

Biosynthesis and transport of lysosomal α -glucosidase in the human colon carcinoma cell line Caco-2: secretion from the apical surface

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Summary

The human adenocarcinoma cell line Caco-2 was used for studies on the biosynthesis and transport of lysosomal acid α -glucosidase in polarized epithelial cells. Metabolic labelling revealed that in Caco-2 cells α -glucosidase is synthesized as a precursor form of $110 \times 10^3 M_r$. This form is converted into a precursor of slightly higher M_r (112×10^3) by the addition of complex oligosaccharide chains. Via an intermediate form of $95 \times 10^3 M_r$, this precursor is processed into a mature form of $76 \times 10^3 M_r$.

Combination of metabolic labelling with subcellular fractionation showed that the $112 \times 10^3 M_r$ precursor of α -glucosidase is transported to the lysosomes. However, the same form is secreted into the culture medium (20% of newly synthesized enzyme after 4 h of chase). Immunoprecipitation of α -glucosidase from culture medium derived from either the apical

or basolateral site of radiolabelled Caco-2 cells, showed that 70–80% of the total amount of precursor form present in the medium is secreted from the apical membrane. Measurement of enzyme activities also showed that α -glucosidase, unlike other lysosomal enzymes, is mainly secreted via the apical pathway. Furthermore, immunocytochemistry showed the presence of a precursor form of α -glucosidase on the apical, but not the basolateral, membrane of the Caco-2 cells. We conclude that α -glucosidase is, unlike all other secretory proteins studied so far, secreted preferentially from the apical membrane of Caco-2 cells.

Key words: brush border, Caco-2, immunocytochemistry, lysosomal enzymes, polarized secretion.

Introduction

All soluble lysosomal enzymes studied so far are synthesized as high molecular weight precursors on polyosomes attached to the endoplasmic reticulum, where synthesis of secretory proteins and membrane glycoproteins also occurs (for reviews, see Kornfeld, 1987; von Figura and Hasilik, 1986; Creek and Sly, 1984; Pfeffer, 1988; Dahms *et al.* 1989). During transport through the endoplasmic reticulum and the Golgi apparatus, the oligosaccharides of the precursor forms of soluble lysosomal enzymes are modified by the generation of mannose 6-phosphate moieties; this provides a signal that is recognized by mannose 6-phosphate receptors (MPRs) and leads to sorting from secretory proteins. The ligand receptor complex leaves the *trans* Golgi in a coated vesicle and is delivered to an acidified endocytotic compartment. After dissociation from the MPR, which probably occurs in a late endosomal compartment (Griffiths *et al.* 1988; Geuze *et al.* 1988), the enzyme is packaged into a lysosome. The MPR either returns to the Golgi (Duncan and Kornfeld,

1988) or moves to the plasma membrane, where it can participate in a second pathway, the endocytotic pathway. Usually, a small portion of lysosomal enzyme is secreted. MPRs present on the plasma membrane, usually accounting for 10% of the total receptor pool, mediate the specific uptake of these enzymes (Vladutiu and Ratazzi, 1979; Hickmann *et al.* 1974; Sando and Neufeld, 1977; Kaplan *et al.* 1977).

Acid α -glucosidase is a lysosomal hydrolase that hydrolyses both α 1-4 and α 1-6 glycosidic linkages of the natural substrates glycogen, maltose and isomaltose. In fibroblasts the enzyme is synthesized as a precursor of $110 \times 10^3 M_r$, which is phosphorylated on its mannose residues in the Golgi apparatus (Oude Elferink *et al.* 1985; van der Horst *et al.* 1987). Recently, we demonstrated by means of immunocytochemistry that in human enterocytes a substantial amount of this precursor form is present in the microvillar membrane (Fransen *et al.* 1988). A similar localization was found in kidney proximal tubule epithelial cells (Oude Elferink *et al.* 1989) and the human colon carcinoma cell-line HT29 (Klumperman,

unpublished results). This localization suggests the involvement of the apical plasma membrane in the routing of lysosomal enzymes to the lysosomes. Alternatively, the precursor form of α -glucosidase in the microvilli might be actively secreted from the apical domain, as has been previously suggested for lysosomal enzymes in kidney epithelial cells (Paigen and Peterson, 1978). The localization of a glycoprotein in the apical membrane is also of interest in respect of sorting of glycoproteins in intestinal epithelial cells. Rindler and Traber (1988) postulated that glycoproteins without a specific recognition signal are transported to the basolateral membrane of intestinal cells. Furthermore, all glycoproteins secreted by the enterocyte-like cell line Caco-2 have been found mainly in the basolateral medium (Rindler and Traber, 1988; Traber *et al.* 1987).

To investigate the transport of α -glucosidase, we studied the biosynthesis, the subcellular distribution and the secretion of this enzyme in the human colon carcinoma cell line Caco-2 (Fogh *et al.* 1977; Pinto *et al.* 1983). Caco-2 cells form a tight monolayer with transmonolayer resistance and exhibit enterocyte-like characteristics (Hauri *et al.* 1985; Rindler and Traber, 1988). Our work provides evidence that a precursor form of α -glucosidase is secreted into the culture medium, predominantly from the apical membrane.

Materials and methods

Caco-2 cell culture

The human colon carcinoma-derived cell-line Caco-2, kindly provided by Dr A. Zweibaum (Paris), was cultured on surfactant-free nitrocellulose membrane filters (Millipore type HA, pore size 0.45 μ m, Millipore Products Div., Bedford, USA) in mini Marbrook chambers in an atmosphere of 95% air and 5% CO₂. Some of the experiments on the polarity of the secreted enzymes were performed with Caco-2 cells cultured on Millicell chambers (Millipore Products Div.), the results obtained with this system did not significantly differ from those obtained with our system. Dulbecco's modified Eagle's medium (with 4.5 g l⁻¹ glucose) supplemented with 1% non-essential amino acids (Gibco Europe, Hoofddorp, The Netherlands), 20% heat-inactivated fetal calf serum (FCS), 50 i.u. ml⁻¹ penicillin and 50 μ g ml⁻¹ streptomycin (Flow Laboratories Inc., Canada) was used as complete medium. The complete medium was added to both the apical and basolateral sides of the cells and was changed daily. The tightness of the monolayer was tested by inducing a higher level of medium in the upper chamber. The cells were only used when this difference was maintained during 4 h. The cells were used between passage 130 and 170, 5–7 days after confluence.

