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Recombinant human acid α-glucosidase: high level production in mouse milk, biochemical characteristics, correction of enzyme deficiency in GSDII KO mice

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Glycogen storage disease type II (GSDII) is caused by lysosomal acid α-glucosidase deficiency. Patients have a rapidly fatal or slowly progressive impairment of muscle function. Enzyme replacement therapy is under investigation. For large-scale, cost-effective production of recombinant human acid α-glucosidase in the milk of transgenic animals, we have fused the human acid α-glucosidase gene to 6.3 kb of the bovine α_{S1}-casein gene promoter and have tested the performance of this transgene in mice. The highest production level reached was 2 mg/ml. The major fraction of the purified recombinant enzyme has a molecular mass of 110 kDa and resembles the natural acid α-glucosidase precursor from human urine and the recombinant precursor secreted by CHO cells, with respect to pH optimum, K_m, V_{max}, N-terminal amino acid sequence and glycosylation pattern. The therapeutic potential of the recombinant enzyme produced in milk is demonstrated *in vitro* and *in vivo*. The precursor is taken up in a mannose 6-phosphate receptor-dependent manner by cultured fibroblasts, is converted to mature enzyme of 76 kDa and depletes the glycogen deposit in fibroblasts of patients. When injected intravenously, the milk enzyme corrects the acid α-glucosidase deficiency in heart and skeletal muscle of GSDII knockout mice.

INTRODUCTION

Investigations into the production of pharmaceutical proteins in genetically engineered cells and organisms are a major activity in the biotechnology field. The application of biotechnology instead of classical production methods often has an economical advantage, but becomes mandatory when natural product sources are extremely scarce. The latter situation exists for lysosomal enzymes. There are >40 of these enzymes constitutively expressed in almost all cell types of the human body, where they act in concert to degrade and recycle a variety of macromolecules derived from the intracellular and extracellular environment. An inherited single enzyme deficiency may prohibit adequate degradation of one or more of these compounds and result in a lysosomal storage disease (1). The phenotypic expression of these diseases is diverse, with early and late onset forms and variable extents of organ involvement. Our present study deals with the development of enzyme replacement therapy for one of these lysosomal storage disorders: glycogen storage disease type II (GSDII; Pompe's disease).

GSDII is an autosomal recessive disorder, characterized by generalized muscle weakness and wasting due to deficiency of the lysosomal enzyme acid α-glucosidase. Respiratory insufficiency is life threatening for affected children and adults with a slowly progressive course. Severely affected infants succumb by cardio-respiratory insufficiency in the first to second year of life (2). In the early 1960s, an attempt was made to compensate lysosomal enzyme deficiencies by intravenous administration of enzyme preparations from a variety of natural sources. The first attempt concerned treatment of GSDII with acid α-glucosidase extracted from *Aspergillus niger* (3). The outcome of clinical trials performed in several lysosomal storage diseases remained disappointing for a long time; the enzyme dosage was too low and the enzyme targeting inadequate (4,5). The first successful attempt at enzyme replacement therapy was published in 1990 for type I Gaucher disease, a glucocerebrosidase deficiency causing glycolipid storage in macrophages (6,7). The glucocerebrosidase used in this trial was purified on a large scale from human placentas and was modified in the carbohydrate side chain in

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order to fit the mannose receptor on the cell surface of macrophages storing the glycolipid. A very similar recombinant form of human glucocerebrosidase, imiglucerase (Cerezyme; Genzyme, Cambridge, MA), presently is produced in genetically engineered Chinese hamster ovary K1 cells (CHO cells). The same cell type is employed for the production of a number of other recombinant human lysosomal enzymes that are overexpressed and purified from the culture medium (8–18).

A characteristic feature of these enzymes is that they have carbohydrate side chains with mannose 6-phosphate groups (M6P). This facilitates their binding and endocytosis by cells with cell surface mannose 6-phosphate receptors (M6P receptor, IGFII receptor). The cardiomyocytes and skeletal muscle cells affected in GSDII by lysosomal glycogen storage express the M6P receptor on their surface (19,20) and are thereby targets for M6P-containing forms of acid α-glucosidase (15,17,21–24). The feasibility of acid α-glucosidase production in CHO cells has been demonstrated and the therapeutic potential of this enzyme has been tested. However, it remains a major effort and a costly procedure to realize large-scale enzyme production in CHO cells. With this in mind, we have initiated an investigation into the possibility of lysosomal α-glucosidase production in the milk of transgenic mammals, and demonstrated that trace amounts of recombinant human acid α-glucosidase are secreted in the milk of transgenic mice carrying the human acid α-glucosidase cDNA linked to the bovine α_{S1}-casein promoter, but the yield was too low to characterize the recombinant enzyme in detail and to study its therapeutic effect (25,26).

In this study, we demonstrate the feasibility of large-scale production of recombinant acid α-glucosidase in the milk of transgenic mice with a transgene containing the intact α-glucosidase gene instead of the cDNA. The enzyme was purified from the milk and characterized with regard to its structural, biochemical and functional features. In all aspects, it resembles the recombinant enzyme produced in the medium of genetically engineered CHO cells. The therapeutic potential is demonstrated by feeding the enzyme to cultured fibroblasts of a GSDII patient and by intravenous administration to GSDII knock-out (KO) mice, that recently were developed in our laboratory for this and other purposes (27).

RESULTS

Generation of transgenic mice

To obtain high level expression of recombinant human acid α-glucosidase in the mammary gland of transgenic mice, we have fused the acid α-glucosidase gene in its genomic context to the bovine α_{S1}-casein promoter in a multi-step cloning procedure (as described in Materials and Methods). The transgene is depicted in Figure 1A and comprises 6.3 kb of the bovine α_{S1}-casein gene [promoter and 5′-untranslated region (5′-UTR)] and 28.5 kb of the human acid α-glucosidase gene including all exons, introns and the 3′-UTR. This construct was injected into the pronucleus of fertilized oocytes to generate transgenic mice. Nine founders were obtained and they were crossed with wild-type CBA/BrA × C57Bl/6 mice. One male founder appeared to be infertile, two others were germline chimeras. The remaining six founders transmitted the transgene in a Mendelian fashion. Litter sizes were normal and littermates were indistinguishable. The copy number of the transgene in the offspring ranged from two to 10 copies as judged from the intensity of the hybridization signal on Southern blots from tail DNA using human acid α-glucosidase cDNA as probe (Table 1).

