Expression and routing of human lysosomal α -glucosidase in transiently transfected mammalian cells

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Previously isolated lysosomal α -glucosidase cDNA clones were ligated to full-length constructs for expression *in vitro* and in mammalian cells. One of these constructs (pSHAG1) did not code for functional enzyme, due to an arginine residue instead of a tryptophan residue at amino acid position 402. The mutation does not affect the rate of enzyme synthesis, but interferes with post-translational modification and intracellular transport of the acid α -glucosidase precursor. Using immunocytochemistry it is demonstrated that the mutant precursor traverses the endoplasmic reticulum and the Golgi complex, but does not reach the lysosomes. Pulse-chase experiments suggest premature degradation. The Trp-402-containing enzyme (encoded by construct pSHAG2) is processed properly, and has catalytic activity. A fraction of the enzyme is localized at the plasma membrane. It is hypothesized that membrane association of the acid α -glucosidase precursor, as demonstrated by Triton X-114 phase separation, is responsible for transport to this location. Transiently expressed acid α -glucosidase also enters the secretory pathway, since a catalytically active precursor is found in the culture medium. This precursor has the appropriate characteristics for use in enzyme replacement therapy. Efficient uptake via the mannose 6-phosphate receptor results in degradation of lysosomal glycogen in cultured fibroblasts and muscle cells from patients with glycogenosis type II.

INTRODUCTION

Human acid α -glucosidase (glucan 1,4- α -glucosidase; EC 3.2.1.3) is a typical lysosomal enzyme with substrate specificity for glycogen, maltose and isomaltose (Rosenfeld, 1975). The enzyme is produced as a glycosylated 110 kDa precursor, and is routed from the endoplasmic reticulum through the Golgi complex to the lysosomes (Hasilik & Neufeld, 1980a; Reuser *et al.*, 1985). Phosphorylation of the precursor has been demonstrated (Hasilik & Neufeld, 1980b; Reuser *et al.*, 1985) and transport of the enzyme to the lysosomes is considered to be mediated by the mannose 6-phosphate receptor. The proteolytic processing of the acid α -glucosidase precursor involves removal of both N- and C-terminal peptides of significant length (Hoefsloot *et al.*, 1988) and leads to the formation of mature enzymes of 76 and 70 kDa. A molecular species of 95 kDa has been identified as a processing intermediate (Hasilik & Neufeld, 1980a; Oude Elferink *et al.*, 1985; Reuser *et al.*, 1985).

About a dozen different defects have been discovered in the synthesis, the intracellular transport and the function of mutant acid α -glucosidase in cells from patients with an inherited enzyme deficiency (Beratis *et al.*, 1983; Reuser *et al.*, 1987). This condition is known as glycogenosis type II, and leads to fatal heart and skeletal muscle dysfunction (Hers, 1963).

Cloning of acid α -glucosidase cDNA has led to the unexpected finding that lysosomal α -glucosidase has probably arisen from the same ancestral gene as sucrase and isomaltase (Hoefsloot *et al.*, 1988). The latter two enzymes are derived from a single 260 kDa precursor by proteolytic cleavage. The enzyme complex is anchored in the apical membrane of intestinal epithelial cells via the uncleaved signal peptide of prosucrase-isomaltase (Hunziker *et al.*, 1986; Semenza, 1989).

In this study we report on the expression of full-length acid α -glucosidase cDNA constructs *in vitro* and in transfected mam-

malian cells. Immuno-electron microscopy and biochemical analyses were combined to investigate the intracellular routing and processing of acid α -glucosidase. Furthermore, the possible use of cDNA-encoded acid α -glucosidase for enzyme replacement therapy was tested with fibroblasts and muscle cells from patients with glycogenosis type II.

