The conservative substitution Asp-645 → Glu in lysosomal α -glucosidase affects transport and phosphorylation of the enzyme in an adult patient with glycogen-storage disease type II

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Glycogen-storage disease type II (GSDII) is caused by the deficiency of lysosomal α -glucosidase (acid maltase). This paper reports on the analysis of the mutant alleles in an American black patient with an adult form of GSDII (GM1935). The lysosomal α -glucosidase precursor of this patient has abnormal molecular features: (i) the molecular mass is decreased, (ii) the phosphorylation is deficient and (iii) the proteolytic processing is impaired. Sequence analysis revealed four mutations leading to amino acid alterations: Asp-645 → Glu, Val-816 → Ile, Arg-854 → Stop and Thr-927 → Ile. By using allele-specific oligonucleotide hybridization on PCR-amplified cDNA we have demonstrated that the Arg-854 → Stop mutation is located in one allele that is not expressed, and that the other allele contains the remaining three mutations. Each of the mutations was introduced in wild-type cDNA and expressed in COS cells

to analyse the effect on biosynthesis, transport and phosphorylation of lysosomal α -glucosidase. The Val-816 → Ile substitution appeared to have no significant effect in contrast with results [Martiniuk, Mehler, Bodkin, Tzall, Hirschhorn, Zhong and Hirschhorn (1991) *DNA Cell Biol.* 10, 681–687] and was therefore defined as a polymorphism. The Thr-927 → Ile substitution deleting one of the seven glycosylation sites was found to be responsible for the decrease in molecular-mass, but not for the deficient proteolytic processing and phosphorylation. It did not cause the enzyme deficiency either. The third mutation leading to the Asp-645 → Glu substitution was proven to account in full for the observed defects in transport, phosphorylation and proteolytic processing of the newly synthesized α -glucosidase precursor of the patient.

INTRODUCTION

Lysosomal glycogen is normally degraded by lysosomal α -glucosidase (acid maltase; EC 3.2.1.3), and accumulates when this enzyme is deficient (Hers, 1963). The disease, known as glycogenosis type II or glycogen-storage disease type II (GSDII), is inherited as an autosomal recessive trait. The clinical manifestations can vary considerably with respect to time of onset and severity of symptoms (McKusick, 1990).

Apart from clinical heterogeneity, there is also a high degree of biochemical and molecular heterogeneity. A multitude of mutant alleles has been suspected from the molecular abnormalities of the lysosomal α -glucosidase species in cultured fibroblasts and muscle cells from several patients (Reuser et al., 1978, 1985, 1987; Beratis et al., 1978, 1983; Reuser and Kroos, 1982; Van der Ploeg et al., 1989; Hoefsloot et al., 1990a). Genetic heterogeneity has been demonstrated more directly by Southern- and Northern-blot analysis (Martiniuk et al., 1986, 1990a; Hoefsloot et al., 1988; Reuser et al., 1988).

The first two reports on mutation analysis in GSDII have appeared recently (Hermans et al., 1991a; Zhong et al., 1991). In both cases it concerned a patient with a severe infantile form of the disease. We discovered in two sibs from a consanguineous couple a glutamic acid to lysine substitution at position 521 in the homozygous form. The mutation was confirmed to be responsible for the increased electrophoretic mobility of the mutant enzyme precursor, the deficient proteolytic processing and catalytic activity, and the defective intracellular transport

(Hermans et al., 1991a). Substitution of threonine for methionine at amino acid position 318 was found by Zhong et al. (1991) in one of the two lysosomal α -glucosidase alleles of their patient. The mutation gives major changes in the predicted secondary structure and creates a potential N-linked glycosylation site, but it remains to be investigated whether the new site is actually used. The second allele of the patient was not expressed.

In this study we report on a case of adult GSDII, which was deliberately chosen for its interesting complexity. Fibroblasts from this American black patient (cell line GM1935) were originally investigated by Beratis et al. (1983). The residual activity of lysosomal α -glucosidase was found to be unusually low compared with the relatively mild clinical phenotype of the patient. When we studied the biosynthesis of lysosomal α -glucosidase in cell line GM1935, two molecular abnormalities were observed (Reuser et al., 1985, 1987). First, the 110 kDa lysosomal α -glucosidase precursor was not processed to mature enzyme. Second, phosphorylation of the precursor was deficient. The latter abnormality prevents formation of the mannose 6-phosphate recognition marker which is assumed to be essential for lysosomal targeting (Creek and Sly, 1984). Indeed, the amount of enzyme in the lysosomes was found to be extremely low.

