Expression, purification and characterization of recombinant caprine *N*-acetylglucosamine-6-sulphatase

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Mucopolysaccharidosis type IIID or Sanfilippo D syndrome is a lysosomal storage disorder caused by the deficiency of *N*-acetylglucosamine-6-sulphatase (Glc6S). In addition to human patients, a Nubian goat with this disorder has been described and the caprine Glc6S (cGlc6S) cDNA cloned. In this study, the full-length cGlc6S cDNA was inserted into the expression vector, pEFNeo, which placed the cGlc6S cDNA under the transcriptional control of the human polypeptide chain elongation factor promoter. The pEFNeo expression vector also contains the human growth hormone polyadenylation signal and the genes encoding resistance to ampicillin and G418. The cGlc6S expression construct was electroporated into Chinese hamster ovary (CHO-K1) cells, and stably transfected clones were isolated. One clone, CHOrcGlc6S.17, which secreted the highest Glc6S activity into the culture medium, was selected and cultured in cell factories. The secreted recombinant cGlc6S (rcGlc6S) precursor was purified to homogeneity from conditioned medium by a two-column procedure which consisted of a Cu^{2+}-chelating Sepharose column followed by TSK G3000SW gel filtration. The native molecular mass of rcGlc6S was estimated to be 102 kDa and the subunit size was 94 kDa. The kinetic properties of cGlc6S were similar to those of human Glc6S isolated from liver. rcGlc6S was endocytosed by fibroblasts from patients with mucopolysaccharidosis type IIID via the mannose 6-phosphate receptor-mediated pathway resulting in correction of the storage phenotype of these cells.

INTRODUCTION

Mucopolysaccharidosis type IIID (MPS IIID) or Sanfilippo syndrome type D is a lysosomal storage disorder caused by the deficiency of *N*-acetylglucosamine-6-sulphatase (Glc6S; EC 3.1.6.14), a lysosomal hydrolase that desulphates the α-linked terminal *N*-acetylglucosamine 6-sulphate residue at the non-reducing end of heparan sulphate [1,2]. The MPS IIID clinical phenotype is characterized by progressive and severe mental retardation, behavioural problems, degeneration of the central nervous system and mild visceral and skeletal involvement [2]. Human Glc6S has been purified from urine and liver [3–5], the cDNA cloned and the chromosomal location of the gene identified [6,7].

Recently, a Nubian goat (*Capra aegagrus*) with this disorder has been described [8], and an MPS IIID goat colony was established. The caprine Glc6S cDNA was subsequently cloned [9] and the molecular defect in the MPS IIID goat was identified as an R102X nonsense mutation [10]. The MPS IIID goat displayed clinical features consistent with a severe phenotype, and biochemical analysis indicated that the type and distribution of storage materials within goat tissues were comparable with those observed in human MPS IIID (M. Z. Jones, unpublished work). The clinical and biochemical similarity between human and goat MPS IIID suggests that the goat will be a relevant animal model of the human Sanfilippo syndromes. Currently, it is the only available animal model for any of the four MPS III subtypes.

In MPS III, a major site of pathology is neuronal tissue, especially the brain. Studies of bone marrow transplantation and enzyme replacement therapy in animal models of lysosomal storage disorders have shown that delivery of sufficient enzyme to the brain is hindered by the blood–brain barrier [11–14]. Therefore, in order to facilitate enzyme-replacement therapy for MPS IIID, purified recombinant caprine (rcGlc6S) may require chemical modification to increase uptake into the brain.

As a first step towards enzyme-replacement therapy for goat (and eventually human) MPS IIID, the aim of this work was to develop a scheme for the production and purification of active rcGlc6S in a form that will be efficiently targeted to tissues at sites of pathology. In this study, we report the expression of the full-length cGlc6S cDNA in CHO cells, the purification of the secreted precursor form of cGlc6S by a two-column procedure and its physical and catalytic properties. We also demonstrate the enzymic correction of human MPS IIID skin fibroblasts mediated by the mannose 6-phosphate (M6P) receptor pathway.