EXPERIMENTAL

Construction of full-length cDNA

Standard techniques were used (Sambrook *et al.*, 1989) to construct full-length cDNA clones coding for human acid α -glucosidase in the *EcoRI* site of the eukaryotic expression vector pSG5 (Green *et al.*, 1988). The unique *StuI* site present in acid α -glucosidase cDNA clones (Hoefsloot *et al.*, 1988) was used to generate pSHAG1 and pSHAG2 (see Fig. 1). pSHAG5 and pSHAG6 were constructed by exchanging the *SmaI* fragments of pSHAG1 and pSHAG2 (Fig. 1).

DNA sequence analysis

Double-stranded pSHAG1 or pSHAG2 DNA was used as a template. Oligonucleotides complementary to the cDNA were used to prime sequence reactions with the Sequenase sequencing kit according to the specifications of the manufacturer (USB, Cleveland, Ohio, U.S.A.).

RNA transcription and translation

Plasmid DNA (1 μ g), linearized with *BglII*, was used as a template in a transcription reaction with 3 units of T7 polymerase (Boehringer, Mannheim, Germany), 10 mM-dithiothreitol, 1 unit of RNAase inhibitor (Promega, Madison, WI, U.S.A.), 0.8 mg of BSA/ml, 500 μ M-ATP, 500 μ M-CTP, 500 μ M-UTP, 50 μ M-GTP and 500 μ M-dGpppG in a buffer with 40 mM-Tris/HCl

Abbreviation used: MU, 4-methylumbelliferone.

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(pH 7.5), 6 mM-MgCl₂ and 2 mM-spermidine (Melton *et al.*, 1984). The reaction was carried out for 60 min at 40 °C, whereafter the template was digested by addition of 1 unit of RNAase-free DNAase (Boehringer) per µg of DNA. Nucleotides were removed by Sephadex G50 filtration (spin-column), and the RNA concentration was measured. Typically, 0.2 µg of RNA was used for translation *in vitro* in a rabbit reticulocyte lysate system (Promega), containing [³⁵S]methionine (sp. radioactivity 1130 Ci/mmol; Amersham International, Amersham, Bucks., U.K.) in the presence or absence of canine pancreatic microsomes (Amersham), according to the protocol provided by the manufacturer. Translations were analysed by electrophoresis on a 12% polyacrylamide gel with 1% cross-linking in the presence of SDS as described by Laemmli (1970). After fixation and drying, gels were exposed to Kodak XAR films for 1–3 days.

Transfection procedures

Cells were cultured routinely in Dulbecco's modified Eagle's medium, supplemented with antibiotics and 10% fetal calf serum, under air/CO₂ (9:1). Acid α-glucosidase in the serum was inactivated by incubating the serum for 4 h at 37 °C and pH 10, followed by neutralization.

Monkey kidney COS-1 cells were transfected using the DEAE-dextran method as described (Oshima *et al.*, 1988). In short, 2 × 10⁵ cells in 3.5 cm tissue-culture dishes were transfected with DNA, at a final concentration of 20 µg of DNA/ml of culture medium, in the presence of DEAE-dextran (200 µg/ml; approx. 500 kDa; Pharmacia, Uppsala, Sweden). After 16 h the cells were incubated for 3 h with 100 µM-chloroquine and harvested with trypsin at different time points, or they were used for pulse labelling.

HeLa cells were transfected using a modification of the calcium phosphate method (Graham & van der Eb, 1973). In short, 3 µg of DNA was added to 2 × 10⁵ cells in 3.5 cm tissue-culture dishes as a calcium phosphate precipitate. After 16 h the cells were treated with 30% (v/v) dimethyl sulphoxide for 30 min and then harvested with trypsin at the desired time.

Measurement of acid α-glucosidase activity

Cell lysates were prepared by repeated freezing and thawing of cell pellets in water, and debris was pelleted by spinning for 15 min at 10000 g. The acid α-glucosidase activity in culture media and cell lysates was measured using the artificial substrate 4-methylumbelliferyl-α-D-glucopyranoside as described (Galjaard, 1980) and expressed as nmol of 4-methylumbelliferone (MU) formed per hour per mg of protein. The protein concentration of cell lysates was determined according to Lowry *et al.* (1951).

