Lysosomal α-glucosidase: cell-specific processing and altered maturation in HT-29 colon cancer cells

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We have previously described the abnormal localization of resident Golgi proteins and O-glycans in the rough endoplasmic reticulum of mucin-secreting HT-29 M6 colon cancer cells, suggesting altered protein trafficking in these cells [Egea, Francí, Gambús, Lesuffleur, Zweibaum and Real (1993) J. Cell Sci. **105**, 819–830]. In the present work, we have chosen lysosomal α-glucosidase as a reporter to examine the intracellular traffic of glycoproteins in M6 cells. We have compared the synthesis and processing of α-glucosidase in mucin-secreting M6 cells and in Caco-2 colon cancer cells, the latter resembling normal absorptive intestinal epithelium. Our results show that α-glucosidase processing and secretion is markedly delayed in M6 cells as compared with Caco-2 cells or normal fibroblasts, and this delay is caused by an accumulation of α-glucosidase precursor form in the trans-Golgi network. Furthermore, treatment of Caco-2 cells with brefeldin A led to changes in α-glucosidase maturation similar to those observed in untreated M6 cells. To determine whether altered processing occurs in other cultured cells, a panel of cancer cell lines and cultures from normal exocrine pancreas were examined. In pancreas-derived cultures, α-glucosidase showed a processing pattern different from that described until now. Only HT-29 cells and HT-29-derived subpopulations displayed a defect in α-glucosidase maturation. In conclusion, α-glucosidase processing is more diverse than has previously been described; this finding may have tissue-specific functional implications.

INTRODUCTION

HT-29 M6 cells (M6), a subpopulation of HT-29 colon cancer cells [1] selected by adaptation to 1 μM methotrexate, display a differentiated mucus-secreting phenotype [2]. Because there is extensive evidence for altered glycosylation of mucins in cancer cells [3], we have used M6 cells to examine the subcellular distribution of mucin-associated epitopes [4] and have shown that two Golgi markers, GalNAc-O-Ser/Thr and a cis-Golgi membrane-resident protein, are abnormally located in the rough endoplasmic reticulum (RER) of M6 cells, where they co-localize with classical RER markers such as protein disulphide-isomerase. In addition, the Golgi complex was disorganized and the RER cisternae were dilated. This phenotype, which was also observed in colon cancer tissues [4], is reminiscent of the effect of brefeldin A (BFA) on normal cells [5–7].

In the present work, we have chosen lysosomal acid α-glucosidase (EC 3.2.1.20) as a reporter to examine the intracellular traffic of glycoproteins in M6 cells. This enzyme was selected because its intracellular transport routes have been well characterized in normal cells and it undergoes post-translational modifications in distinct subcellular compartments [8,9]. Acid α-glucosidase is synthesized as a 110 kDa precursor which is glycosylated in the endoplasmic reticulum (ER) and Golgi apparatus. N-glycans are attached to seven potential glycosylation sites, and at least two of them are phosphorylated on mannose, creating a tag for mannose 6-phosphate-receptor (MPR)-mediated transport to lysosomes [10–12]. After transit through the Golgi apparatus, lysosomal enzymes are segregated from secretory proteins by the mannose 6-phosphate-dependent pathway and directed to lysosomes via clathrin-coated vesicles [13–15]. The maturation of the precursor form of α-glucosidase involves proteolytic processing, resulting in intermediate (95–100 kDa) and mature forms (70 and 76 kDa), the latter localizing to lysosomes [9,16]. A proportion of the precursor form is secreted to the culture medium without further processing [17,18].

Since it is not possible to maintain normal intestinal epithelial cells in culture, we have compared the synthesis and processing of α-glucosidase in mucin-secreting M6 cells with that of Caco-2 cells, the latter resembling normal absorptive intestinal epithelium [19]. Caco-2 is the only epithelial cell line in which α-glucosidase processing has been analysed in detail [18].

Our results show that the maturation of α-glucosidase is markedly delayed in M6 cells, as compared with Caco-2 cells or normal fibroblasts [8], due to a defective exit from the trans-Golgi network (TGN) to both the constitutive and the endosomal–lysosomal pathways. Using a panel of normal and neoplastic epithelial cultures, we show that different cell lines display distinct patterns of α-glucosidase processing, but only HT-29 cells and HT-29-derived subpopulations display this defective phenotype.

EXPERIMENTAL

Cell culture

HT-29, M6 and Caco-2 colon cancer cell lines were obtained from Dr. Alain Zweibaum and Dr. Thécla Lesuffleur (INSERM U178, Villejuif, France). Colon cancer cell lines WiDr, SW 1083, SK-CO-1, SW 620, SW 480, SW 1222, LS174T and ASPC-1

Abbreviations used: BFA, brefeldin A; DMEM, Dulbecco's modified Eagle's medium; endo H, endoglycosidase H; FBS, fetal-bovine serum; M6, HT-29 cells selected in 1 μM methotrexate; 2-ME, 2-mercaptoethanol; MPR, mannose 6-phosphate receptor; NPC, normal pancreas cultures; RER, rough endoplasmic reticulum; TGN, trans-Golgi network.