MATERIALS AND METHODS

Materials

Restriction endonucleases and other enzymes for DNA manipulation were purchased from Boehringer-Mannheim (Dulwich, SA, Australia) and New England Biolabs (Beverly, MA, U.S.A.). DNA oligonucleotides were synthesized with a PCR-mate EP

Abbreviations used: GlcNAc6SIdOA, *O*-(α-*N*-acetylglucosamine 6-sulphate)-(1 → 3)-L-[6-^{3}H]idonic acid; EFlα, elongation factor-1α; rcGlc6S, recombinant caprine *N*-acetylglucosamine-6-sulphatase; COON'S/DMEM, COON'S Dulbecco's modified Eagle's medium; MPS, mucopolysaccharidosis; CHO-K1 cells, Chinese hamster ovary cells; M6P, mannose 6-phosphate; DTT, dithiothreitol; BME, basal medium Eagle's; FCS, fetal calf serum; PSK, penicillin, streptomycin, kanamycin; 4MUS, 4-methylumbelliferyl sulphate; 4MU-α-GlcNAc6S, 4-methylumbelliferyl-α-D-*N*-acetylglucosaminide-6-sulphate; G418, Geneticin.

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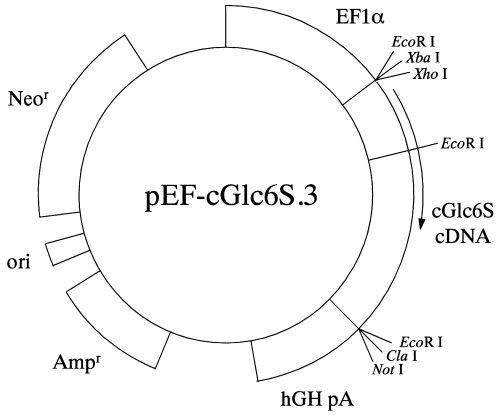

Figure 1 Schematic diagram of the rcGlc6S expression construct

EF1α, human elongation factor-1α promoter; hGH pA, human growth hormone polyadenylation sequence; Amp^r, ampicillin-resistance gene; ori, origin of replication; Neo^r, neomycin resistance gene. The relevant restriction sites are shown and the direction of transcription of the cGlc6S cDNA is indicated by the arrow. For details see the Materials and methods section.

model 391 DNA synthesizer (Applied Biosystems, Foster City, CA, U.S.A.). The plasmid pGEM7Zf(+) was obtained from Promega (Madison, WI, U.S.A.). Tissue culture and cell-factory flasks were from Nunc (Roskilde, Denmark). Kanamycin and streptomycin were purchased from Boehringer-Mannheim and penicillin was from Flow Laboratories (Sydney, NSW, Australia). COON'S/Dulbecco's modified Eagle's medium (COON'S/DMEM) was obtained from CSL Ltd. (Melbourne, Vic., Australia). Fetal calf serum (FCS), Ham's F12 medium and G418 were from Gibco–BRL (Glen Waverley, Vic., Australia). The Cell-Porator was obtained from BRL Life Technologies Inc. (Gaithersburg, MD, U.S.A.). Pluronic F68 was purchased from Fluka (Buchs, Switzerland). The ultrafiltration stirred-cell (model 8200) and Diaflo ultrafiltration membrane YM10 were from Amicon (Danvers, MA, U.S.A.). Dimethylglutarate, dithiothreitol (DTT), butyric acid, 4-methylumbelliferyl sulphate (4MUS) and BSA were obtained from Sigma (St. Louis, MO, U.S.A.). T4 polynucleotide kinase, Chelating Sepharose (fast flow), high- and low-molecular-mass standard kits for SDS/PAGE and gel chromatography were purchased from Pharmacia (North Ryde, NSW, Australia). 4-Methylumbelliferyl-α-D-*N*-acetylglucosaminide-6-sulphate (4MU-α-GlcNAc6S) was from Moscerdam (Rotterdam, The Netherlands). TSK G3000SW Ultrapac was purchased from LKB (Bromma, Sweden). $Na_2{}^{35}SO_4$ (2 mCi/mmol) was obtained from New England Nuclear (Du Pont, North Ryde, NSW, Australia).