Pulse-labelling experiments

Cells were transfected as described above. After 48 h the cells were pre-incubated for 1 h in leucine-free medium. This medium was replaced with leucine-free medium to which [³H]leucine (190 Ci/mmol; Amersham) had been added at a final concentration of 75 µCi/ml. The cells were pulse-labelled for 3 h, and then harvested directly or after a subsequent chase of 16 h. Acid α-glucosidase was immunoprecipitated from cell extracts and media with a polyclonal antibody preparation against human acid α-glucosidase, and analysed by polyacrylamide-gel electrophoresis in the presence of SDS as described previously (Reuser *et al.*, 1985).

Triton X-114 phase separation

For assessment of membrane association of acid α-glucosidase, COS cells were harvested 96 h after transfection by scraping, and

washed twice in phosphate-buffered saline (10 mM-sodium phosphate/150 mM-NaCl, pH 7.2). Cells were solubilized in a buffer containing 10 mM-Tris/HCl (pH 7.4), 150 mM-NaCl, and 0.6% Triton X-114 at 0 °C. Proteins were separated into hydrophilic (water phase) and membrane-associated (detergent phase) fractions as described (Bordier, 1981).

Acid α-glucosidase was immunoprecipitated from the detergent phase and from the water phase with rabbit anti-(human acid α-glucosidase) antibodies. Different molecular forms of acid α-glucosidase were separated by PAGE in the presence of SDS (Laemmli, 1970), and blotted on to nitrocellulose (Towbin *et al.*, 1979). Acid α-glucosidase was visualized using polyclonal rabbit anti-(human acid α-glucosidase) antibodies in combination with ¹²⁵I-labelled protein A, as described earlier (Reuser *et al.*, 1987). Blots were exposed to Kodak XAR films for 1–3 days.

Immunocytochemistry

For application of light microscopy, the cells were seeded at low density on cover slips on the evening before analysis, and were prepared for immunocytochemistry as described previously (Van Dongen *et al.*, 1984). Incubations were performed with mouse polyclonal antibodies against human acid α-glucosidase (de Jonge *et al.*, 1985) and rabbit polyclonal antibodies against β-hexosaminidase (Reuser *et al.*, 1985). Immune complexes were visualized with goat anti-(mouse IgG) conjugated to fluorescein and goat anti-(rabbit IgG) conjugated to rhodamine.

For immuno-electron microscopy, cells were harvested by scraping and fixed in 1% acrolein and 0.4% glutaraldehyde. Cell pellets were embedded in gelatin. Ultra-thin cryosections were immunostained by incubation with rabbit polyclonal antibodies against human acid α-glucosidase, followed by an incubation with 10 nm colloidal gold coupled to goat anti-(rabbit IgG) (Jansen Pharmaceutica, Beerse, Belgium) as described before (Van Dongen *et al.*, 1985).

For demonstration of glycogen in transmission electron microscopy, cells were fixed after 4 weeks of confluency as described (De Bruijn, 1973) and embedded in epon.

Miscellaneous

For assessment of mannose 6-phosphate receptor binding, transfected cells were harvested by scraping and resuspended in 10 mM-sodium phosphate buffer (pH 7.2) containing 150 mM-NaCl, with or without 10 mM-mannose 6-phosphate. They were washed three times in the same buffer, and prepared for immuno-electron microscopy as described above.

Acid α-glucosidase was isolated from bovine testis and human placenta as described previously (Reuser *et al.*, 1985).