Transgene expression

Northern and western blotting were performed to demonstrate transcription and translation of the transgene. Mice from different transgenic lines were tested. Figure 1B and C shows as a representative example the results obtained with mouse 4312 of line 2585 with an approximate copy number of 10. The northern blot (Fig. 1B) reveals very high expression of the transgene in the lactating mammary gland, and low expression in various other organs, such as brain, lungs, liver, salivary gland and intestine. Also, mammary gland-specific overexpression is shown by western blotting. Some other tissues contain human acid α-glucosidase at a much lower concentration (Fig. 1C).

Table 1. Characteristics of transgenic mouse lines

Line number	Estimated copy number[a]	mRNA level[b]	Acid α-glucosidase concentration in milk samples			
			Average[c]	Range	*n*	*m*
2470	10	++	248	11–1095	38	3
2471	2	+++	113	13–260	8	2
2474	2	+	31	4–99	9	2
2475	4	++	81	20–238	15	4
2477	4	+	72	5–215	8	3
2585	10	+++	514	24–2048	38	8
Non-transgenic	–	–	3	2–3	7	

[a]The copy number of the transgene was estimated by Southern blotting.
[b]The level of mRNA in the mammary gland of lactating females was estimated by northern blotting.
[c]The concentration of acid α-glucosidase in the milk (μg/ml) was calculated from the enzymatic activity measured with the artificial substrate 4-methylumbelliferyl-α-D-glucopyranoside.
n, number of milk samples (sample collection was started 7 days after birth); *m*, number of mice used for sample collection.

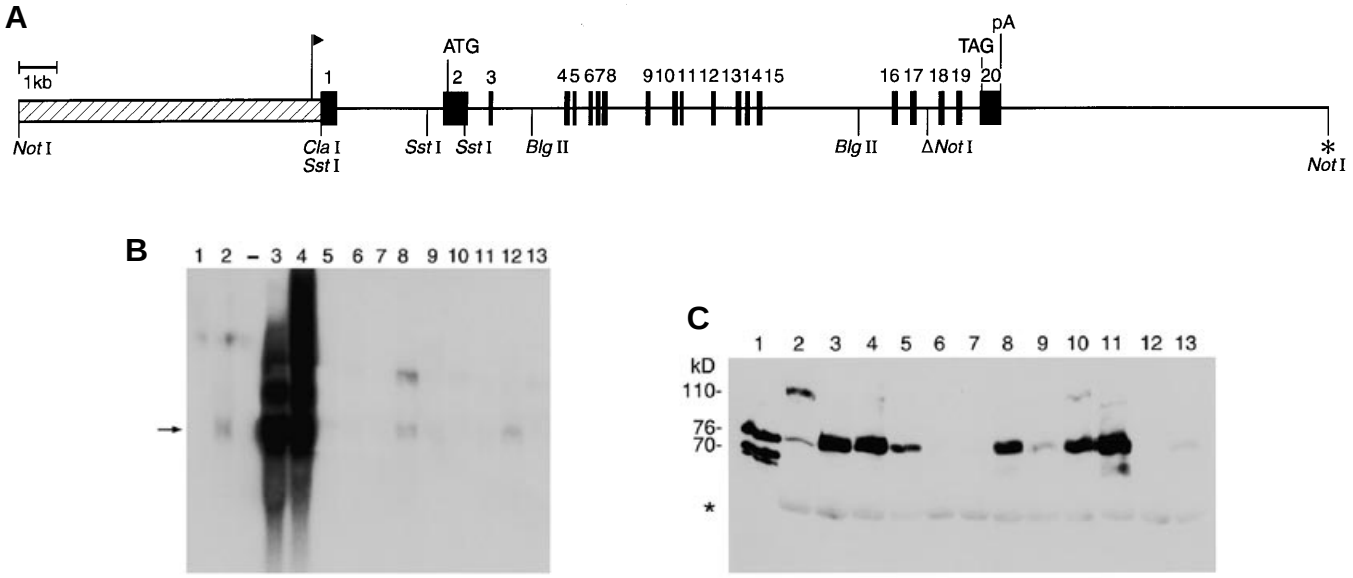

Figure 1. The acid α-glucosidase transgene and its expression. (**A**) Structure of the bovine α_{S1}-casein–human acid α-glucosidase transgene. Solid boxes, exons of the human acid α-glucosidase gene; line, introns and adjacent 3′ sequences; striated box, bovine α_{S1}-casein promoter region; flag, transcription initiation site; ATG, translation initiation site; TAG, translation stop site; pA, polyadenylation site; Δ*Not*I, deleted *Not*I site; *, location of the *Bam*HI–*Bgl*II fusion site. The essential restriction sites *Cla*I, *Sst*I, *Bgl*II and *Not*I are indicated. (**B**) Northern blot analysis of transgene expression. Twenty micrograms of RNA extracted from various tissues of a lactating transgenic mouse (mouse 4312 of line 2585, with a copy number of 10) was loaded in each lane and transferred onto a Hybond-N filter. The filter was hybridized with a ^{32}P-labelled human specific acid α-glucosidase cDNA probe as described in Materials and Methods. Lane 1 contains 1 µg of bovine mammary gland RNA mixed with 19 µg of mammary gland RNA of a non-transgenic mouse. Lane 2 contains 20 µg of human fibroblast RNA. The other lanes contain RNA samples from the left mammary gland (lane 3), the right mammary gland (lane 4), brain (lane 5), lungs (lane 6), kidney (lane 7), liver (lane 8), salivary gland (lane 9), muscle (lane 10), heart (lane 11), intestine (lane 12), ovaries and uterus (lane 13). The arrow points to the human acid α-glucosidase messenger. Higher bands are probably caused by aberrant splicing. (**C**) Immunoblot analysis of human acid α-glucosidase expression in various tissues of mouse 4312 (line 2585). The acid α-glucosidase activity in tissue homogenates was measured with 4-methylumbelliferyl-α-D-glucopyranoside, and the human enzyme was immunoprecipitated selectively with mouse anti-human acid α-glucosidase antibodies from a sample containing 100 nmol of activity. The precipitate was applied to SDS–PAGE and subsequently blotted onto a nitrocellulose filter. Acid α-glucosidase was visualized with rabbit antibodies using chemilimunescence detection. Human placental acid α-glucosidase (0.1 µg, lane 1) and 5.5 µl of milk of the transgenic mouse (lane 2) were used as markers. The molecular masses of the 110 kDa precursor and the 76 and 70 kDa mature forms of acid α-glucosidase are given in kDa. Immunoglobulins are indicated with an asterisk. Lanes 3–13 show the same panel of tissues as described in (B).

