samples were carbamylated at room temperature overnight (16). 7 μl of the carbamylated samples were run against rabbit anti-mouse IgG1 serum, goat anti-mouse IgG2a serum or rabbit anti-mouse IgG2b serum (100 μl antiserum/12 ml agarose solution; Nordic Immunology) at 80 V overnight in a Pharmacia flat bed apparatus FBE-3000. Purified monoclonal antibodies of different subclasses were used as standards in concentrations between 6.5 to 52.0 μg protein/ml rocket buffer. Antibody concentrations in ascites fluids ranged from 14 to 22 mg/ml. The contribution of endogenous nonimmune IgG1 to the overall measured IgG1 concentration was small and did not exceed 0.8 mg/ml as determined by rocket experiments in which ascites fluids containing non-IgG1-type monoclonal antibodies were run against anti-IgG1.

Immunofluorescence: Microvillar hydrolases were localized by an indirect immunofluorescence technique using monoclonal antibodies (ascites fluid 1:100) and human intestinal cryosections (17). Normal small-intestinal biopsies were fixed with 2% paraformaldehyde and 0.1% glutaraldehyde for 2 h at room temperature before freezing in Tissue-Tek II (Miles Laboratories Inc., Elkhart. IN). The second antibody was a rhodamine-conjugated rabbit anti-mouse IgG (Nordic Immunolgy). Expression of microvillar hydrolases in Caco 2 cells was studied with cells grown on coverslips 3 to 9 d after confluence. The cells were fixed with 2% paraformaldehyde and immunolabeling was performed as described (18).

Immunoisolation of Antigens and SDS PAGE: Antigens were immunoisolated using the above protein A-Sepharose assay with the modification that 0.2% (wt/vol) BSA (final concentration) was added to buffer 2. 100 μl antibody (10-times concentrated culture medium or ascites-fluid 1:100) was

adsorbed to 40 μl protein A-Sepharose and then incubated with 100 μl to 200 μl Triton X-100-solubilized microvillus membranes (1 mg protein/ml). After the final wash the beads were transferred to an Eppendorf tube, washed twice with 100 mM phosphate, pH 8.0, and once with 10 mM phosphate, pH 8.0, and the adsorbed antigen–antibody complexes were solubilized with electrophoresis sample buffer containing 2% (wt/vol) SDS and 100 mM dithiothreitol at 100°C for 4 min. Proteins were separated on 7.5% SDS-polyacrylamide slab gels (19) and stained with Coomassie Brilliant Blue R 250. [^{35}S]Methionine-labeled or [^{3}H]fucose-labeled proteins were visualized by fluorography using EN^{3}HANCE (New England Nuclear).

Immunoblotting: The method of Towbin et al. (20) was used to transfer microvillus membrane proteins from SDS gels to nitrocellulose sheets (Schleicher & Schuell, Keene, NH). The blots were stained with 0.1% amido black, destained with 20% methanol-7.5% acetic acid, photographed with a Polaroid camera, and the immunoreaction was carried out with 3-mm nitrocellulose strips in eight-well plates (Inotech AG, Wohlen, Switzerland) using fish gelatin as blocking agent (21). The sequence of incubations (at room temperature) was as follows: (*1*) 30 min in blocking solution (PBS containing 3% [wt/vol] fish gelatin, 0.05% Nonidet P-40, and 0.05% azide); (*2*) monoclonal antibody (2 μl if ascites fluid, 5–10 μl if 10-times-concentrated culture supernatant was used) in blocking solution overnight; (*3*) rabbit anti-mouse immunoglobulins, 2.5 μg/ml, for 2 h; (*4*) ^{125}I-protein A, 200,000 cpm/ml, for 4 h. The strips were washed three times for 20 min with blocking solution after steps *2*, *3*, and *4*, and after a final rinse with PBS they were processed for autoradiography.

Iodination of Intestinal Microvillus Membranes: The lactoperoxidase method (22) using glucose oxidase to generate peroxide (23) was adapted for the iodination of Triton X-100–solubilized membranes. 200 μg membrane protein was solubilized in 200 μl 1% Triton X-100, 10 mM K_2HPO_4, pH 7.2, for 1 h on ice. Nonsolubilized particles were removed by ultracentrifugation (100,000 *g*, 1 h) and to the resulting supernatant were added 200 μl 0.5 M potassium phosphate, pH 7.2, 45 μl 10 mM glucose in water, 8 μl of a 1 mg/ml PBS stock solution lactoperoxidase (Boehringer Mannheim Biochemicals, Mannheim, Federal Republic of Germany), 10 μl (173 mU) in PBS diluted glucose oxidase (type V, 1730 U/ml; Sigma Chemical Co.). This mixture was transfered to an Eppendorf tube containing 1 or 2 mCi carrier-free Na^{125}I (Eidgenössisches Institut für Reaktorforschung, Würenlingen, Switzerland). After 10 min at 20°C the reaction was stopped by the addition of 200 μl saturated tyrosine in 0.3 M potassium phosphate, pH 7.2. Protein-associated radioactivity was immediately separated from free iodine on a 0.7 × 30 cm Sephadex G 25 disposable column (Econocolumn; Bio-Rad Laboratories, Richmond, CA), which was equilibrated and developed with 0.1 M potassium phosphate, pH 7.2. Approximately 30 to 50% of the ^{125}I was recovered in the eluting protein peak. The ^{125}I-protein fraction was frozen in aliquots at −20°C and used within 2 wk.

Caco 2 Cell Culture and Labeling with [^{35}S]Methionine or [^{3}H]Fucose: Caco 2 cells (24) were cultured in 100-mm Falcon Optilux dishes (Falcon Labware, Oxnard, CA) in Iscove's modified Dulbecco's medium (4.5 g/L of glucose) containing 20% fetal calf serum, 1% nonessential amino acids (Gibco Latoratories Inc., Grand Island, NY), 50 U/ml penicillin, and 50 μg/ml streptomycin. This medium is designated complete medium. Cells were subcultured weekly at a 1:10 dilution by the EDTA method (25). The cells were used between passages 65 and 80. Caco 2 cells have not been cloned, but all of the cells display a polygonal shape. Labeling with [^{35}S]methionine was performed 5 to 9 d after confluence of the cells. The dishes were rinsed once with 10 ml methionine-free medium containing 20% dialyzed fetal calf serum, 1% nonessential amino acids, and antibiotics as above. This medium is called met-free medium. The cells were incubated in 10 ml met-free medium for 10 min at 37°C to deplete their methionine pool. Thereafter the cells were labeled with 100–500 μCi L-[^{35}S]methionine (70–85 Ci/mM; Amersham Corp., Arlington Heights, IL) in 2 ml met-free medium for 15 min at 37°C, and then a chase was performed with complete medium containing 10 mM unlabeled L-methionine for up to 20 h. Incorporation of [^{3}H]fucose was studied with 9-d confluent cells. A 100-mm culture plate was incubated with 500 μCi L-[5,6-^{3}H]fucose (70 Ci/mmol; Amerhsam Corp.) in 2 ml complete medium for 3 h at 37°C.

Preparation of a Crude Membrane Fraction of Caco 2 Cells and of Fetal Small Intestines: Caco 2 cells were placed on ice, rinsed three times with 10 ml ice cold PBS, scraped in 5 ml 2 mM Tris-50 mM mannitol, 40 μg/ml phenylmethylsulfonyl fluoride, pH 7.1, and homogenized in a glass-Teflon potter (Elvehjem) for 65–70 s with 11 strokes at 1,100 rpm. Sonication for 5 s at position 8 (Kontes Sonicator, Kontes Glass Co., Vineland, NJ) was necessary for complete disruption of cells. The resulting homogenate was centrifuged at 2,800 *g* for 10 min to remove the nuclei. The supernatant was spun at 100,000 *g* for 1 h yielding a pellet designated the "crude membrane fraction." Such a "crude membrane fraction" was also prepared from a 1-cm piece of small intestine obtained from a 14-wk-old human fetus after legal abortion.

Endoglycosidase H (endo H)[1] Treatment: Equal-radioactivity aliquots of sucrase-isomaltase adsorbed on to HBB2/614/88-protein A-Sepharose were boiled in 50 μl 0.1 M citric acid-NaOH pH 5.2 for 3 min in the presence of a cocktail of protease inhibitors containing antipain (1 μg/ml final concentration), benzamidine (17.5 μg/ml), pepstatin (1 μg/ml), aprotinin (10 μg/ml), and phenylmethylsulfonyl fluoride (1 mM) (26). 3 μl (45 μg/ml) endo H (New England Nuclear) together with a fresh aliquot of the protease inhibitor cocktail was added and the samples were incubated for 24 h at 37°C, reduced with 25 μl three-times-concentrated sample buffer (containing dithiothreitol) for 3 min at 100°C, and loaded onto an SDS gel. Controls were incubated at 37°C without endo H and yielded protein patterns identical to those of the original samples.