RESULTS

Translation *in vitro*

cDNA constructs containing the complete coding sequence for human acid α-glucosidase (Fig. 1) were cloned in the eukaryotic expression vector pSG5 (Green *et al.*, 1988). mRNA was synthesized from the T7 promoter in front of the cDNA insert, and used to direct protein synthesis in a reticulocyte translation system. Fig. 2 shows the products obtained by transcription and translation of pSHAG2 (sense) and pSHAG2a (anti-sense). Only translation of sense mRNA led to protein production. The proteins formed in the absence and in the presence of microsomes corresponded in their molecular masses to the previously identified unglycosylated and glycosylated precursors of acid α-glucosidase obtained by translation *in vitro* of total RNA isolated from human fibroblasts (Van der Horst *et al.*, 1987). Identical results were obtained with the other cDNA constructs depicted in Fig. 1.

Fig. 1. Acid α -glucosidase cDNA clones and constructs

Depicted are cDNA clones (3.1 kb, 3.3 kb and 2.6 kb) (Hoefsloot *et al.*, 1988) and constructs in pSG5 (pSHAG-1, -2, -5 and -6). cDNA constructs containing the entire coding sequence of acid α -glucosidase were obtained by fusing the 5' end of the 2.6 kb clone to the 3' ends of the 3.1 kb clone (pSHAG1) or the 3.3 kb clone (pSHAG2) at the unique *StuI* site. pSHAG5 and pSHAG6 were derived from pSHAG1 and pSHAG2 respectively by exchanging the internal *SmaI* fragments. The arrows in the partial restriction map of the entire cDNA indicate the positions of the start and stop codons.

Functional analysis of cDNA-encoded acid α -glucosidase

pSHAG1 and pSHAG2 were used for transient expression of acid α -glucosidase in COS cells (monkey) and HeLa cells (human). Transfection with a bacterial β -galactosidase cDNA (pGA293; An *et al.*, 1982) served as a negative control. Table 1 shows that the acid α -glucosidase activity of pSHAG2-transfected COS cells increased between 48 and 96 h after transfection. The resulting enzyme level was approx. 5 times higher than in pGA293-transfected cells. The maximal activity in transfected HeLa cells was reached at 48 h after transfection, and was much lower than the maximal activity in COS cells. Transfection of both cell types with pSHAG2 led to a dramatic increase of acid α -glucosidase activity in the culture media.

There was no increase of acid α -glucosidase activity in cells or medium after transfection with pSHAG1 (Table 1). The failure of pSHAG1 to code for functional enzyme was unexpected. Therefore two other cDNA constructs (pSHAG5 and pSHAG6; Fig. 1) were made to search for the difference between pSHAG1 and pSHAG2. With pSHAG5 the same results were obtained as with pSHAG2, whereas pSHAG6 resembled pSHAG1 (Table 1).

Fig. 2. Synthesis of human acid α -glucosidase *in vitro*

pSHAG2 (sense) and pSHAG2a (anti-sense) derived RNA was translated in a rabbit reticulocyte lysate in the absence (–) or presence (+) of canine pancreatic microsomes. Products were labelled with [35 S]methionine and separated by SDS/PAGE. The relative molecular masses of the unglycosylated and glycosylated acid α -glucosidase precursor are indicated.

In this way the difference between pSHAG1 and pSHAG2 could be assigned to the region between the *StuI* and 3' *SmaI* sites (Fig. 1). On sequence analysis of this region, only one nucleotide difference was found (Fig. 3). It concerned a C in pSHAG1 versus a T in pSHAG2 at position 1423, resulting in an arginine residue instead of a tryptophan residue at amino acid position 402.

Biosynthesis of acid α -glucosidase

To characterize further the effect of the pSHAG1 mutation, the biosynthesis of pSHAG1- and pSHAG2-encoded acid α -glucosidase was studied in transiently transfected cells. The results of a typical pulse-chase experiment are shown in Fig. 4. In pSHAG2-transfected COS cells the 110 kDa precursor was the predominant form of acid α -glucosidase after a 3 h pulse period, whereas the 95 kDa intermediate and the 76 kDa mature enzyme

Table 1. Acid α -glucosidase activity in transfected cells

nt, untransfected cells.