Antibodies

Immunoprecipitation of α -glucosidase was carried out with monoclonal antibody 118G3, which was generated against urinary α -glucosidase essentially as described by Hilkens *et al.* (1981). Because this antibody fails to react with α -glucosidase under the conditions used for immunocytochemistry (Sips *et al.* 1985), monoclonal antibody 43D1 was used to immunolocalize α -glucosidase (Fransen *et al.* 1988). The reactivity of the different forms of α -glucosidase with this antibody was not affected by preincubation of the enzyme with *N*-glycanase to remove oligosaccharide chains; this treatment led to a loss of reactivity with concanavalin A. In addition, α -glucosidase was localized with monoclonal antibody 43G8 (Oude Elferink *et al.* 1984b). Both in Western blots and in immunoprecipitates of post-nuclear supernatants of Caco-2 cells, the 95 and 76 $\times 10^3$ M_r forms only were recognized by this antibody (not shown). All antibodies were raised against acid α -glucosidase derived from human placenta (Hilkens *et al.* 1981) and were used in the ascites form. Despite the high degree of homology between α -glucosidase and sucrase-

isomaltase (Hoefsloot *et al.* 1988) the monoclonal antibody 43D1 does not cross-react with sucrase-isomaltase in our immunocytochemical procedures. In enterocytes in human jejunal biopsies from patients with sucrase-isomaltase deficiency, which do not synthesize sucrase-isomaltase at all, the labelling obtained with 43D1 remains unaltered (Fransen, unpublished observations).

Immunolabelling and immunoprecipitation of cathepsin D were carried out with rabbit antiserum (Oude Elferink *et al.* 1985).

Metabolic labelling and immunoprecipitation

For the studies on the biosynthesis of α -glucosidase, Caco-2 cells were metabolically labelled with [³⁵S]methionine (Radiochemical Centre, Amersham, England). After preincubation for 10 min at 37°C in phosphate-buffered saline (PBS) containing 20% dialyzed FCS followed by a 1 h pulse at 37°C with 100 μ Ci [³⁵S]methionine per filter (added to the basolateral side in 300 μ l PBS/FCS), the cells were washed with PBS, cultured for various chase times in complete medium supplemented with 10 mM methionine, and rinsed twice with ice-cold PBS and once in 0.1 M phosphate buffer (pH 8). The cells were then scraped off the filter in 1 ml 100 mM Na₂HPO₄ (pH 8), 1% Triton X-100 and 40 μ g ml⁻¹ phenylmethylsulphonyl fluoride (PMSF) and homogenized by being passed 10 times through a 25G needle connected to a 1-ml syringe.

Culture medium – either total medium or, where indicated, apical and basolateral medium separately – was collected, and Triton X-100 and PMSF were added. After 1 h of solubilization on ice, the homogenates and media were centrifuged for 1 h at 100 000 g. The supernatants were immunoprecipitated (2 h, 4°C) with the antibodies absorbed to protein A–Sepharose beads (Oude Elferink *et al.* 1984a,b). After precipitation the beads were washed 4 times with a mixture of 100 mM Na₂HPO₄ (pH 8), 0.2% bovine serum albumin (BSA), 1% Triton X-100, and PMSF, twice with 100 mM Na₂HPO₄ without Triton and BSA, and once with 10 mM Na₂HPO₄.

Finally, denaturing sample buffer containing 12% (v/v) sodium dodecyl sulphate (SDS), 4% (v/v) β -mercaptoethanol, and 125 mM Tris–HCl (pH 6.8) was added to the beads and the suspension was boiled for 3 min. After centrifugation the supernatant was loaded onto a 10% SDS/polyacrylamide gel for electrophoresis. Radioactive bands were visualized by fluorography, making use of preflashed films. For quantification the bands were scanned with a LKB 2202 ultrascan laser densitometer.

Digestion with endoglycosidase H

After immunoprecipitation as described above, the beads were boiled for 5 min in 100 μ l 100 mM NaH₂PO₄ buffer (pH 6.1) containing 50 mM EDTA, 1% (v/v) Triton X-100 and 0.1% (w/v) SDS. Then the suspension was cooled to room temperature and 1% β -mercaptoethanol and a mixture of 10 μ g leupeptin, 10 μ g pepstatin and 200 μ g PMSF was added. Finally, 1 μ l endoglycosidase H (74 μ g ml⁻¹, Nenzymes) was added and the samples were incubated for 24 h at 37°C. Incubation was stopped with denaturing sample buffer and the beads were treated as described above.

Preparation of a lysosomal fraction

This isolation was carried out according to the method recently developed by Matter *et al.* (1990c). In short, homogenates of metabolically labelled Caco-2 cells were scraped in buffer B (2 ml 250 mM sucrose, 1 mM Na₂EDTA, 10 mM triethanolamine acetic acid, pH 6.5) and centrifuged for 10 min at 370 g in a SS34 rotor (Sorvall Instruments Div.). The supernatant was brought to 30 ml, 4.66 ml stock solution of iso-osmotic Percoll (density of Percoll = 1.129 g ml⁻¹, initial density = 1.048 g ml⁻¹) was added, and the gradient was centrifuged for 41 min at 36 900 g. From the bottom of the gradient, a 2 ml sample was collected and processed as follows: 2 g of this fraction was mixed with 2 g 60% (w/w) metrizamide (Nycodenz) and overlaid with 2.5 ml each of 21.5 and 14.5% metrizamide. Gradients were run for 5 h at 70 600 g, after which the 21.5 and 14.5% interphase (LII) is enriched in lysosomes. This fraction was diluted with the buffer needed for a given experiment and centrifuged at 105 000 g for 1 h.

Immunocytochemistry

Five to seven days after confluence of the cells, the filters were taken out of the chambers and rinsed 3 times in phosphate-buffered saline (PBS). The cells were then fixed in a mixture of 0.1% glutaraldehyde and 1% freshly prepared formaldehyde in 0.15 M sodium bicarbonate buffer (pH 7.4) for 1 h at room temperature. The use of sodium bicarbonate buffer gave a better preservation of glycogen (Artvinli, 1975). After fixation, the cells were washed again and gently scraped off the filter with a spatula with a rubber tip, pelleted in 10% gelatin, post-fixed and stored for at least 24 h at 4°C in 1% formaldehyde in 0.1 M phosphate buffer (pH 7.4). Ultrathin cryosections were cut on a Reichert-Jung ultracut E with cryoattachment FC 4D, and were incubated with the specific antibody. For monoclonal antibodies, a second incubation step with rabbit-anti-mouse IgG was used. The antibodies were visualized with colloidal gold particles complexed to protein A (Fransen *et al.* 1985).

