Endocytosis, Recycling, and Lysosomal Delivery of Brush Border Hydrolases in Cultured Human Intestinal Epithelial Cells (Caco-2)*

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Lyosomes of intestinal epithelial cells in vivo and in culture display strong immunoreactivity with monoclonal antibodies against various brush border enzymes as visualized by immunoelectron microscopy. Novel subcellular fractionation procedures were developed to study, by the pulse-chase technique and by internalization assays, the pathway along which two microvillar hydrolases, sucrase-isomaltase and dipeptidylpeptidase IV, are transported to lysosomes in the differentiated colon adenocarcinoma cell line Caco-2. 7-9% of metabolically labeled sucrase-isomaltase of differentiated colon adenocarcinoma cell line Caco-2. 7-9% of metabolically labeled sucrase-isomaltase of differentiated colon adenocarcinoma cell line Caco-2. 7-8 h of chase as intact complex-glycosylated molecules. Appearance of these enzymes in lysosomes was biphasic. Endocytosis studies with radioiodinated anti-enzyme monoclonal antibodies (monovalent antigen-binding fragments) and by means of cell surface iodination revealed only slow transport of the enzymes to lysosomes at a low level. However, both enzymes were internalized with different efficiencies and recycled to the cell surface via endosomes. These results suggest that in Caco-2 cells a significant amount of newly synthesized sucrase-isomaltase and dipeptidylpeptidase IV is directly imported into lysosomes bypassing the brush border membrane.

Newly synthesized proteins that pass through the Golgi apparatus have been classified into three major groups characterized by their exit pathways from the trans-Golgi network, plasma membrane and constitutively secreted proteins, secretory proteins that are released from the cell in a regulated fashion, and lysosomal enzymes (Griffiths and Simons, 1986). In kidney epithelial cells, but not in hepatocytes (Bartles et al., 1987; Bartles and Hubbard, 1988), the first pathway is further subdivided into an apical and a basolateral route that split at the exit site of the Golgi apparatus (Matlin and Simons, 1983; Misek et al., 1984, Griffiths et al., 1985; Rindler et al., 1985; Wandering-Ness and Simons, 1988). It is possible that this general framework of trans-Golgi sorting needs to be modulated in order to accommodate multiple localization patterns observed for a number of different proteins including the cell surface localization of the mannose 6-phosphate receptor (Geuze et al., 1984, 1985; Griffiths et al., 1988) and of a lysosomal membrane glycoprotein (Lippincoott-Schwartz and Fambrough, 1987) as well as the brush border localization of lysosomal acid α-glucosidase (Fransen et al., 1988). Presently, it is unclear if these localizations are due to missorting of a subset of a given protein or whether there is a dynamic equilibrium between cell surface and lysosomes as proposed by various authors (Kornfeld, 1987; Lippincoott-Schwartz et al., 1987).

An interesting case of unexpected protein localization concerns the apparent presence of intestinal brush border enzymes in lysosomes of intestinal epithelial cells as visualized by immunoelectron microscopy (Fransen et al., 1985; Sips et al., 1985; Lorenzsonn et al., 1987; Fransen et al., 1989). To date at least two brush border enzymes, sucrase-isomaltase and dipeptidylpeptidase IV (DPPIV), have been localized in this organelle. The lysosomal immunoreactivity is not due to carbohydrate epitopes, which may be common to nonrelated glycoproteins, since most studies were performed with well-characterized protein epitope-specific mAbs. On the basis of rather indirect evidence it has been speculated that a fraction of newly synthesized brush border enzymes may be directly imported into lysosomes from the Golgi apparatus (Blok et al., 1984).

In the present study we have addressed the question of whether brush border enzymes in intestinal lysosomes originate from the brush border membrane and are transported to this organelle by means of endocytosis, or whether they have bypassed the apical membrane on their way to lysosomes. We approached this question by studying the trafficking of brush border sucrase-isomaltase, a representative disaccharidase, and DPPIV, a representative peptidase, in the differentiated colon adenocarcinoma cell line Caco-2 (Pinto et al., 1983; Zweibaum et al., 1988).

Disaccharidases and peptidases are the major glycoproteins of the small intestinal brush border membrane (Kenny and Maroux, 1982; Semenza, 1986; Noren et al., 1986; Hauri, 1985) some of which are expressed in Caco-2 cells (Zweibaum et al., 1988; Hauri, 1988). In these cells the biogenesis and intracellular transport of sucrase-isomaltase and DPPIV have been studied in detail. The two enzymes were found to mature at different rates (Hauri et al., 1985a). This asynchronism is due to at least two rate-limiting steps along the rough endoplasmic reticulum to trans-Golgi pathway as established by subcellular fractionation (Stieger et al., 1988). To study the transport of brush border enzymes to lysosomes, we have now developed an isolation procedure for lysosomes of Caco-2 cells. Using

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1 The abbreviations used are: DPPIV, dipeptidylpeptidase IV; Fab, monovalent antigen-binding fragment; NHS-SS-Biotin, sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; sulfo-SHPP, sulfoalkylated biotinyl-200; PBS, phosphate-buffered saline.
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This method in conjunction with metabolic and cell surface labeling experiments, we provide evidence that a significant amount of newly synthesized sucrase-isomaltase and DPPIV is directly targeted to lysosomes and bypasses the brush border membrane. Furthermore, evidence is presented for endocytosis and recycling of brush border hydrolases.

MATERIALS AND METHODS

Cell Culture and Labeling with \[^{35}S\]Methionine—Caco-2 cells, kindly provided by Dr. A. Zweibaum (Paris), were grown in Optilux Petri dishes as described (Pinto et al., 1985; Hauri et al., 1985a) or on Mini-Marbrook filters in medium Optilux (Stieger et al., 1988). Labeling with \[^{35}S\]magnesium was performed with filter-grown cells 5–15 days after confluence (Stieger et al., 1988). In all pulse-chase experiments a labeling time of 30 min was used. All the experiments were carried out with Caco-2 cells of passage 137 to 150.