DNA manipulation

All DNA manipulations were standard techniques [15]. The full-length Glc6S cDNA was assembled from 5′ and 3′ sequences [9] (K. Friderici, unpublished work). The 5′ sequence was obtained from clone 1-1, an 890 bp cDNA PCR product derived from goat brain RNA containing 18 bp of 5′ non-coding sequence and the first approximately 870 bp of cGlc6S coding sequence, subcloned into the pCRII vector (Invitrogen, San Diego, CA, U.S.A.). The 3′ sequence was obtained from clone 2-1, a 1.23 kb *Eco*RI cDNA fragment in pBluescript containing nt 553 to nt 1694 of the cGlc6S coding region and 93 bp of 3′ non-coding sequence. Clone 2-1 was isolated from a custom-made λZAP caprine kidney cDNA library [9]. The expression vector pEFNeo has been described previously [16].

CHO cell transfection

The cGlc6S cDNA expression construct in pEFNeo (Figure 1) was electroporated into CHO cells using a Cell-Porator, as previously described [17]. Stably transfected cells were selected with G418 (750 μg/ml total mass).

CHO cell culture

CHO-K1 cells were grown in COON'S/DMEM supplemented with 10% (v/v) FCS, 0.1% (w/v) Pluronic F68 and antibiotics PSK (100 μg/ml penicillin, 100 μg/ml streptomycin sulphate, 120 μg/ml kanamycin sulphate) at 37 °C in a 5% CO_2/air atmosphere. When the cells reached confluency, the cell monolayers were thoroughly rinsed with PBS and the medium was replaced with FCS-free COON'S/DMEM supplemented with 0.1% (w/v) Pluronic F68 and PSK. In order to increase the yield of rcGlc6S, 1 mM butyric acid and 10 mM NH_4Cl were added to the medium. Cells were cultured in 75 cm² culture flasks or in cell factories (1200 cm² two-layer system) at 37 °C typically for 4–5 days between changes of medium.

Purification of rcGlc6S

The rcGlc6S was purified from the conditioned medium using a two-column procedure. The CHOrcGlc6S-conditioned medium was clarified by centrifugation (750 ***g*** for 10 min), the supernatant filtered through 0.2 μm filter units (Sartorius, Göttingen, Germany) and the filtrate was stored at 4 °C. Batches of filtered conditioned medium were concentrated and dialysed against buffer A [15 mM sodium dimethylglutarate, 10% (v/v) glycerol, 0.1 mM DTT, 3 mM NaN_3, pH 6.0] using Amicon stirred-cell ultrafiltration units with Diaflo YM10 membranes. All procedures were performed at 4 °C unless otherwise stated.

Chelating Sepharose (fast flow) (10 cm × 1 cm column) was complexed with Cu^{2+} cations and equilibrated with buffer B [15 mM sodium dimethylglutarate, 500 mM NaCl, 10 mM Tris/HCl, 10% (w/v) glycerol, 0.1 mM DTT, 3 mM NaN_3, pH 6.0]. The concentrated and dialysed CHOrcGlc6S-conditioned medium was adjusted to the composition of buffer B but at pH 7.0, and applied to the Cu^{2+}-chelating Sepharose column (flow rate 0.5 ml per min). The column was first washed with 15 ml of buffer B at pH 7.0 and then with successive 14 ml batches of buffer B of decreasing pH (pH 5.0, 4.8, 4.6, 4.45, 4.2, 4.0, 3.8). The column was then washed with 10 ml of 1 M NaCl/100 mM EDTA, pH 8.0, which chelates Cu^{2+} ions and consequently elutes any remaining bound proteins from the column. Each eluate was collected into 2 ml of 1 M dimethylglutarate pH 7.0. Fraction samples were dialysed against buffer A at 4 °C overnight. Aliquots (5 μl) of dialysed samples were assayed for Glc6S activity using the fluorogenic substrate 4MU-α-GlcNAc6S (see below), and total protein was determined.