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pancreas cancer cell line were obtained from the tumour bank of the Ludwig Unit at Memorial Sloan-Kettering Cancer Center (New York, U.S.A.). SK-PC-1, SK-PC-3, IMIM-PC-1 and IMIM-PC-2 pancreas cancer cell lines are described in a separate paper [20]. MZ-PC-4 pancreas cancer cells were kindly provided by Dr. Alex Knuth (Nordwest Krankenhaus, Frankfurt, Germany). Normal human exocrine pancreas cultures (NPC), displaying a ductal-cell phenotype, were obtained as described elsewhere [21]. HT-29 cell populations were cultured as described previously [2,22]. Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, U.S.A.) supplemented with 20% fetal-bovine serum (FBS) (Gibco), 1% non-essential amino acids, penicillin (50 units/ml) and streptomycin (50 μg/ml). All other colon and pancreas cancer cell lines were cultured in DMEM supplemented with 10% FBS, non-essential amino acids, penicillin and streptomycin. Cultures were regularly tested for mycoplasma contamination by a DNA-hybridization method (Genprobe, San Diego, CA, U.S.A.) and only mycoplasma-free cultures were used.

Metabolic labelling and immunoprecipitation

All experiments were carried out 3–5 days after cultures reached confluence, unless otherwise indicated. Cells were metabolically labelled with Tran^{35}S-label (ICN Biomedicals, Costa Mesa, CA, U.S.A.) (100 μCi/ml). For pulse–chase experiments, cells cultured in 35 mm-diam. dishes were maintained for 30 min in methionine-free MEM containing 10% dialysed FBS, pulse-labelled for 1 h or 4 h, and chased for different time periods in DMEM supplemented with 10% FBS and 2 mM methionine. Cellular proteins were solubilized in lysis buffer [1% Triton X-100, 50 mM Tris/HCl, pH 8, 62.5 mM EDTA, 2 mM PMSF, aprotinin (1 μg/ml) and leupeptin (1 μg/ml)] for 30 min at 4 °C, and lysates were centrifuged at 10000 ***g*** for 30 min at 4 °C. Supernatants were immunoprecipitated with rabbit anti-α-glucosidase polyclonal antiserum in combination with formalin-fixed *Staphylococcus aureus* (GIBCO BRL, Gaithersburg, MD, USA). Immunoprecipitates were washed three times with 10 mM Tris/HCl (pH 7.4)/0.1% SDS/1% sodium deoxycholate/1% Nonidet P-40/150 mM NaCl, three times with 10 mM Tris/HCl (pH 7.4)/1 mM EDTA/0.5% Nonidet P-40/0.1% SDS/0.5 M NaCl, and twice with PBS. Finally, denaturing sample buffer containing 2% SDS and 5% 2-mercaptoethanol (2-ME) was added to the immunoprecipitates, and the suspension was boiled for 3 min. After centrifugation, immunoprecipitated proteins were resolved by SDS/PAGE (8% gels). Radioactive bands were revealed by fluorography. The following protease inhibitors were used: 10 mM NH_4Cl, 2 mM PMSF, 0.1 mM leupeptin, 5 μM pepstatin. BFA (0.5 μg/ml) was kindly provided by Dr. A. Takatsuki (University of Tokyo, Tokyo, Japan).

Digestion with glycosidases

Immunoprecipitates were boiled for 5 min in 0.1 M phosphate buffer, pH 6.1, containing 1% SDS and 50 mM EDTA, diluted 10-fold in 0.1 M phosphate buffer, pH 6.1, containing protease inhibitors (leupeptin 10 μg/ml, aprotinin 10 μg/ml, PMSF 1 mM, pepstatin 10 μg/ml, benzamidine HCl 100 μg/ml, soybean trypsin inhibitor 100 μg/ml), and 1% 2-ME, and divided in two portions, to one of which 2 m-units of endoglycosidase H (endo H) (Genzyme, Boston, MA, U.S.A.) was added. Samples were incubated overnight at 37 °C, the reaction was stopped with sample buffer, and immunoprecipitates were analysed by SDS/PAGE as described above. For digestion with N-glycosidase F (Boehringer Mannheim, Mannheim, Germany), immunoprecipitates were boiled for 5 min in 0.1 M phosphate buffer (pH 7)/50 mM EDTA/1% SDS and diluted 10-fold in 0.1 M phosphate buffer, pH 7, containing 0.5% Nonidet P-40, 1% 2-ME and protease inhibitors (leupeptin 10 μg/ml, aprotinin 10 μg/ml, 1 mM PMSF, pepstatin 1 μg/ml, benzamidine HCl 20 μg/ml). N-glycosidase F (1 unit) (Boehringer Mannheim) was added to one of the samples, and digestion was performed as described above. For digestions with neuraminidase from *Arthrobacter ureafaciens* (Boehringer Mannheim), immunoprecipitates were resuspended in 20 mM acetate buffer, pH 5, containing 5 mM $CaCl_2$ and 20 m-units of neuraminidase, incubated at 37 °C for 3 h, washed in 10 mM Tris/HCl, and resuspended in sample buffer.

Triton X-114 fractionation

Triton X-114 phase separation was performed as described elsewhere [23]. Briefly, cells were solubilized in 1% Triton X-114 (Sigma, St. Louis, MO, U.S.A.)/10 mM Tris/HCl (pH 7.4)/150 mM NaCl/aprotinin (1 μg/ml)/leupeptin (1 μg/ml)/2 mM PMSF. Lysates were centrifuged at 10000 ***g*** for 30 min at 4 °C and supernatants were warmed to 30 °C; 5 min later, detergent and water phases were separated by centrifugation at 2000 rev./min for 1 min at room temperature.