Enzyme Assays: Sucrase, isomaltase, maltase, lactase, glucoamylase, and trehalase were measured according to Dahlqvist (27) using sucrose, isomaltose, maltose, lactose, glycogen, and trehalose as substrates, respectively. The lactase assay was performed in the presence of *p*-chloromercuribenzoate, an inhibitor of lysosomal β-galactosidase (28). Aminopeptidase N was measured according to Roncari and Zuber (29) with L-leucine-*p*-nitroanilide as substrate. Aminopeptidase A, dipeptidylpeptidase IV, and γ-glutamyltranspeptidase were measured with L-α-glutamyl-*p*-nitroanilide, glycyl-L-proline-*p*-nitroanilide-*p*-tosylate, and L-γ-glutamyl-*p*-nitroanilide as substrates, respectively. Angiotensin I-converting enzyme was measured according to a modification of the method of Cushman and Cheung (30) using *N*-α-hippuryl-L-histidyl-L-leucine as substrate. The liberated His-Leu was assayed fluorometrically after the addition of Fluorescamine (1 mg/ml in dioxane; Fluka AG, Buchs, Switzerland). The excitation wavelength was 395 nm and the fluorescence was measured at 465 nm. *p*-Aminobenzoic acid (PABA) peptide hydrolase was measured according to Sterchi et al (31) with *N*-benzoyl-L-tryrosyl-PABA as substrate. Endopeptidase was assayed according to Wilk et al. (32) with *N*-carboxybenzoxy-glycyl-glycyl-L-leucine-*p*-nitroanilide as substrate. Carboxypeptidase was determined according to Auricchio et al. (33) using *N*-carboxybenzoxy-L-prolyl-L-methionine as substrate.

Competitive Enzyme-linked Immunosorbent Assay with Monoclonal Antibodies: Purified monoclonal antibodies (5 mg/ml) were coupled to horseradish peroxidase (type V Sigma Chemical Co.) according to Engvall (34). Peroxidase-linked antibodies were made 50% in glycerol and stored at −20°C. The competitive enzyme-linked immunosorbent assay was performed in flexible 96-well polyvinyl chloride plates by means of the following steps: (*1*) antigen coating with 50 μl/well of a suspension of microvillus membranes (20 μg/ml) in PBS overnight at 4°C; (*2*) blocking with 250 μl 2% BSA-PBS for 1 h at 37°C; (*3*) incubation with a mixture of 25 μl peroxidase-labeled monoclonal antibody (1 μg/ml) and 25 μl competitor monoclonal antibody (ascites form in serial dilutions) for 1 h at 37°C; (*4*) the peroxidase reaction performed with *o*-phenylendiamine according to Uotila et al (35) and measured with an enzyme-linked immunosorbent assay reader (Kontron SLT 210, Kontron Elektronik GmbH). The wash steps were as for the solid-phase radioimmunoassay described above.

Characterization of Monoclonal Antibodies against Microvillar Hydrolases of the Adult Human Small Intestine: Three independent fusions yielded 1,177 growing hybridoma cultures in total, 262 of which produced antibodies binding to human microvillus membranes as detected by the solid-phase radioimmunoassay. The immunization schedule appeared to influence the percentage of positive cultures, which increased from 4% (after 6 wk) to 42% (after 47 wk). The positive cultures were expanded to 100-mm dishes, and their ten-times concentrated media were screened for noninhibitory antibodies to various enzymes. Many cultures displayed more than a single activity. This can be ascribed in part to antibodies against disaccharidase enzyme complexes having two different active sites. Thus, cultures that exhibited both sucrase and isomaltase activities were indicative of antibodies against sucrase-isomaltase. Both subunits of this heterodimer also have maltase activities (1). Cultures that have both maltase and glucoamylase, but no sucrase, activities contained antibodies to maltase-glucoamylase. A total of 31 cultures (designated non-specific) were positive for virtually all of the enzyme activities tested. Further analysis of these media by immunoblotting using whole microvillus membranes showed immunoreaction with all the major high M_r proteins known to be glycoproteins (36, 37) including microvillar enzymes. We suspect that these cultures synthesized antibodies to carbohydrate antigens common to many microvillus glycoproteins. Indeed, it is well known that microvillar hydrolases of various species, including man, carry blood group antigens (5, 38). No specific antibodies were obtained against alkaline phospha-

[1] *Abbreviations used in this paper:* endo H, endoglycosidase H; PABA, *p*-aminobenzoic acid.

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TABLE I. *Properties of Selected Monoclonal Antibodies to Adult Human Microvillar Hydrolases*

Antibody code	Enzyme specificity	IgG class	Reaction on Western blots	Efficiency in immunoprecipitation*	
				HBB‡	Caco 2§
				%	
HBB 1/451/82	Sucrase-isomaltase	1	−	63	60
HBB 1/691/79	Sucrase-isomaltase	1	−	72	63
HBB 2/614/88	Sucrase-isomaltase	1	+	90	62
HBB 2/219/20	Sucrase-isomaltase	2b	−	95	93
HBB 3/705/60	Sucrase-isomaltase	1	+	1	5
HBB 2/143/17	Maltase-glucoamylase	1	−	10–30 (maltase)	—
HBB 2/962/94	Maltase-glucoamylase	1	−	10–30 (maltase)	—
HBB 1/909/34/74	Lactase-phlorizin hydrolase	2a	−	95	93
HBB 3/153/63	Aminopeptidase N	1	−	90	49
HBB 3/775/42	Dipeptidylpeptidase IV	1	−	96	98
HBB 3/264/18	Angiotensin I-converting enzyme	1	−	96	—
HBB 3/716/36	PABA peptide hydrolase	2a	−	90	—
HBB 3/850/164	Brush border	1	+	—	—

* The efficiency is defined as the maximum percentage of immunoprecipitable enzyme activity from Triton X-100–solubilized membranes in a single step with antibody in excess (see Materials and Methods).
‡ HBB, purified human small-intestinal brush border membranes.
§ Caco 2, Caco 2 "crude membrane" fraction.

tase, trehalase, aminopeptidase A, γ-glutamyltranspeptidase, carboxypeptidase, or endopeptidase, which might be due to their low abundance in the microvillus membrane.

Table I summarizes the features of 12 monoclonal antibodies against three disaccharidases and four peptidases which were obtained from selected hybridoma cultures after cloning. It is apparent that the five antibodies against sucrase-isomaltase precipitated the active enzyme with different efficiencies. Antibody HBB2/219/20 was most efficient, whereas HBB3/705/60 did not precipitate significant amounts of catalytically active enzyme. However, this latter antibody specifically precipitated inactive sucrase-isomaltase from Triton X-100–solubilized "aged" microvillus membranes that had lost 95% of their original sucrase activity due to prolonged storage at −20°C for 8 mo in diluted form. Furthermore, HBB3/705/60 was the only antibody which bound to its antigen under denaturing conditions of immunoprecipitation as used by Matsuura et al. (39; see Fig. 7). To establish whether the other four antibodies to sucrase-iosomaltase bound to the same or a different epitope of the native enzyme competitive enzyme-linked immunosorbent assay were carried out as described above. It was found that antibodies HBB 2/219/20 and HBB 1/451/82 were the only ones to compete substantially with each other. Competition was particularly prominent if HRP-HBB 1/451/82 was incubated together with unlabeled antibody HBB2/219/20 in excess, and in the inverse situation HRP-HBB 2/219/20 was only slightly displaced by HBB 1/451/82. This suggests that the former antibody has a higher affinity for sucrase-isomaltase than does the latter, which is in line with the different efficiencies of the two antibodies in immunoprecipitation (see above). Binding of these two antibodies was not inhibited by antibodies HBB 2/614/88 or HBB 1/691/79. Likewise, antibody HBB 2/614/88 did not compete with antibody HBB 1/691/79. These findings suggest that HBB 2/614/88, HBB 1/691/79, and HBB 2/219/20 probably recognize different epitopes on sucrase-isomaltase, whereas HBB 2/219/20 and HBB 1/451/82 bind to an identical or closely adjacent epitope.

The antibodies against maltase-glucoamylase, as expected, precipitated only a fraction of the total maltase activity (Table I). Thus, as in other mammals, in the human maltase-glucoamylase plays a minor role in the digestion of maltose. Immunoisolated maltase-glucoamylase could not hydrolyze isomaltose. All other antibodies very efficiently precipitated their corresponding antigen, as judged by enzyme activity measurements (Table I).

