The cation-independent mannose 6-phosphate receptor is not involved in the polarized secretion of lysosomal α-glucosidase from Caco-2 cells

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In the human adenocarcinoma cell line Caco-2 a substantial amount of a precursor form of the lysosomal enzyme α-glucosidase is not segregated into lysosomes, but instead secreted from the apical membrane. In this study we addressed the question whether this process is mediated by mannose 6-phosphate receptors. The subcellular distribution of the cation-independent mannose 6-phosphate receptor was studied by means of electron microscopic immunocytochemistry. The bulk of label was found in the perinuclear region in electron-lucent and dense vesicles, some of the latter bearing a coat. Receptor-containing dense vesicles were also found throughout the cytoplasm. In the apical part of the cells, label for the receptor was present over the surrounding membrane and the interior vesicles of multivesicular bodies, but not over lysosomes. Label on the plasma membrane was mainly restricted to the apical domain.

In contrast to α-glucosidase, the secreted forms of the lysosomal enzymes cathepsin D, β-hexosaminidase and β-glucuronidase are mainly found in the basolateral medium. Enzyme activity measurements and immunoprecipitation of metabolically labeled cells showed that incubation with NH_4Cl leads to an enhanced secretion of these enzymes into the basolateral medium, but has no effect on the basolateral secretion of α-glucosidase. In addition, NH_4Cl caused a minor decrease in the secretion of these enzymes from the apical side and had little or no effect on the secretion of α-glucosidase. When buffer of low *p*H or medium supplemented with 20 mM mannose 6-phosphate to the apical side of the cells was added, no effect was found on the amount of immunocytochemically detectable α-glucosidase in the microvilli.

We conclude that although mannose 6-phosphate receptor-mediated transport is functionally active in Caco-2 cells, an additional or alternative mechanism contributes to the transport of α-glucosidase to the apical membrane.

Introduction

It is now well established that mannose 6-phosphate receptors (MPRs) function in the targeting and transport of newly synthesized soluble lysosomal enzymes (for a review, see [26]). Lysosomal enzymes are synthesized on polysomes attached to the rough endoplasmic reticulum, translocated into the lumen of the endoplasmic reticulum and transported to the Golgi apparatus where they acquire M6P residues, which act as recognition markers for binding to the MPR. The receptor-ligand complex moves to an acidified compartment of endosomal origin [11, 14], where the low *p*H causes the complex to dissociate. The receptor can recycle to the trans Golgi region [7, 12, 23] or to the cell surface, whereas the lysosomal enzymes are packaged into lysosomes. Most cells secrete a small percentage of newly synthesized lysosomal enzymes, and a portion of these molecules may bind to MPRs present on the cell surface (10–20% of the total receptor pool). These receptor-ligand complexes are internalized via coated pits and delivered to endosomes, where dissociation of the complex occurs.

The first MPR to be identified was an integral membrane glycoprotein of 215 kDa [40]. Immunolocalization studies performed in a variety of cell types (for a review, see [26]) located the receptor in the Golgi region, endosomal compartments, the plasma membrane, and coated pits emerging from the plasma membrane, but not in lysosomes. A number of cell lines were found to lack the receptor [20, 21]; this led to the discovery of a second MPR. This second receptor has a relative molecular mass of 46 kDa and re-

Abbreviations. CI Cation independent. — IGF Insulin-like growth factor. — M6P Mannose 6-phosphate. — MPR Mannose 6-phosphate receptor. — MVB Multivesicular body. — sd Standard deviation.

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quires divalent cations for the efficient binding of lysosomal enzymes. In addition to its function in the targeting of lysosomal enzymes to the lysosomes, this receptor may also be involved in the secretion of lysosomal enzymes [4], but not in their endocytosis [42].

In earlier studies we reported on the pathways taken by newly synthesized lysosomal α-glucosidase in the polarized epithelial cells lining the small intestine [8] and the kidney proximal tubule [32], as well as in the enterocyte-like cell line Caco-2 [25]. We found that in all three cell types a precursor form of lysosomal acid α-glucosidase is associated with the apical brush border membrane. For Caco-2 cells we obtained evidence that a considerable amount of this precursor is secreted from the apical domain, whereas all other enzymes are preferentially secreted from the basolateral side of these cells [25, 38].

In the present study we investigated the subcellular distribution of the cation-independent receptor (CI-MPR) and the functional importance of MPRs in the transport and secretion of α-glucosidase in Caco-2 cells.