RESULTS

Intracellular processing of α-glucosidase in M6 cells is delayed compared with that in Caco-2 cells

After 16 h of continuous labelling, the major form of α-glucosidase immunoprecipitated from M6 cells showed a mobility corresponding to 120 kDa, typical for an early precursor. In contrast, the major components immunoprecipitated from Caco-2 cells had mobilities corresponding to 96 and 76 kDa, characteristic of the intermediate and mature forms (Figure 1A). To examine the biosynthesis and processing of α-glucosidase in greater detail, pulse–chase experiments were performed. After 1 h of pulse, a 110 kDa precursor was identified in both M6 and Caco-2 cells. In M6 cells, the apparent size of the precursor increased gradually to 120 kDa within the following 5 h of chase (Figure 1B). After 3 h, a processing intermediate was detectable in Caco-2 cells, but not in M6 cells; after 5 h, 41% and 8% of immunoprecipitated α-glucosidase corresponded to the intermediate form in Caco-2 and M6 cells, respectively (Figures 1B and 2). The different rate of α-glucosidase processing in M6 and Caco-2 cells was also demonstrated at longer chase periods. After a 4 h pulse and 24 h of chase, the precursor, intermediate and mature forms were detected in M6 cells, whereas the precursor was no longer detectable in Caco-2 cells (Figure 1C). At this point, densitometric analysis indicated that the precursor and mature forms constituted 38% and 37% of α-glucosidase in M6 cells (Figure 2), respectively, whereas in Caco-2 cells the precursor form was undetectable and the mature form represented 62% of all α-glucosidase (Figure 2). At longer chase time points (48 and 72 h) almost complete processing of α-glucosidase was observed in both cell lines.

In Caco-2 cells, a 177 kDa component was co-immunoprecipitated with the precursor form, but not with the processed molecules (Figure 1B).

Biosynthesis and glycosylation of α-glucosidase in M6 and Caco-2 cells

To characterize further the delay in maturation observed in M6 cells compared with Caco-2 cells, we studied the glycosylation of α-glucosidase in both cell lines.

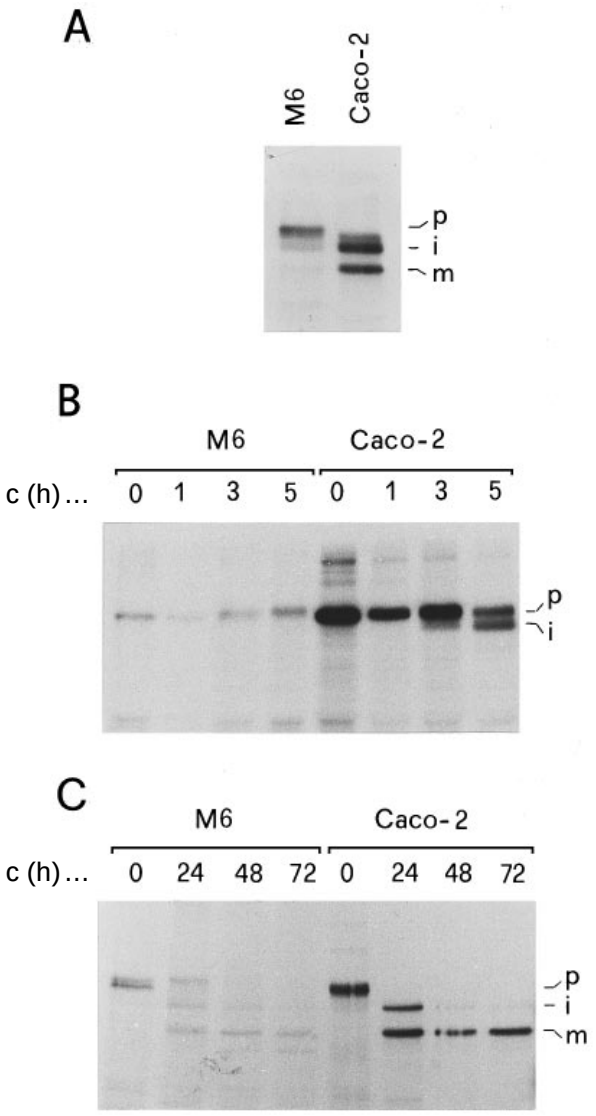

Figure 1 Biosynthesis of α-glucosidase in M6 and Caco-2 cells

Cells were labelled for 16 h (**A**), or pulse-labelled for 1 h (**B**) or 4 h (**C**) and chased (c) as indicated. Labelled cell lysates were immunoprecipitated and analysed by SDS/PAGE as described in the Experimental section. Positions of the precursor (p), intermediate (i) and mature (m) forms are indicated.

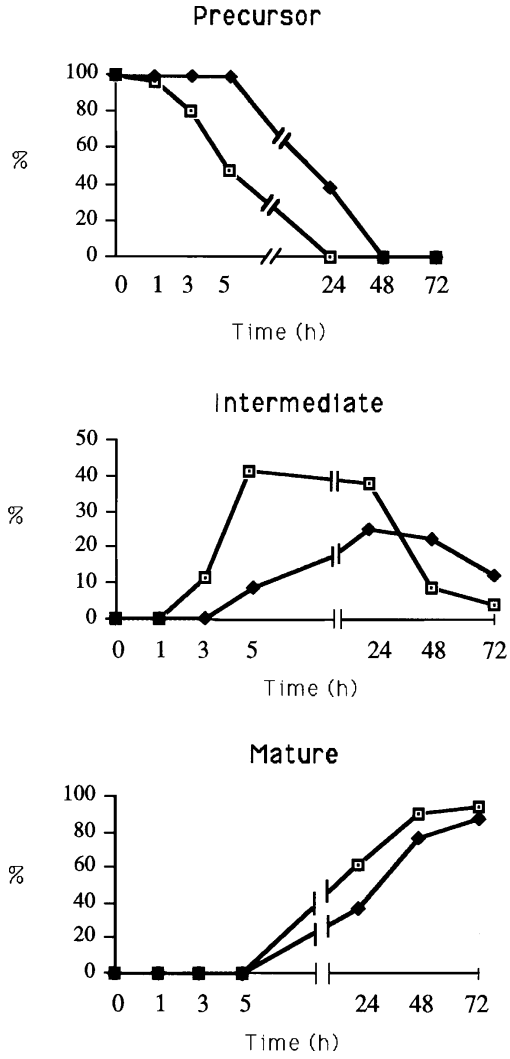

Figure 2 Densitometric analysis of α-glucosidase precursor, intermediate, and mature forms