Cell type	Transfected construct	Time after transfection (h) ...	α -Glucosidase activity (nmol of MU/h per mg of protein)					
			48		72		96	
			Cells	Medium	Cells	Medium	Cells	Medium
COS	nt		50.5	0.2	61.1	0.3	52.5	0.6
	pGA293		14.2	0.2	22.0	0.5	32.2	1.1
	pSHAG2		30.5	1.5	112.3	23.6	187.2	57.8
	pSHAG1						20.1	0.5
	pSHAG5						174.2	64.4
	pSHAG6						36.6	0.9
HeLa	nt		4.5	0.1				
	pGA293		2.2	0.1				
	pSHAG2		26.1	3.2

Fig. 3. Nucleotide sequences of pSHAG1 and pSHAG2 cDNA inserts between nucleotides 1421 and 1427

Sequence reactions were performed using double-stranded plasmid DNA as a template and a cDNA specific 21-mer (nucleotides 1329–1349) as the primer. The mutated nucleotide is indicated with an arrowhead.

Fig. 4. Processing of pSHAG1- and pSHAG2-encoded acid α -glucosidase

pSHAG1- and pSHAG2-transfected COS cells were labelled for 3 h with [3 H]leucine, and were harvested directly (0 h) or after an additional chase period of 16 h (16 h). Acid α -glucosidase was immunoprecipitated from cell homogenates and culture media and separated on SDS/PAGE. In the pulse, acid α -glucosidase was precipitated from double the volume of medium compared with the chase. The relative molecular masses are indicated. 14 C-labelled marker proteins (M) (Amersham) were used.

became the major species after a subsequent chase of 16 h. In the culture medium of pSHAG2-transfected cells only the precursor of acid α -glucosidase was detectable. The molecular mass did not change during the chase period. In pSHAG1-transfected cells normal synthesis of the 110 kDa precursor was observed, but maturation and secretion did not occur.

Intracellular transport of acid α -glucosidase

The intracellular routing of the pSHAG1- and pSHAG2-encoded enzymes was compared using immunocytochemistry. Typical punctate lysosomal labelling was obtained in pSHAG2-transfected cells using immuno-light microscopy (Fig. 5a). In contrast, the pattern of labelling in pSHAG1-transfected cells was that of a diffuse network spreading from the nucleus into the cytoplasm (Fig. 5b). Immuno-electron microscopy confirmed the difference in subcellular localization. In Fig. 6 the results are shown for pSHAG2. For both pSHAG1 and pSHAG2, labelling of the rough endoplasmic reticulum, including the nuclear envelope, was observed (Figs. 6a–6c). Also, the Golgi complex was labelled in both cases (shown in Fig. 6c for pSHAG2). However, lysosomal labelling (Figs. 6b and 6c) and labelling of

Fig. 5. Localization of acid α -glucosidase in pSHAG1- and pSHAG2-transfected COS cells studied by light-microscopy

Transfected cells were incubated with rabbit antibodies against human acid α -glucosidase. Immune complexes were visualized with goat anti-rabbit-fluorescein. (a) pSHAG2-transfected cell; (b), pSHAG1-transfected cell. Bars = 20 μ m.

the plasma membrane (Fig. 6b) were only obtained in pSHAG2-transfected cells.

The plasma membrane localization was rather unexpected, and therefore was studied in more detail. To this end, pSHAG2-transfected COS cells were harvested by scraping and washed three times in iso-osmotic buffer with or without 10 mM-mannose 6-phosphate. Immuno-electron microscopy revealed that mannose 6-phosphate did not abolish membrane labelling (results not shown).