Materials and methods

Caco-2 cell culture

Caco-2 cells [35] were cultured on surfactant-free nitrocellulose filters (Millipore type HA, pore size 0.45 μm; Millipore Products Div., Bedford, MA/USA) in mini-Marbrook chambers at 37 °C in an atmosphere of 95% air and 5% CO_2. Dulbecco's modified Eagle's medium (Gibco Europe, Hoofddorp/The Netherlands) containing 4.5 g/l glucose, and supplemented with 1% non-essential amino acids (Gibco), 20% heat-inactivated fetal calf serum (Flow Laboratories Inc., Irvine/Scotland), 50 U/ml penicillin and 50 μg/ml streptomycin (Flow), was added daily. The cells were used between passages 130 and 170, 5 to 7 days after confluency. The tightness of the monolayer was tested by inducing a higher medium level in the apical chamber, which had to be maintained for at least 4 h.

Antibodies

The antibody used to immunolocalize the CI-MPR was raised in a goat and generously given to us by Prof. Kurt von Figura, Dept. of Biochemistry II, University of Göttingen/Germany [3]. The immunocytochemical labeling of α-glucosidase was performed with monoclonal antibody 43D1 [8] or with antiserum raised in rabbit [8]. Immunoprecipitation of α-glucosidase was performed with antibody 118G3 [17, 25, 29, 30] and of cathepsin D with an antiserum raised in rabbit [25].

Immunocytochemistry

Immunolabeling of cryosections was performed as described elsewhere [24]. In short, the cells were fixed in a mixture of 0.1% glutaraldehyde and 1% freshly prepared formaldehyde in 0.15 M sodium bicarbonate buffer at *p*H 8.3 for 1 h at room temperature, gently scraped off the filter, pelleted in 10% gelatin, and postfixed and stored for at least 24 h at 4 °C in 1% formaldehyde in 0.1 M phosphate buffer. Ultrathin cryosections were incubated with goat anti-(CI-MPR) or with 43D1 or rabbit anti-(α-glucosidase). With the first two of these antibodies, a second step was performed with rabbit anti-(goat IgG) or rabbit anti-(mouse IgG), respectively. The complexes were visualized with colloidal gold particles complexed to protein A [41].

Effect of low pH and surplus M6P on the presence of α-glucosidase in the microvilli

To study the effect of low *p*H on the presence of the precursor form in the microvilli of Caco-2 cells, we incubated the cells with 0.1 M phosphate buffer of *p*H 3, 4 or 5. To investigate the effect of surplus M6P, we added 20 mM M6P to the standard medium. Medium or buffer were added to the cells, and the petri dishes were rotated frequently during 5 or 30 min at 4 °C.

Enzyme assays

To study the involvement of MPR in the transport and secretion of lysosomal enzymes, 20 mM NH_4Cl was added to both the apical and basolateral media, and the secreted enzymes were assayed enzymatically. Enzyme assays were performed as described elsewhere [25], with the use of 4-methylumbelliferyl-conjugated artificial substrates. The amount of liberated 4-methylumbelliferone was measured fluorimetrically at 445 nm.

Metabolic labeling and immunoprecipitation

Cells were metabolically labeled for 1 h with 0.1 mCi [^{35}S]methionine (Radiochemical Centre, Amersham/UK) as described elsewhere [25]. Where indicated, 20 mM NH_4Cl was added to both the apical and basolateral chase media. Immunoprecipitation and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were carried out as described [25].

Results

Immunocytochemical localization of the 215 kDa MPR in Caco-2 cells

The subcellular distribution of the CI-MPR in Caco-2 cells was investigated by immunoelectron microscopy. The bulk of label was found in dense vesicular and tubular structures in the perinuclear zone (Figs. 1a, b). The average diameter of the vesicles was 85 nm (sd: 20 nm) and some of them were coated (Fig. 1c). Between these small dense vesicles larger vesicles (average diameter: 250 nm, sd: 70 nm) with an electron-lucent matrix were present, which also could contain label representing the CI-MPR. Occasionally, a connection between these two types of vesicles was seen (Fig. 1b). Golgi cisternae showed little or no label (Fig. 1c). Occasionally, MVBs containing the CI-MPR were observed between the vesicles in the perinuclear region (Fig. 1a).

In the apical part of the cytoplasm of Caco-2 cells we saw the CI-MPR in electron-lucent and dense vesicles, as well as in MVBs (Fig. 2a). The label in the MVBs was associated with both the surrounding membrane and the interior vesicles. The MPR was not detectable in dense lysosomes (Fig. 2b).