α-Glucosidase was immunoprecipitated from cells which had been pulse-labelled and chased as shown in Figures 1(B) and 1(C). Cells: ◆, M6; □, Caco-2. Similar results were obtained in two independent experiments. % indicates the proportion of each molecular form in cell lysates, with total α-glucosidase = 100%.

The glycosylation of the three intracellular forms of α-glucosidase was analysed by treatment of the immunoprecipitates with N-glycosidase F and endo H (Figure 3). N-glycosidase F cleaves all N-glycans [24], whereas endo H cleaves only high-mannose N-glycans [25]. After a 1 h pulse, the precursor form of M6 cells was completely sensitive to endo-H treatment. All N-glycans are of the high-mannose type, since the same mobility shift was observed after N-glycosidase F or endo-H treatment. In Caco-2 cells, a minor proportion of immunoprecipitated molecules had already become partially resistant to endo H after a 1 h pulse (Figure 3B), indicating the acquisition of some complex carbohydrate chains. After 3 h of chase, the precursor form had become partially endo-H resistant in both cell lines. At this time point, there was still 15% of precursor molecules in M6 cells containing exclusively high-mannose N-glycans, but none in Caco-2 cells, indicating a slower ER–Golgi transit of α-glucosidase in M6 cells. After 6 and 24 h of chase, the intermediate and mature forms showed partial endo-H resistance in both cell types, independently of the rate of maturation. This partial endo-H resistance is due to the high-mannose-type oligosaccharide chains that carry the mannose 6-phosphate recognition marker [9].

In summary, the following three differences were observed with regard to the biosynthesis of α-glucosidase in M6 cells compared to Caco-2 cells: (1) a precursor form with slower electrophoretic mobility, (2) a delay in maturation of the precursor, and (3) a slight delay in the acquisition of partial endo-H resistance.

α-Glucosidase secretion in M6 and Caco-2 cells

To examine α-glucosidase secretion, immunoprecipitation of culture media was performed and compared with the total amount of α-glucosidase synthesized (secreted + cell-associated)

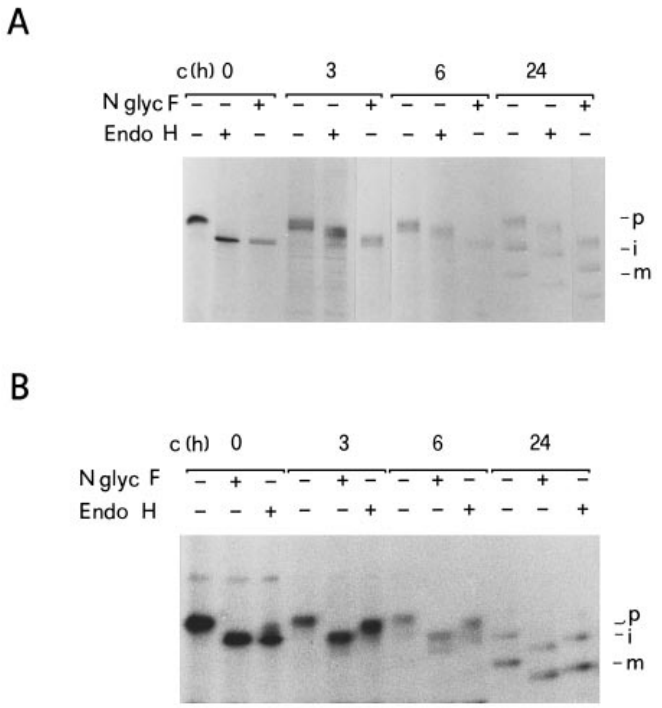

Figure 3 Biosynthesis and glucosidase digestion of α-glucosidase

Cells were pulse-labelled for 1 h and then chased (c) for the indicated time periods. Immunoprecipitates were digested overnight with 2 m-units of endo H or 1 unit of N-glycosidase F (N glyc F). Cells: (**A**) M6; (**B**) Caco-2. Migration positions of precursor (p), intermediate (i) and mature (m) forms are indicated.