In order to investigate the possible existence of membrane-associated forms of acid α -glucosidase, transfected COS cells were homogenized and a phase separation was performed with Triton X-114 (Fig. 7). The major component in the water phase of pSHAG2-transfected cells was the 95 kDa processing intermediate. In addition, a soluble precursor of approx. 110 kDa and the 76 kDa mature form of acid α -glucosidase were present. No soluble forms of acid α -glucosidase were precipitable from the water phase of pSHAG1-transfected cells. The detergent phase of both pSHAG1- and pSHAG2-transfected cells contained almost exclusively the 110 kDa precursor (Fig. 7).

Enzyme uptake and correction

To analyse further the structural and functional characteristics of acid α -glucosidase encoded by pSHAG2, fibroblasts and myoblasts from patients with an acid α -glucosidase deficiency were cultured in the medium of transfected COS cells. Fig. 8 shows that pSHAG2-produced enzyme precursor was taken up by fibroblasts with the same efficiency as mannose 6-phosphate containing acid α -glucosidase purified from bovine testis, whereas the uptake of non-phosphorylated human placental enzyme was much less efficient. Moreover, the uptake of enzyme from the COS cell culture medium was inhibited by 10 mM-mannose 6-phosphate (results not shown). This suggests that the secreted enzyme precursor is equipped with the mannose 6-phosphate recognition marker. After 4 days the acid α -glucosidase activity

Fig. 6. Localization of acid α -glucosidase in pSHAG2-transfected COS cells studied by immuno-electron microscopy

Ultra-thin cryosections were incubated with rabbit polyclonal antibodies directed against acid α -glucosidase, and immune complexes were visualized with goat anti-(rabbit IgG) coupled to colloidal gold. Acid α -glucosidase was found at the nuclear envelope (*a, b* and *c*), in the endoplasmic reticulum (*a, b*), at the plasma membrane (*b*), in the Golgi complex (*c*) and in the lysosomes (*b, c*). The untransfected cell in (*b*) on the left did not contain any label. M, mitochondrion; N, nucleus; R, rough endoplasmic reticulum; PM, plasma membrane; L, lysosomes; G, Golgi complex. Bars = 0.2 μ m.

of the fibroblasts had increased from 0.9 to 47.8 nmol of MU/h per mg of protein, and the activity in myoblasts went up from 0 to 5.1 nmol of MU/h per mg of protein.

Immunoblot analysis revealed that the 110 kDa acid α -glucosidase precursor secreted by COS cells was converted after

internalization by fibroblasts to mature enzyme of 76 kDa via the known 95 kDa processing intermediate (Fig. 9). Culture medium from pGA293-transfected cells served as a control.

Immuno-light microscopy was employed to establish the intracellular localization of endocytosed acid α -glucosidase.

Fig. 7. Triton X-114 phase separation of pSHAG1- and pSHAG2-transfected COS cells

A phase separation using Triton X-114 was performed on transfected COS cells. Acid α -glucosidase was immunoprecipitated from both the water phase (lanes 1 and 2) and the detergent phase (lanes 3 and 4) and analysed on SDS/PAGE followed by immunoblotting (lanes 1 and 3, pSHAG1-transfected cells; lanes 2 and 4, pSHAG2-transfected cells). Relative molecular masses are indicated. Prestained high-molecular-mass protein markers (M) from BRL were used.

Before enzyme addition, the fibroblasts of the patient were completely devoid of acid α -glucosidase (Fig. 10a), but had a normal lysosomal localization of β -hexosaminidase (Fig. 10b). Uptake of acid α -glucosidase (Fig. 10c) resulted in a labelling pattern identical with that of β -hexosaminidase (Fig. 10d). Fibroblasts treated with COS/pGA293-conditioned culture medium were taken as a control and were negative for acid α -glucosidase (Fig. 10e), but positive for β -hexosaminidase (Fig. 10f). A characteristic lysosomal distribution of label was also obtained in myoblasts from a glycogenosis type II patient incubated with culture medium conditioned by pSHAG2-transfected COS cells (Fig. 10g). Exposure of the enzyme-deficient

Fig. 8. Uptake kinetics of acid α -glucosidase

Enzyme-deficient fibroblasts from a patient with glycogenosis type II were maintained in culture medium containing acid α -glucosidase secreted by pSHAG2-transfected COS cells (●), bovine testis acid α -glucosidase (○) or human placental acid α -glucosidase (▲), or in culture medium without enzyme addition (△). Equal amounts of enzyme (equivalent to 100 nmol of MU/h) were added. The intracellular acid α -glucosidase activity was measured at different time points.