Fractions containing Glc6S activity (pH 5.0 and 4.8) were pooled (Figure 2a) and concentrated in an Amicon ultrafiltration stirred-cell. A 200 μl sample of the concentrated pool was applied to a TSK G3000SW Ultrapac column (30 cm × 0.8 cm) which was equilibrated in 15 mM dimethylglutarate/0.5 M NaCl/3 mM NaN_3, pH 6.0 (flow rate 0.5 ml/min and a pressure of 150 kPa). Native molecular mass was determined using the above FPLC system which was calibrated with molecular-mass standards as described previously [18]. A sample from the fraction with peak

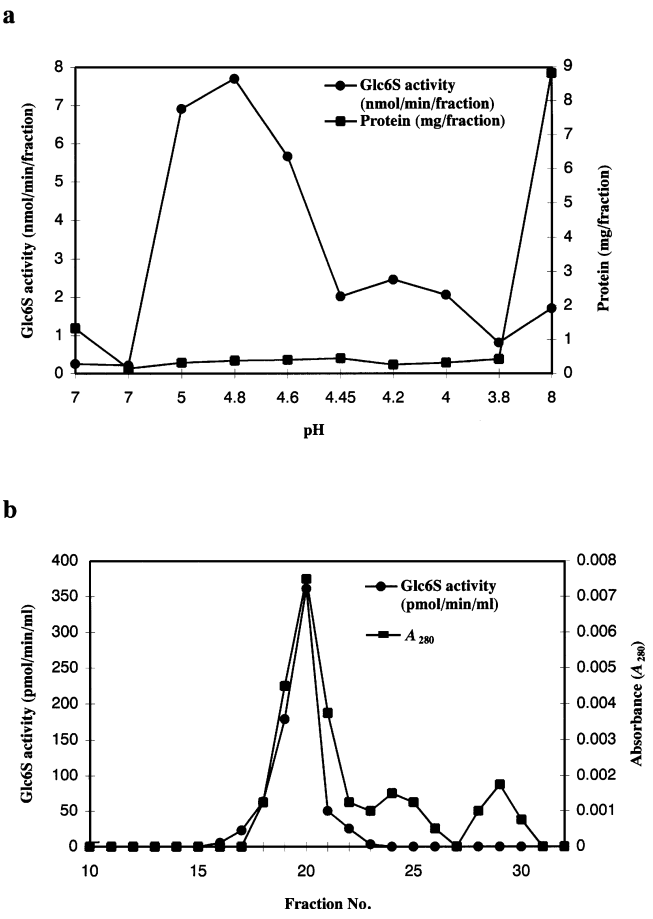

Figure 2 Chromatography of rcGlc6S

For experimental details refer to the Materials and methods section. (**a**) Cu^{2+}-chelating Sepharose and (**b**) TSK G3000SW chromatography. Fractions indicated were assayed for Glc6S activity and protein/A_{280}.

activity (fraction 20) on FPLC (Figure 2b) was run on SDS/PAGE (12 % acrylamide) [19] under reducing conditions (32 mM DTT) to estimate apparent subunit size.

Glc6S assays

Three different assays were used to determine Glc6S activity. The first utilized the fluorogenic substrate 4MU-α-GlcNAc6S and was performed as described by He et al. [20], except that the first incubation of the two-step assay was at 37 °C for 14.5 h. Glc6S was also assayed using the radiolabelled disaccharide substrate GlcNAc6SIdOA [4,5] as follows; the total assay volume of 12 μl included 1 μl of 17 μM GlcNAc6SIdOA, 3 μl of 0.1 M dimethylglutarate pH 6.0, 1 μl of BSA (6 mg/ml) and 1 μl of enzyme sample either diluted in 25 mM dimethylglutarate, pH 6.0, or dialysed against 5 mM sodium acetate pH 4.0. The assay volume was made up to 12 μl with water. Assay tubes were incubated at 37 °C for the appropriate time to give between 10 and 30 % breakdown of substrate to product. High-voltage electrophoresis was used to separate substrate from product [4]. The fluorogenic substrate 4MUS was also used to assay rcGlc6S under conditions of 5 mM 4MUS, 50 mM sodium acetate, pH 5.2, and BSA (50 μg/ml) in a total volume of 110 μl. The first assay was used to measure Glc6S activity in culture medium and partially purified fraction samples, and the second and third assays were used to measure activity in purified fractions.