RESULTS

Characterization of Immunoisolated Microvillar Hydrolases by SDS PAGE

Starting with 100 μg adult jejunal microvillar protein per tube was sufficient for the visualization of all the antigens on Coomassie Blue–stained gels after immunoisolation, except for the PABA peptide hydrolase. Since the Coomassie Blue–stained gels did not allow the visualization of microvillar proteins in the M_r 50,000 to 70,000 range, due to the presence of IgG heavy chain and traces of albumin and its breakdown products, the antigens were also isolated after radioiodination and visualized by autoradiography. Fig. 1 demonstrates that all of the tested antibodies precipitated antigens of M_r >100,000. The M_r's of the immunoisolated antigens are listed in Table II. All five antibodies to sucrase-isomaltase yielded an identical pattern of three proteins two of which ran closely together (only one antibody is shown [Fig. 1, lane *2*]). The M_r 146,000 protein was positively identified as the sucrase subunit by NH_2-terminal sequencing and by comparison with the rat and rabbit sucrase (Sterchi, E., H. Wacker, and H.-P. Hauri, unpublished observation). We can conclude therefore that the M_r 151,000 protein is the isomaltase subunit and, in analogy to sucrase-isomaltase of the rat (40), that the M_r 231,000 is the common precursor (pro-sucrase-isomaltase) of both subunits. This latter assumption was confirmed by immunoblotting experiments. Fig. 2 shows that antibody HBB2/614/88 is specific for the sucrase subunit and HBB3/705/60 is specific for the isomaltase subunit. However, both antibodies also recognize the M_r 231,000 protein. The antibodies against maltase-glucoamylase precipitated a major protein of very high M_r (Fig. 1, lane *3*), which suggests that in the human intestine, in contrast to that of other mammals (41), the major form of maltase-glucoamylase is not cleaved into subunits or, less likely, is not dissociated under our SDS PAGE conditions. The antibody against lactase (Fig. 1, lane *4*) precipitated a major protein of M_r 156,000 which represented the subunits of this enzyme (7) and a minor M_r 262,000 protein which may be the precursor, pro-lactase (see below). The antibody against aminopeptidase N gave a major band of M_r 162,000 and occasionally a minor band in a high M_r range, which was more pronounced after radioiodination (Fig. 1). The latter band probably arose by aggregation, an assumption supported by biosynthesis studies described below. The antibodies against angiotensin I-converting enzyme, dipeptidylpeptidase IV, and the PABA peptide hydrolase gave a single band each (Fig. 1) with characteristic M_r's (Table II). It is particularly noteworthy that, for the first time, specific antibodies against human microvillar peptidases were obtained. The finding that activities of aminopeptidase N, dipeptidylpeptidase IV, angiotensin I-converting enzyme, and PABA peptide hydrolase

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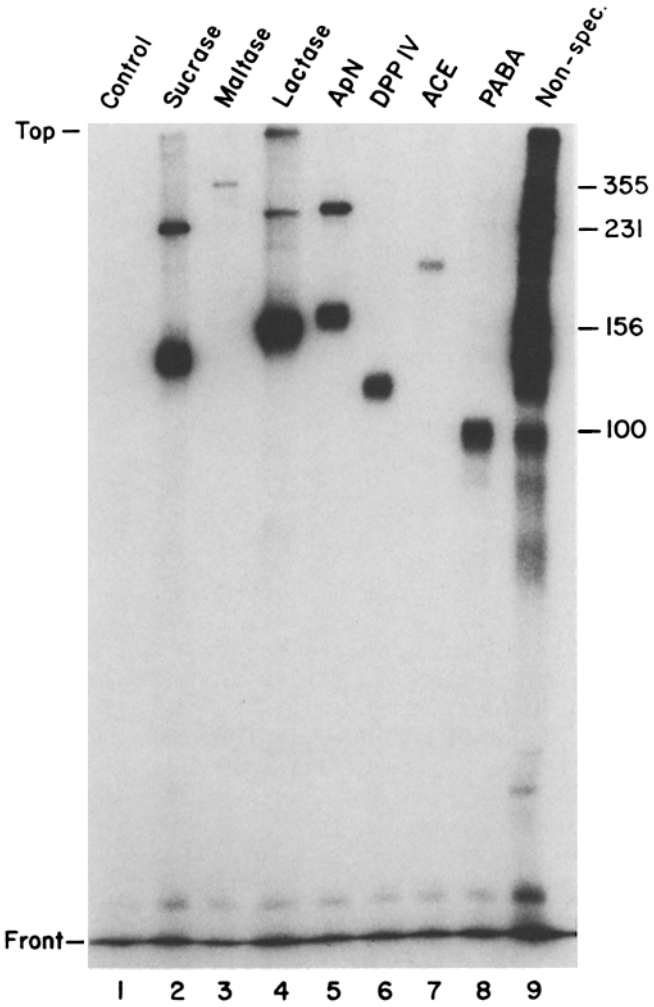

FIGURE 1 Identification of immunoisolated ^{125}I-labeled microvillar hydrolases from human jejunal microvillar membranes by SDS PAGE (autoradiogram). Triton X-100–solubilized microvillar proteins were iodinated by the lactoperoxidase method, and 2 × 10^6 cpm/tube were used for the immunoisolation of the individual hydrolases by monoclonal antibodies adsorbed to protein A-Sepharose. Lane *1*, monoclonal antibody PB10/205/44 against the major phenobarbital-inducible cytochrome P450 of rat liver (control; see reference 55); lane *2*, antibody HBB2/614/88; lane *3*, antibody HBB2/143/17; lane *4*, antibody HBB1/909/34/74; lane *5*, antibody HBB3/153/63; lane *6*, antibody HBB3/775/42; lane *7*, antibody HBB3/264/18; lane *8*, antibody HBB3/716/36; lane *9*, antibody HBB3/850/164. Molecular weights (in thousands) of maltase-glucoamylase, pro-sucrase-isomaltase, lactase subunits, and PABA peptide hydrolase are indicated as calculated from M_r marker proteins (Pharmacia high M_r marker kit). *ApN*, aminopeptidase N. *DPP IV*, dipeptidylpeptidase IV. *ACE*, angiotensin I-converting enzyme. *Non-spec.*, non-specific.

were each immunoprecipitable, in a quantitative manner, by individual monoclonal antibodies suggests that they are due to single, separate enzymes. Antibody HBB3/850/164, which showed high binding to microvillar membranes in the solid-phase radioimmunoassay, recognized a set of high M_r proteins, including all of the major hydrolases (Fig. 1, lane *9*). This antibody, designated anti–brush border, binds to an epitope that appears to be common to most high M_r microvillus membrane proteins.

TABLE II. *Apparent M_r Values (in Thousands) of Human Adult and Fetal (14 wk) Small Intestinal Hydrolases and of Hydrolases Expressed in Caco 2 Cells*

Enzyme	Adult	Fetal	Caco 2
Sucrase-isomaltase			
-pro-SI	231	217	217*
			210‡
-Isomaltase	151	NE	NE
-Sucrase	145	NE	NE
Maltase-glucoamylase	355	NE	NE
Lactase-phlorizin hydrolase			
-pro-LP	262	NE	214*
			200‡
-Lactase	156	NE	143
Aminopeptidase N	162	158	158*
			139‡
Dipeptidylpeptidase IV	136	124	124*
			114/110‡
Angiotensin I-converting enzyme	184	NT	NE
PABA peptide hydrolase	100	NT	NE

NT, not tested. NE, not expressed. pro-SI, pro-sucrase-isomaltase. pro-LP, pro-lactase-phlorizin hydrolase.
* endo H-resistant form.
‡ endo-H sensitive form(s).

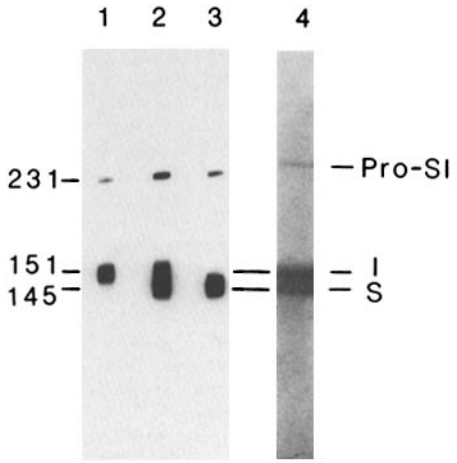

FIGURE 2 Subunit specificity of two antibodies to sucrase-isomaltase. Sucrase-isomaltase was immunoisolated from human jejunal microvillar membranes with antibody HBB2/219/20, separated by PAGE, and electrophoretically transfered to nitrocellulose. Nitrocellulose strips were either stained with amido black (lane *4*) or incubated with antibody HBB3/705/60 (lane *1*), antibody HBB2/614/88 (lane *3*), or an equal mixture of the two antibodies (lane *2*), and then rabbit anti-mouse immunoglobulin and ^{125}I-protein A were added. Note that antibody HBB3/705/60 labels the isomaltase subunit (*I*) and antibody HBB2/614/88 labels the sucrase subunit (*S*). Pro-SI, pro-sucrase-isomaltase. Molecular weights are indicated in thousands.