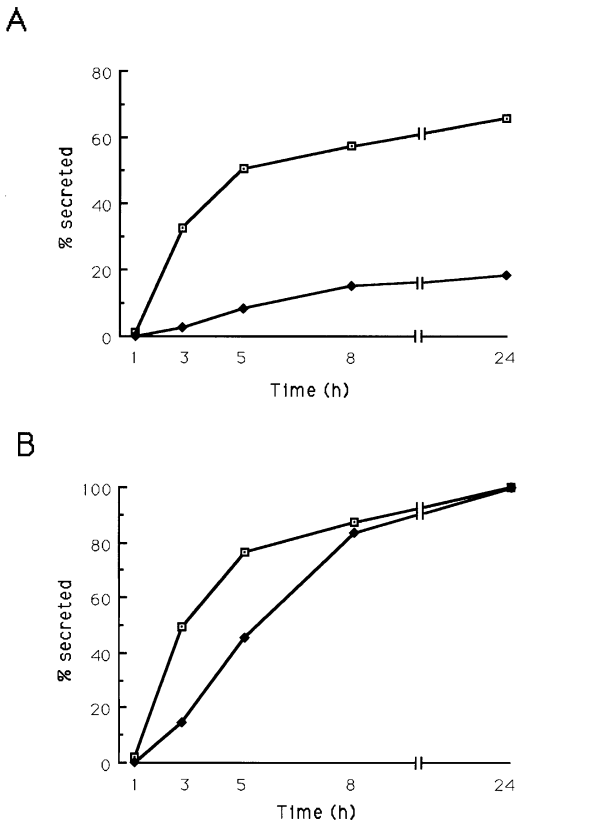

Figure 4 Densitometric analysis of α-glucosidase secretion by M6 and Caco-2 cells

Cell-associated and secreted α-glucosidase were immunoprecipitated from lysates and culture medium of cells which had been pulse-labelled for 1 h and chased for 1, 3, 5, 8 and 24 h. (**A**) Proportion of secreted α-glucosidase at each time point (calculated as 100 × secreted/secreted + cell-associated). (**B**) Secretion kinetics of α-glucosidase, taking the total amount of secreted molecules during the 24 h of chase as reference (100%). Cells: ◆, M6; ⊡, Caco-2.

by using densitometric analysis (Figure 4). There was no detectable secretion of α-glucosidase after a 1 h pulse in M6 cells; no secretion was detected after a 1 h chase, and only 2.7% of newly synthesized α-glucosidase was secreted at 3 h chase. In contrast, 1.3 and 32.5% of synthesized α-glucosidase was detected in the medium after 1 and 3 h of chase, respectively, in Caco-2 cells. For both cell types, secretion increased up to 24 h of chase; however, the proportion of α-glucosidase that was secreted by M6 cells was considerably lower than by Caco-2 cells, 18.4% versus 65.7% (Figure 4A). To compare secretion kinetics in both cell lines, the proportion of α-glucosidase secreted at each time point was calculated by taking the amount secreted during the 24 h of chase as reference (100%). As shown in Figure 4(B), secretion was slower in M6 cells than in Caco-2 cells. In both cell lines, the precursor was the only secreted form of α-glucosidase, and its molecular mass was greater in M6 cells than in Caco-2 cells (results not shown).

Proteolytic processing to the intermediate form takes place in a post-TGN compartment

To determine in which compartment processing to the intermediate form takes place in M6 cells, we took advantage of the temperature-dependence of the protein transport system: when cells are cultured at 20 °C, transport of viral glycoproteins proceeds beyond the Golgi stacks, envelope proteins acquire terminal sugars, and glycoproteins are arrested in the TGN [26–28]. M6 cells were pulse-labelled for 1 h at 37 °C and chased for 24 h either at 37 °C (Figure 5A) or at 20 °C (Figure 5B, lanes 1 and 2); at each time point neuraminidase was used to determine sialic acid acquisition. After a 1 h pulse, precursor molecules were neuraminidase-resistant (Figure 5A); during the 24 h chase at 37 °C, partial processing was demonstrated and the remaining precursor molecules had become neuraminidase-sensitive (Figure 5A). When cells were chased at 20 °C for 24 h, proteolytic processing was arrested and the precursor molecules became neuraminidase-sensitive (Figure 5B, lanes 1 and 2). When cells were pulse-labelled for the same period of time but chased for 12 h at 20 °C and for an additional 12 h at 37 °C, maturation was resumed (Figure 5B, lanes 3 and 4). In these experiments, precursor molecules remaining after 24 h of chase showed a slower electrophoretic mobility and were sensitive to neuraminidase digestion (Figure 5A and 5B), suggesting that increased sialylation is a consequence of a longer residence in distal Golgi compartments.

A similar experiment was performed with Caco-2 cells, except that shorter periods of chase were used, because, as shown above, α-glucosidase processing takes place more rapidly in these cells. After a 6 h chase at 20 °C, the precursor had not been processed (Figure 5C, lane 1), whereas an additional 6 h of chase at 37 °C resulted in its processing (Figure 5C, lane 2).

These results indicate that: (1) proteolysis of the precursor takes place beyond the TGN in both cell lines, and (2) in M6 cells the precursor form accumulates in the TGN, since its processing

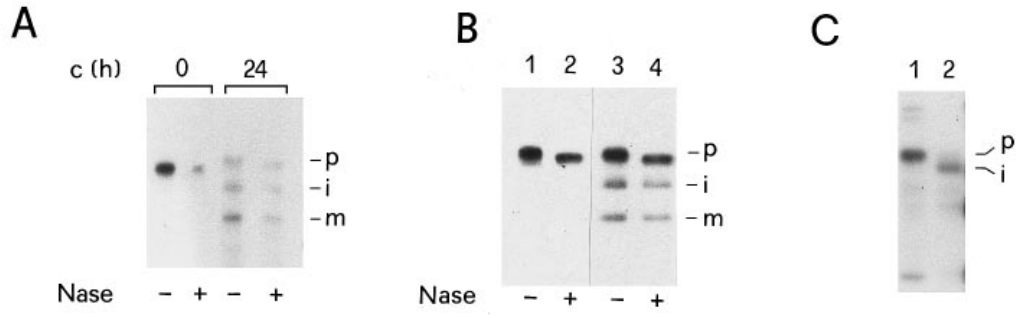

Figure 5 Effect of temperature on the processing and sialylation of α-glucosidase