In an attempt to explain these findings we have now analysed the genetic phenotype of the patient. Different mutations were detected in the two lysosomal α -glucosidase alleles. One mutant allele with a premature stop codon was found to be silent. The second allele appeared to harbour two polymorphisms in addition

Abbreviations used: GSDII, glycogen-storage disease type II; 4-MU, 4-methylumbelliferyl α -D-glucopyranoside; 1 × SSC, 0.15 M NaCl/0.015 M sodium citrate; 1 × SSPE, 15 mM NaCl/10 mM NaH₂PO₄/1 mM EDTA.

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to the mutation causing the abnormal properties of the patient's lysosomal α -glucosidase.

The same patient was studied independently by Martiniuk et al. (1991) who reported on the silent allele and ascribed the lysosomal α -glucosidase deficiency to a Val $\rightarrow$ Ile substitution at amino acid position 816. Two other substitutions in the same allele (Asp-645 $\rightarrow$ Glu and Thr-927 $\rightarrow$ Ile) were identified as polymorphisms. The analysis described in this paper, however, predicts the Asp-645 $\rightarrow$ Glu substitution to be the mutation and the Val-816 $\rightarrow$ Ile substitution to be a polymorphism.

MATERIALS AND METHODS

Pulse-chase experiments

The fibroblast cell line GM1935 was obtained from the Human Mutant Cell Repository Institute for Medical Research, Camden, NJ, U.S.A. The control cell line from a healthy individual was from Dr. M. F. Niermeijer, Department of Clinical Genetics, University Hospital, Rotterdam, The Netherlands.

Cells were cultured in Dulbecco's modification of Eagle's medium, supplemented with 10% fetal calf serum and antibiotics, under 10% CO₂, at 37 °C. Labelling conditions with [³H]leucine or carrier-free [³²P]P_i were as described by Reuser et al. (1985). Cells were pulse-labelled for 2 or 6 h and harvested either directly or after a subsequent chase of 9 h. In some experiments, tunicamycin (final concentration 10 µg/ml) was added 1 h before labelling and during labelling to inhibit glycosylation of newly synthesized proteins. Lysosomal α -glucosidase was immunoprecipitated from cell extracts and analysed by SDS/PAGE as described (Hasilik and Neufeld, 1980; Reuser et al., 1985).

DNA amplification and sequencing

DNA and RNA were extracted from cultured fibroblasts by standard procedures (Sambrook et al., 1989). cDNA synthesis was carried out using Amersham's cDNA Synthesis System Plus and oligo(dT) primers. PCR amplification using intronic or exonic primers specific for lysosomal α -glucosidase was as described (Hermans et al., 1991a). Each 100 µl of PCR reaction mixture contained approx. 0.5 µg of DNA, 100 pmol of each primer and 2 units of *Taq* polymerase (BRL) in the standard PCR buffer recommended by Sambrook et al. (1989). Amplification was performed in a Perkin-Elmer Cetus DNA amplifier. The first cycle was: 5 min denaturation at 94 °C, 5 min annealing at 59 °C and 5 min DNA synthesis at 72 °C. Subsequent cycles (25–30) consisted of 2 min at 94 °C, 2 min at 59 °C and 5 min at 72 °C. The DNA-synthesis step of the final cycle was extended to 15 min. In some instances, PCR-amplified DNA fragments were cloned in a T-tailed Bluescript plasmid vector. This so-called T-vector was constructed as described by Marchuk et al. (1991). DNA fragments were sequenced using the T7 polymerase sequencing kit (Pharmacia LKB Biotechnology Inc.) with appropriate primers.

Slot-blot analysis and allele-specific oligonucleotide hybridization

PCR products were denatured in 0.4 M NaOH/25 mM EDTA for 10 min and applied directly to Hybond N⁺ membranes using a slot-blot apparatus. The filters were air-dried, prehybridized for 1 h at 42 °C in 5 × SSPE/1% SDS/50 µg/ml salmon sperm DNA, and subsequently hybridized for 3 h in the same solution to ³²P-labelled oligonucleotides. The filters were washed in 6 × SSC for 5 min at 2–3 °C below the estimated melting temperature (Wallace et al., 1981).