Isolation of brush border membranes

The method used for the isolation of brush border membranes of Caco-2 cells has been described in detail by Stieger *et al.* (1988). In short: cells were homogenized by nitrogen cavitation, and brush border membrane vesicles were isolated by differential centrifugation in the presence of MgCl₂ and CaCl₂, successively. The pellet, P₄, was resuspended in the appropriate buffer.

Enzyme assays

To measure enzyme activities in the medium of Caco-2 cells we first inactivated enzymes present in the FCS by adding 900 μ l 5 M NaOH per 100 ml serum (30 min, 37°C), after which the pH was adjusted to 7.4 with 2 M Hepes. Caco-2 cells were grown for 24 h in medium with 20% pH-inactivated serum. For the determination of α -glucosidase activity, the reaction mixture contained 100 mM sodium acetate buffer (pH 4.0) and 0.4 mM 4-methylumbelliferyl- α -D-glucoside (Sigma Chemical Co., St Louis, MO) in a total volume of 0.5 ml. The reaction was started by adding 100 μ l medium and was stopped after 60 min by adding 2 volumes of 300 mM glycine/NaOH buffer (pH 10.6). The 4-methylumbelliferone liberated was quantified fluorometrically at an excitation wavelength of 445 nm. β -Hexosaminidase activity was assessed with 4-methylumbelliferyl-N-acetyl- β -D-glucosaminide in 100 mM acetate buffer (pH 5.0) at a final concentration of 1.6 mM. β -Glucuronidase was measured with the use of 4-methylumbelliferyl- β -glucuronide as substrate at a final concentration of 0.7 mM in 100 mM acetate buffer (pH 3.5).

Results

Biosynthesis of α -glucosidase in Caco-2 cells

For investigation of the biosynthesis of α -glucosidase, 100 μ Ci [³⁵S]methionine was added to the basolateral side of the cells for 1 h at 37°C, after which the medium was replaced and the cells were cultured for various intervals without radiolabelled methionine. The newly synthesized α -glucosidase was precipitated with antibody 118G3 conjugated to protein A-Sepharose 4B beads, and subjected to SDS-PAGE (Fig. 1).

After a 1 h pulse, a single band with an apparent M_r of 110×10^3 was observed (Fig. 1A), which after 4 h of chase was first converted into a form of approximately $112 \times 10^3 M_r$, and then *via* a $95 \times 10^3 M_r$ intermediate into a mature form of $76 \times 10^3 M_r$. After a chase period of 8 h the $110 \times 10^3 M_r$ precursor of α -glucosidase was no longer detectable in the cells and after 24 h only the intermediate and mature forms were precipitated.

Sensitivity of the precursor forms of α -glucosidase to endoglycosidase H digestion

To find out whether the conversion of the $110 \times 10^3 M_r$

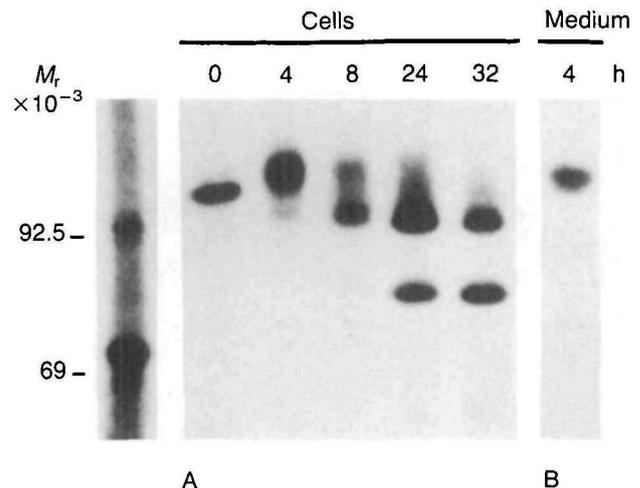


Fig. 1. Fluorograph showing the biosynthesis of α -glucosidase in Caco-2 cells. Cells were labelled for 1 h with 100 μ Ci [³⁵S]-methionine, which was added to the basolateral side. The medium was then changed and the cells were chased for the indicated intervals. The radiolabelled enzyme was precipitated with antibody conjugated to protein A-Sepharose beads and applied to SDS-PAGE. (A) α -Glucosidase is synthesized as a precursor form with an apparent M_r of 110×10^3 . After a 4 h chase the molecular weight of the precursor has increased and an intermediate form of $95 \times 10^3 M_r$ starts to appear. At longer chase times the precursor forms disappear and a mature form of $76 \times 10^3 M_r$ is formed. (B) After a 4 h chase α -glucosidase was precipitated from the total culture medium in a manner analogous to the isolation of intracellular α -glucosidase. The amount of the precursor form of α -glucosidase present in the medium after this chase interval represents 20% of the newly synthesized enzyme. M_r standards are shown on the left.

precursor into the $112 \times 10^3 M_r$ form is due to differential glycosylation of the oligosaccharide chains, we determined the sensitivity of both precursor forms to endoglycosidase H digestion. After a 1 h pulse with radiolabelled methionine we detected only the $110 \times 10^3 M_r$ precursor form of α -glucosidase, which proved to be sensitive to endoglycosidase H digestion (Fig. 2A). In cells cultured for 5 h the $112 \times 10^3 M_r$ precursor of α -glucosidase was formed. This form too was sensitive to endoglycosidase H digestion, but the shift in M_r was distinctly smaller than that seen for the $110 \times 10^3 M_r$ form, which indicates that the $112 \times 10^3 M_r$ precursor had acquired some oligosaccharide chains of the complex type.