(**A**) M6 cells were pulse-labelled for 1 h at 37 °C, chased (c) for the indicated time periods, and immunoprecipitated α-glucosidase molecules were digested with neuraminidase. (**B**) M6 cells were pulse-labelled for 1 h at 37 °C, then chased at 20 °C during 24 h (lanes 1 and 2) or chased for 12 h at 20 °C and for an additional 12 h at 37 °C (lanes 3 and 4). Portions of each sample were digested with neuraminidase (Nase) as indicated. (**C**) Caco-2 cells were pulse-labelled for 1 h at 37 °C and chased for 6 h at 20 °C (lane 1) or chased for 6 h at 20 °C and for an additional 6 h at 37 °C (lane 2). The mobility of precursor (p), intermediate (i) and mature (m) forms is indicated.

rate after release from the 20 °C incubation period is indistinguishable from that of M6 cells continuously cultured at 37 °C.

Effect of ammonia and protease inhibitors on the processing of α-glucosidase in M6 cells and Caco-2 cells

To determine which steps of α-glucosidase proteolysis take place in acidic compartments, we have examined the effect of ammonia and protease inhibitors on its processing. NH_4Cl did not affect the synthesis of the precursor form in M6 cells, but it completely inhibited its processing as detected after 24 h of chase. In Caco-2 cells, an incomplete processing of the precursor form was observed at 24 h of chase in the presence of NH_4Cl: a broad band with a molecular mass ranging from 95 to 100 kDa was detected. No clear-cut effect on secretion was observed. In both cell lines, leupeptin inhibited the conversion of the 95 kDa intermediate into the mature form without impairing secretion. PMSF and pepstatin did not have any effect on α-glucosidase processing or secretion in either cell line (results not shown). These results indicate that processing of α-glucosidase precursor takes place in acidic compartments and that proteolysis of the intermediate form is dependent on thiol proteases.

Membrane association of α-glucosidase

Because α-glucosidase is mainly routed to the lysosomes, but is also targeted to the cell membrane and secreted [9,18], we analysed its membrane association in M6 and Caco-2 cells using Triton X-114 fractionation [23]. After a 1 h pulse, the precursor form was detected in both the aqueous and detergent phases; after a 24 h chase, a proportion of precursor molecules remained membrane-associated in M6 cells, whereas the intermediate and mature forms were exclusively detected in the aqueous phase. The 177 kDa component co-immunoprecipitated with precursor molecules in Caco-2 cells distributed in the aqueous phase (results not shown).

BFA treatment induces changes in α-glucosidase processing that are reminiscent of the pattern observed in untreated M6 cells

Since the immuno-ultrastructural alterations observed in M6 cells [4] are similar to those induced by BFA on normal cells, we examined the effect of BFA on the processing of α-glucosidase in Caco-2 cells.

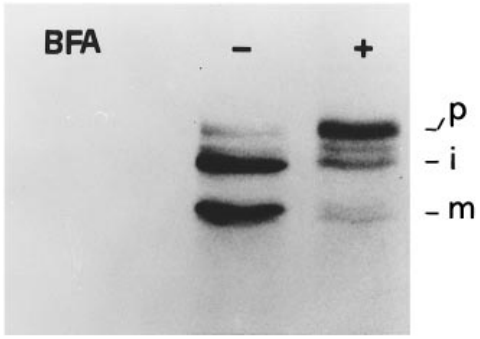

Figure 6 Effect of BFA on the biosynthesis of α-glucosidase

Caco-2 cells were continuously labelled for 24 h in the absence or presence of BFA (0.5 μg/ml). The migration of precursor (p), intermediate (i) and mature (m) forms is indicated.

Caco-2 cells were continuously labelled for 20 h in the presence or in the absence of BFA (0.5 μg/ml) (Figure 6) and compared with untreated M6 cells labelled for the same period of time (Figure 1A). Treatment of Caco-2 cells with BFA induced: (1) an accumulation of a precursor form with a lower electrophoretic mobility than that of control cells, (2) a marked decrease in processing to the mature form, and (3) the appearance of an additional component of approx. 100 kDa. This pattern of processing is similar to that observed in untreated M6 cells (Figure 1A). BFA did not affect the distribution of the precursor form after Triton X-114 fractionation (results not shown).

Different cell types display distinct patterns of α-glucosidase processing

To determine whether the delayed processing observed in mucin-secreting M6 cells is a more general phenomenon occurring in other epithelial cells, we examined α-glucosidase processing in a panel of colon and pancreas cancer cell lines and in normal exocrine pancreas cultures (NPC).

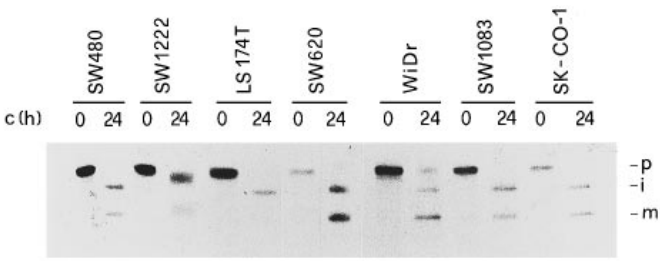

Figure 7 Biosynthesis of α-glucosidase in cultured colon cancer cells

After a 1 h pulse, cells were chased for 24 h and used for immunoprecipitation. The migration of precursor (p), intermediate (i) and mature (m) forms is indicated.