Site-directed mutagenesis

Site-directed mutagenesis was carried out as described by Hermans et al. (1991b). The following oligonucleotides, synthesized on an Applied Biosystems 381A DNA synthesizer, were used: Glu-645, TCGGGGCCGAAGTCTGCGGCT; Ile-816, ACACCATCAACATCCACCTCC; Stop-854, TGGA-GAGGCCTGAGGGGAGCT; Ile-927, TCCAACCTTCATC-TACAGCCCC.

Transient expression in COS cells

Wild-type and mutant cDNAs were cloned in the eukaryotic expression plasmid pSG5. The transfection of COS cells was carried out as described by Hoefsloot et al. (1990b). The activity of lysosomal α -glucosidase in the cell homogenates and the culture media was measured with 4-methylumbelliferyl α -D-glucopyranoside (4-MU) and with glycogen as described by Hermans et al. (1991b). Radioactive labelling and analysis of lysosomal α -glucosidase expressed in COS cells was as mentioned above.

Immunocytochemistry

Immunocytochemistry on transiently transfected COS cells was performed exactly as described before (Hoefsloot et al., 1990b).

RESULTS AND DISCUSSION

Delineation of the molecular phenotype

The lysosomal α -glucosidase activity in the fibroblast cell line GM1935 derived from a black American adult with GSDII is 1–3% of the average control value (Beratis et al., 1983; Reuser et al., 1985, 1987). Figure 1(a) reveals the molecular nature of the enzyme deficiency. In control fibroblasts, the enzyme is synthesized as a precursor of approximately 110 kDa, and is proteolytically processed to a long-lived 95 kDa intermediate. In a later stage, mature enzyme of 76 and 70 kDa is formed (Hasilik and Neufeld, 1980; Oude Elferink et al., 1985; Reuser et al., 1985). These major molecular forms of lysosomal α -glucosidase

Figure 1 Labelling of lysosomal α -glucosidase (a) and lysosomal β -hexosaminidase (b) with [³H]leucine or [³²P]P_i in control and GM1935 fibroblasts

The fibroblasts were labelled for 6 h with [³H]leucine or [³²P]P_i and harvested directly. Lysosomal α -glucosidase (a) or β -hexosaminidase (b) were immunoprecipitated from the cell homogenates and analysed by SDS/PAGE. The molecular masses (kDa) of wild-type and mutant protein species are indicated. Lanes 1 and 4, control; lanes 2 and 5, GM1935; lane 3, marker.

are labelled in a 6 h pulse period with [^3H]leucine (Figure 1a, lane 1). The Figure reveals, in addition, short-lived processing intermediates with molecular masses between 110 kDa and 95 kDa, which are not always observed. From Figure 1(a) it is evident that the post-translational modification of the lysosomal α -glucosidase precursor is impaired in cell line GM1935. The mutant precursor is, in addition, about 2 kDa smaller than normal (108 kDa). A deficient phosphorylation of the mutant precursor became apparent when the labelling was performed via incorporation of ^{32}P into the mannose 6-phosphate recognition marker. A phosphorylation defect was not observed when the [^3H]leucine and [^{32}P]i incorporation were compared for the reference lysosomal enzyme β -hexosaminidase (Figure 1b). It is therefore an intrinsic feature of the mutant lysosomal α -glucosidase precursor.

These results confirm our earlier findings except that the decrease in molecular mass of the mutant precursor escaped our attention in the high-percentage polyacrylamide gels used previously (Reuser et al., 1985).

A decrease in the apparent molecular mass of a polypeptide can have several causes. It can result from a deletion, an aberrant splicing of the transcript or a point mutation introducing a premature stop codon. Also a mutation causing the loss of a glycosylation site has to be considered. We have demonstrated recently that even a single amino acid substitution can give rise to a significant difference in the electrophoretic mobility of lysosomal α -glucosidase on SDS/PAGE (Hermans et al., 1991a,b). The possible lack of a glycosylation site was assessed by comparing the size of the unglycosylated and glycosylated wild-type and mutant precursors. To this end, glycosylation was inhibited with tunicamycin. Figure 2 illustrates that the wild-type and mutant lysosomal α -glucosidase precursors are the same size (97 kDa) when synthesized in the presence of tunicamycin. On the basis of these results our working hypothesis was that the lysosomal α -glucosidase precursor of mutant GM1935 lacks one of the seven glycosylation sites. In the preceding paper (Hermans et al., 1993), we have demonstrated by site-directed mutagenesis that individual elimination of six of these seven sites does not lead to defective processing and loss of enzyme function. Only the loss of the glycosylation site at amino acid position 233 was found to interfere with enzyme maturation and transport, but the phosphorylation was not affected. Therefore we conclude that the lack of a glycosylation site alone cannot account for the abnormalities observed in cell line GM1935.