Assay of other lysosomal enzymes

β-Hexosaminidase was assayed with 4-methylumbelliferyl 2-acetamido-deoxy-β-D-glucopyranoside [21]. Iduronate-2-sulphatase [18], α-L-iduronidase [22], *N*-acetylgalactosamine-4-sulphatase [23], *N*-acetylgalactosamine-6-sulphatase [24], sulphamidase [25] and glucuronate-2-sulphatase [26] were assayed by previously published procedures.

Protein assay

Total protein was determined using either the Lowry method [27] or the Bio-Rad protein assay kit according to the manufacturer's instructions, using BSA as the standard.

Correction of human MPS IIID fibroblasts

Fibroblasts from two normal individuals (SF4292 and SF4294) and from a clinically severe MPS IIID patient (SF1941), who had undetectable levels of Glc6S activity in both fibroblasts and leucocytes, were grown to confluency in 25 cm^2 flasks in basal medium Eagle's (BME)/10 % (v/v) FCS and antibiotics PS (penicillin and streptomycin sulphate). Before labelling of fibroblasts with $Na_2{}^{35}SO_4$, the medium was removed, the cells rinsed with Ham's F12/10 % (v/v) FCS and penicillin only and re-fed with 3 ml/flask of the same medium to deplete the $SO_4{}^{2-}$ pools. After 4 h, the medium was replaced with fresh medium [Ham's F12/10 % (v/v) FCS and penicillin] supplemented with $Na_2{}^{35}SO_4$ at 10 μCi/ml. After 48 h the cells were rinsed with PBS and the medium changed to BME/10 % (v/v) FCS and PS, and rcGlc6S was added at 0.5 and 5.0 μg/ml in the presence or absence of M6P (5 mM final concentration). Cells were harvested 48 h later by trypsin treatment, and washed three times with 0.15 M NaCl by centrifugation (750 ***g***, 5 min)/resuspension. Cell pellets were resuspended in 100 μl of 20 mM Tris/HCl, 0.25 M NaCl, pH 7.0, the cells frozen and thawed six times and the cell lysates clarified by microcentrifugation (13000 ***g***; 5 min; 4 °C). Cell lysates were dialysed against 5 mM sodium acetate, pH 4.0, overnight at 4 °C before being assayed for Glc6S and β-hexosaminidase activities, protein content and radioactivity.

Kinetics

Kinetic data (K_m, V_{max} and pH optimum) for Glc6S were obtained from Lineweaver–Burk plots using the radiolabelled substrate and 4MUS. The assays were performed within a concentration ranged of 3.3–17 μM for the disaccharide substrate and 0.5–5 mM for 4MUS.

RESULTS AND DISCUSSION

Assembly of Glc6S expression construct

The full-length cGlc6S cDNA was assembled from two fragments before insertion into the expression vector. The 5′ sequence of the cGlc6S cDNA was excised from clone 1-1 as a 0.6 kb *Xho*I–*Eco*RI fragment and contained 18 bp of 5′ non-coding sequence and 552 bp of 5′ coding sequence. The *Xho*I–*Eco*RI

fragment was subcloned into the corresponding sites of the cloning vector pGEM7Zf(+) to generate pGEM-5′cGlc6S.1. The 3′ sequence of the cGlc6S cDNA was excised as a 1.23 kb *Eco*RI fragment from clone 2-1 and contained nt 553 to nt 1694 of the cGlc6S coding region and 93 bp of 3′ non-coding sequence. The 1.23 kb *Eco*RI fragment was then subcloned into the *Eco*RI site of pGEM-5′cGlc6S.1, and clones with the correct orientation of the 5′ and 3′ cGlc6S cDNA sequences were identified by restriction analysis. Clone pGEM-cGlc6S.8 was selected and the complete cGlc6S cDNA was excised from the polylinker as an approx. 1.8 kb *Xba*I(5′)–*Cla*I(3′) fragment.