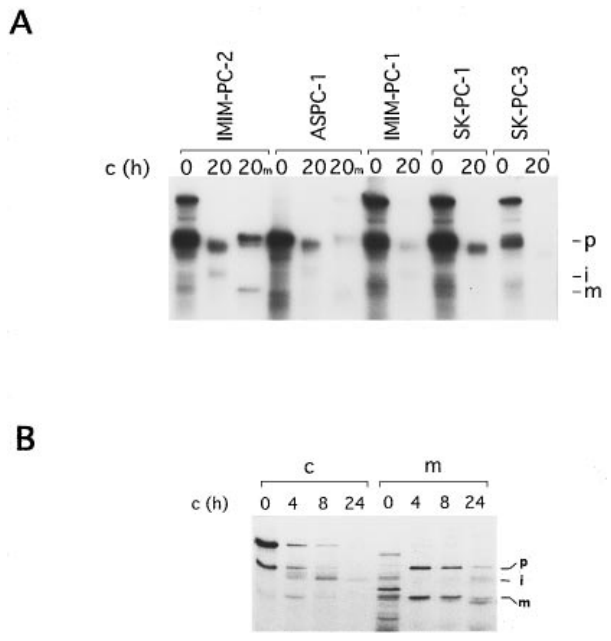

Figure 8 Biosynthesis and secretion of α-glucosidase in exocrine pancreas cultures

(**A**) Pancreas cancer cells. Cells were labelled for 1 h, chased for 20 h, and used for immunoprecipitation. In some cases, α-glucosidase was immunoprecipitated from the culture medium (m). Bars indicate the migration of precursor (p), intermediate (i) and mature (m) forms. (**B**) Normal exocrine pancreas cultures. Cells were labelled for 1 h and chased for the indicated time periods. Cells (c) and culture media (m) were used for immunoprecipitation. Cell integrity was monitored in all experiments. The migration of precursor (p), intermediate (i) and mature (m) forms is indicated. Similar results were obtained in two independent experiments using different primary NPC.

Figure 7 shows the results of immunoprecipitation assays after a 1 h pulse and 24 h chase, using seven independent colon cancer cell lines. In SW 1222 cells, a component of approx. 100 kDa accumulates after 24 h of chase, which may correspond to an intermediate form and which is partially processed to an 80 kDa mature form. In LS174T cells, the precursor form is completely processed to the intermediate form, but the mature form is undetectable. SW 480, SW 620, SW 1083 and SK-CO-1 cells displayed a maturation pattern similar to that of Caco-2 cells. The pattern observed in WiDr cells (Figure 7) was very similar to that of M6 cells. However, it must be noted that WiDr may indeed be the same cell line as HT-29 (American Type Culture Collection, Rockville, MD, U.S.A.; Catalogue 1988). Parental HT-29 cells and other HT-29-derived subpopulations selected by adaptation to 1 mM methotrexate (M3) or to 10 μM 5-fluorouracil showed the same α-glucosidase processing pattern as M6 cells (results not shown).

The results obtained with pancreas cancer cell lines and NPC are shown in Figure 8. In cancer cells (Figure 8A), a precursor of approx. 115 kDa was detected after a 1 h pulse. After 20 h of chase, a major component of approx. 100 kDa was immunoprecipitated from IMIM-PC-2, ASPC-1, IMIM-PC-1 and SK-PC-1 cells. This molecule is likely to correspond to a partially processed form of α-glucosidase. In addition, a weak band of approx. 72 kDa was immunoprecipitated from IMIM-PC-2, ASPC-1 and IMIM-PC-1 cell lysates. With regard to secretion, the precursor and a 63 kDa component were immunoprecipitated from the culture medium of IMIM-PC-2 and ASPC-1 cells.

The processing of α-glucosidase in pancreas cancer cells was compared with that of normal exocrine pancreas (NPC), since these cells can be consistently maintained in culture for up to 4 weeks [21]. In NPC cell lysates, the precursor form of α-glucosidase was detected after a 1 h pulse (Figure 8B, lane 1), and the intermediate and mature forms were detected after a 4 h chase, earlier than in any other cell type studied. Interestingly, after 8 h of chase the predominant form detected was the intermediate (Figure 8B). Secretion of α-glucosidase was more prominent in NPC than in other cell types studied, and both the precursor and mature form were secreted (Figure 8B). Cell integrity was monitored, and these findings did not result from cell damage. Therefore, the processing and secretion of α-glucosidase in NPC are different from what has been described in any other cell type.

A protein with an apparent molecular mass of 166 kDa was co-immunoprecipitated with precursor α-glucosidase from NPC cell lysates, but not from culture media. A component of the same apparent molecular mass was also detected in four out of five cancer pancreas cell lines (Figure 8A, lanes 1, 7, 9, 11). Since α-glucosidase, but not this protein, was detected by Western blotting with the same antiserum, the 166 kDa component may represent a protein associated with α-glucosidase precursor.

DISCUSSION

In this paper we show that α-glucosidase is processed differently in a variety of cultured epithelial cells, both normal and neoplastic. This diversity has not previously been recognized, and it may have a currently unknown functional significance.

Previous studies of α-glucosidase processing have mainly used Caco-2 colon cancer cells [18], normal human fibroblasts [8], fibroblasts from patients with type II glycogenosis [8], and COS cells transiently transfected with the α-glucosidase cDNA [9]. The studies described here demonstrate two types of differences in α-glucosidase processing: (1) the appearance of novel intermediates and secreted forms, occurring mainly in cultures derived from pancreatic epithelium, and (2) changes in the rate of processing without affecting the intermediates.