Figure 2 Labelling of lysosomal α -glucosidase with [^3H]leucine in the presence or absence of tunicamycin

The fibroblasts were labelled for 6 h with [^3H]leucine in the presence (+) or absence (–) of tunicamycin and harvested directly. Lysosomal α -glucosidase was immunoprecipitated from the cell homogenates and analysed by SDS/PAGE. The molecular masses (kDa) of wild-type and mutant lysosomal α -glucosidase species are indicated. Lanes 1 and 4, control; lanes 2 and 3, GM1935. The asterisk indicates a molecular species of unknown identity that is only formed in the presence of tunicamycin.

Sequence analysis

To identify the mutations responsible for the molecular phenotype of GM1935, we PCR-amplified all exonic regions of the lysosomal α -glucosidase gene. Most PCR products were sequenced directly. In some instances, however, it was necessary to clone the PCR fragments before sequencing. In these cases at least four clones were analysed.

Sequence analysis of the complete coding region revealed several differences from the wild-type cDNA sequence (Hoefsloot et al., 1988; Martiniuk et al., 1990b) (EMBL entry number Y00839). All these differences were heterozygous, indicating that GM1935 carries two distinct lysosomal α -glucosidase alleles. Besides several known and two new polymorphisms, we discovered four mutations leading to an amino acid alteration: an Asp to Glu substitution at amino acid position 645 in exon 14, a Val to Ile substitution at position 816 in exon 17, a Thr to Ile substitution at position 927 in exon 19, and a premature stop at position 854 in exon 18 (Table 1). Except for the last mutation, the same substitutions were reported by Martiniuk et al. (1991).

Since several mutations were found, the alleles had to be defined more precisely. We started out with the Val-816 $\rightarrow$ Ile, the Arg-854 $\rightarrow$ Stop and the Thr-927 $\rightarrow$ Ile mutations. Exons

Table 1 Base-pair substitutions: comparison with the lysosomal α -glucosidase cDNA sequence in EMBL/Genbank (Y00839)

Nucleotide position is counted from the initiation codon of the precursor. References: 1, Hoefsloot et al. (1990b); 2, Martiniuk et al. (1990b).

Nucleotide position	Exon	cDNA	GM1935	Amino acid alteration	Reference
Mutations					
1935	14	C	A	Asp-645 $\rightarrow$ Glu	
2446	17	G	A	Val-816 $\rightarrow$ Ile	
2560	18	C	T	Arg-854 $\rightarrow$ Stop	
2780	19	C	T	Thr-927 $\rightarrow$ Ile	
Polymorphisms					
596	3	G	A	Arg-199 $\rightarrow$ His	1,2
1203	8	A	G	–	2
1374	9	C	T	–	
1581	11	A	G	–	1,2
2553	18	A	G	–	1,2
2862	20	G	A	Non-coding

Figure 3 Allele-specific oligonucleotide hybridization of PCR-amplified cDNA

PCR products were denatured and applied to Hybond N⁺ membranes using a slot-blot apparatus and probed with ³²P-labelled oligonucleotides for the presence of either normal (Wt) or mutant cDNA sequences

16–19 containing the latter three mutations were PCR-amplified in one fragment from genomic DNA and this fragment was cloned. Six different clones were sequenced. Three were found to have the Arg-854 → Stop mutation in combination with a wild-type sequence at position 816 and 927. The other three clones were wild-type at position 854, but contained both the Val-816 → Ile as well as the Thr-927 → Ile mutation. From these results we conclude that the Val-816 → Ile and the Thr-927 → Ile mutations are located together in one allele, and the Arg-854 → Stop mutation in the other.

The substitution of Ile for Thr-927 eliminates the consensus sequence (Asn-Phe-Thr) for N-linked glycosylation of the seventh glycosylation site. Therefore this mutation is expected to be responsible for the lower molecular mass of the GM1935 lysosomal α -glucosidase precursor. The mutation resulting in the premature stop of enzyme synthesis at amino acid position 854 leads hypothetically to a protein of approximately 98 kDa, because 98 amino acids and two glycosylation sites are lacking. We did not detect a lysosomal α -glucosidase polypeptide of this size in cell line GM1935, but such a mutant protein might be unstable. Another reason for the absence of a 98 kDa polypeptide in GM1935 could be that the allele carrying this mutation is not expressed.