In various cell types 10 to 20% of the CI-MPR occur on the plasma membrane [26]. In Caco-2 cells we found the plasma membrane-associated receptor mainly in the microvilli (Figs. 3a, b). The amount of label in the microvilli varied considerably between cells; this heterogeneity did not seem to be related to obvious changes in the overall ultrastructure. Little or no label was seen on the basolateral plasma membrane (Figs. 4a, b), but in the vicinity of the basolateral membrane we observed label in both smooth and coated dense vesicles, as well as electron-lucent vesicles (Figs. 4a, b).

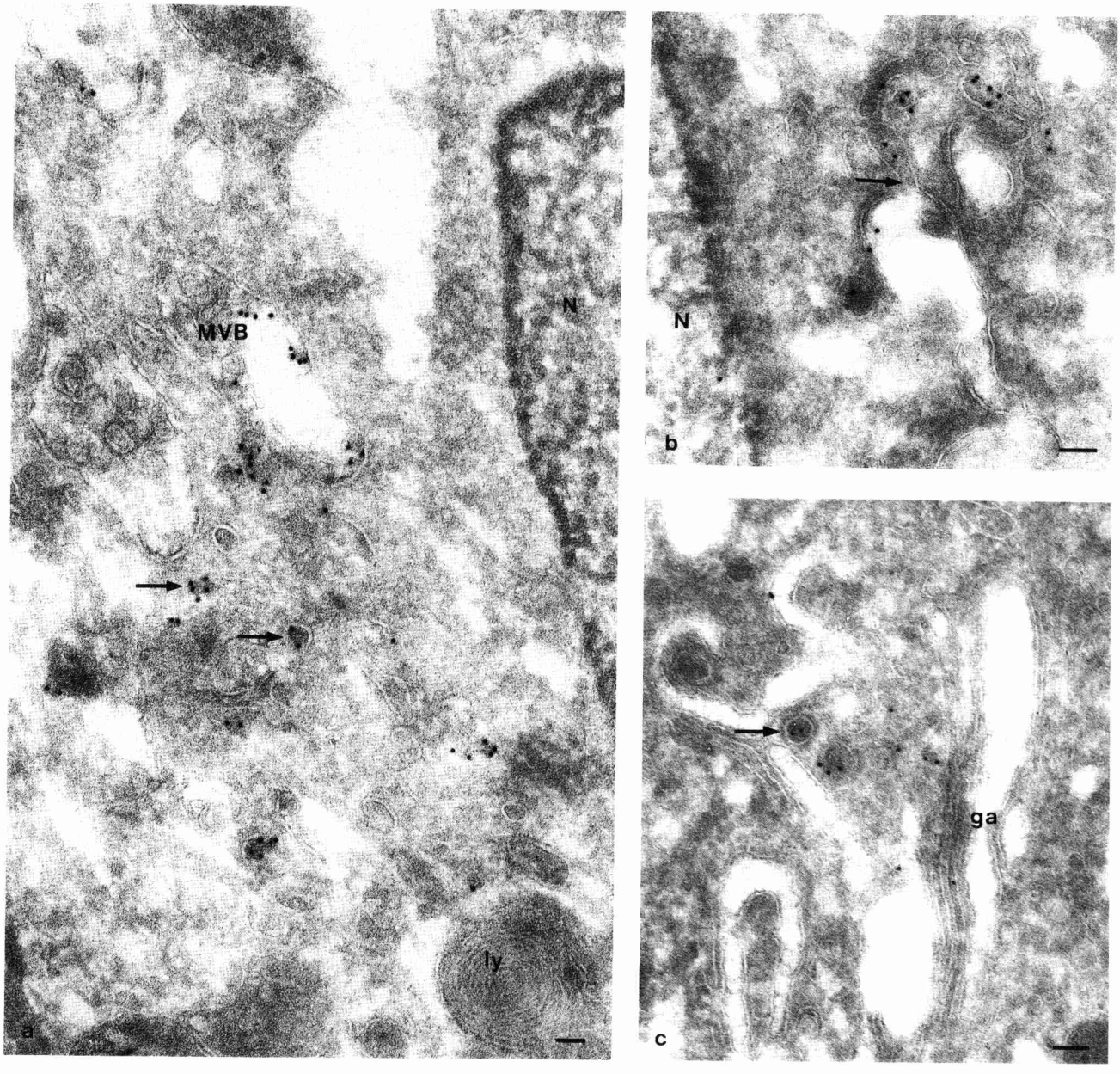

Fig. 1. Electron micrographs of cryosections of Caco-2 cells labeled for the CI-MPR showing details of the perinuclear area. — **a.** CI-MPRs are present in dense vesicles (*arrows*) and MVBs. — **b.** Occasionally, a connection between a dense and a larger electron-lucent vesicle is observed (*arrow*). — **c.** Only little label is found over the Golgi cisternae. Some of the adjacent positively labeled dense vesicles are coated (*arrow*). — ga Golgi apparatus. — N Nucleus. — MVB Multivesicular body. — ly Lysosome. — Bars 0.1 μm.