Immunolocalization of Microvillar Hydrolases in Jejunal Biopsies

Immunofluorescence studies with cryosections of human intestinal biopsies demonstrated that for all of the antibodies the microvillus border was the principal site of immunoreaction (Figs. 3 and 4). With antibody HBB3/153/63 no immunofluorescence was observed after fixation of the tissue with glutaraldehyde and paraformaldehyde. However, with paraformaldehyde alone the antigenicity was preserved and strong immunofluorescence was found associated with the microvil-

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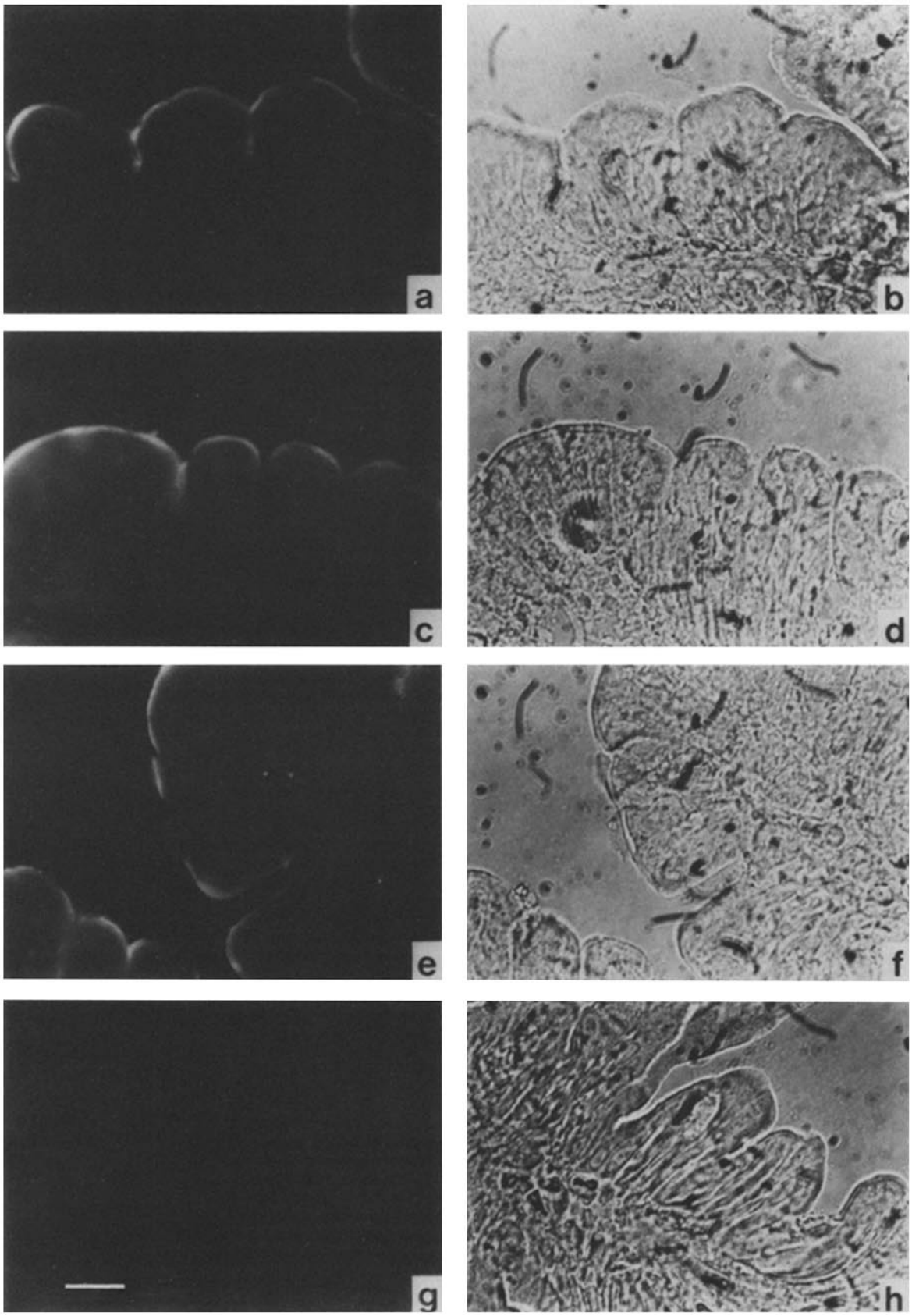

FIGURE 3 Immunofluorescence staining of human small-intestinal biopsies with monoclonal antibodies. Biopsies were fixed with 2% paraformaldehyde and 0.1% glutaraldehyde. After cryosections were incubated with monoclonal antibodies (ascites fluid 1:100), rhodamine-conjugated rabbit anti-mouse IgG, diluted 1:400, was added (*a*, *c*, *e*, *g*) or the cryosections were directly photographed (phase contrast, *b*, *d*, *f*, *h*). Antibodies used were (*a*) HBB2/614/88 against sucrase-isomaltase, (*c*) HBB2/143/17 against maltase-glucoamylase, (*e*) HBB1/909/34/74 against lactase-phlorizin hydrolase, (*g*) no antibody (control). Identical control fluorescence was obtained with a monoclonal antibody against the major phenobarbital-inducible cytochrome P450 of rat liver (reference 55) which does not react with any protein of human brush border membranes. Bar, 20 μm. × 500.

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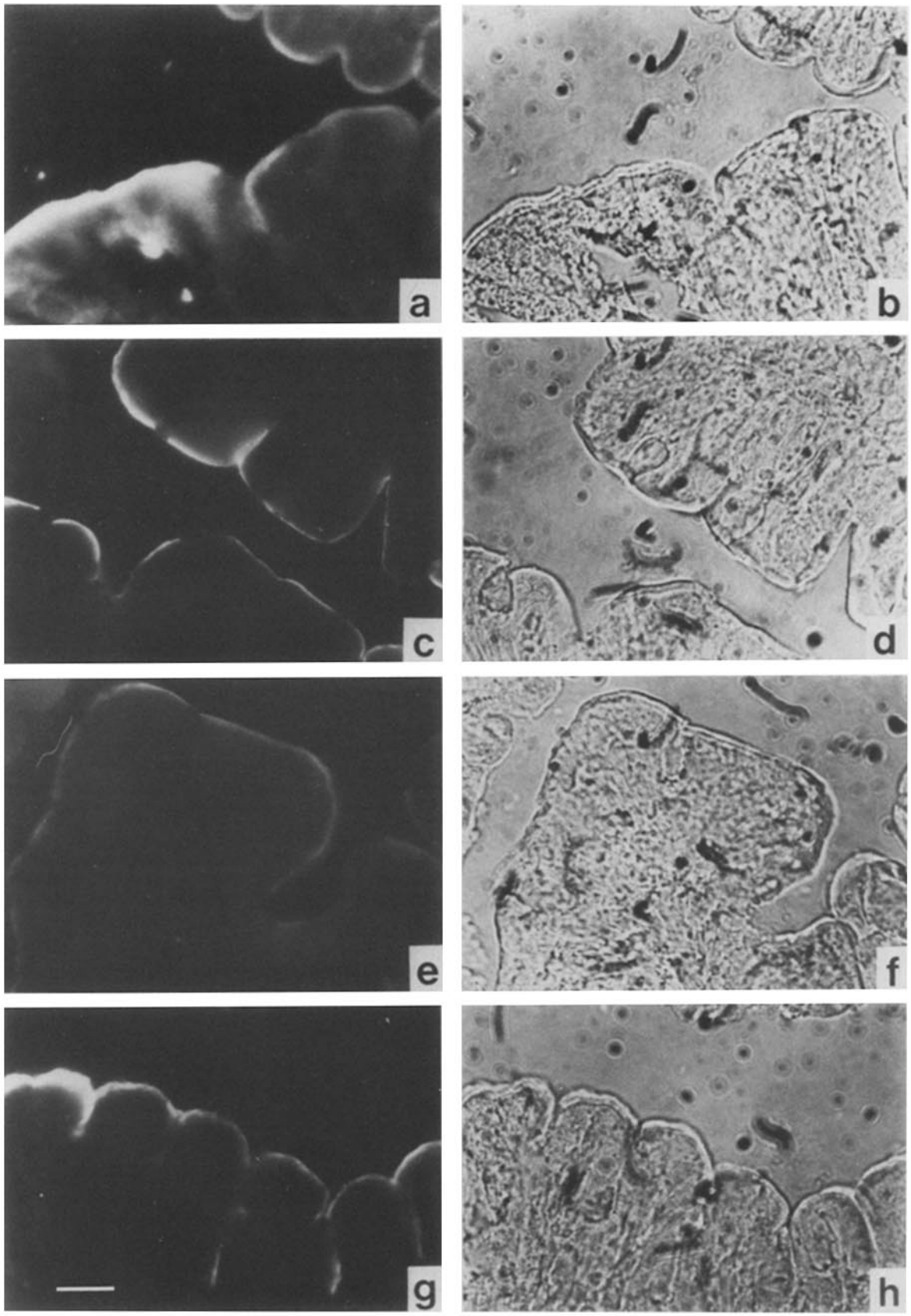