High level expression of the transgene in the mammary gland

The six different transgenic mouse lines were compared by northern blot analysis to select those with the highest transgene expression in the mammary gland. RNA was extracted from the mammary gland of lactating F1 females at day 11 post-partum of their second litter. As a reference, we used mammary gland RNA from a lactating transgenic mouse producing human lactoferrin under control of the bovine α_{S1}-casein promoter and from a lactating non-transgenic cow. The results are shown in Figure 2. The blot was hybridized sequentially with a probe recognizing the 5′-UTR of the bovine α_{S1}-casein gene, identifying both the acid α-glucosidase and the reference mRNAs (Fig. 2A), with a human acid α-glucosidase cDNA probe exclusively recognizing the human acid α-glucosidase messenger (Fig. 2B), and a murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe specific for GAPDH mRNA as a control for gel loading (Fig. 2C). A phosphoimager was used to digitize the hybridization signals. The acid α-glucosidase mRNA level appeared to be lowest in mouse lines 2477 and 2474 and highest in lines 2585 and 2471 (Fig. 2A and B), but in the latter two lines is still significantly lower than the level of human lactoferrin mRNA in the human lactoferrin transgenic mice. There is no strict correlation between the copy number of the transgene and the acid α-glucosidase mRNA steady-state level in the mammary gland (Table 1).

The acid α-glucosidase mRNA expressed in the mammary gland of transgenic mice is translated as shown by western blotting (Fig. 1C, lanes 3 and 4) and immunocytochemistry (Fig. 3). High expression of endogenous β-casein is observed in all epithelial cells in the lactating mammary gland of transgenic (data not shown) and non-transgenic animals (Fig. 3A), while human acid α-glucosidase is not detectable in the latter mammary gland (Fig. 3B). A mosaic expression pattern of the acid α-glucosidase transgene is detected in the mammary gland of mouse 2785 (line 2474) with low mRNA expression (Fig. 3C), whereas intermediate mRNA expression in mouse 4316 (line 2470) leads to staining of all epithelial cells (Fig. 3D).

Secretion of recombinant acid α-glucosidase

The milk of founders, F1 and F2 females was assayed for acid α-glucosidase content by measuring the enzyme activity (Table 1 and Fig. 4) and by western blotting (Fig. 1C, lane 2). Figure 4 illustrates for mouse 3616 (line 2585) that the acid α-glucosidase concentration in the milk is low in the first few days after birth of the litter, but increases from day 7 and reaches an optimum at day 13–14 of the lactation period. This expression curve holds for all mouse lines that were obtained, with slight variations in time of onset and number of days to optimal production. The data are summarized

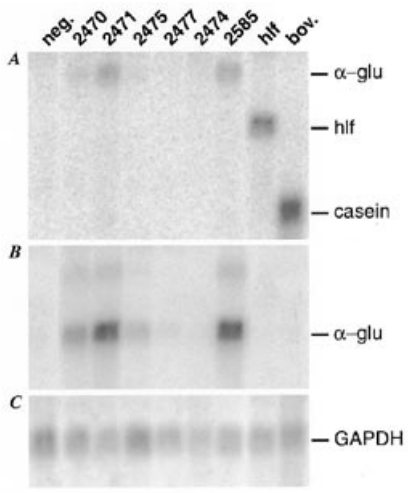

Figure 2. Northern blot analysis of human acid α-glucosidase expression in the mammary gland of different transgenic lines. Each lane contains 20 µg RNA. The blot was hybridized with (**A**) a bovine α_{S1}-casein oligo (18 bp of exon 1), recognizing the bovine α_{S1}-casein 5′-UTR, which is part of the acid α-glucosidase and human lactoferrin transgenes; (**B**) a human acid α-glucosidase probe; and (**C**) a GAPDH probe for comparing the quantities of mRNA loaded. The numbers above the lanes refer to the different transgenic mouse lines. neg., non-transgenic mouse (negative control); hlf, mouse transgenic for human lactoferrin. One microgram of bovine mammary gland RNA, mixed with 19 µg of RNA from a non-transgenic mouse, was loaded in the lane marked bov. The positions of human acid α-glucosidase (α-glu), human lactoferrin (hlf), bovine α_{S1}-casein (casein) and GAPDH transcripts are indicated.

in Table 1. The acid α-glucosidase concentration in the milk ranges from 4 µg/ml to 2 mg/ml. The acid α-glucosidase in the milk is predominantly of the 110 kDa precursor type. A minor amount represents 76 kDa mature enzyme (Fig. 1C, lane 2).

Characterization of the recombinant enzyme

The recombinant acid α-glucosidase was purified from transgenic mouse milk by a combination of concanavalin A–Sepharose and G200 Sephadex affinity chromatography as described in Materials and Methods. Two fractions were obtained: one (I) containing the 110 kDa precursor and the other (II) containing the 76 kDa mature form of acid α-glucosidase (Fig. 5). The biochemical and structural features of the recombinant enzyme purified from the milk were compared with those of natural and recombinant forms of acid α-glucosidase obtained from human placenta and culture medium of genetically engineered CHO cells (Table 2). There are no major differences between the various precursor preparations. The various preparations of the mature enzyme also have very similar characteristics, with respect to pH optimum, K_m and V_{max} values and specific activity. The precursor from both mouse milk and CHO medium typically has a several-fold higher K_m for the natural substrate than do the mature forms from the respective sources.