Secretion of α -glucosidase precursor into the culture medium

To determine the total amount of α -glucosidase secreted from Caco-2 cells we precipitated the enzyme from the chase medium (apical and basolateral media taken together). Quantification of the fluorogram shown in Fig. 1B revealed that 20% of the total amount of newly synthesized α -glucosidase is present in the culture medium after a 4 h chase. To compare the secreted form with the intracellular precursors, we assessed the sensitivity of the secreted form to endoglycosidase H digestion (Fig. 2B) and found that the precursor form of α -glucosidase secreted into the medium had the same reduced sensitivity to endoglycosidase H as the intracellular $112 \times 10^3 M_r$ precursor.

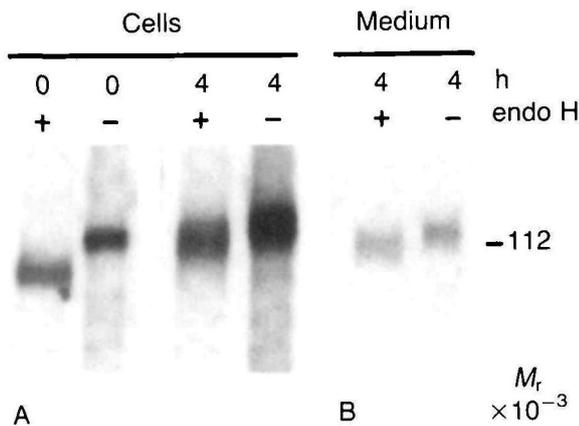


Fig. 2. Fluorograph showing the sensitivity of the precursor forms of α -glucosidase to endoglycosidase H digestion. Cells were labelled for 1 h with [35 S]methionine and chased for the indicated periods. α -Glucosidase was precipitated from both the cellular homogenate and the total medium. Some of the precipitated enzyme was incubated with endoglycosidase H during 24 h. (A) The $110 \times 10^3 M_r$ precursor form of α -glucosidase, which is present after 1 h of synthesis, is clearly sensitive to endoglycosidase H digestion, as shown by the shift of M_r . The $112 \times 10^3 M_r$ form, which is present after a 4 h chase, also shows a decrease in M_r after endoglycosidase H digestion. However, the sensitivity of the $112 \times 10^3 M_r$ precursor to endoglycosidase H is distinctly lower as observed for the $110 \times 10^3 M_r$ precursor. (B) The precursor form of α -glucosidase, which is secreted into the culture medium, shows the same sensitivity to endoglycosidase H as the intracellular $112 \times 10^3 M_r$ precursor.

Polarity of secretion of α -glucosidase

The use of filters mounted on mini Marbrook chambers allowed us to collect the apical media and the basolateral culture medium separately. In the following experiments we labelled the cells metabolically for 1 h and immunoprecipitated selectively the enzymes secreted into either the apical or the basolateral media after various time intervals. In addition to α -glucosidase we also investigated the secretion of the lysosomal enzyme cathepsin D. In Caco-2 cells cathepsin D was immunocytochemically detectable in lysosomes, multivesicular bodies and apical vesicles, but not in the microvillar membrane (not shown).

A representative fluorograph of such an experiment is given in Fig. 3. Both α -glucosidase and cathepsin D were found to be secreted from the apical and basolateral sides of the cells, but the distribution between the two media differed markedly for the two enzymes. These data were quantified by scanning fluorograms of various experiments (Fig. 4). For all chase times, α -glucosidase occurred predominantly in the apical medium. The polarity of secretion was most pronounced after 2 and 4 h of chase, after which 71 and 83%, respectively, of the total amount of secreted precursor was present in the apical medium. Cathepsin D was found to be preferentially secreted into the basolateral medium after 2 and 4 h of chase, and after longer chase periods the enzyme was found in equal amounts in the apical and basolateral media.

The secretion of α -glucosidase was strictly limited to the $112 \times 10^3 M_r$ precursor form, whereas, for cathepsin D a form with a lower M_r began to appear in the apical but not the basolateral medium after chase periods of 6 h and longer. The presence of processed forms could be due either to proteolytic degradation events after secretion or to the

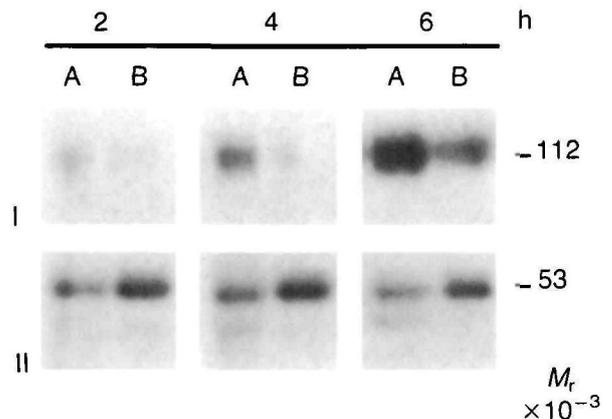


Fig. 3. Fluorograph showing the distribution of the secreted precursor forms of α -glucosidase and cathepsin D over the apical (A) and basolateral (B) media. Cells were metabolically labelled for 1 h and chased for the indicated periods. The media derived from the apical and basolateral sides of the cells were collected separately, after which the respective enzymes were precipitated. I, α -glucosidase; II, cathepsin D.

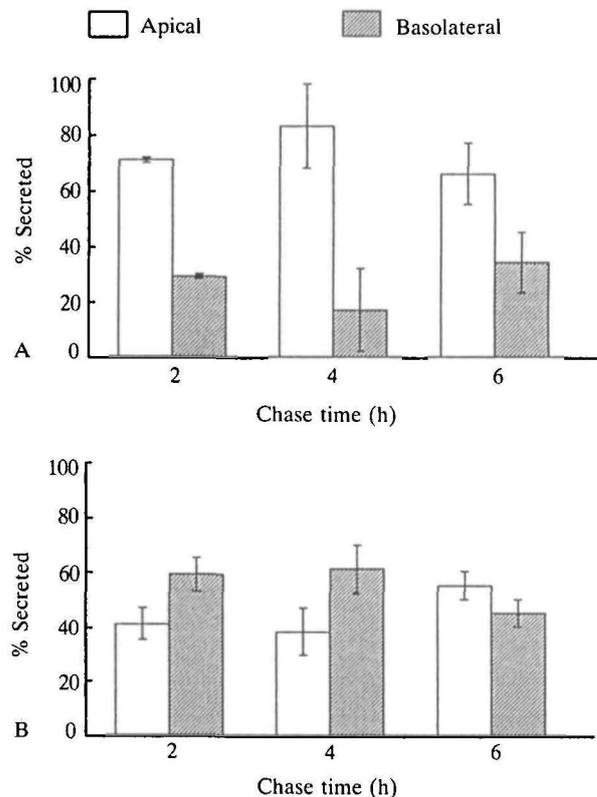


Fig. 4. Quantification of the distribution of secreted α -glucosidase and cathepsin D over the apical and basolateral media, based on scanning of fluorograms of experiments as described in Fig. 3. Each value is the mean of at least 3 experiments. After all chase periods studied, α -glucosidase was preferentially found in the apical medium. In contrast, cathepsin D occurred preferentially in the basolateral medium after a chase of 2 and 4 h and was distributed equally over the 2 media after 6 h chase. (A) α -glucosidase; (B) cathepsin D.