FIGURE 4 Immunofluorescence staining of human small-intestinal biopsies with monoclonal antibodies. Cryosections were immunolabeled as described in the legend to Fig. 5. Antibodies used were (a) HBB3/775/42 against dipeptidylpeptidase IV, (c) HBB3/716/36 against PABA peptide hydrolase, (e) HBB3/264/18 against angiotensine I-converting enzyme, (g) HBB3/850/164 against the brush border (non-specific). *b*, *d*, *f*, and *h* are corresponding phase-contrast pictures. Bar, 20 μm. × 500.

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lus border (not shown). An interesting finding was that the anti-brush border monoclonal antibody HBB3/850/164 showed a fluorescence pattern that was essentially indistinguishable from that obtained with the most reactive antibodies against individual hydrolases (Fig. 4, *g* and *h*). Most notable, the antibody appeared not to recognize either basolateral membrane proteins or goblet cell mucus, suggesting that it indeed reacted with a microvillus-specific determinant. Since this antibody precipitated virtually all of the high M_r proteins known to be glycoproteins, we suspect that the antibody is probably directed against a carbohydrate antigen.

Immunolocalization of Microvillar Hydrolases in Caco 2 Cells

It has been reported that Caco 2 cells express small-intestinal hydrolase activities (24). Using our monoclonal antibodies and the indirect immunofluorescence technique we found

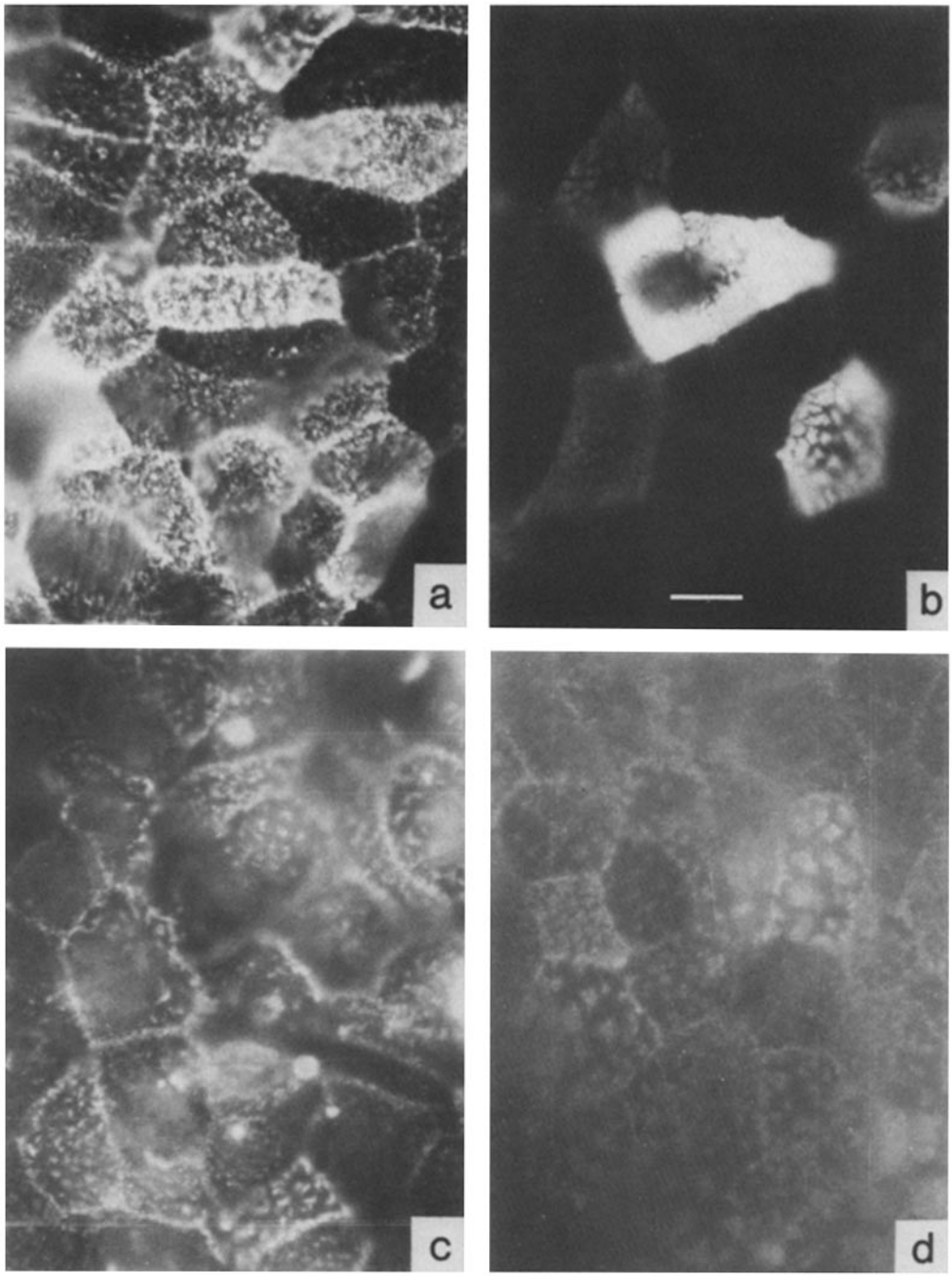

FIGURE 5 Immunofluorescence staining of Caco 2 cells with monoclonal antibodies. Caco 2 cells were cultured on coverslips and fixed with 2% paraformaldehyde 7 d after confluence. Immunolabeling was as described in Fig. 5. Antibodies used were (*a*) HBB2/614/88 against sucrase-isomaltase, (*b*) HBB1/909/34/74 against lactase-phlorizin hydrolase, (*c*) HBB3/775/42 against dipeptidylpeptidase IV, (*d*) HBB3/153/63 against aminopeptidase N. Although the amiopeptidase N panel had to be printed more softly than the others due to the weak immunofluorescence, the immunoreaction was clearly above the background of controls (not shown). Bar, 14 μm. × 714.

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that 5 to 9 d after confluence virtually all of the cells expressed sucrase-isomaltase (Fig. 5 *a*), dipeptidylpeptidase IV (Fig. 5 *c*), and aminopeptidase N (Fig. 5 *d*) at their luminal surface. A few cell clusters were also positive for lactase (Fig. 5 *b*), whereas no fluorescence was detectable with the antibodies against maltase-glucoamylase, angiotensin I-converting enzyme or PABA-peptide hydrolase. Surprisingly, the anti-brush border antibody HBB3/850/164 was also negative. The patterns of immunofluorescence were characteristic for the individual hydrolases. The antibody against dipeptidylpeptidase IV gave a punctuated surface fluorescence with little variation among the different cells of the monolayer. The antibody against aminopeptidase N essentially resulted in the same pattern, but the fluorescence was weak. Strong immunofluorescence was obtained with antibody HBB2/614/88 against sucrase-isomaltase. However, the intensity of fluorescence differed considerably among the individual cells of the monolayer. Immunofluorescence carried out with 1-μm cryosections of fixed and scraped monolayers confirmed the exclusive localization of the hydrolases on the luminal surface (not shown). The basolateral membrane was negative. The expression of the four hydrolases was confirmed by enzyme activity measurements in cell homogenates and in immunoprecipitates. The following enzyme activities (mU/mg protein, mean ± SD, n = 4) were found in Caco2 cell homogenates (67th passage) 7 to 9 d after confluence: sucrase, 38 ± 2; lactase, 10 ± 2; dipeptidylpeptidase IV, 1,105 ± 125; and aminopeptidase N, 312 ± 11. Since we intended to carry out biosynthetic studies it was important to compare the efficiencies of the different antibodies in precipitating the catalytically active Caco 2 enzymes with those for human jejunal enzymes. With two exceptions (HBB2/614/88 and HBB3/153/63) the efficiencies were comparable (Table I). An equal mixture of the five anti-sucrase-isomaltase antibodies precipitated almost 100% of sucrase activity.