The two fractions of acid α-glucosidase from murine milk were characterized further with regard to their N-terminal amino acid sequence. The precursor fraction I contains two molecular species in equimolar amounts. One starts at amino acid position 70 counted from the first methionine, the other seven residues further (Table 3). The same starting positions and an additional start at position 68 were found for precursor preparations from CHO cell medium. Mature acid α-glucosidases from mouse milk, human placenta and CHO cell medium all start at amino acid 123, although the latter two enzyme preparations also contain isoforms starting at position 122. With respect to the glycosylation pattern, the acid α-glucosidase species from the different fractions and sources are very similar. Removal of the carbohydrate chains with endoF leads to a comparable mass reduction on SDS–PAGE (Fig. 6).

Therapeutic potential of recombinant acid α-glucosidase; *in vitro* studies with GSDII fibroblasts

Precursor (I) and mature (II) enzyme from milk of transgenic mice was fed to cultured fibroblasts of a patient with GSDII to investigate its therapeutic potential. Fraction I enzyme was internalized in an M6P receptor-dependent manner. The acid α-glucosidase activity in the cells increased from 0.5 to 344 nmol of 4-methylumbelliferyl-α-D-glucopyranoside converted per mg protein/h by addition of enzyme over a period of 48 h. The effect obtained with acid α-glucosidase precursor preparations from human urine and CHO cell medium and with mature acid α-glucosidase isolated from bovine testes was much the same (15,21,28). No increase of activity was obtained in the presence of 5 mM M6P. In contrast to fraction I, fraction II enzyme was not internalized by the fibroblasts. Uptake of recombinant fraction I enzyme is documented further by the molecular mass reduction from 110 kDa to 95 and 76 kDa (Fig. 7A) and most of all by the disappearance of the lysosomal glycogen storage in cells of the patient, as demonstrated by electron microscopy (Fig. 7C).

Table 2. Characteristics of purified forms of acid α-glucosidase

Source	Mouse milk	CHO medium[a]	Mouse milk	CHO medium[a]	Placenta
Isoform (kDa)	110	110	76	76	76/70
Sp. act. (µmol 4-MU/mg/h[b])	369 ± 181 (6)[c]	276 ± 72 (3)[c]	261 ± 57 (3)[c]	351 ± 71 (5)[c]	331 ± 89 (7)[c]
pH optimum, 4-MU substrate	4.5–5.0	4.5–5.0	4.5–5.0	4.5–5.0	4.5–5.0
pH optimum, glycogen substrate	4.0–4.5	4.0–4.5	4.0–4.5	4.0–4.5	4.0–4.5
K_m for 4-MU (mM)	0.63	0.71	0.86	0.57	0.76
K_m for glycogen at pH 4.3 (mg/ml)	484.6	425.3	54.8	43	42.2
V_{max} for 4-MU (mmol/mg/h)	0.21	0.36	0.43	0.38	0.54
V_{max} for glycogen (mmol glucose formed/mg/h)	6.58	9.72	5.25	5.14	6.63

[a]Details about these recombinant forms of human acid α-glucosidase are described by Fuller *et al*. (15).
[b]4-MU, 4-methylumbelliferyl-α-D-glucopyranoside.
[c]The number of different purifications is given in parentheses.