Effect of low pH and M6P on the occurrence of α-glucosidase in the microvilli

In Caco-2 cells a precursor form of lysosomal acid α-glucosidase is associated with the microvillar membrane [25]. The presence of the MPR in the microvilli suggests that microvillar α-glucosidase may be receptor bound. We investigated this possibility by incubating the cells with either buffer of low *p*H or medium containing 20 mM M6P. When cells were incubated for 5 min at 4 °C with buffer at *p*H 3, the microvilli were still extensively labeled for α-glucosidase (Fig. 5). Similar results were obtained after 30 min of incubation and when buffers were used at *p*H 4 and 5. When 20 mM M6P was added to the culture medium and the cells were exposed for 30 min, there was no significant decrease in the degree of intensity of labeling of the microvilli either (not shown).

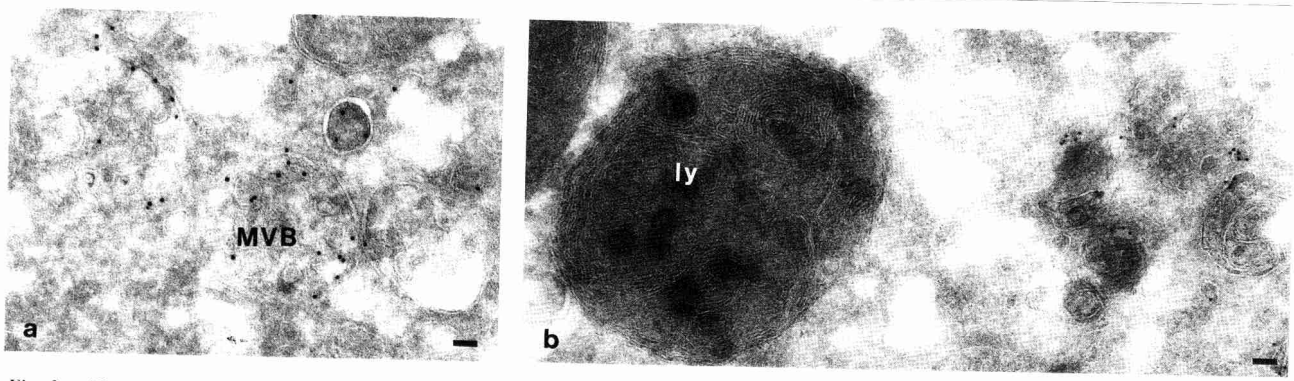

Fig. 2. Electron micrographs of the apical cytoplasm of Caco-2 cells labeled for the CI-MPR. — **a.** Label in small vesicles and MVBs. The label in the latter is associated with both the surrounding membrane and the luminal vesicles. — **b.** Dense lysosomes are devoid of label. — MVB Multivesicular body. — ly Lysosome. — Bars 0.1 μm.

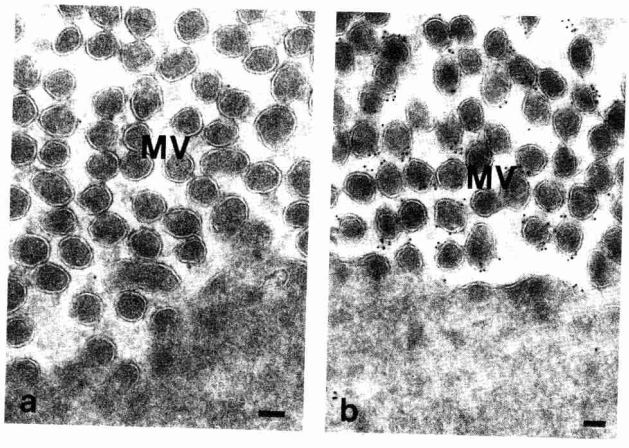

Fig. 3. Electron micrographs of cryosections of Caco-2 cells labeled for the CI-MPR. The amount of label in the microvilli varies considerably between cells. — **a.** Microvilli of a cell which contain only few CI-MPRs. — **b.** Microvilli which are extensively labeled for the CI-MPR. — MV Microvilli. — Bars 0.1 μm.