The expression vector pEFNeo-EXCN.1 was constructed by linearizing pEFNeo with *Eco*RI and *Not*I and then inserting a pair of adaptor linkers containing *Eco*RI, *Xba*I, *Cla*I and *Not*I restriction sites (5′ → 3′). The full-length cGlc6S cDNA *Xba*I–*Cla*I fragment was then ligated into the corresponding sites of pEFNeo-EXCN.1 to generate pEF-cGlc6S.3 (Figure 1).

In pEF-cGlc6S.3, the cGlc6S cDNA is under the transcriptional control of the human polypeptide chain elongation factor (EF1α) promoter. The expression vector also contained the human growth hormone polyadenylation signal and the genes for ampicillin and neomycin resistance.

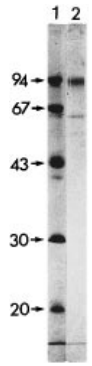

Figure 3 SDS/PAGE of rcGlc6S

Lanes 1 and 2 are molecular-mass standards and purified rcGlc6S respectively, electrophoresed under reducing conditions with DTT and Coomassie-stained. The sizes of the molecular-mass standards are indicated in kDa.

6S expression in CHO cells

The Glc6S expression construct pEF-cGlc6S.3 was introduced into CHO-K1 cells by electroporation, and stably transfected clonal lines were isolated by G418 selection. In order to select the clone secreting the highest levels of cGlc6S activity into the culture medium, 39 clonal CHO lines were individually cultured to confluence, and Glc6S activity was assayed in the medium using 4MU-α-GlcNAc6S [20].

The highest activity clone, CHOrcGlc6S.17, was found to secrete 32-fold higher Glc6S activity into the culture medium relative to untransfected CHO-K1 (results not shown). Preliminary experiments confirmed that the addition of butyric acid and NH_4Cl increased the yield of Glc6S activity in conditioned medium (results not shown). NH_4Cl causes the rerouting of lysosomally destined enzymes to the secretory pathway [28]. Butyric acid was added because it has been shown to increase expression of transfected genes [29].

Large-scale production and purification of rcGlc6S

Clone CHOrcGlc6S.17 was expanded and cultured in a cell factory system as described in Materials and methods. The yield of rcGlc6S secreted into the medium was about 5 mg/litre (range 2–7 mg) or 3–4 % of total medium protein, based on Coomassie staining of bands on SDS/PAGE (results not shown). Recombinant cGlc6S was purified to homogeneity from the culture medium of CHOrcGlc6S.17 using a two-column procedure consisting of Cu^{2+}-chelating Sepharose and gel filtration.

All the rcGlc6S applied to the Cu^{2+}-chelating column at pH 7.0 was bound and required a drop in pH for elution. The majority of rcGlc6S activity was eluted at pH 5 and pH 4.8, but activity was not completely eluted until pH 3.8. Fractions at pH 5.0 and 4.8, which together contained approx. 20 % of the total activity applied to the column (Figure 2a), were pooled, concentrated and dialysed. The majority of the contaminating protein was in the EDTA elution (33 %), which also contained some activity ($\sim$ 2 %). The original flow-through had 5 % of the initial contaminating proteins.