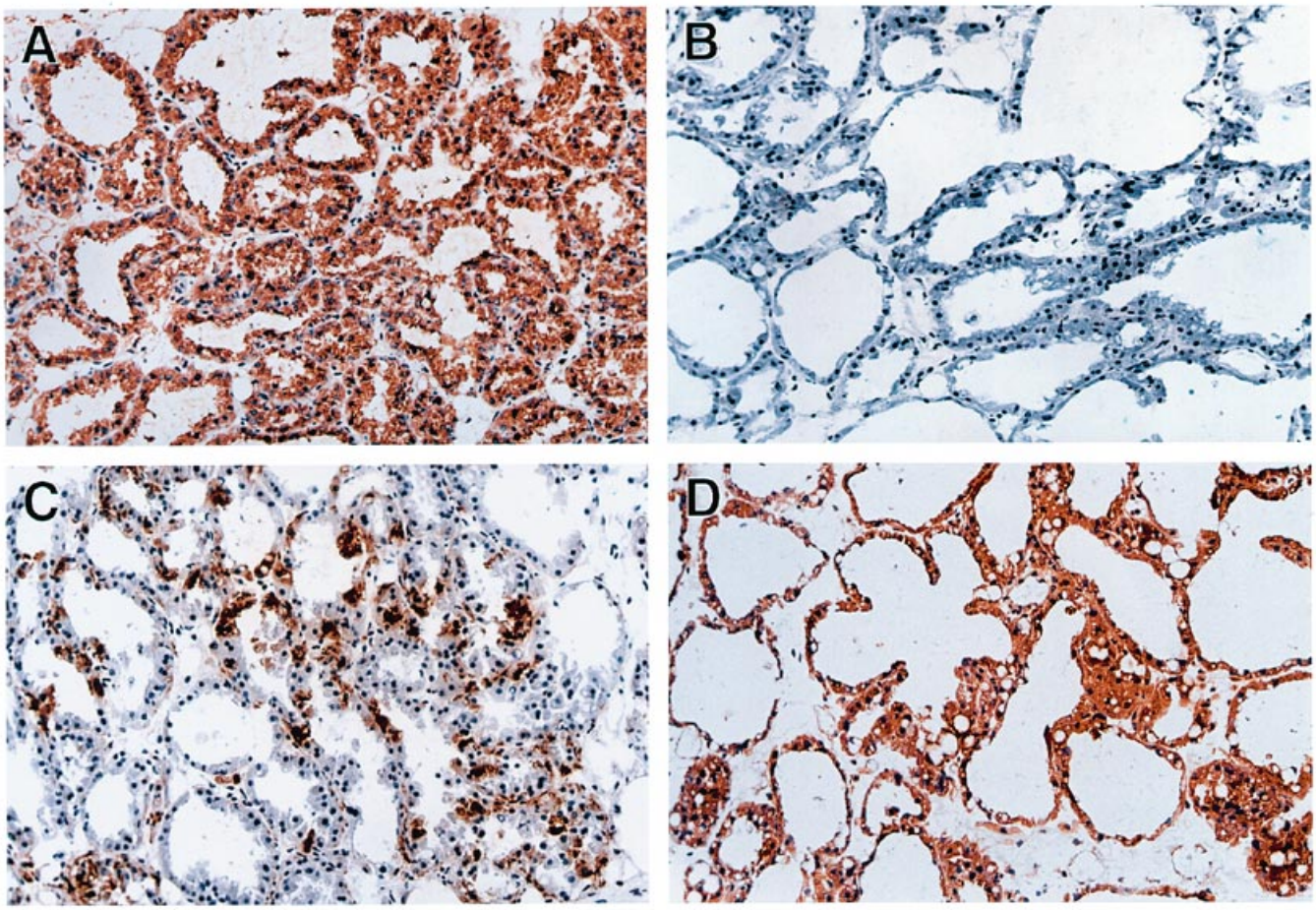

Figure 3. Immunocytochemistry of the lactating mammary gland. (**A** and **B**) Sections from a non-transgenic mouse incubated with an antibody against murine β-casein (A) and human acid α-glucosidase (B). (**C**) A section of a transgenic mouse with low acid α-glucosidase mRNA expression (mouse 2785, line 2474) and (**D**) with intermediate expression (mouse 4316, line 2470) both incubated with rabbit anti-human acid α-glucosidase antibodies. Immunoperoxidase conjugates were used for staining.

Table 3. N-terminal amino acid sequence of purified forms of acid α-glucosidase

Source	Isoform	N-terminus	
Mouse milk	precursor	77	AVPTQXDV...
		70	AHPGRPRA...
CHO medium	precursor	70	AHPGRPRA...
		77	AVPTQXDV...
		68	AQAHPGRP...
Human urine	precursor[a]	70	AHPGRPRA...
Mouse milk	mature	123	GQPWXFFP...
CHO medium	mature	122	MGQPWXFF...
		123	GQPWXFFP...
Placenta	mature	122	MGQPWXFF...
		123	GQPWXFFP...

[a]The N-terminal sequence of human urine acid α-glucosidase precursor was described by Wisselaar *et al*. (45).

Therapeutic potential of recombinant acid α-glucosidase; *in vivo* studies with GSDII KO mice

We were able to collect enough enzyme from the mouse milk to perform a pilot experiment on the *in vivo* efficacy of the recombinant enzyme in our recently developed KO mouse model of GSDII. The model mimics the severe infantile form of human GSDII in that the acid α-glucosidase activity is fully deficient, and both heart and skeletal muscles are involved. The GSDII KO mice were given a single 100 µg dose of enzyme intravenously and they were sacrificed 2 days later to measure the acid α-glucosidase activity in tissues. Affected and non-affected littermates received injections of phosphate-buffered saline (PBS) as placebo. The results are shown in Table 4. The increase in acid α-glucosidase acivity resulting from enzyme administration is highest in liver and spleen, but is also significant in heart, skeletal muscle and other organs. Notably, the activity is not increased in brain and spinal cord of treated animals. Western blot analysis of heart and triceps brachii (Fig. 8A) shows that the injected 110 kDa milk precursor is converted in the tissues of treated animals to mature enzyme of 76 kDa. In this respect, the milk enzyme also behaves the same as the recombinant acid α-glucosidase produced by CHO cells (shown for quadriceps, Fig. 8B).

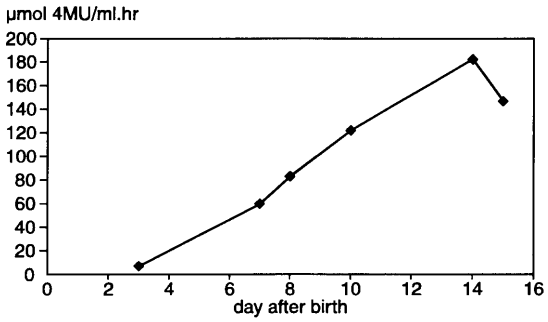

Figure 4. A representative curve of the acid α-glucosidase activity during lactation. The acid α-glucosidase activity in the milk of a transgenic mouse was measured with the artificial substrate 4-methylumbelliferyl-α-D-glucopyranoside during lactation (see Materials and Methods); 1 µmol of activity corresponds to 3.3 µg of enzyme.

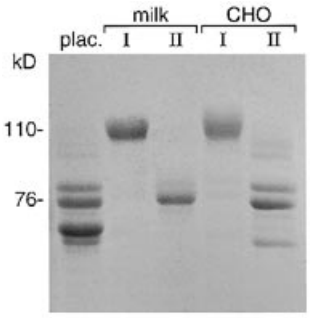

Figure 5. SDS–PAGE analysis of acid α-glucosidase purified from human placenta, milk of transgenic mice and medium of genetically engineered CHO cells. Fraction I contains the 110 kDa precursor and fraction II the 76 kDa mature form. The gel was stained with Coomassie brilliant blue. Plac., human placental enzyme.

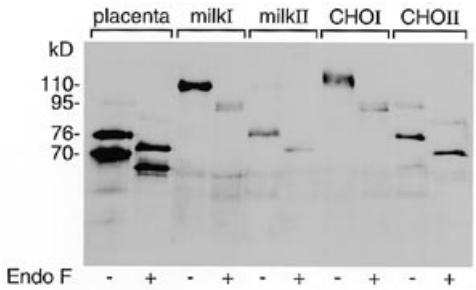

Figure 6. The glycosylation of acid α-glucosidase purified from human placenta, milk of transgenic mice and medium of genetically engineered CHO cells compared by endoF digestion. After endoF (+) or mock (–) treatment, the enzyme preparations were applied to SDS–PAGE and subsequently analysed by western blotting. Acid α-glucosidase was visualized with rabbit antibodies in combination with chemiluminescence staining. Fraction I contains the precursor and fraction II the mature enzyme.