Fig. 9. Processing of acid α -glucosidase after uptake by enzyme-deficient fibroblasts

Enzyme-deficient fibroblasts from a patient with glycogenosis type II were cultured in medium conditioned by untransfected COS cells (lane 2), pGA293-transfected COS cells (lane 3) or pSHAG2-transfected COS cells (lane 4). Acid α -glucosidase was immunoprecipitated from cell homogenates and separated on SDS/PAGE followed by immunoblotting. Acid α -glucosidase isolated from human placenta was applied as a marker (lane 1).

myoblasts to culture medium from pGA293-transfected COS cells had no effect (Fig. 10h). Fig. 11 illustrates that enzyme uptake resulted in the degradation of accumulated lysosomal glycogen.

DISCUSSION

Two full-length acid α -glucosidase cDNA constructs differing in only one base pair were expressed in mammalian cells. pSHAG1 represents the originally cloned cDNA (Hoefsloot *et al.*, 1988) and codes for an arginine residue at position 402 of the amino acid sequence. pSHAG2-encoded enzyme had a tryptophan residue at this position. It is shown that the synthesis and post-translational modification of pSHAG2-encoded acid α -glucosidase in transfected cells proceeds as in human fibroblasts (Hasilik & Neufeld, 1980a; Reuser *et al.*, 1985). A glycosylated 110 kDa precursor is initially formed and then converted stepwise to the mature 76 kDa enzyme. Expression of pSHAG2 leads to an increase in the acid α -glucosidase activity and secretion of a catalytically active precursor. In contrast, pSHAG1-encoded acid α -glucosidase does not mature and is not catalytically active. The combined results of pulse-chase experiments and immuno-electron microscopy suggest that the precursor is degraded after it has reached the Golgi complex. Therefore we conclude that pSHAG2 contains a wild-type cDNA sequence, whereas pSHAG1 cDNA is mutated.

The mutation in pSHAG1 may represent either a natural variant or a cloning artifact. Amino acid 402 is not obviously located near proteolytic cleavage sites used during maturation, nor near the putative active site (Hoefsloot *et al.*, 1988). Therefore we assume that the lack of maturation and the deficiency of catalytic activity are secondary effects. The change from tryptophan to arginine is from a hydrophobic to a hydrophilic amino acid, and influences the secondary structure of acid α -glucosidase locally. Computer analysis of the secondary structure according to Chou & Fasman (1978) predicts the loss of a β -sheet and the introduction of a turn. This mis-folding may decrease the stability of the precursor.

Fig. 10. Intracellular localization of acid α -glucosidase studied by immunofluorescence microscopy

Cultured fibroblasts and muscle cells were fixed and incubated with antibodies directed against acid α -glucosidase and β -hexosaminidase. Immune complexes were visualized with goat anti-mouse or goat anti-rabbit antibodies coupled to fluorescein and rhodamine respectively. Panels (a)–(f) show enzyme-deficient fibroblasts from a patient with glycogenosis type II stained for acid α -glucosidase (a, c, e) and the same cells stained for *N*-acetyl- β -hexosaminidase (b, d, f). Panels (a) and (b) show untreated cells; (c) and (d) show cells treated with culture medium from pSHAG2-transfected COS cells; and (e) and (f) show cells treated with culture medium from pGA293-transfected COS cells. Panels (g) and (h) show enzyme-deficient myoblasts from a patient with glycogenosis type II treated with culture medium from pSHAG2- (g) or pGA293- (h) transfected COS cells and stained for acid α -glucosidase. Bars = 20 μ m.