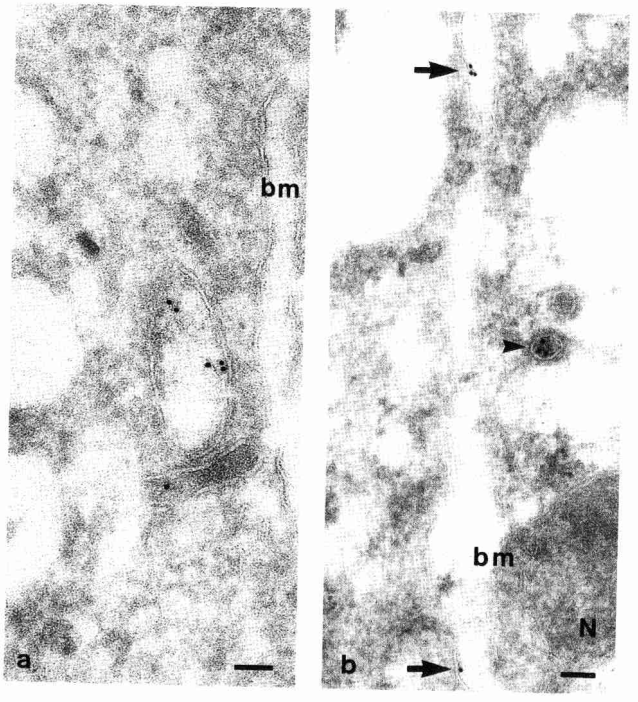

Fig. 4. Electron micrographs of cryosections of Caco-2 cells labeled for the CI-MPR. — **a.** In the vicinity of the basolateral membrane, there are electron-dense and lucent vesicles containing CI-MPRs. — **b.** Coated vesicles labeled for the MPR may also be located close to the basolateral plasma membrane (*arrowhead*). Little label is present on the membrane itself (*arrows*). — N Nucleus. — bm Basolateral membrane. — Bars 0.1 μm.

Effect of NH_4Cl on the secretion of lysosomal enzymes by Caco-2 cells

To establish the actual involvement of the MPRs in the transport of lysosomal enzymes in Caco-2 cells, we assessed the effect of NH_4Cl on the secretion of several acid hydrolases. Weak bases accumulate in the lumen of acid compartments, which increases the luminal *p*H [34]. Under these conditions receptor-ligand complexes no longer dissociate, which leads to the depletion of unoccupied receptors and the enhanced secretion of newly synthesized soluble lysosomal hydrolases. Confluent Caco-2 cells cultured on nitrocellulose filters in mini-Marbrook chambers were metabolically labeled for 1 h with 0.1 mCi [^{35}S]methionine and then chased in culture medium, supplemented with 10 mM methionine and 20 mM NH_4Cl. After 4 h of chase the apical and basolateral media were collected separately, and α-glucosidase and cathepsin D were immunoprecipitated and subjected to SDS-PAGE. Figure 6 shows a representative fluorogram from such an experiment. Under standard culture conditions most of the secreted α-glucosidase was found in the apical chamber, whereas cathepsin D was secreted preferentially from the basolateral side. In the presence of NH_4Cl the secretion of cathepsin D from the basolateral side of the cells increased markedly, but there was