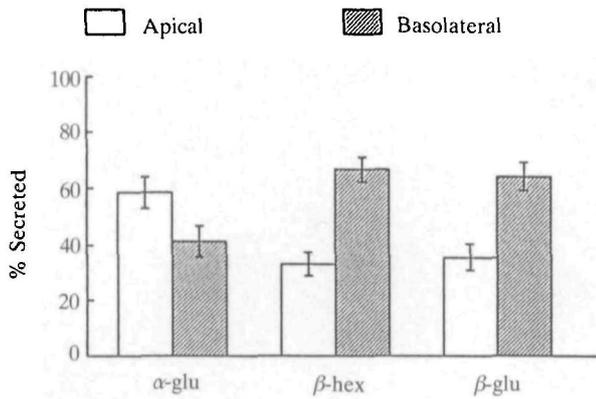


Fig. 5. Quantification of the distribution of lysosomal enzymes over the culture media derived from the apical and basolateral sides of Caco-2 cells, based on enzyme assays. The cells were cultured for 8 h in medium supplemented with pH-inactivated FCS. Enzyme assays were performed with artificial substrates conjugated to 4-methylumbelliferyl. The amount of liberated substrate was determined with a fluorimeter. Each value is the mean of 6 experiments. α -glu, α -glucosidase; β -hex, β -hexosaminidase; β -glu, β -glucuronidase. The distribution over the two media is similar for β -hexosaminidase and β -glucuronidase, which occur preferentially in the basolateral chamber. Again, most of the α -glucosidase activity was found in the apical chamber.

release of enzymes from lysosomes of exfoliated cells. Analysis of lactate dehydrogenase activity showed, however, that release into the medium during the experiments was negligible (not shown).

The distribution of secreted lysosomal enzymes over the apical and basolateral media was also assessed on the basis of enzyme activity. This approach provided additional data on the secretion of β -hexosaminidase and β -glucuronidase. Caco-2 cells were cultured for 24 h in medium with 20% pH-inactivated FCS before the enzyme assays were performed. Fig. 5 shows the results of quantification for one of these experiments. For both β -hexosaminidase and β -glucuronidase most of the activity was present in the basolateral chamber. Again, α -glucosidase showed a different distribution, with a greater proportion of the activity in the apical medium. The polarization was, however, lower than that found in the pulse-chase experiments, which is concomitant with the slight decrease in polarity of secretion after longer chase periods.

Immunocytochemical localization of α -glucosidase

Two antibodies with different specificities for the distinct molecular forms of α -glucosidase were used to localize the enzyme by immunocytochemistry. Antibody 43G8 recognizes only the processed forms of the enzyme, e.g. the $95 \times 10^3 M_r$ intermediate and the mature forms of 76 and $70 \times 10^3 M_r$ (Oude Elferink *et al.* 1984b), whereas antibody 43D1 recognizes all molecular forms of the enzyme (Fransen *et al.* 1988).

With antibody 43G8 the principal sites of labelling were the lysosomes (Fig. 6). Multivesicular bodies and small vesicles were only occasionally labelled. The microvillar and basolateral membranes and the Golgi apparatus were always devoid of label (Figs 7 and 8).

The application of antibody 43D1 led to similar labelling of lysosomes and multivesicular bodies (Fig. 9). However, additional labelling was found over the microvillar

membrane (Fig. 10) and the Golgi apparatus (Fig. 11), but not the basolateral membrane (Fig. 10). In cross-sections through the microvilli, the majority of the gold particles in the brush border were located at the extracellular side of the microvillar membrane. The amount of label in the microvilli varied considerably between cells, and there was no label at all in about 15% of the cells. This divergence was not related to any obvious changes in the morphology or the intracellular labelling of the cells. A similar heterogeneity of Caco-2 cells has been reported concerning the microvillar expression of the brush border enzymes lactase and sucrase-isomaltase (Hauri *et al.* 1985).

In view of the distinct specificities of both antibodies, the labelling of the Golgi apparatus and the microvilli must be attributable to a precursor form of α -glucosidase. However, no conclusion can be reached as to whether the label indicates the presence of either the $110 \times 10^3 M_r$ precursor or $112 \times 10^3 M_r$ precursor or both.

Precipitation of α -glucosidase from a brush border fraction of Caco-2 cells

To obtain more information about the precursor form occurring in the microvilli, we precipitated radiolabelled α -glucosidase from a brush border fraction prepared according to Stieger *et al.* (1988). This method has already been proved to be appropriate for studies on the transport of integral membrane proteins (e.g. the brush border hydrolase sucrase-isomaltase) to the microvillar membrane.