Biosynthesis of Microvillar Hydrolases in Caco 2 Cells

In the first experiment the five monoclonal antibodies against sucrase-isomaltase were tested for their suitability to precipitate newly synthesized antigen. All of the antibodies recognized a single protein after a 15-min pulse with [^{35}S]-methionine at 37°C, but differences in their efficiencies were apparent (not shown). Although antibody HBB2/614/88 was the most efficient one, immunoprecipitation was still incomplete as compared with when an equal mixture of the five monoclonal antibodies was used. 3 h after continuous labeling four of the five antibodies also precipitated a slower-migrating protein that is the mature form of sucrase-isomaltase in Caco 2 cells (see below). Antibody HBB 3/705/60 could not recognize the complex glycosylated enzyme under these conditions (see Fig. 7).

When Caco 2 cells were labeled with [^{35}S]methionine for 15 min at 37°C and then chased with unlabeled methionine, sucrase-isomaltase was found to be synthesized as an endo H-sensitive M_r 210,000 protein, which was gradually converted to an endo H-resistant M_r 217,000 form (Fig. 6; see Table II for M_r's). Only the latter protein incorporated [^{3}H]fucose, and it is therefore the complex-glycosylated mature form. Both forms were immunoprecipitable by either the sucrase-specific (nondenaturing conditions) or the isomaltase-specific (denaturing conditions) antibody, which suggests that, as in the rat intestine, sucrase-isomaltase is synthesized as a single polypeptide that comprises both subunits (17). However, unlike in the normal gut, in Caco 2 cells no cleavage into subunits

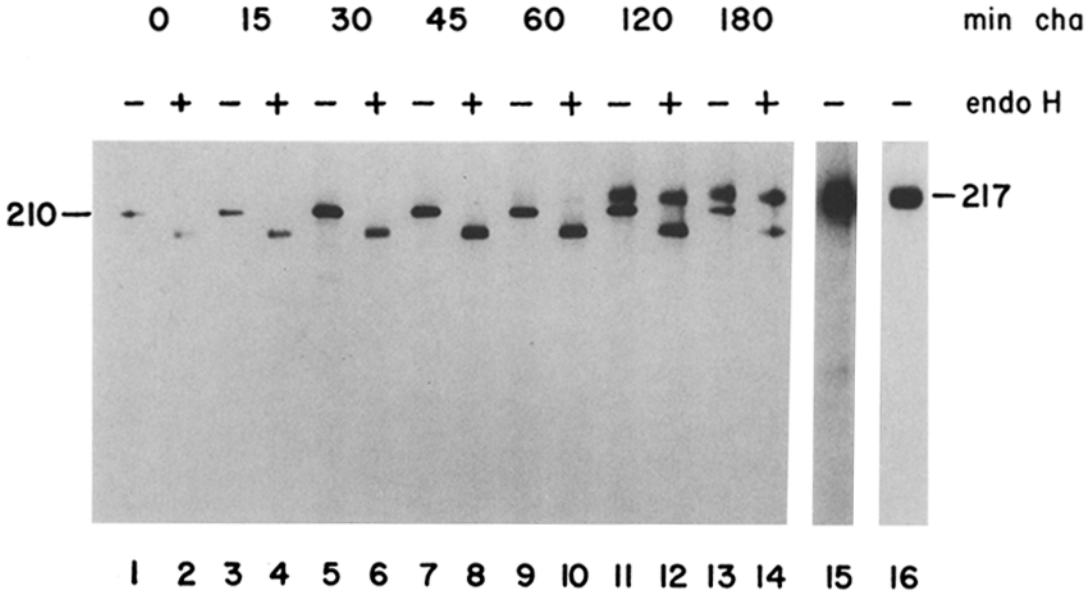

FIGURE 6 Pulse-chase experiment with sucrase-isomaltase (fluorogram). Caco 2 cells were labeled with [^{35}S]methionine for 15 min and then a chase was performed with complete medium containing unlabeled methionine in excess. After the indicated intervals sucrase-isomaltase was immunoprecipitated from a crude membrane fraction with an equal mixture of the five monoclonal antibodies to sucrase-isomaltase (Table II). Immunoprecipitates were subjected to SDS PAGE. In lanes *2*, *4*, *6*, *8*, *10*, *12*, and *14* the immunoprecipitates were treated with endo H before SDS PAGE. Lane *15*, the chase was extended to 20 h and the amount of radioactivity was threefold higher than in lanes *1–14*. Lane *16*, continuous 3-h labeling with [^{3}H]fucose. M_r's are indicated in thousands. Every lane represents the [^{35}S]methionine-labeled sucrase-isomaltase of a single 100-mm culture. The apparent differences in the intensities of the radioactivity bands are due to the variability of incorporation among the individual culture dishes.

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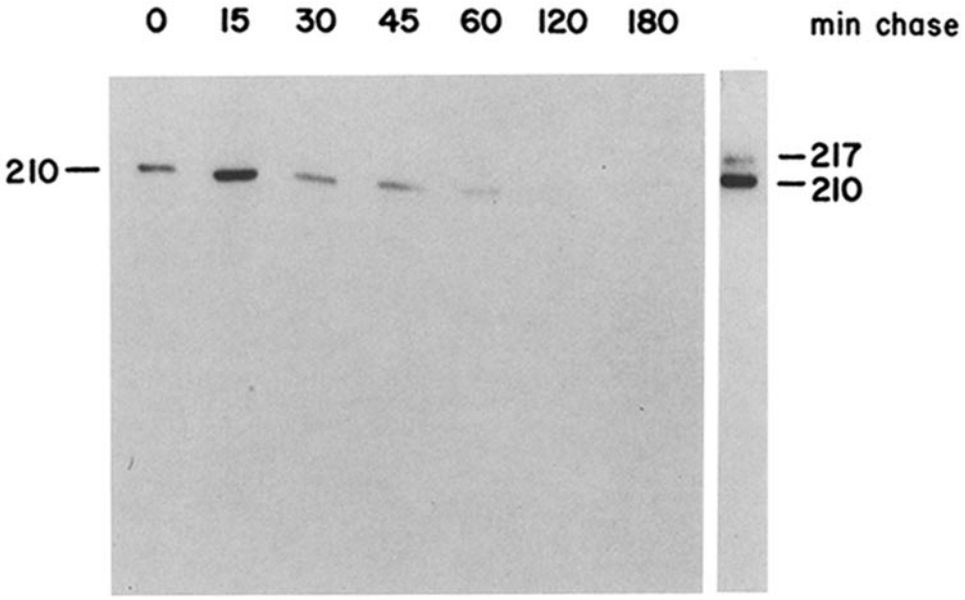

FIGURE 7 Pulse-chase experiment with sucrase-isomaltase (fluorogram). The experiment was performed as described in the legend to Fig. 6 with the exception that sucrase-isomaltase was immunoprecipitated with a single antibody (HBB3/705/60). Lane *8*, sucrase-isomaltase was immunoprecipitated under denaturing conditions (39) after continuous labeling with [^{35}S]methionine for 3 h.