Antibodies, Immunooisolation of Antigens, and SDS-PAGE—Details of the features of mAbs and of the immunooisolation procedure are given in Hauri et al. (1985a). All the antibodies used in this study are specific for their corresponding antigen. They are directed against protein epitopes and do not recognize any carbohydrate antigens. None of the mAbs against sucrase-isomaltase cross-reacts with lysosomal α-glucosidase that has been shown by cDNA cloning to share homologous sequences with sucrase-isomaltase (Hauri et al., 1988). Sucrase-isomaltase was precipitated with a mixture of the four mAbs HBB2/614, HBB3/705, HBB3/210, and HBB1/601 directed against different epitopes, and DPPIV was precipitated with antibody HBB3/775 and HBB3/456. For the immunogold-labeling of cytoskeleton prepared with the lysosomal fraction LII mAb HBB3/ 775 against DPPIV and mAb 4G8 against lysosomal form of acidic α-glucosidase (Hilkens et al., 1981) kindly provided by Prof. J. M. Tager, Amsterdam, The Netherlands) were used. In controls for the cell surface labeling experiments, three other antibodies were used. Non-specific binding was tested with \[^{125}I\]-Fabs prepared from CPI/126, a mAb directed against an antigen of the rat colon brush border membrane (Gorr et al., 1988). This mAb does not recognize any protein in Caco-2 cells. Furthermore, antibody G1/139 against a lysosomal membrane glycoprotein (Schweizer et al., 1988), and HBB3/456 a new mAb against DPPIV were used. The latter antibody was derived from the same fusion as HBB3/775 and was found to precipitate 52% of the DPPIV activity of solubilized Caco-2 membranes (using glycyl-L-proline-p-nitroanilide-p-tosylate as substrate). The antigen recognized by HBB3/456 has the same molecular weight as that of the HBB3/775 antigen. SDS-PAGE was performed as described (Hauri et al., 1985a) using 7.5%-gels. \[^{35}S\]Methionine-labeled proteins were visualized by fluorography using En3Hance (Du Pont-New England Nuclear). Bands on fluorograms were quantified by means of a Camag LTC Scanner II connected on a Camag SP4290 integrator.

Isolation of a Fraction Enriched in Lysosomal Membranes—All the steps of the isolation procedure were carried out at 4 °C unless indicated otherwise. Caco-2 cell monolayers were washed once with PBS containing 100 mM potassium phosphate, pH 7.4, containing 250 mM sucrose, 10 mM triethanolamine-acetate, pH 7.4 (buffer A) with a clearance of 20 μm. The resulting homogenates were resuspended and centrifuged for 10 min at 2,000 rpm (370 × g), in an SS34 rotor (Servall Instruments Division). The supernatant was brought to exactly 30 ml with buffer B, and 4.66 ml stock isoosmotic Percoll were added as carriers. To this was added 3 μl of a solution of 700 μg/ml of HBB3/775, HBB2/614, and CPI/126 were produced by papain digestion (Mage, 1980). In brief, the immunoglobulin Gs (4 mg/ml) were preincubated at 37 °C in PBS containing 2 mM EDTA and 25 mM β-mercaptoethanol. After 10 min papain (Merck, Darmstadt, Germany) was added to a final concentration of 40 μg/ml. The digestion was stopped after 1 h at 37 °C by the addition of iodoacetamide (30 mM final concentration), and the sample was dialyzed against 10 mM NaH2PO4, pH 7.6. DPPIV fragments were precipitated with DEAE-52 column chromatography using 0.1 M NaH2PO4, pH 7.6. Purity of the Fab fragment was tested by SDS-PAGE (10% gels) that were stained with silver (Merril et al., 1984). After dialysis against PBS the fragments were stored at −20 °C.

Fab fragments were iodinated by the chloramine-T method (Hunter and Greenwood, 1962) for the labeling of 5 μg of protein 0.5 mCi Na[125I] was used, and free iodine was removed by gel filtration on Sephadex G25. The labeled fragments were stored at 4 °C and used within 2 weeks.

Cell Surface Labeling with \[^{125}I\]F Fab Fragments—Mini-Marbrook chambers containing the filter-grown Caco-2 cells were transferred to 6-well plates and labeled at 4 °C as follows. The cells were rinsed once in PBS containing 0.1% bovine serum albumin and were incubated in the same buffer. After 10 min the chambers were transferred to a fresh 6-well plate containing Caco-2 medium. The \[^{125}I\]F Fab fragments (5 × 10^6 cpm in 350 μl of medium) were added to the apical chamber containing the filter-grown Caco-2 cells transferred to 6-well plates and labeled at 4 °C as follows. The cells were rinsed once in PBS containing 0.1% bovine serum albumin and were incubated in the same buffer. After 10 min the chambers were transferred to a fresh 6-well plate containing Caco-2 medium. The \[^{125}I\]F Fab fragments (5 × 10^6 cpm in 350 μl of medium) were added to the apical medium. After 1 h the chamber was rinsed twice with PBS, disassembled, and washed three times for 3 min each with PBS containing 0.1% bovine serum albumin. Depending on the experiment the labeled cells were either harvested directly or incubated in Caco-2 medium for 24 h at 37 °C. In some experiments the cell surface label was removed by a low pH treatment. For this purpose the cells were rinsed twice with 100 mM glycine, 250 mM succrose, pH 2.0, followed by three 20-min incubations in the same buffer. By this procedure more than 98% of cell surface radioactivity was removable from cells that were labeled and acid-treated at 4 °C. Thereafter, the cells were washed once more with the acidic buffer and twice with PBS. After incubating the cells twice in PBS for 10 min, they were harvested in buffer A for subcellular fractionation or in PBS for direct determination of radioactivity in a γ-counter (GAMMAmatic, Kontron Elektronik GmbH, Zurich).

Cell Surface Labeling—Sufo-SHPP (sulfosuccinimidyl (hydroxypyrrolidin-2-ypropionate; Pierce, The Netherlands), a membrane impermeable form of the Bolton-Hunter reagent (Thompson et al., 1987) was used for cell surface iodination. Iodination of the reagent and the labeling of the cell surface was done according to Borel and Lutter (1988). Briefly, to 0.5 μg of H2O2 and 5 μl of Na[125I] (0.5 mCi) was added 1 μl of 3 M NaCl, 2 μl of 1 M K3HPO4, pH 7.0, 1.5 μl of 0.1 M sodium acetate (1 mg/ml chloramine T and this mix was kept on ice for 15 min. To this was added 3 μl of a solution of 700 μm hydroxyphenylacetic acid, pH 7.0, containing 0.1 M NaCl. After this mix on ice the labeled reagent was diluted with 50 μl of 10 mM tris(hydroxymethyl)aminomethane/HC1, pH 7.4, containing 250 mM sucrose and 2 mM CaCl2.