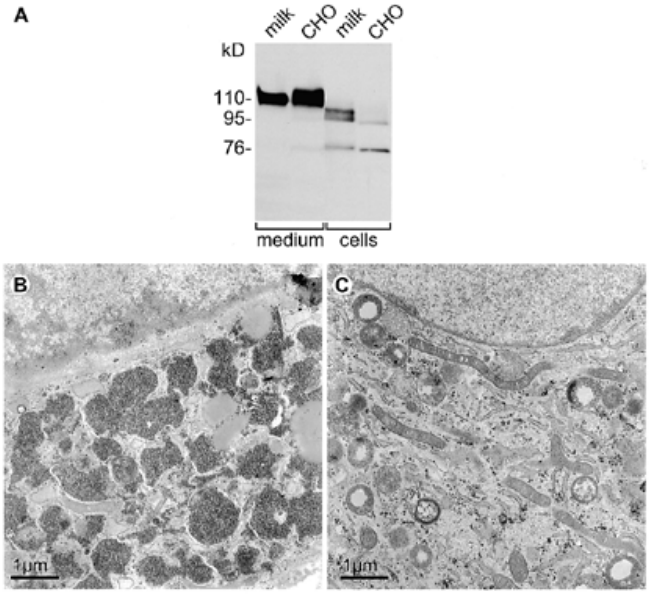

Figure 7. Uptake and processing of recombinant acid α-glucosidase by fibroblasts of a patient with GSDII, and clearance of lysosomal glycogen. (**A**) Western blot analysis of acid α-glucosidase from treated fibroblasts and culture medium 48 h after enzyme addition. Fraction I enzyme purified from the milk of transgenic mice is compared with fraction I enzyme purified from the medium of genetically engineered CHO cells. The enzyme is visualized with rabbit antibodies against human placental acid α-glucosidase and chemiluminescence staining. (**B** and **C**) Electron micrographs of untreated (B) and treated (C) cultured fibroblasts.

DISCUSSION

Receptor-mediated enzyme replacement therapy has proven its value for patients with type 1 Gaucher disease (6,7) and is under development for a number of other lysosomal storage diseases. The M6P receptor is foreseen as the enzyme target in most of these other diseases because of its role in lysosome-directed transport and its widespread tissue distribution (29). The requirement for phosphorylated mannose residues on the therapeutic enzyme limits the choice of enzyme source or biotechnological production system. The glycosylation and subsequent mannose 6-phosphorylation of lysosomal proteins is lacking or inadequate in bacteria, yeast, fungi, baculovirus-infected insect cells and plants (30). Due to the lack of natural sources, production of lysosomal proteins by genetically engineered CHO cells or in the milk of transgenic animals are two remaining practical options. The first production system has been explored broadly, and products have been tested *in vitro* and in animal models (10–12,15–17,24,31–36). The results are promising, but the predicted high production costs are a major concern. Potentially cheaper production in milk of transgenic animals is under development for typical milk and secretory proteins, but has hardly been explored for lysosomal enzymes (37). In our initial attempt to express and produce acid α-glucosidase in the mammary gland of mice, we used the human cDNA linked to the bovine α_{S1}-casein promoter (26). Although we succeeded in providing proof of the principle, the expression levels were low. By linking the entire genomic DNA sequences to the casein

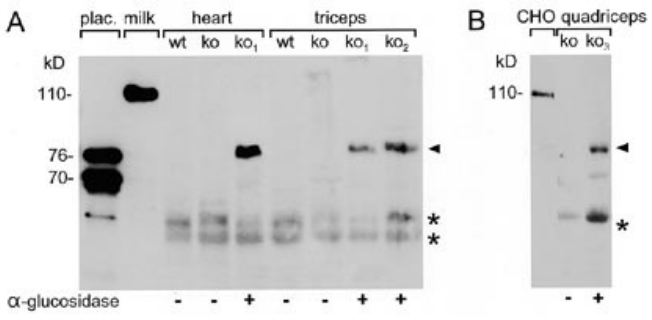

Figure 8. Uptake and processing of human recombinant acid α-glucosidase by heart and skeletal muscle tissue of GSDII mice. Mice were injected intravenously with 100 µg of the 110 kDa human precursor purified from milk of transgenic mice (**A**) and medium of transfected CHO cells (**B**). The mice were killed 2 days later with an overdose of anaesthetics and the circulation was perfused with PBS. Homogenates of heart muscle (2 mg of protein) and skeletal muscle (3.5 mg of protein) were analysed for the presence of recombinant human acid α-glucosidase by western blotting after immunoprecipitation as described in Figure 1C, except that rabbit anti-human acid α-glucosidase antibodies were used for precipitation and human specific mouse anti-acid α-glucosidase antibodies for detection on the blot. Enzyme preparations from milk, CHO cell medium and human placenta were loaded as reference samples. Wt, wild-type; KO, GSDII knockout mice that received PBS (–); KO_1, KO_2 and KO_3, GSDII KO mice that received enzyme (+). The arrowhead points to the 76 kDa human mature enzyme. Immunoglobulins are marked with an asterisk.

promoter we have increased the expression levels by 1000-fold. Up to 2 mg acid α-glucosidase/ml were measured in the milk of the highest producers. Other proteins also have an increased yield, when genomic instead of cDNA sequences are used for transgene construction. In general, the expression is highest when the gene locus is left intact and the vector system is designed to operate as an integration site-independent entity (30,38,39). Differences in expression levels of acid α-glucosidase in the six transgenic mouse lines described here point to integration site-dependent transgene expression. To achieve the most optimal production level of acid α-glucosidase, one can experiment by adding insulator elements at both ends of the acid α-glucosidase transgene, which may eliminate effects of the integration site, and remove the transcription/translation inhibitory elements in intron 1 of the gene (40).

The acid α-glucosidase production during lactation follows a curve similar to that of milk proteins such as α_{S1}-casein (41). The transgene expression is not fully mammary gland specific: other tissues also contain human acid α-glucosidase mRNA and protein, but in much lower concentrations than the mammary gland. This leaky expression is not unique, it has been described for other transgenes even for those encoding milk proteins (30). We have little concern that the (ectopic) transgene expression causes any harm to the animals, because acid α-glucosidase is an endogenous household enzyme that is naturally present in all cell types and body fluids. In addition, the overproduced acid α-glucosidase is thought to be secreted at the apical side of the epithelial cells along with the bulk of milk proteins. Ultimately, the transgenic mice look perfectly healthy. They give birth to healthy litters, with Mendelian distribution of the trait, and they have a normal average life span.