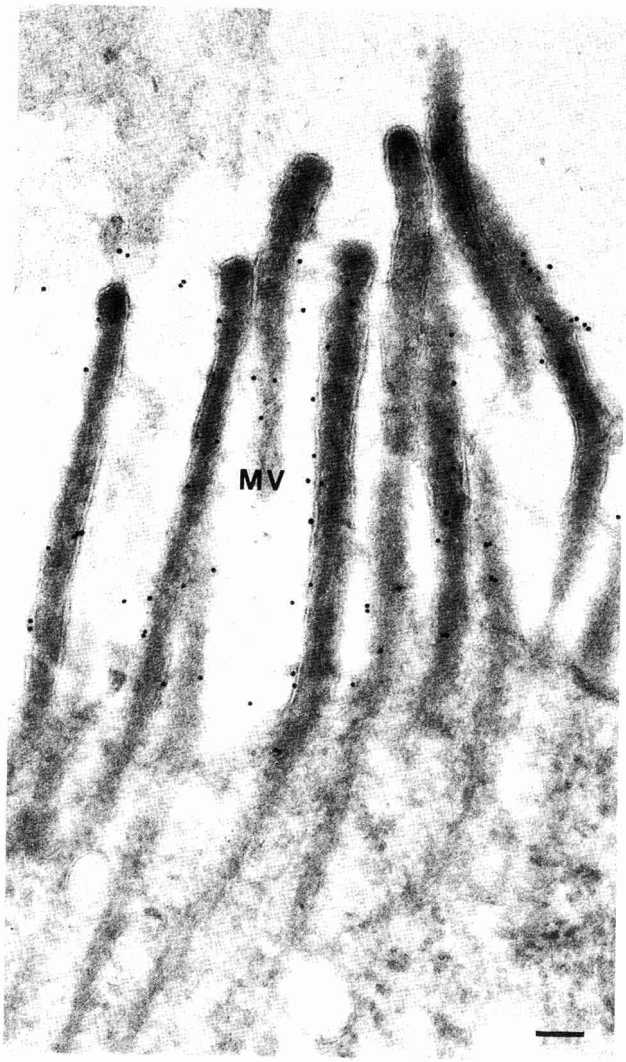

Fig. 5. Electron micrograph of a cryosection of a Caco-2 cell labeled for α-glucosidase. Prior to fixation the cells were incubated for 5 min with buffer of *p*H 3 at 4 °C. Although the overall ultrastructure is less well preserved, the microvilli are extensively labeled for α-glucosidase. — MV Microvilli. — Bar 0.1 μm.

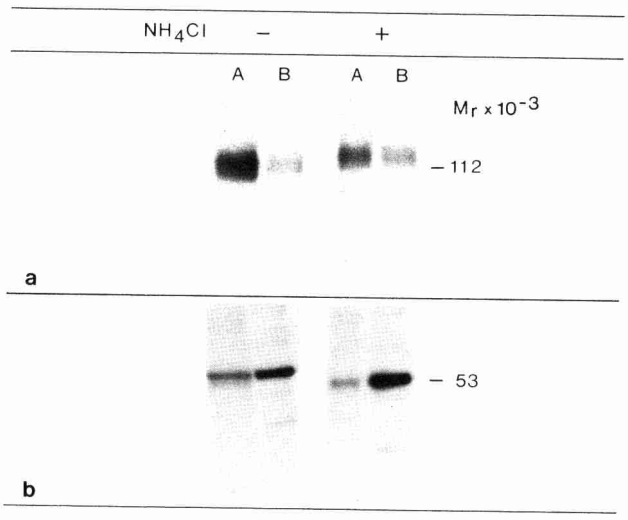

Fig. 6. Fluorograph of α-glucosidase (**a**) and cathepsin D (**b**) precipitated from the culture medium of Caco-2 cells. Filter-grown cells were metabolically labeled with 0.1 mCi [^{35}S]methionine for 1 h. After a 4-h chase in the absence or presence of 20 mM NH_4Cl, the apical and basolateral media were collected separately, and after immunoprecipitation the enzymes were applied to SDS-PAGE. — A Apical. — B Basolateral.

only a slight increase in the amount of basolaterally secreted α-glucosidase. The amount of both cathepsin D and α-glucosidase secreted from the apical side of the cells was only slightly reduced by NH_4Cl.

Comparable results were obtained when enzyme activity assays were performed. Caco-2 cells were cultured for either 8 or 24 h in medium with *p*H inactivated fetal calf serum in the presence or absence of 20 mM NH_4Cl, after which the enzyme activities of α-glucosidase, β-hexosaminidase, and β-glucuronidase were assayed. After both chase periods, only a minor decrease in the amount of α-glucosidase and β-hexosaminidase secreted from the apical membrane was found (Fig. 7; Tab. I), whereas the basolateral secretion of β-hexosaminidase and β-glucuronidase increased markedly in the presence of NH_4Cl. Surprisingly, the amount of β-glucuronidase secreted from the apical surface increased. The reason for this is not clear. Again, there was no or little effect on the secretion of α-glucosidase.

Discussion

The plasma membrane of polarized epithelial cells displays a striking polarization into an apical and a basolateral domain (for a review, see [2]). Both domains contain specific sets of proteins and lipids which are prevented from intermingling by the presence of tight junctions. The human colon carcinoma-derived cell line Caco-2 exhibits characteristics of the epithelial cells of the small intestine [16, 35]. The cells grow in a monolayer, form tight junctions and express brush border hydrolases. In a previous study we revealed the occurrence of a biosynthetic precursor form of the lysosomal enzyme α-glucosidase in the microvillar membrane of Caco-2 cells and the secretion of a considerable amount of α-glucosidase precursor from their apical side [25]. This finding is consistent with the concept that lysosomal enzymes can be secreted from the apical side of polarized cells, e.g., epithelial cells of the proximal tubule [32, 33]. However, the mechanism underlying this pathway is unknown, which led us to the question whether MPRs in Caco-2 cells are involved in the transport of α-glucosidase to the apical membrane.