Before labeling, the cells grown in mini-Marbrook chambers were washed twice with PBS for 5 min each. Then the above described diluted reagent mixture was transferred to the inside of the mini-Marbrook chamber that was placed in a 6-well plate containing the same buffer as used for diluting the labeled reagent. After 30 min on ice, the cells were rinsed three times for 5 min with...
lysosomes, we developed an isolation procedure for Caco-2 DPPIV and to study the transport of these hydrolases to lysosomes indeed due to intact polypeptides of sucrase-isomaltase and DPPIV. In order to investigate whether these cytochemical signals are small-intestinal enterocytes that lysosomes of Caco-2 cells may harbor sucrase-isomaltase and DPPIV (Fransen et al., 1985) was dissolved in 7.25 ml of water and 1 ml of 10-times concentrated TBS. Immediately before use 750 ml of 1 N NaOH and then 1 ml of 10% BSA were added to the dissolved glutathione. The 6-well plate was placed on a horizontal shaker for 20 min at 4 °C. Then the cells were transferred to fresh glutathione solution and shaken for an additional 20 min. This step was repeated once, and the cells were rinsed twice in PBS containing 0.1% BSA and twice in Caco-2 medium. Cell surface biotin was detected by the use of streptavidin (Pierce, The Netherlands) which was iodinated as described for Fab fragments. The cells were labeled at 4 °C for 1 h. After 1 h the cells were washed and harvested as described for the 125I-Fab experiments. 125I-streptavidin was not limiting in this assay.

Enzyme Assays—The following marker enzymes were used: alkaline phosphatase (measured according to Stieger et al., 1986) for the brush border membrane, glucosaminidase (measured according to Scalera et al., 1980) for lysosomes, galactosyltransferase (measured according to Venable and Coggeshall, 1965; Stieger et al., 1988) for trans-Golgi (see Schweizer et al., 1986 for the localization of this enzyme in Caco-2 cells). K+-stimulated p-nitrophenylphosphatase (measured according to Stieger et al., 1986 using buffer I and III) for basolateral membranes, KCN-resistant NADH oxidoreductase (measured according to Scotta et al., 1967) for the endoplasmic reticulum, and horseradish peroxidase for endosomes (measured according to Marsh et al., 1987). Caco-2 cells were incubated with horseradish peroxidase, type II (Sigma, 10 mg/ml in Caco-2 medium) for 15 min at 37 °C after which time the cells were washed extensively with ice-cold PBS before harvesting. Protein was determined with the Bio-Rad protein assay kit using protein standard I (Bio-Rad).

Immunoelectron Microscopy—To a sample of the lysosomal fraction an equal volume of a solution of 2% formaldehyde, 0.2% glutaraldehyde, and 0.1 M sodium bicarbonate (23 g/liter) was added. After 30 min at room temperature, the fixed membranes were centrifuged for 45 min at 105,000 g in a T7T 75.13 rotor (Kontron Elektronik GmbH, Zurich). The pellet was resuspended in 100 mM phosphate buffer, pH 7.4, containing 1% formaldehyde. After 5 min on ice, the sample was centrifuged for 5 min at 15,000 g. The resulting pellet was resuspended at 37°C in 10% gelatin dissolved in PBS and immediately centrifuged as above. The supernatant was removed and the sample was postfixed with 1% paraformaldehyde in 100 mM phosphate buffer. Cryosectioning and immunogold-labeling was done as described (Fransen et al., 1985; Schweizer et al., 1988).

RESULTS

Appearance of Metabolically Labeled Sucrase-Isomaltase and DPPIV in Lysosomes—Recent immunocytochemical labeling studies at the ultrastructural level with mAbs have suggested that lysosomes of Caco-2 cells may harbor sucrase-isomaltase and DPPIV (Fransen et al., 1988b) analogous to lysosomes of small-intestinal enterocytes in vivo (Fransen et al., 1985; Lorenzsonn et al., 1987; Hauri et al., 1985b; Sips et al., 1985). In order to investigate whether these cytochemical signals are indeed due to intact polypeptides of sucrase-isomaltase and DPPIV and to study the transport of these hydrolases to lysosomes, we developed an isolation procedure for Caco-2 lysosomes (Fig. 1). Up to the Percoll gradient centrifugation, this method is identical to the preparation of Golgi-derived membranes (Stieger et al., 1988). Percoll gradient centrifugation already led to a good separation of lysosomes from membranes derived from other organelles as deduced from marker enzyme measurements (not shown). To achieve a higher purity of lysosomes the bottom fraction of the Percoll gradient was fractionated on a Metrizamide gradient (Fig. 1).

Yield and recovery of marker enzymes in a fraction enriched in lysosomal membranes (LII)

The values are given as percentage of the amount determined in the homogenate.

Enzyme Assays—The values are given as mU/mg protein.
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analysis of the lysosomal fraction revealed dense organelles resembling lysosomes (Fig. 2). The lysosomal origin of these organelles was confirmed by immunoelectron microscopy using a mAb against the lysosomal enzyme α-glucosidase (Fig. 2A). This antibody is specific for the intermediate and mature forms of α-glucosidase but does not react with the 110-kDa precursor form (Fransen et al., 1988). The lysosome-like structures also showed immunoreactivity with a mAb against DPPIV (Fig. 2B) and a mAb against sucrase-isomaltase (not shown). The signal with the latter antibody was weak but clearly above background.