Table 4. Uptake of acid α-glucosidase by tissues of GSDII knockout mice

	Acid α-glucosidase activity (nmol 4-MU/mg protein/h)			
	Wild-type placebo	Knockout placebo	Knockout milk α-glucosidase[a]	Knockout CHO α-glucosidase[a]
Liver	49.4	0.8	209.7 (428)	286.6 (580)
Spleen	58.4	0.7	187.5 (321)	249.2 (426)
Kidney	31.3	1.9	6.7 (21)	10.9 (35)
Thymus	27.6	0.5	7.9 (29)	10.5 (38)
Lung	28.7	0.8	5.0 (17)	ND
Heart	21.9	0.5	7.7 (35)	27.8 (127)
Triceps	13.2	0.2	1.6 (12)	3.4 (26)
Quadriceps femoralis	10.3	0.3	3.0 (29)	3.6 (35)
Gastrocnemius	14.8	0.2	2.4 (16)	6.2 (42)
Tongue	17.6	0.3	7.8 (44)	18.6 (106)
Cerebellum	66.0	0.2	1.0 (1.5)	0.6 (0.9)
Cerebrum	51.2	0.2	0.8 (1.5)	0.7 (1.4)
Spinal cord	63.8	ND	1.0 (1.5)	ND

Enzyme activity was assayed, at pH 4.0, 2 days after intravenous injection of acid α-glucosidase or a physiological salt solution (placebo). The endogenous activities in the wild-type mice are the means of three individual mice. The activities in the GSDII knockout mice injected with either placebo or acid α-glucosidase are each from a single animal, except for heart and muscle injected with milk acid α-glucosidase where it concerns the mean activity in two animals.
[a]The figures within parentheses are the activities in the treated GSDII knockout mouse as a percentage of wild-type mice.
ND, not done.

More intriguing is the finding of a mosaic expression pattern of human recombinant acid α-glucosidase in the mammary gland of a relatively low producer (Fig. 3C). The underlying mechanism is unclear. One possible explanation is irreversible inactivation of the transgene, by methylation, rearrangement or excision processes during the developmental stages. Another explanation can be differential activation of milk protein genes in individual mammary gland cells. Similar mosaic expression patterns have been reported for endogenous and transgenically expressed milk proteins in sheep, cattle and mice (42–44). Mice with intermediate and high acid α-glucosidase expression in the milk produce human acid α-glucosidase in all the epithelial cells of the alveoli.

Obviously, the relatively high concentration of recombinant acid α-glucosidase in the milk facilitates extraction and purification. In essence, pure enzyme was obtained in just two purification steps. Of the two fractions obtained, fraction I has the same properties as the acid α-glucosidase precursor extractable from human urine and the medium of genetically engineered CHO cells. These properties concern the substrate specificity, the pH optimum, the molecular mass on reducing SDS–PAGE (before and after deglycosylation) and N-terminal amino acid sequence (15,21,45). Similarly, fraction II resembles, in terms of its properties, the 76 kDa mature acid α-glucosidase from human placenta and the 76 kDa fraction of CHO cell medium. Thus, it appears that the post-translational modifications of recombinant human acid α-glucosidase proceed in the mouse mammary gland in the same way as in human fibroblasts, muscle cells and CHO cells (45–47).

The therapeutic potential of the recombinant enzymes produced in milk and by CHO cells was compared *in vitro* and *in vivo*. Only the precursor but not the mature form of acid α-glucosidase from both sources is taken up by cultured fibroblasts of a patient with GSDII. Uptake is in both cases mediated by the M6P receptor and is followed by natural proteolytic conversion of the 110 kDa precursor to 76 kDa mature enzyme. The activity towards the natural substrate increases by this process. As a result, the lysosomal glycogen accumulation is diminished. In all these features, the recombinant enzymes are not different from the natural precursor isolated from human urine (21).

In vivo, the acid α-glucosidase produced in mouse milk gave results similar to those of the enzyme produced by CHO cells. Two days after treatment of GSDII KO mice, the enzyme activity in heart and skeletal muscle samples had increased from <3% to >12% of the wild-type activity. The same proteolytic maturation is observed *in vivo* as *in vitro*, suggesting that the enzyme has entered the cardiomyocytes and skeletal muscle fibres.

Altogether, the production of recombinant human acid α-glucosidase in the mammary gland of transgenic animals seems a good alternative to production by CHO cells because of lower intrinsic costs and similar therapeutic potential. Guided by these positive results, we have started large-scale production of recombinant acid α-glucosidase in the milk of transgenic rabbits.

MATERIALS AND METHODS

Construction of the expression cassette and generation of transgenic mice

The expression cassette (Fig. 1A) was constructed by fusion of the human acid α-glucosidase gene (48) to 6.3 kb of the bovine α_{S1}-casein gene promoter (49). The human acid α-glucosidase gene was cloned by Hoefsloot *et al*. in 1990 (48) and is available as three *Bgl*II fragments: a 5′ fragment of 10.5 kb with 5 kb upstream sequences plus exons 1–3, an 8.5 kb middle fragment containing exons 4–15, and a 14 kb 3′ fragment containing exons 16–20 plus 9 kb of 3′-flanking sequences. The three fragments were subcloned into the *Bgl*II site of plasmid pKUN1 derivatives with an adapted polylinker (50) and the plasmids were named p10.5αglu, p8.5αglu and p14αglu, respectively. The 5′-UTR was first removed from p10.5αglu by a partial digestion with *Sst*I followed by self-ligation. This resulted in plasmid p5′αgluSstI starting at the *Sst*I site in exon 1. Since the *Not*I site in intron 17 interfered with the cloning strategy, it was removed by digestion of p14αglu with *Not*I and re-ligation after blunting the sticky ends. This adapted 3′ fragment was excised from p14αglu with *Bgl*II and *Bam*HI, and inserted into the (remaining) 3′ *Bgl*II site of p5′αgluSstI, resulting in p5′3′αgluEx1. The orientation of the fragment was checked by restriction enzyme analysis. Subsequently, the middle fragment was excised from p8.5αglu with *Bgl*II and inserted in the de-phosphorylated *Bgl*II site between the 5′ and 3′ α-glucosidase gene fragments of p5′3′αgluEx1, resulting in pαgluEx1. The correct orientation of the fragment was confirmed with enzyme digestions. The acid α-glucosidase gene was excised from the plasmid with *Cla*I and *Not*I double digestion and ligated together with the 6.3 kb *Not*I–*Cla*I bovine α_{S1}-casein promoter fragment into cosmid pWE15, on *Not*I digestion and dephosphorylation, resulting in cosmid c8αgluEx1. The expression cassette was excised from the vector by *Not*I digestion, isolated and microinjected into the pronuclei of fertilized mouse oocytes (CBA/BrA × C57Bl6) using standard procedures (51).