To investigate whether both alleles of GM1935 were expressed, we extracted RNA from the cells and reverse-transcribed it into cDNA. We then PCR-amplified a fragment spanning all four mutations. Allele-specific oligonucleotide hybridization of slot-blot was performed to screen for the presence of the mutations. cDNA from GM1935 was found to hybridize with the Glu-645 and the Ile-927 oligonucleotides, but not with the Stop-854 oligonucleotide. When oligonucleotides complementary to the wild-type sequences at positions 645, 854 and 927 were used, hybridization was only obtained at position 854. cDNA amplified from RNA extracted from human HepG2 cells hybridized only with the wild-type oligonucleotides (Figure 3). As suitable positive and negative controls for hybridization conditions, plasmid constructs containing the wild-type and mutant sequences were used (results not shown). Two conclusions can be drawn from these results. First, the GM1935 allele characterized by the Arg-

854 → Stop mutation is not expressed. Second, the allele which we found to harbour the Val-816 → Ile and the Thr-927 → Ile mutations also contains the Asp-645 → Glu mutation, and is expressed at the RNA level. Thus this second allele must encode the mutant protein. This is in agreement with the results reported by Martiniuk et al. (1991) which were obtained with a different methodology.

The question remains of which of the mutations is responsible for the defect in post-translational modification. The Thr-927 → Ile mutation eliminating the glycosylation site at Asn-925 seems unlikely, since we have demonstrated that the loss of this site does not interfere with enzyme processing or function (Hermans et al., 1993). Moreover, the Chou-Fasman and the Garnier algorithms did not predict a significant change in the secondary structure of the protein (Chou and Fasman, 1978; Garnier et al., 1978). No significant differences were observed either when the Val-816 → Ile substitution was analysed. In contrast, the replacement of Asp-645 by Glu disturbs a β -strand according to the Chou-Fasman algorithm.

The mutations and their effects

To study the effects of the various mutations on the biosynthesis and function of lysosomal α -glucosidase, they were introduced in the wild-type cDNA by site-directed mutagenesis and expressed in COS cells. Besides single amino acid substitutions, a double mutant was made containing both the Asp-645 → Glu and the Thr-927 → Ile substitutions. At least two independent clones of each construct were tested.

COS cells were harvested 60 h after transfection and the lysosomal α -glucosidase activity was determined with the artificial 4-MU substrate and with the natural substrate glycogen. At least three transfection experiments were performed for each mutation. Typical results of one such experiment are given in Table 2 which also includes data on the average outcome of three independent experiments. COS cells transfected with *Escherichia coli* β -galactosidase cDNA were used as a negative control. Transfection with the wild-type, the Val-816 → Ile or the Thr-927 → Ile cDNAs resulted in a significant expression of activity for both substrates, indicating that these substitutions represent polymorphisms rather than mutations. In contrast, the activity measured after transfection with the Asp-645 → Glu or the double-mutant construct was hardly above background. The small additive effect of the Thr-927 → Ile on the Asp-645 → Glu substitution was not significant. No increase in activity was measured after transfection with cDNA containing the Arg-854 → Stop mutation.

Our results are at this point at variance with those reported by Martiniuk et al. (1991) who expressed their constructs in SV40-immortalized human fibroblasts. In the one experiment they report on, the Thr-927 → Ile substitution was without effect, the Asp-645 → Glu substitution resulted in a 67% decrease in lysosomal α -glucosidase activity, and the enzyme activity in fibroblasts transfected with the Val-816 → Ile mutant cDNA was not above background.

The synthesis and proteolytic processing of the wild-type and mutant lysosomal α -glucosidase species were assayed by pulse-chase labelling at 60 h after transfection. The results are illustrated in Figure 4. In COS cells transfected with either the wild-type cDNA or the cDNA containing the Val-816 → Ile mutation, the usual 110 kDa precursor was synthesized in a 2 h pulse with [³H]leucine. COS cells transfected with the Thr-927 → Ile mutant cDNA produced a 108 kDa precursor. Transfection with the Asp-645 → Glu mutant construct resulted in the synthesis of an apparently normal 110 kDa precursor in the 2 h

Table 2 Catalytic activities of lysosomal α -glucosidase mutants in transiently transfected COS cells 60 h after transfection

The activity is expressed as nmol of 4-MU/h per mg of protein with MU as substrate or nmol of glucose/h per mg of protein with glycogen as substrate. The activities compared with the wild-type are given within parentheses as percentages $\pm$ S.E.M. ($n = 3$).