A pulse-chase protocol was used to study the arrival in the lysosomal fraction of newly synthesized sucrase-isomaltase and DPPIV. A typical fluorograph of such an experiment is given in Fig. 3 which shows that the molecular mass of the hydrolases in lysosomes is indistinguishable from the complex-glycosylated enzyme forms of the homogenate suggesting that the visible polypeptides represent intact enzymes. The fact that no degradation products appeared on the gels may be due to an inability of our mAbs to recognize proteolytic fragments of the enzymes. The kinetics of appearance are drawn in Fig. 4. The kinetic behavior of the two digestive hydrolases is relatively similar during the first 10 h of chase. DPPIV showed a peak after 7 h and sucrase-isomaltase after 8 h. This delayed appearance is likely to be due to the asynchronous transport of the two enzymes to and through the Golgi apparatus (Stieger et al., 1988). The maximal fraction of newly synthesized enzyme in lysosomes was about 9% for sucrase-isomaltase and 7% for DPPIV (values are corrected for the yield of the fraction). It is unlikely that this result is due to cross-contamination of the lysosomal fraction with membranes of other organelles for two reasons. First, the enzymatic data of the fraction LII (Tables I and III) do not indicate such cross-contamination problems and second, the kinetics of appearance of DPPIV and sucrase-isomaltase in lysosomes is strikingly different from that in other organelles including the Golgi apparatus and the brush border membrane. (Stieger et al., 1988).

The finding that both enzymes arrive in lysosomes later than in the brush border membrane would be compatible with a mechanism whereby the proteins are first exported to the cell surface followed by endocytosis. Interestingly, the profiles of metabolically labeled sucrase-isomaltase and DPPIV in LII are biphasic, in particular for DPPIV, suggesting that the hydrolases enter lysosomes in two waves. In subsequent experiments we tested the assumption that the first wave reflects a pathway which bypasses the brush border membrane and that the second wave originates from endocytosis.

FIG. 2. Electron micrographs of the lysosomal fraction. Cryosections of the lysosomal fraction LII were labeled either with a mAb against lysosomal acid α-glucosidase (A), or with mAb HBB3/775 against DPPIV (B). Bar, 0.24 μm.

FIG. 3. Appearance of newly synthesized DPPIV and sucrase-isomaltase (SI) in the lysosomal fraction of Caco-2 cells. The cells were pulse-labeled with [35S]methionine for 30 min and fractionated after different time intervals of chase. Sucrase-isomaltase and DPPIV were immunoprecipitated from the solubilized lysosomal fraction LII and from an aliquot of the homogenate (H). Immunoprecipitates were analyzed by SDS-PAGE followed by fluorography.

FIG. 4. Arrival of newly synthesized DPPIV (x) and sucrase-isomaltase (o) in the lysosomal fraction deduced from pulse-chase experiments (quantification of fluorograms). The highest amount of radioactivity in either DPPIV or sucrase-isomaltase in the lysosomal fraction relative to the amount of radioactivity in the enzymes in the corresponding homogenate was set to 100%.
Endocytosis of Sucrase-Isomaltase and DPPIV—Assuming that all of the newly synthesized sucrase-isomaltase or DPPIV would first be inserted into the brush border and subsequently internalized to lysosomes one would expect the maximal appearance in lysosomes of cell surface-labeled brush border enzymes (that is the maximal lysosomal radioactivity relative to the homogenate) to be in the same order of magnitude as that found after metabolic labeling with [35S]methionine. To study a possible endocytosis of brush border enzymes, we established a cell surface labeling assay. In this assay Caco-2 cells were labeled at 4°C with 125I-Fab fragments of anti-enzyme mAbs. After removal of the unbound antibodies the cells were returned to the 37°C incubator and the time-dependent uptake of radioactivity into the cell was measured.

The use of Fab fragments instead of the divalent intact immunoglobulin G is important since cross-linking of brush border enzymes by divalent antibodies may induce endocytosis (Louvard et al., 1980). The availability of a highly specific cell surface labeling assay is crucial for endocytosis experiments. Additional labeling of unrelated antigens that are static in the brush border membrane may mask a low rate of endocytosis or, in the opposite case, nonspecifically labeled components, if efficiently endocytosed, may lead to an artificially high apparent rate of endocytosis. In a first control experiment, Caco-2 cells were separately labeled with the Fab fragments of HBB2/614 against sucrase-isomaltase, HBB3/775 against DPPIV and CP1/126 against an antigen of rat proximal colon (Gorr et al., 1988). The latter antibody does not react with any antigen of Caco-2 cells and was used as a negative control. The incubations with anti-sucrase-isomaltase-Fab or anti-DPPIV-Fab led to a high cell-associated radioactivity whereas the control antibody produced a signal only minimally above the background of the y-counter (data not shown). This indicates high specific binding of the anti-hydrolase antibodies. However, different mAbs may respond differently to the limited proteolysis conditions of the Fab preparation, i.e. some may become sticky due to partial denaturation, and hence the above result may simply reflect different degrees of stickiness. To rule out this possibility we performed competition experiments. The cells were incubated with either IgG HBB2/614 or HBB3/775 in caeco, or without antibody, before labeling with either Fab HBB2/614 (Fig. 5A) or Fab HBB3/775 (Fig. 5B). The results show that the binding of an 125I-Fab can only be inhibited by its corresponding intact IgG confirming the specificity of the cell surface binding assay.

The total rate of endocytosis was measured in experiments that are analogous to a metabolic pulse-chase protocol. The cells were labeled with 125I-Fab fragments at 4°C and were then cultured at 37°C for different time intervals. Thereafter, the cells were cooled down to 4°C and the Fab fragments specifically bound to the cell surface were removed by a low pH treatment. In this assay, acid-resistant radioactivity reflects internalized enzyme. The results of these experiments showed internalization of DPPIV and sucrase-isomaltase (Fig. 6). The shape of the two curves are quite similar and level off after 60-90 min at 37°C. Endocytosis of DPPIV reaches a plateau after 60 min. It is worth noting, however, that the magnitude of endocytosed radioactivity was different for the two enzymes. While the plateau for DPPIV was at 18% of total radioactively labeled DPPIV, the endocytosis of sucrase-isomaltase did not exceed 2% of totally labeled sucrase-isomaltase even after 6 h at 37°C. The leveling off of the curves indicates that, if at all, only a small fraction of endocytosed sucrase-isomaltase and DPPIV are transported all the way to the lysosomes. Rather both enzymes appear to recycle to the cell surface (see below).