Southern blot hybridization of genomic DNA

DNA was extracted from tail biopsies, digested with *Eco*RI and subjected to agarose gel electrophoresis and Southern blotting to detect the transgene. An *Nsi*I–*Nco*I bovine α_{S1}-casein fragment was used as probe. The copy number of the transgene was determined by Southern blot analysis on digestion with *Sac*I and hybridization with acid α-glucosidase cDNA PCR fragments as probes. The band intensities were compared with those of a serial dilution of plasmid DNA. A phosphoimager with Image Quant program (Molecular Dynamics) was used for quantitation.

Collection and storage of tissue and milk samples

Bovine mammary gland samples were obtained through the local slaughterhouse. Mouse milk was collected from day 7 after birth, unless stated otherwise, with a hand-made milking apparatus and stored at –20°C. Mice were killed by cervical dislocation. Tissue samples were quick-frozen in liquid nitrogen and stored at –20°C for subsequent isolation of RNA and biochemical analyses.

RNA isolation and northern blotting

Total RNA was isolated using the RNA-zol B method (Tel-Test). RNA (20 µg) was separated on a 1% agarose–formaldehyde gel, and transferred to Hybond-N membranes (Amersham). To detect the human acid α-glucosidase mRNA, three PCR fragments covering the human acid α-glucosidase cDNA were used as probe. An oligonucleotide of exon 1 of the bovine α_{S1}-casein gene was used as a probe to detect the acid α-glucosidase and hlf mRNA levels in transgenic mice and the endogenous α_{S1}-casein

mRNA level in cows. A GAPDH probe was used to quantify the total amount of mRNA in the different tissue samples.

Acid α-glucosidase activity and protein assay

Mouse tissues were homogenized in PBS using an ultra turrax (TP 19–10, 20.000 Upm; 170 W; Janke & Kunkel KG). After removal of the large debris at 10 000 *g* (twice for 15 min), the supernatant was stored at –20°C. The supernatants, crude milk and purified milk fractions were assayed for acid α-glucosidase activity with 4-methylumbelliferyl-α-D-glucopyranoside as substrate at pH 4.3 unless stated otherwise. The hydrolysis of glycogen was measured as described (52). The protein content of the samples was determined using the bicinchoninic acid (BCA) protein assay (Pierce).

SDS–PAGE and western blotting

Acid α-glucosidase was immunoprecipitated from tissue homogenates or cell lysates with mouse or rabbit antibodies raised against human placental acid α-glucosidase complexed to protein A–Sepharose 4B as described (26). The complex was washed, boiled in sample buffer and applied to 8% SDS–PAGE. Acid α-glucosidase was blotted onto nitrocellulose filters and visualized with rabbit or mouse anti-human placental acid α-glucosidase antibodies using the ECL detection kit (Amersham).

Immunocytochemistry

Mammary gland tissue was taken from transgenic and non-transgenic mice at day 11 of the second lactation period. The tissue was embedded in tissue tek (Sakura Fine tek Europe BV, Zoeterwoude, The Netherlands) and frozen in liquid nitrogen. Cryostat sections (6 µm) were incubated with rabbit anti-human placental acid α-glucosidase antiserum in combination with swine anti-rabbit IgG antibodies conjugated to horseradish peroxidase (Dako) and stained with diaminobenzidine (Dako). The sections were counterstained with Gill's haematoxylin.

Acid α-glucosidase purification and characterization

Recombinant human acid α-glucosidase was extracted from milk. The extraction and purification procedure consisted of three steps. Milk fat and casein were removed by centrifugation at 1000 *g* for 45 min. The whey fraction was applied to concanavalin A–Sepharose 4B. Bound proteins were eluted with 100 mM methyl α-D-mannopyranoside (Sigma) and, after concentration, purified to homogeneity on a Sephadex G200 column (46). The acid α-glucosidase-containing fractions were pooled in such a way that fraction I contained the 110 kDa acid α-glucosidase precursor and fraction II the 76 kDa mature enzyme. These fractions were characterized further. The pH optimum of the recombinant acid α-glucosidase was determined in a buffer system of sodium acetate (0.1 M) and sodium phosphate (0.1 M). The K_m and V_{max} were determined at pH 4.3. N-terminal amino acid sequence analysis was performed as described (15). Samples for endoF digestion (*N*-glycosidase F; Boehringer Mannheim) were brought to pH 7.5 (100 mM sodium phosphate, 10 mM EDTA and 0.1% SDS). After boiling for 5 min, cooling and addition of NP-40 in a final concentration of 0.5% (BDH), 0.2 U of endoF was added and the samples were incubated for 16 h at 37°C, according to the manufacturer's recommendations. The samples were analysed by SDS–PAGE followed by western blotting as described above.

Addition of recombinant acid α-glucosidase to cultured fibroblasts

Confluent fibroblasts in 35 mm diameter tissue culture dishes were incubated with acid α-glucosidase equivalent to 500 nmol of 4-methylumbelliferyl-α-D-glucopyranoside converted/h/1.5 ml medium. Endocytosis via the M6P receptor pathway was assessed by competing with 5 mM M6P. Media and cells were harvested 48 h later. Cell lysates were prepared by four cycles of freeze–thawing of cell pellets in 150 µl of PBS. Both cell lysates and media were assayed for acid α-glucosidase activity and analysed by western blotting.

Enzyme administration

Fraction I (110 kDa acid α-glucosidase) was sterilized through microfiltration and administered intravenously via the tail vein to 8- and 16-week-old GSDII KO mice in a single dosage of 100 µg per 130 µl PBS. Control animals received 130 µl PBS. Two days after enzyme administration, the mice were anaesthetized, and the circulation was perfused with PBS. Tissues were collected and processed as described above.

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