SDS/PAGE of the pooled fractions from the Cu^{2+}-chelating Sepharose step indicated a major protein band at 94 kDa with a few minor-molecular-mass species between 43 kDa and 67 kDa. These contaminants were successfully removed by gel filtration on TSKG3000SW as shown in Figure 3. The faint bands observed at apparent molecular masses of approx. 54 and 62 kDa in purified cGlc6S were also present in the molecular-mass standards lane and also in a buffer-only lane (results not shown) and therefore were considered to be artifacts of SDS/PAGE and not contaminants within the cGlc6S preparation. Recovery of cGlc6S activity from this step was 30 % of the activity loaded. The overall recovery of rcGlc6S from medium after the two-column purification was relatively low. Higher yields may be obtained by pooling pH eluates in addition to fractions pH 5.0 and 4.8 from the Cu^{2+}-chelating Sepharose column.

When a sample from fraction 20 was submitted to SDS/PAGE under reducing conditions, there was one major band at 94 kDa (Figure 3). cGlc6S has 13 potential N-glycosylation sites, and, as the molecular mass of cGlc6S deduced from the amino acid sequence derived from the cGlc6S cDNA is 57.7 kDa, it would appear that most, if not all, are utilized. The 36 kDa difference between the core protein mass and the precursor molecular mass is presumably due to oligosaccharide chains. The diffuseness of the band was considered to be due to heterogeneity in the N-linked oligosaccharides and has been observed in other recombinant enzymes [16]. The oligosaccharide content of the precursor was indirectly confirmed by the observation that rcGlc6S bound strongly to concanavalin A–Sepharose (results not shown).

Mature Glc6S isolated from human liver comprises several forms [4,7]. The two major forms are a form-A polypeptide with a single subunit of molecular mass 78 kDa and a form B consisting of two non-disulphide-linked subunits derived from the proteolytic processing of form A to a 32 kDa N-terminal and 48 kDa C-terminal species.

The majority of lysosomal enzyme precursors undergo a series of post-translational 'maturation' modifications such as limited *endo*- and *exo*-proteolytic cleavage and partial trimming of oligosaccharide side chains during transit through the pre-lysosomal organelles and residence in the lysosome [30]. Lysosomal enzyme precursors can also be secreted extracellularly via an alternative pathway in which these maturation processes are absent or reduced. High-level expression of lysosomal enzymes in cell culture favours the secretion of lysosomal enzymes, and consequently precursor forms predominate in the medium. Therefore the rcGlc6S species purified from conditioned

Table 1 Comparison of the catalytic properties of rcGlc6S and mature Glc6S isolated from human liver using the radiolabelled substrate GlcNAc6SIdOA

For details, see the Materials and methods section. The results for the human liver Glc6S are taken from Freeman et al. [4].

	K_m (μM)	V_{max} (nmol/min per mg)	pH optimum
rcGlc6S	48.2	49	6.0
Human Glc6S form A	11.7	105	5.0

Table 2 Comparison of the catalytic properties of rcGlc6S with recombinant human iduronate-2-sulphatase (rhIDS), recombinant human *N*-acetylgalactosamine-4-sulphatase (rhGal4S) and recombinant human *N*-acetylgalactosamine-6-sulphatase (rhGal6S) using the fluorogenic substrate 4MUS

For details, see the Materials and methods section. The results for rhIDS, rhGAl4S and rhGal6S are taken from Bielicki et al. [24].

	K_m (mM)	V_{max} (nmol/min per mg)	pH optimum
rcGlc6S	5.8	10	5.2
rhIDS	12.4	300	5.6
rhGal6S	4.4	120	4.7
rhGal4S	1.18	48485	5.6

medium is a precursor form that has not undergone the maturation process characteristic of enzymes in transit to the lysosome.

After gel filtration, rcGlc6S was free of detectable amounts of other lysosomal enzymes including iduronate-2-sulphatase, α-L-iduronidase, *N*-acetylgalactosamine-4-sulphatase, glucuronate-2-sulphatase, sulphamidase and *N*-acetylgalactosamine-6-sulphatase (results not shown).