Delivery of Hydrolases from Brush Border to Lysosomes—To measure directly the transport of sucrase-isomaltase and DPPIV from the plasma membrane to lysosomes the cell surface labeling approach was combined with subcellular fractionation. Cell surface sucrase-isomaltase or DPPIV were labeled with 125I-Fab at 4°C and after various time intervals at 37°C the cells were subjected to the isolation of lysosomes. These experiments showed a low rate of enzyme transport to lysosomes (Fig. 7) which was 1-2% after 3 h and 2.5-4.5% after 18 h (values are corrected for the yield of the preparation). Since metabolically labeled sucrase-isomaltase and DPPIV need about 7-8 h for their maximal appearance in the lysosomal fraction (Fig. 4) and 5 h (sucrase-isomaltase) or 3 h (DPPIV) for maximal appearance in the brush border (Stieger et al., 1988) the important time window of the endocytosis experiment is about 3 h. At that time the percentage of endocytosed DPPIV and sucrase-isomaltase in the lysosomal fraction is way below the 7-9% (values are corrected for the yield of the preparation) of metabolically labeled sucrase-isomaltase and DPPIV in that fraction (see Fig. 4). This low delivery to lysosomes of iodinated Fab fragments

![FIG. 5. Competition of the cell surface labeling.](https://example.com/figure5.png)

![FIG. 6. Endocytosis of cell surface-bound [125]I Fab.](https://example.com/figure6.png)
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Fig. 7. Appearance of cell surface-labeled DPPIV (x) and sucrase-isomaltase (O) in the lysosomal fraction of Caco-2 cells. 125I-Fab-labeled cells were incubated at 37 °C for 3 h. After different time intervals the cells were subjected to subcellular fractionation. Radioactivity was measured in lysosomes (LII-fraction) and in aliquots of the homogenate. Values are given as percent of radioactivity in LII relative to the corresponding homogenate corrected for the yield of the preparation.

Fig. 8. Appearance of cell surface-iodinated DPPIV in the lysosomal fraction of Caco-2 cells. The cells were labeled with 125I-Sulfo-SHPP at 4 °C and then incubated at 37 °C. After different time intervals the cells were subjected to subcellular fractionation. DPPIV (A) and sucrase-isomaltase (B) were immunoprecipitated from solubilized lysosomes (LII) and from a sample of the homogenate (H). Immunoprecipitates were analyzed by SDS-PAGE followed by autoradiography.

could be due to a rapid degradation of the antibody fragments in lysosomes and loss of the iodinated peptides or tyrosine residues from lysosomes. However, in neither the incubation medium nor the cells were we able to detect trichloroacetic acid-soluble radioactivity even after incubations of up to 6 h at 37 °C. Another potential problem with this assay concerns the possibility that there might be a rapid release of Fab fragments into the medium upon warming up the cells. Indeed, there is a low but slow release of the Fab into the medium which levels off after 1 h at 37 °C. Maximal release into the medium was 15% for sucrase-isomaltase and 18% for DPPIV. However, this does not critically affect the results of Fig. 7 since the radioactivity in lysosomes was compared with the counts obtained in the corresponding homogenate.

Nevertheless, to circumvent these potential problems we used an alternative method. Cell surface DPPIV and sucrase-isomaltase were covalently modified by iodinated sulfo-SHPP which was recently introduced as an ideal reagent for the labeling of cell surface proteins (Thompson et al., 1987). The experiment was done analogous to that shown in Fig. 7. After radioiodination the cells were incubated at 37 °C for the indicated times and then lysosomes were purified. The lysosomes were solubilized and sucrase-isomaltase and DPPIV were immunoprecipitated and analyzed by SDS-PAGE followed by autoradiography (Fig. 8). The results of this experiment confirm that the two hydrolases are transported to lysosomes at a low rate. While low amounts of DPPIV appeared in lysosomes (A), no sucrase-isomaltase was detectable in this fraction even after an overnight incubation at 37 °C (B). Quantification of these autoradiographs (Fig. 9) showed that the uptake of cell surface iodinated DPPIV was comparable to that of 125I-Fab fragments bound to the cell surface. After an overnight incubation the amount of DPPIV delivered to lysosomes was slightly higher when determined by the direct iodination procedure than by the Fab method. This might indicate that in lysosomes the antibodies have a shorter half-life than DPPIV. On the other hand, the experiment with sucrase-isomaltase suggests that the Fab assay is somewhat more sensitive than the iodination assay.

Another important result emerging from Fig. 8 is that without an incubation at 37 °C neither DPPIV nor sucrase-isomaltase were detected in the lysosomal fraction LII. Thus, the [35S]methionine-labeled molecules found in the LII fraction after metabolic labeling cannot be due to contamination with brush border membranes.

Assuming that newly arriving enzyme molecules in the brush border are indistinguishable from those that already reside in this membrane these results suggest that endocytosis cannot fully explain the relatively high amount of brush border enzymes in lysosomes (see "Discussion").

Recycling of Hydrolases to the Cell Surface via Endosomes—The low rate of endocytic delivery of sucrase-isomaltase and DPPIV to lysosomes (Figs. 7-9) contrasts with the total rate of endocytosis (Fig. 6) and supports the notion that the two hydrolases recycle to the cell surface via endosomes. In order to test if brush border enzymes are internalized into endosomes, we applied Percoll gradient centrifugation to separate endosomes from lysosomes (Marsh et al., 1987). Our separation procedure was similar to that for lysosomes with the exception that a higher Percoll concentration was used (see "Materials and Methods" for details). Fig. 10 demonstrates that the separation of endosomes (labeled by allowing fluid phase endocytosis of horseradish peroxidase for 15 min) from lysosomes (detected by measuring glucosaminidase activity) was efficient in Caco-2 cells. Lysosomes were found near the bottom of the gradient while endosomes almost cofractionated with the brush border membrane marker alkaline phosphatase at the top of the gradient.