In NPC, α-glucosidase processing was faster, and precursor, as well as mature molecules, were secreted into the culture medium. In addition, novel intermediates were detected in cell lysates of pancreas cancer cells. Whether this pattern of α-glucosidase secretion is related to the secretory nature of exocrine pancreatic epithelium is currently unknown. More work is necessary to establish the mechanisms involved in the processing and secretion of α-glucosidase in this cell type.

In another group of lines, those derived from HT-29 colon cancer cells, the major finding was a marked delay in α-

glucosidase processing and secretion. The approximate half-life of the α-glucosidase precursor in Caco-2 cells and normal fibroblasts ranges from 2 to 5 h. In contrast, its half-life in HT-29 cells and its derivatives is > 12 h. However, the processing intermediates did not differ in apparent molecular mass and glycosylation pattern from those of normal fibroblasts or Caco-2 cells. The changes in α-glucosidase processing do not seem to be related to the mucin-secreting phenotype, since the same processing pattern was observed in M6 cells, in undifferentiated parental HT-29 cells, and in differentiated M3 cells which synthesize very low levels of mucins [22].

The cause of the delay in α-glucosidase processing was studied in greatest detail in M6 cells, because we have previously demonstrated in them an abnormal distribution of Golgi markers in the RER [4], a finding which could reflect alterations in the intracellular traffic of glycoproteins. Indeed, a slight delay in the acquisition of partial endo-H resistance was demonstrated in M6 cells as compared with Caco-2 cells. However, there are stronger indications that in M6 cells the precursor accumulates in the TGN. First, incubation of M6 and Caco-2 cells at 20 °C results in an accumulation of the precursor form, indicating that its processing takes place beyond the TGN. Furthermore, once the temperature is shifted to 37 °C, the rate of processing of the precursor is slower in M6 than in Caco-2 cells, as occurs when cells are continuously maintained at 37 °C. Second, the higher molecular mass of the precursor form that accumulates in M6 cells chased for 24 h at 37 °C, or after culture at 20 °C, is due to increased sialylation, which can be explained as the result of a longer stay in the TGN. Third, treatment of M6 cells with NH_4Cl results in a blockade of processing to the intermediate form, indicating the participation of acidic compartments in this step. Finally, the secretion kinetics is slower in M6 than in Caco-2 cells, indicating that the delay affects both the constitutive and late-endosome–lysosome pathways. These results support the contention that exit from the TGN is impaired in M6 cells, leading to precursor accumulation.

Which mechanisms may account for this phenomenon? Our previous immuno-ultrastructural observations led us to propose a BFA-like phenotype in M6 cells [4]. This hypothesis is supported by the observation that, in Caco-2 cells, BFA induced changes in α-glucosidase processing that are similar to those observed in untreated M6 cells. Although the main described effect of BFA is blocking ER-to-Golgi transport [5–7], effects on the distal compartments of the exocytic pathway have also been documented: (1) BFA causes TGN-derived membrane tubules to fuse with early endosomes to form an extensive TGN–early-endosomal network [29]; (2) a 5-fold increase in the cell surface levels of MPR has been demonstrated in BFA-treated cells [29], and it has been proposed that BFA impairs recycling of receptors to the TGN [30]; and (3) traffic from TGN to early endosomes is carried out by clathrin-coated vesicles [13,31] and BFA inhibits formation of the clathrin coat *in vitro* [32]. Although we have not observed morphological abnormalities of the TGN in M6 cells, immuno-electron microscopy may be of help to unravel more subtle alterations in this compartment.

The mechanisms involved in TGN–lysosome traffic have not been fully characterized. Rab9, a *ras*-like GTPase, may be involved in the recycling of MPRs between endosomes and the TGN *in vivo* [33]. Stable expression of a rab9 dominant negative mutant strongly inhibited delivery of the lysosomal enzyme cathepsin D to lysosomes, due to impaired MPR recycling to the TGN. Recently, a role for phosphatidylinositol 3-kinase in the sorting and transport of lysosomal enzymes has been proposed on the basis of the effects of wortmannin on cathepsin D processing and secretion [34,35].

Further work is necessary to determine whether up-regulation of MPR cell-surface levels, impaired clathrin-coated vesicle formation, or mutations in rab9 or phosphatidylinositol kinases, may contribute to the abnormalities in processing described above.

Altered processing of mutant lysosomal α-glucosidase has been described in COS cells transfected with cDNAs isolated from patients with glycogen storage disease type II. However, in this case complete processing to the mature form could not be demonstrated (A. J. J. Reuser, unpublished work). This finding argues against mutations being the cause of the delayed processing observed in M6 cells.

The use of a variety of cultured cells has also allowed the identification of proteins of approx. 166–177 kDa which were detected in immunoprecipitates of Caco-2 cells, NPC and pancreas cancer cell lines. These molecules, which have not previously been reported to be co-immunoprecipitated with α-glucosidase, were only detected in association with the precursor form, suggesting that they may participate in its transport and/or processing. Their molecular mass was slightly different in a variety of cell lines, and their relatedness is currently being analysed.

The results described here show an extensive flexibility in the post-translational modification of a lysosomal enzyme, i.e. the variability of the molecular phenotype brought about by influences of the intracellular environment. It remains to be investigated which are the functional implications of these cell-type-specific features of lysosomal enzyme processing, as well as their contribution to the expression of the disease phenotype.

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