Fig. 11. Lysosomal glycogen content of fibroblasts studied by transmission electron microscopy

(a) Lysosomal glycogen storage in cells from a patient with glycogenosis type II; (b) lysosomes in cells from the same patient after 4 days of culture in medium conditioned by pSHAG2-transfected COS cells. Bars = 0.2 μ m.

The difference between pSHAG1 and pSHAG2 is instructive for the intracellular routing of acid α -glucosidase. As a consequence of the mutation, pSHAG1-encoded enzyme does not reach the lysosomes, is not secreted and is not localized at the plasma membrane. Thus the transport pathways to these three locations diverge after passage of the precursor through the Golgi complex.

The localization of acid α -glucosidase at the plasma membrane has not been observed previously in normal human fibroblasts, and was therefore a rather unexpected finding (Van Dongen *et al.*, 1985; Reuser *et al.*, 1987). It is possible that the membrane association of the acid α -glucosidase precursor (as demonstrated by Triton X-114 phase separation) is responsible for transport to the plasma membrane. This alternative route may gain significance when the enzyme is overexpressed, as in COS cells, or when a specific transport pathway to the apical membrane exists, as in polarized epithelial cells. Plasma membrane localization for acid α -glucosidase has been demonstrated recently for the latter cell type (Fransen *et al.*, 1988; Oude Elferink *et al.*, 1989). Membrane association may also explain the high residual activity of acid α -glucosidase in I-cells, in which the mannose 6-phosphate receptor-dependent pathway is not functional (Van Dongen *et al.*, 1985; Tsuji & Suzuki, 1987). In this situation acid α -glucosidase is also diverted to an extra-lysosomal compartment (Van Dongen *et al.*, 1985).

The nature of the membrane association is still unclear. One hypothesis is that it is related to the proposed homology between acid α -glucosidase and the typical brush border enzyme sucrase-isomaltase (Hoefsloot *et al.*, 1988). The latter enzyme complex is anchored in the apical membrane of intestinal epithelial cells via the uncleaved signal peptide (Hunziker *et al.*, 1986; Semenza, 1989). It is conceivable that the signal peptide of acid α -glucosidase has a similar dual function, despite the presence of a potential cleavage site (Von Heijne, 1986). Another possibility is binding via a glycosphospholipid membrane anchor, shown to act as an apical targeting signal in polarized epithelial cells (Lisanti *et al.*, 1989). We consider that binding via the mannose 6-phosphate receptor is less likely, since mannose 6-phosphate did not release the enzyme from the surface of transfected cells.

The experiments described in Figs. 10 and 11 form the basis for the possible application of enzyme replacement therapy in glycogenosis type II. We have demonstrated in the past that lysosomal glycogen storage in cultured fibroblasts and muscle cells from patients with glycogenosis type II can be prevented or reversed by adding mannose 6-phosphate-containing acid α -glucosidase to the culture medium (Van der Ploeg *et al.*, 1988a,b). In another series of experiments evidence was obtained for uptake of acid α -glucosidase in heart and skeletal muscle of mice after intravenous enzyme administration (Van der Ploeg *et al.*, 1990). This indicates that enzyme replacement therapy for glycogenosis type II is feasible. Our present data show that the cDNA-encoded acid α -glucosidase which is secreted by transfected COS cells is taken up by fibroblasts and muscle cells with the same efficiency as mannose 6-phosphate-containing enzyme from bovine testis. The uptake is inhibited by mannose 6-phosphate and is apparently mediated by the mannose 6-phosphate receptor. Once internalized, the acid α -glucosidase precursor is converted to mature enzyme and lysosomal glycogen is degraded. These features make cDNA-encoded human acid α -glucosidase suitable for enzyme replacement therapy in glycogenosis type II.

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