Kinetics of rcGlc6S

Kinetic data (Table 1) indicate that both rcGlc6S and the mature form of Glc6S isolated from human liver have the same order of magnitude K_m, V_{max} and pH optima with respect to the disaccharide substrate. Results with 4MUS (Table 2) demonstrate that Glc6S, although not an arylsulphatase, is capable of binding 4MUS but its rate of substrate turnover is 4800 times slower than recombinant human *N*-acetylgalactosamine-4-sulphatase which is an arylsulphatase. This has been observed with other non-arylsulphatases such as recombinant human iduronate-2-sulphatase and *N*-acetylgalactosamine-6-sulphatase [24] and with mature human Glc6S purified from liver [7].

Enzymic correction of MPS IIID fibroblasts

MPS IIID skin fibroblasts (SF1941) cultured in the presence of purified rcGlc6S at 0.5 μg/ml showed a level of Glc6S activity comparable with that seen in normal control skin fibroblasts, and this was elevated approximately 3-fold above normal levels when 10-fold the enzyme concentration was used (Table 3). The presence of M6P abolished this effect by between 98 and 99%, thus indicating that endocytosis of rcGlc6S was via the M6P receptor. Untreated MPS IIID cells store approximately 2-fold the amount of [^{35}S]sulphated material as control fibroblasts. Proper cellular targeting of the enzyme to the lysosome was achieved as this storage was corrected by uptake of Glc6S activity. Interestingly, even at very low levels of enzyme (as seen in fibroblasts treated with M6P) there is sufficient enzyme taken up by these cells to correct the storage phenotype. The 2-fold increase in storage in human MPS IIID cells relative to normals, although not large, was identical with that observed in caprine MPS IIID cells relative to normals [8].

Table 3 Endocytosis of rcGlc6S by MPS IIID fibroblasts

Normal and MPS IIID fibroblasts were labelled with $Na_2{}^{35}SO_4$ and incubated with 0.5 μg/ml (*) or 5.0 μg/ml (†) of rcGlc6S in the presence or absence of M6P, as described in the Materials and methods section. Dialysed cell lysates were assayed for Glc6S activity, β-hexosaminidase (β-Hex) activity and radioactivity. nd., none detected. Results are means ± S.D. for three replicates in each case.

Cell line	Glc6S activity (pmol/min per mg)	β-Hex activity (nmol/min per mg)	^{35}S radioactivity (c.p.m./mg of cell protein)
SF4292 (normal)	27.3 ± 6.1	36.6 ± 7.7	29400 ± 10100
SF4294 (normal)	18.2 ± 1.8	31.2 ± 3.8	41600 ± 2800
SF1941 (MPS IIID)	n.d.	33.9 ± 11.4	60200 ± 17700
SF1941 + Glc6S*	14.9 ± 0.8	35.4 ± 4.5	48200 ± 9700
SF1941 + Glc6S†	71.6 ± 11.6	39.7 ± 11.3	46700 ± 6000
SF1941 + Glc6S* + M6P	0.2 ± 0.1	31.0 ± 7.4	45700 ± 2400
SF1941 + Glc6S† + M6P	0.6 ± 0.3	31.8 ± 5.8	43400 ± 8600

Conclusion

In this paper, we have successfully expressed rcGlc6S in CHO culture and purified the precursor form from conditioned medium using a two-column procedure. The kinetic properties of the rcGlc6S precursor (K_m, V_{max} and pH optima) were similar to the mature form A of human Glc6S purified from liver, with respect to the disaccharide substrate. The rcGlc6S precursor was taken up by cultured cells via the M6P receptor-mediated pathway and efficiently corrected lysosomal storage in human MPS IIID cells.

A major site of pathology in MPS III is neuronal tissue, in particular the brain. The blood–brain barrier, however, prevents uptake of exogenous enzymes from the circulation. The availability of large quantities of the rcGlc6S precursor will facilitate the investigation of modifications to the enzyme in an effort to increase transport across the blood–brain barrier. Such modifications may include the addition of polylysine, treatment with ethylenediamine or linking the enzyme to insulin fragments [31]. The effect of modifications of rcGlc6S on transport to sites of pathology can then be appropriately evaluated in the MPS IIID goat model.

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