By means of immunoelectron microscopy we have now shown that the CI-MPR is present in the apical microvilli of Caco-2 cells. However, the association between the pre-

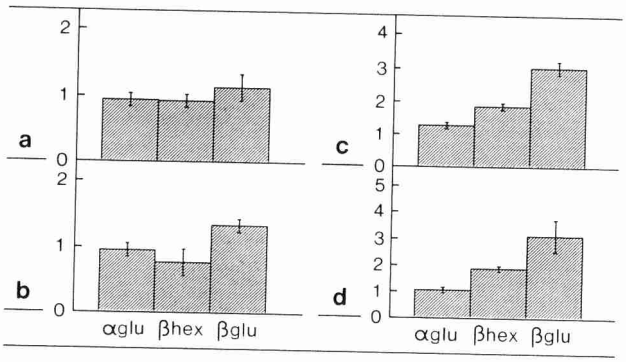

Fig. 7. Quantitation of the effect of 20 mM NH_4Cl on the secretion of lysosomal enzymes by Caco-2 cells. Filter-grown cells were cultured for 8 or 24 h in the absence or presence of 20 mM NH_4Cl. The media were collected separately from the apical and basolateral chambers, and enzyme activities were measured with the use of 4-methylumbelliferyl-conjugated substrates. The bars represent the ratio between activities measured in the medium in the presence of NH_4Cl over the activities measured in the absence of NH_4Cl. — **a.** Apical medium after 8 h. — **b.** Apical medium after 24 h. — **c.** Basolateral medium after 8 h. — **d.** Basolateral medium after 24 h. Note the differences in scale between the apical and basolateral media. — α-glu α-Glucosidase. — β-hex β-Hexosaminidase. — β-glu β-Glucuronidase. All values are the means of two experiments.

Tab. I. Amount of enzyme (in mU) secreted into the apical or basolateral culture medium, collected after 8 or 24 h of culture in the presence or absence of 20 mM NH_4Cl.

	Apical			Basolateral		
	+	−	Ratio	+	−	Ratio
NH_4Cl 8 h						
α-glu	0.03±0.01	0.04±0.01	0.89	0.03±0.01	0.03±0.01	1.07
β-hex	1.30±0.20	1.60±0.20	0.81	5.20±0.60	3.20±0.40	1.63
β-glu	0.23±0.04	0.24±0.03	0.96	0.82±0.23	0.32±0.05	2.56
NH_4Cl 24 h						
α-glu	0.94±0.11	0.97±0.11	0.97	0.74±0.03	0.70±0.10	1.06
β-hex	12.90±1.30	16.80±4.20	0.77	34.90±2.00	20.80±2.20	1.68
β-glu	1.34±0.25	1.00±0.20	1.34	2.59±0.49	0.95±0.06	2.73

The ratio is the amount of enzyme secreted in the presence over the amount secreted in the absence of NH_4Cl. — α-glu α-Glucosidase. — β-hex β-Hexosaminidase. — β-glu β-Glucuronidase.

cursor form of α-glucosidase and the microvillar membrane is not disturbed by extensive washing with surplus M6P or low-*p*H buffer. The persistence of this association under these conditions strongly suggests that the bulk of α-glucosidase present in the microvilli is associated with the membrane in a MPR-independent manner. The same conclusion can be drawn from the effect of ammonium chloride on the secretion of α-glucosidase. If the transport of α-glucosidase to the apical membrane were regulated by MPRs, a significant decrease would be expected in the amount of enzyme that reaches the apical surface. However, secretion of α-glucosidase from the apical surface, and also of the other lysosomal enzymes studied, was not or only slightly reduced in the presence of ammonium chloride. Previous studies have shown that the addition of a weak base to the culture medium can lead to a significant decrease in the rate of vesicular transport [27, 34], but this effect can apparently be mimicked by an increase in the amount of enzymes incorporated in the transport vesicles. Although our results do not allow us to discriminate unequivocally between these two possibilities, we believe that the marginal reduction in the amount of lysosomal enzymes secreted from the apical membrane is due to a general retardation of the vesicular transport.