This fractionation procedure was applied to cells after

Fig. 9. Appearance of cell surface-iodinated DPPIV in the lysosomal fraction LII of Caco-2 cells (quantification of autoradiograms). The amount of DPPIV in LII was plotted relative to the amount of this protein in the homogenate correcting for yield of the preparation.
surface labeling with $^{125}$I-Fab fragments as already described in the experiments illustrated in Fig. 6. After centrifugation the Percoll gradients were fractionated and the fractions were pooled as indicated in Fig. 10. The time-dependent appearance of radioactivity in pools I (endosomal fraction) and III (lysosomal fraction) is given in Fig. 11. The results demonstrate that most of the endocytosed radioactivity entered endosomes but not lysosomes. The shape of the curves for pool I is similar to that found for total endocytosis, with the exception that both hydrolases reached a plateau in the endosomal fraction. However, when for each time point the radioactivity of pools I and III were added (correcting for the different yields of markers in the two fractions which were twice as large in pool I than pool III) curves were obtained that displayed identical shapes as those in Fig. 6 (not shown). These data are in line with the assumption that the majority of endocytosed sucrase-isomaltase and DPPIV recycles to the cell surface rather than being transported to lysosomes.

In order to test this presumed reappearance at the cell surface directly, we combined the Fab assay with NHS-SS-biotin, a reagent which allows reversible biotinylation of proteins. The experiment was started by labeling the cells at 4 °C with reversibly biotinylated Fab fragments specific for DPPIV. This experiment was performed with DPPIV only since the amount of internalized sucrase-isomaltase is too low to be analyzed. After incubating the cells at 37 °C for 75 min, the biotin of Fab fragments that had remained at the cell surface was cleaved off by reduction with glutathione and the cells were returned to the 37 °C incubator. After different times the cells were cooled to 4 °C and labeled with $^{125}$I-streptavidin. Thereafter the cells were harvested and the cell-associated radioactivity was measured (Fig. 12). The results show that there is a time-dependent reappearance of biotinylated Fab fragments at the cell surface which levels off after 1 h. This flattening of the curve was to be expected since the internal pool of biotinylated Fab fragments becomes depleted after a certain time and since the Fab fragments reappearing at the cell surface are endocytosed again. It is important to note that $^{125}$I-streptavidin is not limiting in this assay.

**DISCUSSION**

This study demonstrates that newly synthesized microvillar hydrolases are transported to lysosomes along two different pathways. A first fraction is delivered directly to lysosomes bypassing the brush border membrane, whereas some enzyme molecules endocytosed from the apical membrane appear in lysosomes in a second later wave. The majority of endocytosed sucrase-isomaltase and DPPIV was found to recycle to the brush border membrane.

The purity of the lysosomal fraction LII is critical for the validity of the conclusions drawn in this study. For instance, the appearance of metabolically labeled sucrase-isomaltase and DPPIV in LII may simply be due to brush border vesicles or Golgi-derived membranes cofractionating with lysosomes. Four observations argue against such a problem. First the enzymatic properties of the LII fraction suggest little cross-contamination by organelles of non-lysosomal origin. Second, transport rates (i.e. half-maximal appearance) of these enzymes to the Golgi apparatus and to the brush border membrane are different from those to the lysosomal fraction. In fact, the rate of transport of the two hydrolases to the brush border (Stieger et al., 1988) was at least 2 h faster in comparison to that to lysosomes. Third, when Caco-2 cells were surface-labeled at 4 °C with $^{125}$I-Fab fragments specific for
either DPPIV or sucrase-isomaltase and were fractionated without a chase the radioactivity associated with the lysosomal fraction was close to the detection limit of the γ-counter. Fourth, it was not possible to immunoprecipitate cell surface iodinated proteins from the lysosomal fraction without a chase. The results obtained by the cell surface labeling approach also argue against a cross-contamination by endosomes. At steady state, 18% of the initially labeled DPPIV was found in kinetically early endosomes whereas this fraction was only 2% for sucrase-isomaltase. In contrast, the percentage of newly synthesized sucrase-isomaltase and DPPIV in lysosomes was comparable. These results rule out the possibility of a significant contamination of the lysosomal fraction by early endosomes. We are confident therefore that our lysosomal fraction is sufficiently pure to draw valid conclusions on the trafficking of brush border enzymes.

Lysosomal Delivery of Brush Border Hydrolases—Since metabolically labeled sucrase-isomaltase and DPPIV appeared in lysosomes later than in the microvillar membrane it was initially not possible to decide whether they are transported to lysosomes via the brush border membrane or by a direct intracellular pathway. The biphasic profile of appearance in the LII fraction, however, already indicated that the two hydrolases may be transported to lysosomes along two different pathways. Therefore, we have also studied the endocytosis route by using 125I-Fab fragments and covalent iodination to label cell surface sucrase-isomaltase and DPPIV in the microvillar membrane of intact cells. The Fab cell surface labeling procedure is highly selective as assessed by a number of control experiments that have been discussed under “Results.” The kinetics of transport of cell surface sucrase-isomaltase and DPPIV to lysosomes was found to be different from that of metabolically labeled enzymes. Indeed, endocytic transport to lysosomes is too low and too slow to account for all of the metabolically labeled sucrase-isomaltase and DPPIV measured in this organelle. However, the endocytic kinetics were roughly concordant with the second wave of 35S-labeled hydrolases in lysosomes (that is at chase times longer than 10 h). This suggests that the first wave of 35S-labeled hydrolases in lysosomes was imported along a route that bypasses the brush border membrane. The only low level of delivery to lysosomes of cell surface-bound Fab fragments is unlikely to be due to dissociation of the immunocomplexes in acidic endocytic organelles since more than 80% of 125I-Fab remained associated with the corresponding antigen after prolonged exposure to pH 3.0 (not shown). It is known that lysosomes, the most acidic organelles of the endocytic pathway, have a pH not lower than 4.5 (Mellman et al., 1986). Furthermore, the same degree of lysosomal delivery was obtained by iodinating cell surface DPPIV covalently. These biochemical findings were confirmed by immunoelectron microscopy using ultrathin cryosectioning. It was found that surface bound anti-sucrase-isomaltase or anti-DPPIV antibodies were internalized into endosomal structures while transport to lysosomes was slow.3 These experiments also showed that antibodies to DPPIV or sucrase-isomaltase label the microvillar membrane of intact Caco-2 cells in a uniform manner.