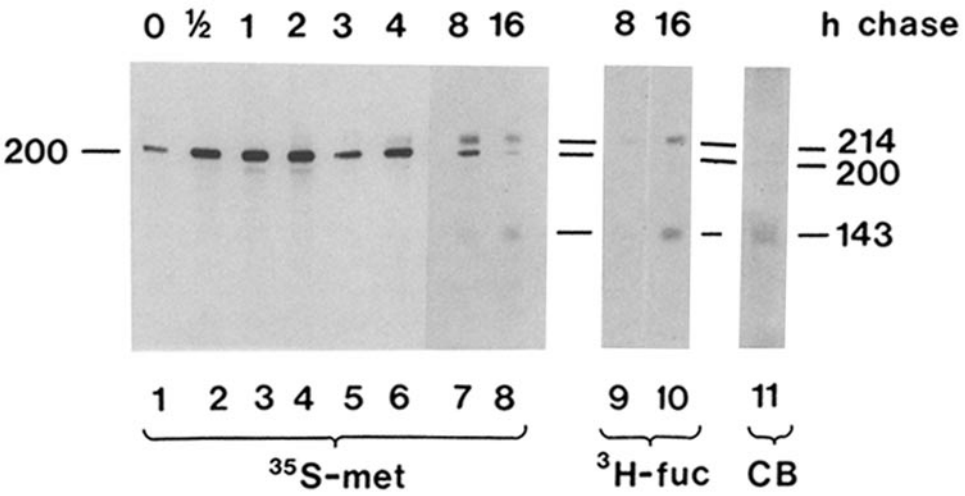

FIGURE 8 Pulse-chase experiment with lactase-phlorizin hydrolase. The experiment was carried out as for Fig. 6. Lactase-phlorizin hydrolase was immunoporecipitated with antibody HBB1/909/34 74. CB, Coomassie Blue–stained gel lane obtained after SDS PAGE of immunoisolated lactase-phlorizin hydrolase from five 100-mm culture dishes.

occurred even after a 20-h chase (Fig. 6, lane *15*). 120 to 150 min were required for conversion of half of the pulse-labeled enzyme to the endo H-resistant form. These kinetics were the same if the experiments were performed 1, 5, or 9 d after the cells had reached confluence. Antibody HBB3/705/60 was able to recognize the enzyme only up to 60 min under nondenaturing conditions (Fig. 7). However, since HBB3/705/60, under denaturing conditions, can also precipitate complex glycosylated sucrase-isomaltase (Fig. 7, lane *8*), we conclude that this antibody binds to an epitope that becomes inaccessible during maturation by a conformational change possibly induced by reglycosylation in the Golgi complex. Note that this change of conformation occurs ~60 min before acquisition of half-maximal endo H resistance. This interval may reflect the transit time of sucrase-isomaltase from the *cis*-face to the *trans*-face of the Golgi apparatus. Further experiments are, however, necessary to validate this interpretation.

Fig. 8 illustrates that lactase-phlorizin hydrolase is synthesized as a single M_r 200,000 protein which is very slowly converted into a M_r 214,000 form that appears only after a 4 to 8 h chase. This latter protein is the complex glycosylated lactase since it incorporated [^{3}H]fucose (Fig. 8, lane *10*). Surprisingly, the high-mannose form was still detectable after a 16-h chase (Fig. 8, lane *8*). At that time an additional fucose-incorporating protein of M_r 143,000 was also precipitated which co-migrated with the principal Coomassie Blue–stainable protein of the electrophoretically separated immunoprecipitates (Fig. *8*, lane 11). These results suggest that intracellular transport of newly synthesized lactase is extremely slow. Furthermore, they indicate that lactase is cleaved into subunits after acquisition of terminal sugars in the Golgi apparatus or later.

Pulse-labeled dipeptidylpeptidase IV displayed an endo H-sensitive protein doublet (M_r 114,000 and 110,000), the upper band of which was more pronounced and therefore might be the initial translation product (Fig. 9). The faster-migrating band may have been generated by proteolytic cleavage or carbohydrate trimming. The half-time for the conversion of the doublet into the endo H-resistant M_r 124,000 form was 20 to 25 min.

Aminopeptidase N was synthesized as a single M_r 139,000 protein, which was converted to the mature M_r 158,000 form with an apparent half-time of 30 to 45 min (Fig. 10). The

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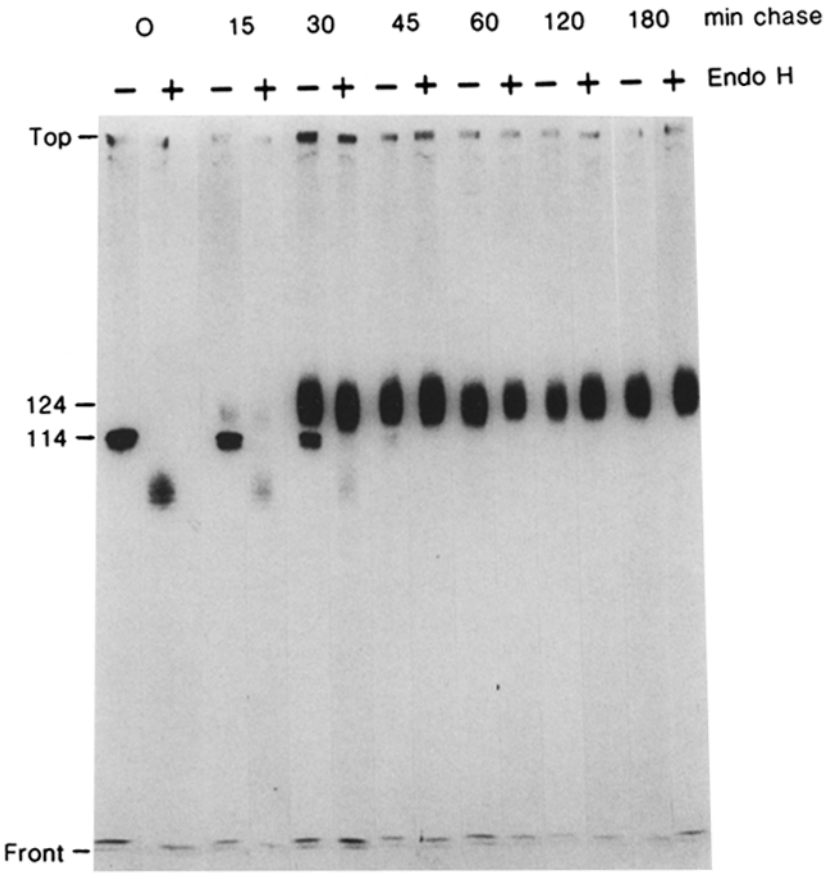

FIGURE 9 Pulse-chase experiment with dipeptidylpeptidase IV. The experiment was carried out as for in Fig. 6. Dipeptidylpeptidase IV was immunoprecipitated with antibody HBB3/775/42. The samples were run on the same gel slab but not in consecutive order, so for clarity they have been rearranged in the figure.

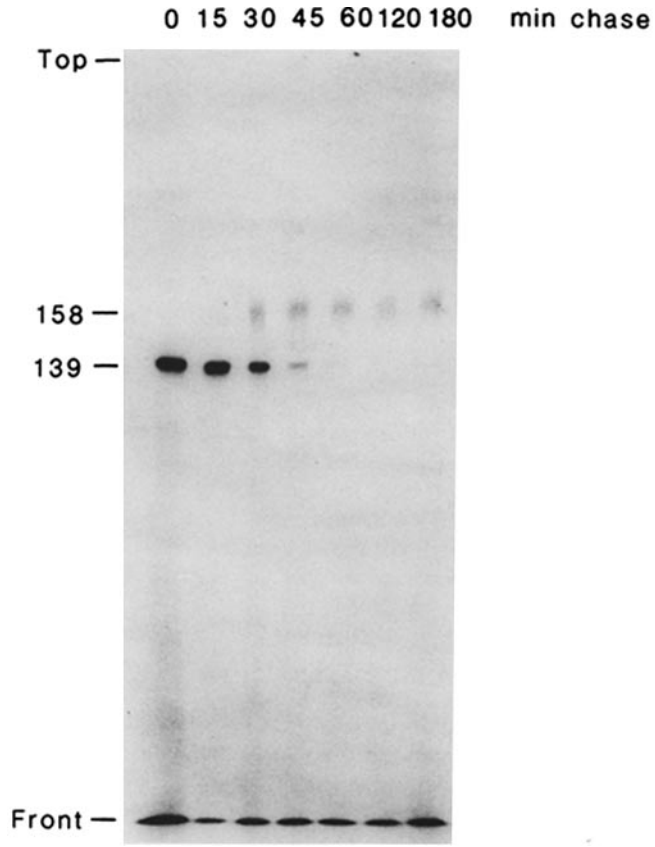

FIGURE 10 Pulse-chase experiment with aminopeptidase N. The experiment was carried out as for Fig. 6. Aminopeptidase N was immunoprecipitated with antibody HBB3/153/63.

actual time of conversion probably is somewhat shorter since only ~50% of the radioactivity initially incorporated into the high-mannose form was recovered in the mature form which is due to fact that the antibody precipitated the mature Caco 2 aminopeptidase with only ~50% efficiency (Table I).

A striking observation concerns the apparent molecular size of Caco 2 hydrolases being slightly smaller than that of the corresponding adult microvillar enzymes in all instances. A comparative study revealed that the Caco 2 hydrolases co-migrated with the fetal-type hydrolases (Table II). Since all of the monoclonal antibodies (except HBB3/850/164) reacted equally well with both the adult and the Caco2 hydrolases, we suspect that the different mobilities on gels might reflect differences of their carbohydrate moiety. It has been postulated that fetal hydrolases, unlike those of the adults, carry terminal sialic acid, leading to increased mobilities on SDS polyacrylamide gels (42). However, neuraminidase treatment could not completely abolish these differences, suggesting that additional carbohydrate structures are involved.