Type of mutation	Catalytic activity	
	4-MU (nmol/h per mg)	Glycogen (nmol/h per mg)
COS cells	106.8 (0)	360 (0)
Wild-type	582.7 (100)	2180 (100)
Asp-645 $\rightarrow$ Glu	133.8 (2.9 ± 2.7)	340 (1.5 ± 1.5)
Val-816 $\rightarrow$ Ile	535.6 (105.0 ± 14.9)	1832 (74.4 ± 6.4)
Arg-854 $\rightarrow$ Stop	113.0 (1.3 ± 1.2)	320 (0)
Thr-927 $\rightarrow$ Ile	245.2 (68.0 ± 38.9)	1830 (72.4 ± 8.4)
Asp-645 $\rightarrow$ Glu/Thr-927 $\rightarrow$ Ile	122.4 (1.6 ± 1.6)	240 (0.5 ± 0.5)

Figure 5 Labelling of wild-type and mutant lysosomal α -glucosidase species in transfected COS cells with $[^3\text{H}]$ leucine and with $[^{32}\text{P}]\text{P}_i$

COS cells transfected with wild-type or mutant cDNA constructs were labelled for 6 h with $[^3\text{H}]$ leucine or $[^{32}\text{P}]\text{P}_i$ and harvested directly. Lysosomal α -glucosidase was immunoprecipitated from the cell homogenates and analysed by SDS/PAGE. The molecular masses (kDa) of wild-type and mutant lysosomal α -glucosidase species are indicated. Lanes 1, 3, 5 and 7, wild-type; lanes 2 and 6, Asp-645 $\rightarrow$ Glu mutant; lanes 4 and 8, Arg-854 $\rightarrow$ Stop mutant.

Figure 4 Synthesis and processing of wild-type and mutant lysosomal α -glucosidase species in transfected COS cells

COS cells transfected with wild-type or mutant cDNA constructs were labelled for 2 h with $[^3\text{H}]$ leucine and harvested directly (pulse) or after a subsequent period of either 9 or 30 h (chase). Lysosomal α -glucosidase was immunoprecipitated from the cell homogenates and analysed by SDS/PAGE. To study secretion, lysosomal α -glucosidase was immunoprecipitated from the chase media. The molecular masses (kDa) of wild-type and mutant lysosomal α -glucosidase species are indicated. Lanes 1 and 6, wild-type; lanes 2 and 7, Asp-645 $\rightarrow$ Glu mutant; lanes 3 and 8, Asp-645 $\rightarrow$ Glu/Thr-927 $\rightarrow$ Ile double mutant; lanes 4 and 9, Thr-927 $\rightarrow$ Ile mutant; lanes 5 and 10, Val-816 $\rightarrow$ Ile mutant.

pulse. The double-mutant precursor was decreased in size like the Thr-927 $\rightarrow$ Ile mutant (Figure 4, pulse).

Within 9 h of the chase, the wild-type precursor was converted into the 95 kDa intermediate and the 76 kDa mature form of lysosomal α -glucosidase (Figure 4, chase 9 h). Also the Val-816 $\rightarrow$ Ile precursor was normally processed to 95 and 76 kDa, confirming our earlier conclusion that the replacement of Val by Ile is a polymorphism. The 108 kDa Thr-927 $\rightarrow$ Ile mutant precursor was converted into a 93 kDa intermediate whereafter a 76 kDa mature species of normal size was formed. This processing confirms our conclusions in the preceding paper that the glycosylation site at Asn-925 is located in a C-terminal propeptide that is cleaved off when the 95 kDa intermediate matures to 76 kDa. When Thr-927 was replaced by Ile the rate of

maturation from 95 kDa to 76 kDa seemed slightly increased. The same effect was observed when this glycosylation site was deleted by an Asn-925 $\rightarrow$ Gln substitution (Hermans et al., 1993). The proteolytic processing of the precursor containing the Asp-645 $\rightarrow$ Glu substitution was delayed as was the processing of the double mutant precursor. (The respective 110 kDa and 108 kDa precursor species were still visible after 9 h of chase.)