Recently, we have demonstrated that newly synthesized proteins are transported to the brush border membrane of Caco-2 cells along two different pathways: a direct intracellular and an indirect pathway via the basolateral membrane.2 Therefore, the first wave of newly synthesized microvillar enzymes in lysosomes may be due to endocytosis from the basolateral membrane rather than a direct intracellular pathway. We have approached this possibility by a combination of the here described subcellular fractionation procedure and a selective cell surface biotinylation assay. Unfortunately, no conclusive result was obtained due to extremely low radioactivity signals which were close to the detection limit. However, the following observations argue against the possibility that the lysosomal appearance is mainly due to a delivery of newly synthesized DPPIV and sucrase-isomaltase from the basolateral membrane. 1) Whereas much more DPPIV than sucrase-isomaltase was found in the basolateral membrane, similar amounts of the two enzymes were found in lysosomes. 2) DPPIV inserted into the basolateral membrane was not to be transcytosed to the brush border membrane quantitatively. 3) Appearance in lysosomes occurs before disappearance in the basolateral membrane. Of course, a minor contribution from the basolateral membrane cannot be excluded. Since the apparent molecular mass of the hydrolases in lysosomes is indistinguishable from that of the complex-glycosylated forms in the homogenate it can be concluded that the hydrolases traverse all subcompartments of the Golgi apparatus before they are sorted to lysosomes. Therefore, they may migrate along the same route as resident lysosomal proteins. Lysosomal enzymes (Kornfeld, 1987; Griffiths and Simons, 1986) and lysosomal membrane glycoproteins (Green et al., 1987) are sorted from plasma membrane proteins at a trans- or post-Golgi intracellular site, most likely in the trans-Golgi network and are transported to lysosomes via a specialized endosomal structure (prelysosome) (Griffiths et al., 1988). It is interesting to note that the transport of lysosomal membrane glycoproteins in kidney cells, macrophages, and Caco-2 cells is considerably faster than the delivery of sucrase-isomaltase and DPPIV to lysosomes (Green et al., 1987).4 Recently, a striking homology was found between lysosomal α-glucosidase, a soluble enzyme, and sucrase-isomaltase (Hoejstool et al., 1988). In contrast to α-glucosidase, which carries the lysosomal targeting signal phosphomannose, the sucrase-isomaltase (like DPPIV) is not phosphorylated in Caco-2 cells and thus a mis-sorting of sucrase-isomaltase to lysosomes due to sequence homology with a lysosomal enzyme appears unlikely. Furthermore, it is important to note that

3 J. Klumperman, manuscript in preparation.
4 K. Matter and H.-P. Hauri, unpublished observations.
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our mAbs to sucrase-isomaltase do not cross-react with lysosomal α-glucosidase. Overall, these kinetic and structural considerations suggest that the mechanism of transport of sucrose-isomaltase and DPP IV to lysosomes differ from that for soluble or membrane-bound lysosomal resident proteins.

What may be the function of a direct intracellular pathway to lysosomes? It has been postulated that the lysosomes may have a regulatory function in the cell surface expression of brush border membrane proteins (Blok et al., 1984) similar to the crinophagic pathway for secretory vesicles in the pituitary of lactating rats (Smith and Farquhar, 1966). Another potential function of this pathway may relate to a late "product-control" mechanism which would remove proteins from the biosynthetic pathway that, for example, are not correctly folded and therefore have not acquired their normal enzymatic activity or have no proper signal for their transport to the microvernular membrane. A quality control system has already been described for the endoplasmic reticulum (Doms et al., 1988; Lippincott-Schwartz et al., 1988). At present it is unknown, however, if the brush border enzymest in lysosomes are enzymatically active. Interestingly, a fraction of newly synthesized complex-glycosylated sucrose-isomaltase and DPP IV has a long apparent residence time in a Golgi fraction of Caco-2 cells (Stieger et al., 1988). A slowly turning over pool of hydrolases in the Golgi apparatus would be in line with both a regulatory or a quality control mechanism.

Endocytosis and Recycling of Brush Border Hydrolases — A remarkable finding is that sucrose-isomaltase and DPP IV undergo interiorization by endocytosis. However, in Caco-2 cells most of the endocytosed sucrose-isomaltase and DPP IV are not delivered all the way to lysosomes but recycle to the cell surface without being degraded as demonstrated for DPP IV. The magnitude of endocytosis is about 10-fold higher for DPPIV than for sucrase-isomaltase. The endocytosis is not due to a cross-linking effect of divalent antibodies as reported by Louvard (1980) for aminopeptidase N in MDCK cells, since we used monovalent Fab fragments. The reason the recycling of brush border hydrolases.

There is no obvious function for such an internal pool nor for the recycling of brush border hydrolases. We have recently demonstrated that newly synthesized brush border hydrolases transiently inserted into the basolateral membrane are transcytosed efficiently to the apical membrane whereas apical-to-basolateral transcytosis did not occur. Together with the results of the present study these data suggest efficient sorting of endocytosed plasma membrane proteins in Caco-2 cells. Interestingly, when the brush border membrane of Caco-2 cells was labeled with a mAb against DPP IV, incubated at 37°C, and then processed for electron microscopic immunocytochemistry the endocytosed antibody was never found in the Golgi apparatus but in various endosomal structures. This finding indicates that sorting of endocytosed plasma membrane proteins does not occur in the trans-Golgi network and supports the hypothesis that endosomes are of high importance for the biogenesis and maintenance of epithelial cell polarity.