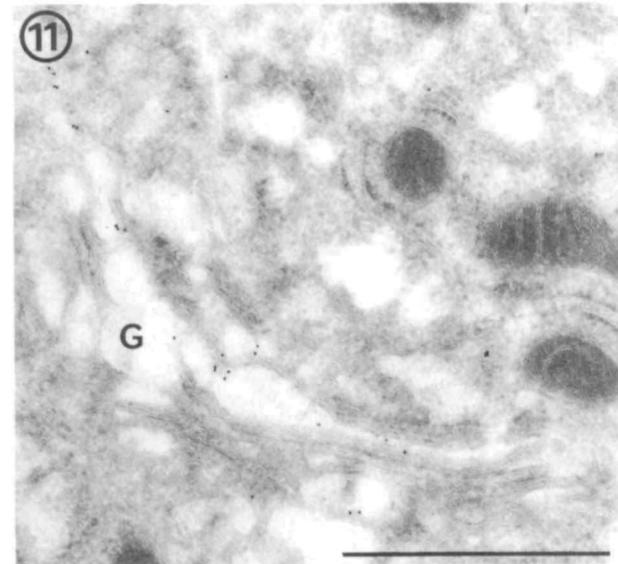
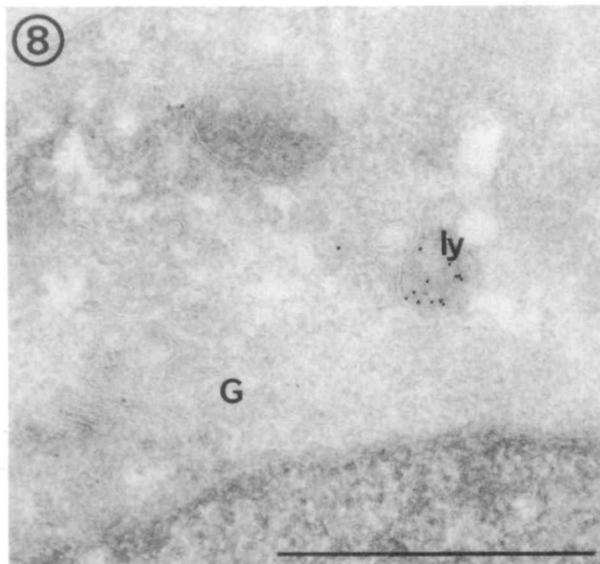
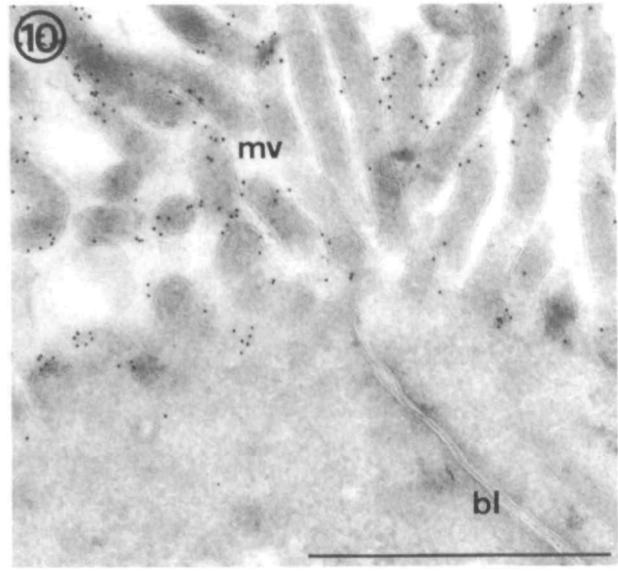
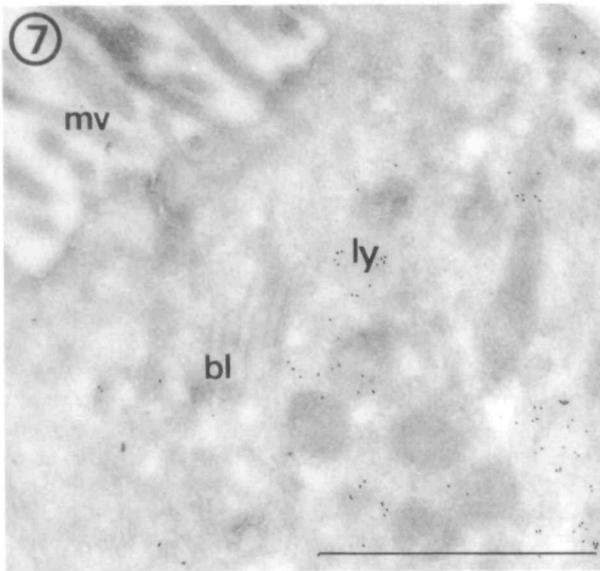
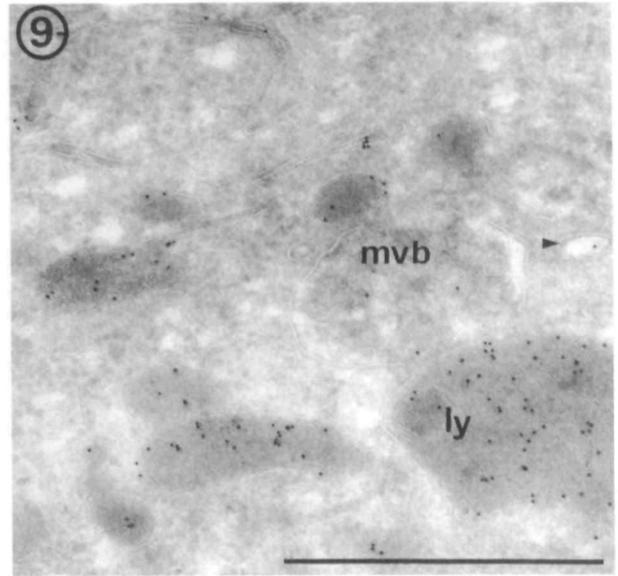
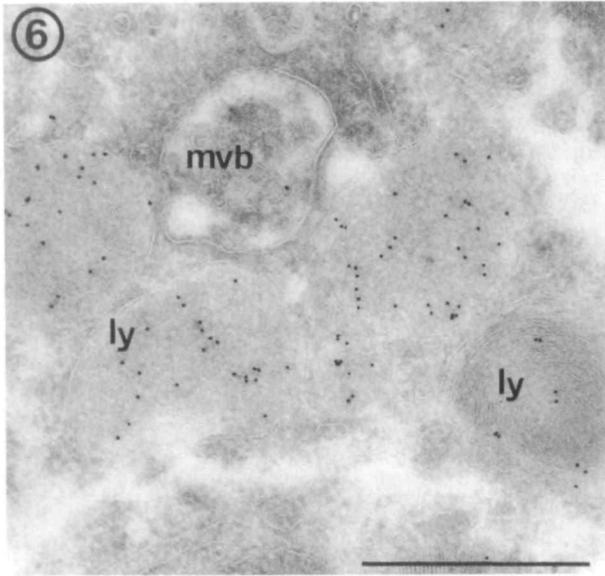
Caco-2 cells were labelled with [35 S]methionine for 2 h and then chased for 4 h. α -Glucosidase was precipitated from both the total cell homogenate and the brush border fraction and subjected to SDS-PAGE. As shown in Fig. 12, α -glucosidase was not detected in the brush border fraction.