A comparable picture was obtained after 30 h of chase, except that the Glu-545 and double mutant precursor were no longer detectable (Figure 4, chase 30 h). The mutant species are apparently degraded since they are not converted into mature enzyme and not secreted either (Figure 4, secretion).

The biosynthesis of lysosomal α -glucosidase containing the Arg-854 $\rightarrow$ Stop mutation is illustrated in Figure 5 (leucine incorporation). In a 6 h pulse period a truncated precursor of 98 kDa was formed. This 98 kDa species is not synthesized in cell line GM1935 since it is derived from the one allele that is not expressed.

Labelling with $[^{32}\text{P}]\text{P}_i$ was performed to determine whether it was the single Asp-645 $\rightarrow$ Glu, the single Thr-927 $\rightarrow$ Ile or the combination of both mutations that interfered with phosphorylation (Figures 5 and 6). The incorporation of phosphate into β -hexosaminidase was taken as a reference. Phosphorylation of all mutant precursors was detectable but the degree of phosphate incorporation was low for the Asp-645 $\rightarrow$ Glu and the double mutant. It is evident that the phosphorylation defect is caused by the Asp-645 $\rightarrow$ Glu substitution rather than by the loss of the glycosylation site at Asn-925. Figure 5 shows that the truncated lysosomal α -glucosidase species also failed to incorporate phosphate. In cell line GM1935, the phosphorylation defect seems to be complete in contrast with that observed in the transfected COS cells. However, the expression of lysosomal α -glucosidase in the transient transfection system was much higher than in fibroblasts. Therefore it could very well be that phosphorylation of the lysosomal α -glucosidase precursor also occurs in cell line GM1935 but remains undetectable.

Figure 6 Labelling of lysosomal α -glucosidase and β -hexosaminidase with [32 P]P_i in COS cells transfected with wild-type or mutant lysosomal α -glucosidase species

COS cells were labelled for 3 h with [32 P]P_i and harvested directly. Lysosomal α -glucosidase and β -hexosaminidase were immunoprecipitated from the cell homogenates and analysed by SDS/PAGE. The molecular masses (kDa) of wild-type and mutant lysosomal α -glucosidase species are indicated. Lanes 1 and 5, wild-type; lanes 2 and 6, Asp-645 $\rightarrow$ Glu mutant; lanes 3 and 7, Asp-645-Glu/Thr-927 $\rightarrow$ Ile double mutant; lanes 4 and 8, Thr-927 $\rightarrow$ Ile mutant.

Figure 7 Localization of mutant lysosomal α -glucosidase in transfected COS cells studied by light microscopy

Transiently transfected COS cells were fixed and incubated with a rabbit polyclonal antiserum against human lysosomal α -glucosidase. Immune complexes were visualized with goat anti-rabbit IgG conjugated to fluorescein. (a) Thr-927 $\rightarrow$ Ile; (b) Asp-645 $\rightarrow$ Glu.

Summarizing the results presented in Table 2 and Figures 4–6, we conclude that it is the double mutant that mimics the molecular defect in cell line GM1936, but the deleterious effect on enzyme biosynthesis and function is caused by the Asp-645 $\rightarrow$ Glu rather than the Thr-927 $\rightarrow$ Ile substitution.

Subcellular localization

Immunocytochemistry was performed to study the intracellular localization of the mutant proteins in the transiently transfected COS cells. Typical punctate lysosomal labelling was obtained for

Table 3 Quantitative data on the lysosomal α -glucosidase labelling of the lysosomes in COS cells transiently transfected with wild-type and mutant cDNA constructs

The units for lysosomal labelling are the number of gold particles per lysosome ($n = 31$). Results are means $\pm$ S.E.M.