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REFERENCES

Bekh, W. E., and Rothman, J. E. (1985) Arch. Biochem. Biophys. 240, 415-425
Bartles, J. R., and Hubbard, A. L. (1988) Trends Biochem. Sci. 13, 181-184
Bartles, J. R., Feracci, H. M., Stieger, B., and Hubbard, A. L. (1987) J. Cell Biol. 105, 1241-1251
Blok, J., Fransen, J. A. M., and Ginsel, L. A. (1984) Cell Biol. Int. Rep. 8, 993-1014
Bretscher, M. S., and Lutter, R. (1988) EMBO J. 7, 4097-4092
Doms, R. W., Rusuia, A., Machamer, C., Helenium, J., Helenius, A., and Rose, J. K. (1988) J. Cell Biol. 107, 89-99
Fransen, J. A. M., Ginsel, L. A., Cambier, P. H., Klumperman, J., Oude Elferink, R. P. J., and Tager, J. M. (1988) Eur. J. Cell Biol. 47, 72-80
Fransen, J. A. M., Klumperman, J., Oude Elferink, R. P. J., Hauri, H.-P., Tager, J. M., and Ginsel, L. A. (1989) International Brush Border Symposium, pp. 12-28, Huber Verlag, Bern
Fransen, J. A. M., Ginsel, L. A., Hauri, H.-P., Sterchi, E. E., and Blok, J. (1985) Eur. J. Cell Biol. 38, 5-13
Geuze, H. J., Slot, J. W., Strous, G. J. A. M., Hasilk, A., and von Figura, K. (1984) J. Cell Biol. 98, 2047-2064
Geuze, H. J., Slot, J. W., Strous, G. J. A. M., Hasilk, A., and von Figura, K. (1985) J. Cell Biol. 101, 2253-2262
Gorr, S.-U., Stieger, B., Fransen, J. A., Kedinges, M., Marxer, A., and Hauri, H.-P. (1988) J. Cell Biol. 106, 1937-1946
Gottlibe, T. A., Gonzales, A., Riusolo, L., Rindler, M. J., Aedenon, M., and Sabatini, D. D. (1986) J. Cell Biol. 102, 1242-1255
Green, S. A., Zimmer, K.-P., Griffiths, G., and Melfin, I. (1987) J. Cell Biol. 105, 1227-1235
Griffiths, G., and Simons, K. (1986) Science 234, 438-443
Griffiths, G., Hoflack, B., Simons, K., Mellman, I., and Kornfeld, S. (1988) Cell 62, 329-341
Griffiths, G., Pfeiffer, S., Simons, K., and Matlin, K. (1985) J. Cell Biol. 101, 943-964
Hauri, H. P., Sterchi, E. E., Bienes, D., Fransen, J. A. M., and Marxer, A. (1985a) J. Cell Biol. 101, 833-851
Hauri, H. P., Roth, J., Sterchi, E., and Lenz, M. J. (1985b) Proc. Natl. Acad. Sci. U.S.A. 82, 4429-4427
Hauri, H.-P. (1988) Subcell. Biochem. 12, 155-219
Hilkeno, J., Tager, J. M., Fujio, B., Brouwer Kelders, B., van Thienen, G. M., Tegelaers, P. F. W., and Hilgers, J. (1981) Biochim. Biophys. Acta 768, 7-11
Hofskot, L. H., Hoogeveen-Westerveld, M., Kroos, M. A., van Boezen, J., Reuser, A. J. J., and Oorstra, B. A. (1988) EMBO J. 7, 1679-1704
Hunter, W. M., and Greenwood, F. C. (1962) Nature 194, 495-496
Kenny, A. J., and Maroux, S. (1982) Physiol. Rev. 62, 91-128
Kornfeld, S. (1983) FASEB J. 1, 462-468
Lippincott-Schwartz, J., Bonifacio, J. S., Yuan, L. C., and Klausner, R. D. (1988) Cell 54, 269-270
Lippincott-Schwartz, J., and Farnham, D. M. (1987) Cell 49, 669-677
Lorenzonz, V., Korsomo, H., and Olsen, W. A. (1987) Gastroenterol. 93, 98-106
Louvard, D. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 4132-4136
Mage, M. G. (1986) Methods Enzymol. 70, 142-150
Marsh, M., Schmidt, K., Kern, H., Harms, E., Maier, P., Melfin, I., and Helenius, A. (1987) J. Cell Biol. 104, 875-886
Matlin, K. S., and Simons, K. (1984) J. Cell Biol. 99, 2131-2139
Melfin, I., Fuchs, R., and Helenius, A. (1986) Annu. Rev. Biochem. 55, 663-700
Merril, C. R., Goldman, D., and van Kurenen, M. L. (1984) Methods Enzymol. 104, 441-447
Misek, D. E., Bard, E., and Rodriguez-Boulan, E. (1984) Cell 39, 537-546
Nelson, W. J., and Veshnock, P. J. (1987) Nature 328, 533-536
Norén, O., Sjostrom, H., Dahlen, E. M., Cowell, G. M., and Skovbjerg, H. (1986) in Molecular and Cellular Basis of Digestion (Desmuelle, P., Sjostrom, H., and Norén, O., eds) pp. 335-365, Elsevier, Amsterdam
Pisto, C., Robina-Lecou, A., Appay, M.-D., Kedinges, M., Triantou, N., Dassaulx, E., Lacroix, B., Simon-Assmann, D., Haften, K., Fogh, J., and Zweibaum, A. (1983) Biol. Cell. 47, 223-330
Rindler, M. J., Ivanov, I. E., Plesken, H., and Sabatini, D. D. (1985) J. Cell Biol. 100, 136-151
Transport of Brush Border Enzymes to Lysosomes

Scalera, V., Storelli, C., Storelli-Joss, C., Hasse, W., and Murer, H. (1980) Biochem. J. 186, 177–181
Schweizer, A., Fransen, J. A. M., Bäch, T., Ginsel, L., and Hauri, H. P. (1988) J. Cell Biol. 107, 1643–1653
Semenza, C. (1986) Annu. Rev. Cell Biol. 2, 255–313
Sips, H. J., Claas, A. H. W., van Dongen, J. M., Willemsen, R., Hoogeveen, A. T., Galjaard, H., Sielasappel, M., Hauri, H.-P., and Sterchi, E. E. (1985) J. Inher. Metabol. Dis. 8, 163–168
Smith, R. E., and Farquhar, M. G. (1986) J. Cell Biol. 106, 1853–1861
Sottocasa, G. L., Kuylenstierna, B., Ernst, L., and Bergstrand, A. (1990) J. Cell Biol. 107, 415–438
Stieger, B., Marxer, A., and Hauri, H.-P. (1986) J. Membr. Biol. 91, 19–31
Stieger, B., Matter, K., Baur, B., Bucher, K., Höchli, M., and Hauri, H. P. (1988) J. Cell Biol. 106, 1853–1861
Thompson, G. A., Lau, A. L., and Cunningham, D. D. (1987) Biochemistry 26, 743–750
Venable, J. H., and Coggeshall, R. (1965) J. Cell Biol. 25, 407–408
Wandinger-Ness, A., and Simon, K. (1988) in Intracellular Transport of Proteins (Hanover, J., and Steer, L., eds) Cambridge University Press (in press)
Zweibaum, A., Laburthe, M., Grasset, E., and Louvard, D. (1989) in Handbook of Physiology, in press