Transport of α -glucosidase precursor to the lysosomes

To find out whether the $112 \times 10^3 M_r$ precursor form is transported to the lysosomes as well as being secreted in the medium, we combined metabolic labelling with subcellular fractionation. A procedure yielding a lysosomal fraction from Caco-2 cells was recently developed by Matter *et al.* (1990c). Caco-2 cells were pulse-labelled for 1 h with [35 S]methionine and chased for 4 h. α -Glucosidase was precipitated from both the homogenate and the lysosomal (LII) fraction. After a chase period of 4 h a major band of $112 \times 10^3 M_r$ and only a very weak band of the $110 \times 10^3 M_r$ band could be discovered in the cell homogenate (Fig. 13). The $112 \times 10^3 M_r$ band could also be visualized in the lysosomal fraction. At shorter chase times we did not obtain precipitable amounts of α -glucosidase in the lysosomal fraction. These findings indicate that in Caco-2 cells the same precursor form of α -glucosidase can be either secreted into the medium or transported to the lysosomes.

Discussion

In the study reported here we applied biochemical and immunocytochemical methods to investigate the intracellular transport of lysosomal α -glucosidase in the human colon carcinoma-derived epithelial cell line Caco-2 (Fogh, 1977; Pinto *et al.* 1983). Our findings show that in Caco-2 cells a partially complex glycosylated precursor form of α -glucosidase is transported to the lysosomes as well as



Figs 6–11. Cryosections of Caco-2 cells labelled according to the protein A–gold technique with monoclonal antibodies 43G8 and 43D1, which exhibit different specificities towards the distinct molecular forms of α -glucosidase. Bar, 0.5 μ m.

Figs 6–8 Labelling with antibody 43G8, which only reacts with the processed forms of α -glucosidase. Lysosomes are heavily labelled, whereas only a little label is seen over the multivesicular bodies (Fig. 6). The apical microvilli and the basolateral membrane are devoid of label (Fig. 7) and also in the Golgi region no processed forms of α -glucosidase can be detected (Fig. 8).

Figs 9–11. Labelling with antibody 43D1, which recognizes all forms of α -glucosidase. Again the lysosomes are the main sites of intracellular labelling (Fig. 9); little label is present on multivesicular bodies and small vesicles (arrowhead). There is also labelling of the microvilli, but not the basolateral membrane (Fig. 10), and the Golgi region (Fig. 11). mv, microvilli; ly, lysosome; mvb, multivesicular body; bl, basolateral membrane; G, Golgi region.

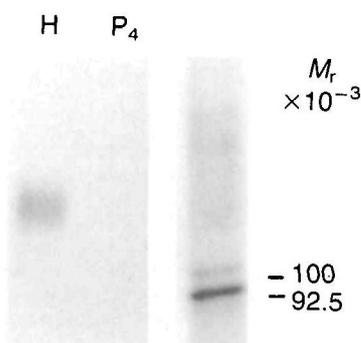


Fig. 12. Fluorograph showing the precipitation of α -glucosidase from a brush border fraction deriving from Caco-2 cells. The cells were metabolically labelled with [35 S]methionine for 2 h and chased for 4 h. Some of the labelled cells were used for the preparation of a brush border fraction (P_4) according to Stieger *et al.* (1988). α -Glucosidase was precipitated from both samples of the total cell homogenate (H) and the P_4 fraction. The fluorogram shows that although the precursor form is unequivocally in the cell homogenate, no α -glucosidase can be seen in the brush border fraction.

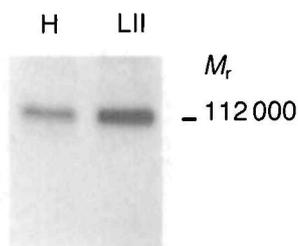


Fig. 13. Fluorograph showing the precipitation of α -glucosidase from a lysosomal (LII) fraction made of Caco-2 cells. The cells were pulse labelled with [35 S]methionine for 1 h and chased for 4 h. Percoll and metrizamide density centrifugation yielded a lysosomal fraction with a high degree of purity. α -Glucosidase was

precipitated from a sample of the cell homogenate (H) as well as from the lysosomal LII fraction. In H both the $110 \times 10^3 M_r$ and the $112 \times 10^3 M_r$ precursor forms of α -glucosidase were detected on SDS–PAGE, but in the LII fraction only the precursor form of $112 \times 10^3 M_r$ was visible.

secreted into the culture medium. This observation has already been described in our publication on the effects of nocodazole on transport routes in Caco-2 cells (Eilers *et al.* 1989). However, the differences between the amounts of precursors of lysosomal enzymes secreted in the apical and basolateral media were not quantified in that study. Here we have further characterized the secretion of lysosomal enzymes by Caco-2 cells, and noticed the preferential apical secretion of the precursor of α -glucosidase. Quantitation of the distribution of secreted enzyme between the apical and basolateral media was assessed by metabolic labelling and subsequent immunoprecipitation, and by measuring enzyme activities. Both methods showed that secretion of α -glucosidase occurs mainly from the apical membrane, whereas other lysosomal enzymes were all found to be secreted preferentially from the basolateral side of the cells.

The secretion of lysosomal enzymes has been described for a number of cultured cells (Braulke *et al.* 1987; Hasilik *et al.* 1981; Rosenfeld *et al.* 1982), but the biological significance of this phenomenon is not clear. The secretion of lysosomal enzymes by Caco-2 cells has been previously reported by Rindler and Traber (1988). Radiolabelling studies showed the secretion of β -hexosaminidase, 85 % of which was found in the basolateral medium. This finding is compatible with our results pertaining to the enzymes other than α -glucosidase, although in our system the polarity of secretion is somewhat lower. The reason for this discrepancy is not clear, but might be the use of different culture conditions. Rindler and Traber (1988) and Traber *et al.* (1987) also showed by trichloroacetic acid precipitation of samples of medium that all glyco- and lipoproteins secreted by Caco-2 cells occurred predominantly in the basolateral medium. On the basis of these results the authors postulated that in intestinal epithelial cells, 'the basolateral pathway represents a default pathway for exocytosis, not requiring a specific signal' (Rindler and Traber, 1988). The preferential apical secretion of α -glucosidase suggests therefore that this enzyme possesses a special signal that leads to its segregation into the apical pathway and the question arises of whether the transport of α -glucosidase to the apical membrane is mediated by the same unknown mechanism as that underlying the transport of brush border enzymes.