DISCUSSION

In this paper we describe the successful production of monoclonal antibodies against microvillus membrane vesicles prepared from the human small intestinal epithelium. These new probes served to identify and characterize seven digestive hydrolases and to study their expression in the differentiated human adenocarcinoma cell line Caco 2.

Characterization of Human Microvillar Hydrolases by Monoclonal Antibodies

Sucrase-isomaltase in the human, as in most other mam-

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malian species (41), was found to be composed of two subunits of unequal size, i.e., an apparently smaller sucrase and a larger isomaltase. Furthermore, a considerable proportion of the enzyme persisted in the noncleaved precursor form which had not been observed in a previous study on the purification of this enzyme (6). Presumably this was due to the lengthy isolation procedure, which included autolysis at 37°C. Previous studies on mammalian sucrase-isomaltase emphasized the similarities of the two subunits, which led to the hypothesis that sucrase-isomaltase has evolved from an ancestoral isomaltase by gene duplication (43). Although such a mechanism seems plausible, our subunit-specific monoclonal antibodies point to antigenic differences that appear to be quite extensive, as none of an additional eight monoclonal antibodies to this hydrolase recognized both subunits on Western blots (Hauri, H. P., unpublished observation). Quantitative immunoprecipitation of sucrase and isomaltase activities with a mixture of the five monoclonal antibodies demonstrated that no other enzyme is important in hydrolyzing these two substrates and the sucrase-isomaltase is responsible for up to 90% of microvillar maltase activity.

The principal molecular form of maltase-glucoamylase under fully denaturing conditions had an apparent M_r of 355,000, suggesting that this enzyme is not proteolytically modified in the microvillus membrane. In this respect it resembles the corresponding enzyme in pigs, which, at least in some preparations, appeared as a single high molecular weight protein (44). The extraordinarily high apparent M_r of human maltase-glucoamylase can be ascribed in part to extensive glycosylation (5). However, we cannot totally exclude the possibility that maltase-glucoamylase was not dissociated under our experimental condition. The immunoisolated enzyme was responsible for up to 30% of total microvillar maltase activity, but it did not have any isomaltase activity.

The immunoisolated lactase-phlorizin hydrolase displayed the same pattern and a similar M_r on SDS gels as that described by Skovbjerg et al. (7). The presence also of an M_r 262,000 protein might suggest that lactase-phlorizin hydrolase, which in Caco 2 cells and in mucosal organ culture is synthesized as a single polypeptide chain, may be cleaved into its two subunits in the microvillus membrane, just as sucrase-isomaltase is (40). However, our biosynthesis studies with Caco 2 cells suggest that the cleavage of lactase may be independent of the presence of pancreatic proteases and might already occur intracellularly, as Skovbjerg et al. proposed (45).

The present study shows that the human small intestine has at least four structurally different peptide hydrolases: aminopeptidase N, dipeptidylpeptidase IV, angiotensin I-converting enzyme (a carboxydipeptidase), and PABA peptide hydrolase. Human aminopeptidase N and dipeptylpeptidase IV closely resemble the corresponding hydrolases in other mammalian species that have similar apparent M_r's (41, 44). Isolation of the intestinal angiotensin I-converting enzyme, which splits the dipeptide histidyl-leucine from the COOH-terminal end of angiotensin I and other peptides, has not been reported previously. Our results show that the angiotensin I-converting enzyme of human jejunum has an apparent M_r of 184,000 and is a major microvillar enzyme. The high abundance, together with the sensitive fluorometric assay, used in the antibody screening may explain why 18 of 228 positive hybridoma cultures were found to produce antibodies against this enzyme. Angiontensin I-converting enzyme has been detected in various human and animal tissues by means of its enzymatic activity (46), and recently a proteolytic fragment of the enzyme was purified from kidney tissue (47). The PABA peptide hydrolase, a recently detected intestinal enzyme (31), has now been identified as a less abundant protein of apparent M_r 100,000. The natural substrate of this hydrolase is unknown. Recent organ culture experiments demonstrate that angiotensin I-converting enzyme and PABA peptide hydrolase are indeed synthesized by the human enterocytes and are not merely adsorbed pancreatic enzymes (Naim, H., and E. Sterchi, unpublished observation).

Expression and Intracellular Transport of Intestinal Microvillar Hydrolases in Caco 2

The present study shows that Caco 2 is a highly useful epithelial cell line with which to investigate the biosynthesis, intracellular transport, and polarized surface expression of at least four endogenous membrane proteins. Progress in our understanding of these processes has come mainly from studies using Madin-Darby canine kidney cells infected with enveloped viruses (48). However, it is important to know to what extent the viral model also applies to endogenous membrane proteins in particular since cytopathic effects that accompany infections might influence the polarized surface expression of viral glycoproteins.

Using our monoclonal antibodies against adult microvillar proteins it was possible to study the biogenesis and intracellular transport of four intestinal hydrolases in Caco 2 cells. The Caco 2 hydrolases show apparent mobilities on SDS gels identical to their fetal (Table II), but different from their adult, counterparts and were not recognized by the microvillus-specific monoclonal antibody HBB3/850/164. This antibody probably recognizes a carbohydrate epitope common to human adult microvillar glycoproteins, which suggests that Caco 2 cells may express fetal hydrolases.

Our pulse-chase experiments show that the individual Caco 2 hydrolases have different intracellular transport kinetics as probed by the acquisition of endo H resistance. The rate of transport from the rough endoplasmic reticulum to the *trans* side of the Golgi complex where endo H resistance is acquired (49) is at least four times lower for sucrase-isomaltase than for aminopeptidase N or dipeptidylpeptidase IV. The occasional appearance after a 60 to 90 min chase of a blur of radioactivity larger than the high-mannose but smaller than the fully glycosylated protein (not shown) suggests that both the transport from the endoplasmic reticulum to the Golgi apparatus and the passage through the Golgi apparatus were slower for sucrase-isomaltase than for the peptidases. This diffuse band is probably due to incompletely reglycosylated enzyme in transit through the Golgi apparatus. The intracellular transport of lactase was even slower than that of sucrase-isomaltase. Although the persistance of significant amounts of high-mannose lactase after a 16-h chase points to the possibility that some cells may synthesize this hydrolase without expressing it at the cell surface, recent immunofluorescence experiments with cryosections of fixed monolayers did not support this interpretation. The immunofluorescence was strictly confined to the luminal side of the cells (our unpublished observation), and no intracellular accumulation of lactase could be marshalled by this method. Furthermore, all the cells were found to express lactase in these experiments although to a highly variable degree.

Overall, our pulse-chase studies suggest that in Caco 2 cells

intracellular transport of newly synthesized microvillar hydrolases is a selective process. It is interesting to note that although the two peptidases were very efficiently transported to the Golgi apparatus, for the two disaccharidases this process was slow. Recent studies with organ-cultured human intestinal biopsies showed transport kinetics for these four enzymes that were very similar to those obtained with Caco 2 cells (Naim, H., E. Sterchi, and H.-P. Hauri, manuscript in preparation). Furthermore, in organ culture two additional peptidases, i.e., angiotensin I-converting enzyme and PABA peptide hydrolase, displayed fast transport kinetics, very much in contrast to the slow transport of the disaccharidases maltase-glucoamylase and sucrase-isomaltase. These results suggest a fast transport mechanism for peptidases and a slow one for disaccharidases. It is presently unknown if this asynchrony of transfer reflects two different transport receptor systems or different affinities of the two classes of hydrolases for a single system. The latter possibility is particularly intruiging when the anchoring to the lipid bilayer of sucrase-isomaltase and aminopeptidase N is compared. It has been shown that sucrase-isomaltase has both its NH_2- and COOH-termini on the luminal side of the microvillar membrane and probably spans the bilayer twice (50). In contrast, it was proposed that aminopeptidase N is a transmembrane protein with a COOH-terminus-out and NH_2-terminus-in orientation (51). Thus the short cytoplasmic segment of aminopeptidase N may mediate its efficient transport to the Golgi apparatus. Alternatively, the transport signals may be confined to the membrane anchor or the extracytoplasmic portion of the hydrolases.

It has recently been reported that also secretory proteins in hepatocytes (52) and hepatoma cells (53), as well as viral membrane proteins (54), also migrate from the endoplasmic reticulum to the Golgi complex at characteristic rates.

In conclusion, this study demonstrates the suitability of the monoclonal antibody approach to identifying integral proteins of the human intestinal microvillus membrane and studing their biosynthesis, intracellular transport, and sorting in Caco 2 cells.

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