Type of mutation	Lysosomal labelling
Wild-type	43 $\pm$ 21
Thr-927 $\rightarrow$ Ile	31 $\pm$ 32
Asp-645 $\rightarrow$ Glu	13 $\pm$ 16
Double mutant	3 $\pm$ 3

Figure 8 Subcellular localization of the Asp-645 $\rightarrow$ Glu mutant protein in transfected COS cells studied by immunoelectron microscopy

Ultrathin cryosections were incubated with rabbit polyclonal antiserum against human lysosomal α -glucosidase and subsequently with goat anti-rabbit IgG coupled to colloidal gold. Mutant protein was found in normal amounts in the endoplasmic reticulum (R) but very little enzyme was detectable in the lysosomes (L). M: mitochondrion. Bar is 0.1 μ m. (a) Lysosome; (b) endoplasmic reticulum.

lysosomal α -glucosidase with the Thr-927 $\rightarrow$ Ile substitution (Figure 7a), but expression of lysosomal α -glucosidase with the single Asp-645 $\rightarrow$ Glu or the double mutation resulted in a labelling pattern characteristic of endoplasmic reticulum. Lysosomal labelling was barely detectable (Figure 7b). Semiquantitative data on the lysosomal labelling were obtained by immunoelectron microscopy on ultrathin cryosections. To this end, the number of gold particles per lysosome was counted in 31 randomly chosen lysosomes. The results are presented in Table 3. The amount of label in the lysosomes was not significantly different in cells expressing the wild-type lysosomal α -glucosidase compared with cells expressing lysosomal α -glucosidase with the Thr $\rightarrow$ Ile substitution at position 927. However, lysosomal labelling was significantly lower when Asp-645 was replaced by Glu or when the Asp-645 $\rightarrow$ Glu and the Thr-927 $\rightarrow$ Ile substitutions were present simultaneously. Both the wild-type and the Thr-927 $\rightarrow$ Ile-substituted lysosomal α -glucosidases were not only present in the lysosomes but also in the various compartments of the glycoprotein-transport pathway, including the endoplasmic reticulum, the Golgi cisternae and the trans-Golgi network (results not shown). The Asp-645 $\rightarrow$ Glu and the double-mutant α -glucosidase precursors were mainly localized in the endoplasmic reticulum (Figure 8). This abnormal enzyme distribution suggests that the mutant precursor is trans-

ported inefficiently from the endoplasmic reticulum to the Golgi. This is in line with the observed phosphorylation defect. The *N*-acetylglucosaminyl phosphotransferase responsible for the incorporation of ^{32}P in the lysosomal α -glucosidase precursor is thought to be located in the cis-most Golgi cisternae (Lazzarino and Gabel, 1988, 1989). Inefficient transfer of the mutant precursor to this compartment will therefore automatically lead to an apparent phosphorylation defect. On the basis of our present findings, we consider it less likely that the mutation in this patient affects the *N*-acetylglucosaminyl phosphotransferase-recognition site directly. The dramatic effect of the Asp to Glu substitution at position 645 is possibly caused by the change in secondary structure, as predicted by the Chou-Fasman algorithm. There are several examples in the literature of mutant proteins that do not exit the endoplasmic reticulum because of wrong folding (Pelham, 1989).

We have no explanation for the apparent discrepancy between our results and those reported by Martiniuk et al. (1991), where it concerns the effect of the amino acid substitutions. The experimental approach is only different in the sense that Martiniuk et al. (1991) used SV40-immortalized fibroblasts from a patient with GSDII for transient expression of lysosomal α -glucosidase cDNA whereas we used COS-1 cells. We have, however, not experienced a difference in the biosynthesis, the post-translational modification and the intracellular location of wild-type and mutant forms of lysosomal α -glucosidase in transfected COS-1 cells compared with human fibroblasts [compare Figures 1(a) and 4, and Hoefsloot et al. (1990b), Hermans et al. (1991a)]. Our conclusions are not solely based on the assay of lysosomal α -glucosidase activity but we have demonstrated in addition that the Asp-645 $\rightarrow$ Glu substitution accounts for delayed intracellular transport and deficient phosphorylation and proteolytic processing. The notion of Martiniuk et al. (1991) that the secondary structure of lysosomal α -glucosidase is not affected by the Asp-645 $\rightarrow$ Glu mutation is not in accordance with the Chou-Fasman analysis performed by us.

The most difficult to explain is the clinical phenotype of the patient. The mutation analysis has shown that only one allele is expressed and that this allele does not produce a substantial amount of functional lysosomal α -glucosidase. The results are in this respect no different from those obtained with fibroblasts from the patient. The low level of residual activity is typical for patients with an infantile form of glycogenosis type II. The relatively slow progression of the disease in this black adult patient remains an enigma, unless the biosynthesis of this mutant lysosomal α -glucosidase is different in muscle from that in fibroblasts or transfected COS cells.

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