The transport of brush border enzymes to the apical membrane occurs in Caco-2 cells *via* either a direct pathway or an indirect pathway by way of the basolateral membrane (Matter *et al.* 1990a). The efficiency with which a protein is segregated into either pathway depends on the protein involved. Interestingly, we found that retardation of the apical transport by nocodazole leads to an enhanced amount of the brush border enzyme aminopeptidase N occurring on the basolateral membrane, but not to an increased secretion of α -glucosidase into the basolateral medium (Eilers *et al.* 1989; Matter *et al.* 1990b). The increased amount of aminopeptidase N at the basolateral membrane is not a result of mis-sorting induced by nocodazole (Matter *et al.* 1990b), but rather an accumulation of this enzyme at its primary delivery side (Matter *et al.* 1990a). The observations that the basolateral secretion of α -glucosidase is not increased by nocodazole, and that α -glucosidase is absent from the basolateral membrane in immunolabelled cryosections, strongly suggest that α -glucosidase is transported directly to the apical membrane. It is interesting in this respect that the brush border enzyme sucrase-isomaltase, which shows a remarkable degree of homology with α -glucosidase (Hoefs-

loot *et al.* 1988), is almost exclusively segregated into the direct apical pathway (Le Bivic *et al.* 1990; Matter *et al.* 1990a).

Cathepsin D was found primarily to be secreted preferentially into the basolateral medium, but the distribution over the two media became more equal after longer chase periods. A speculative explanation of this finding could be the incorporation of basolaterally secreted cathepsin D into the transcytotic pathway to the apical membrane (Matter *et al.* 1990a). The decrease in polarity is accompanied by the appearance of an intermediate form in the apical medium. Additional experiments are required to show whether this intermediate form is derived from an intracellular source or is the product of proteolytic activity in the culture medium. Processed forms of lysosomal enzymes are also found *in vivo* in human urine. The ratio between the amounts of the precursor form and the processed forms in urine can differ from intracellular ratios. For example, 50% of urinary α -glucosidase is in the $110 \times 10^3 M_r$ phosphorylated precursor form (Oude Elferink *et al.* 1984a). To explain this phenomenon, Kress *et al.* (1982) and also Brown *et al.* (1985) suggested that in addition to a direct pathway from the Golgi apparatus to the plasma membrane, which accounts for the secretion of precursor forms of lysosomal enzymes, there is a pathway *via* the lysosomes.

α -Glucosidase has always been considered to be a soluble lysosomal enzyme, because of its enzymological properties. Indeed, like other soluble lysosomal hydrolases, the precursor is phosphorylated (Hasilik and Neufeld, 1980a,b; Reuser *et al.* 1985) and transported *via* the MPR (Oude Elferink *et al.* 1984a,b, 1986). Preliminary experiments in our laboratory showed that the cation-independent MPR is present in the microvilli of Caco-2 cells and that the secreted precursor form is phosphorylated, although it is not yet clear whether this occurs at the carbohydrate moieties (Klumperman and Matter, unpublished results). Transport of α -glucosidase to the microvilli might therefore be mediated by the MPR, in a way similar to that found by Baron *et al.* (1985, 1988) in osteoclasts. However, culturing Caco-2 cells in the presence of weak bases or surplus mannose 6-phosphate did not affect the apical secretion of α -glucosidase or the occurrence in the microvilli (Klumperman *et al.* unpublished results). Recent data pertaining to fibroblasts suggest that the precursor form of α -glucosidase may be synthesized and transported as a membrane-associated enzyme (Tsuji and Suzuki 1987a,b, 1988). Our inability to recover the α -glucosidase precursor from brush border fractions prepared from radiolabelled Caco-2 cells indicates that the association of the enzyme with the microvillar membrane is not stable during the conditions we used for fractionation. Since integral membrane proteins such as sucrase-isomaltase and dipeptidylpeptidase IV can be recovered after the same procedure (Stieger *et al.* 1988), we must assume that the α -glucosidase precursor is not an integral membrane protein. Preliminary results from studies on the association of the α -glucosidase precursor with the membrane show that it also is not anchored *via* glycosylphosphatidylinositol, which is known to provide a signal directing proteins to the microvillar membrane in Caco-2 cells (Lisanti *et al.* 1989).

In the present study we focussed on the secretion of the $112 \times 10^3 M_r$ precursor of α -glucosidase. However, this $112 \times 10^3 M_r$ precursor is, as in fibroblasts (see Hasilik and Neufeld, 1980a,b; Oude Elferink *et al.* 1985), also transported to the lysosomes and processed *via* a $95 \times 10^3 M_r$,

intermediate form into a mature form of $76 \times 10^3 M_r$. It remains possible that some of the microvillar α -glucosidase remains in the microvilli or is endocytosed and transported to the lysosomes. The MPR-mediated (Creek and Sly, 1984) endocytosis of lysosomal enzymes is a well-studied pathway (Hickmann *et al.* 1974; Lemansky *et al.* 1987; Oude Elferink *et al.* 1986; Willingham *et al.* 1981), but it is generally viewed as a salvage mechanism of quantitatively less importance, the major portion of the enzymes being transported *via* a completely intracellular pathway (Pfeffer, 1988; Dahms *et al.* 1989; Geuze *et al.* 1985). Intracellularly we found α -glucosidase in vesicles of possible endocytotic origin and multivesicular bodies. On the basis of the immunocytochemical data alone, however, we cannot decide whether the α -glucosidase present in these organelles is derived from the microvilli or the Golgi region. It is clear that additional experiments must show whether endocytosis of α -glucosidase is an important pathway in Caco-2 cells or whether the transport to the lysosomes occurs mainly *via* an intracellular pathway.

In sum, our results show that in polarized epithelial cells an important portion of the α -glucosidase precursor is not transported to the lysosomes, but rather is incorporated into the secretory pathway to the apical membrane. Future studies will deal with the mechanisms underlying the apical secretion of α -glucosidase, the nature of its association with and dissociation from the membrane, and the pathways to the lysosomes.

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