The basolateral secretion of α-glucosidase only increased slightly after incubation with NH_4Cl, whereas the secretion of other lysosomal enzymes was markedly enhanced. This finding might indicate that the transport of α-glucosidase to the lysosomes too, is, at least partially, independent of the mannose 6-phosphate receptor-mediated pathway. Like other soluble lysosomal enzymes, the precursor form of α-glucosidase is phosphorylated [15, 37], and the involvement of MPRs in its transport to the lysosomes has been shown [31]. However, indications for an additional MPR-independent pathway were previously obtained by Tsuji et al. [44–46], who studied the transport of α-glucosidase in fibroblasts. The 110 kDa precursor was solubilized in vitro by Triton X-100 and trypsin, but not by mannose 6-phosphate or 1 M KCl [45]. Furthermore, in I-cell fibroblasts, in which lysosomal enzymes are not phosphorylated because of the absence of the phosphotransferase, the amount of α-glucosidase reaching the heavy lysosomal fraction did not differ significantly from that in normal fibroblasts [46]. Also, evidence have been obtained for a membrane-associated form of α-glucosidase in COS cells transfected with the gene for human α-glucosidase [18, 19]. Interestingly, it has now also been shown for cathepsin D that at least a portion of the precursor form of this enzyme is transported via an unknown, non-mannose 6-phosphate receptor-mediated, pathway in HepG2 cells [39].This suggests that alternative membrane association of soluble lysosomal enzymes may be a more general phenomenon.

The apical localization of the CI-MPR is rather unique, since the majority of receptors found so far in polarized cells occurred at the basolateral membrane [28]. The distribution of CI-MPRs over the apical and basolateral membranes of polarized epithelial cells differs among the various cell types. In osteoclasts CI-MPRs are found all along the exocytotic pathway up to the apical ruffled border membrane [1], where the receptor-ligand complexes are dissociated due to the low *p*H in the bone-resorbing lumen. In normal and dystrophic rat retinal pigment epithelial cells too, the CI-MPRs are located in the apical plasma membrane domain [43]. In Madin Darby canine kidney cells, however, the CI-MPR was shown to be exclusively present at the basolateral surface, whereas no surface expression of the CD-MPR was found [36].

The function of the CI-MPR in the apical microvilli of Caco-2 cells is not clear. Both Rindler et al. [38] and Hughson et al. [22] provided evidence that in Caco-2 cells newly synthesized proteins that do not interact with the sorting machinery of the cell are sequestered into the pathway leading to the basolateral membrane. Thus, if the receptor located on the plasma membrane functioned solely as a scavenger of missorted enzymes, at least comparable labeling

over the basolateral membrane would be expected. An alternative explanation for the presence of the CI-MPR in the apical membrane may be the shedding of the receptor. It has been shown that the turnover of the CI-MPR does not occur via lysosomal mechanisms [6, 40] and indeed a truncated form of the receptor, which is still able to bind to M6P and IFGII, is found in human urine and serum [3, 5].

Although coated and uncoated vesicles containing the receptor were frequently observed in the vicinity of the basolateral membrane, little or no label was found on the membrane itself. The low labeling intensity of the basolateral membrane relative to the apical membrane may partly be ascribed to differences in accessibility of the antigen in both domains [13], or be due to a short sojourn of the receptor in the basolateral membrane.

Considerable amounts of CI-MPR label were found in dense and electron-lucent vesicles in the perinuclear region, which most likely represent parts of the trans Golgi network. Immunocytochemical studies performed by several groups showed that the CI-MPR is enriched in tubules and coated buds of the trans Golgi network (TGN) [9, 10]. In NRK cells Griffiths et al. [14] found the bulk of CI-MPR in a compartment they defined as intermediate between late endosomes and early lysosomes. This intermediate compartment differed from the TGN and endosomes in that it did not accumulate virus proteins at 20 °C and was not reached by endocytic markers at that temperature. Clearly, comparable studies are needed to define whether Caco-2 cells possess a compartment with the same characteristics. In all cells already studied, the vesicles containing MPR/lysosomal enzyme complexes which exit from the TGN are not directly targeted to mature lysosomes, but rather to compartments of endosomal origin [11, 14]. The MPRs return to the Golgi or plasma membrane, before entering the lysosomes, and thus escape degradation. In agreement with this general concept, we never observed the CI-MPR in dense lysosomes, but we did find label in MVBs and the vesicles surrounding them, which were located either in the Golgi region or in the apical cytoplasm. Elsewhere we showed that MVBs contain lysosomal enzymes and participate in the endocytic pathway [24, 25]. MVBs in Caco-2 cells may therefore be considered an endosomal compartment to which the CI-MPR and lysosomal enzymes are transported prior to reaching the lysosomes. However, our findings give no indication as to where the receptor-ligand complexes enter the endocytic pathway.

In conclusion, the results presented support the concept that in spite of a functional MPR-mediated transport, other mechanisms may account for the transport of soluble lysosomal enzymes. α-Glucosidase provides an interesting model to study this phenomenon.

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