Recombinant human sulphamidase: expression, amplification, purification and characterization

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Mucopolysaccharidosis type IIIA (MPS IIIA, Sanfilippo A syndrome) is a lysosomal storage disease that causes a profound neurological deterioration. The disorder is caused by a deficiency of the lysosomal enzyme sulphamidase which is a requisite for the degradation of heparan sulphate. To facilitate the development of enzyme-replacement strategies for MPS IIIA patients, we have constructed a high-level expression system for recombinant human sulphamidase in Chinese hamster ovary (CHO) cells. An expression construct containing a methotrexate-resistant dihydrofolate reductase (DHFR) gene allowed amplification of expression levels from less than 1 mg of sulphamidase per litre of culture medium to approx. 15 mg/l. Unlike many cell lines made by gene amplification in DHFR-deficient CHO cells, and utilizing the normal DHFR gene, these cell lines appeared to be stable in the absence of selective pressure. Recombinant human sulphamidase was purified from unamplified and amplified cell lines. The native enzyme was found to be a dimer of 115 kDa. Denaturing and reducing SDS/PAGE revealed a subunit size of 62 kDa. Kinetic analysis demonstrated that the recombinant enzyme had broadly similar kinetic characteristics to sulphamidase purified from liver. Recombinant human sulphamidase was able to correct the storage phenotype of MPS IIIA fibroblasts after endocytosis via the mannose-6-phosphate receptor.

INTRODUCTION

Mucopolysaccharidosis type IIIA (MPS IIIA, Sanfilippo A syndrome) is one of the most common of the lysosomal storage diseases, with certain Northern European populations having an incidence of 1 in 24000 births [1,2]. In the Cayman Islands there is an extremely high incidence of MPS IIIA with an estimated carrier frequency of 0.1 [3], presumably the result of a founder effect. The disorder is inherited in an autosomal recessive manner and results from a deficiency of the lysosomal enzyme sulphamidase (NS) (sulphamate sulphohydrolase, EC 3.10.1.1). Both genomic and cDNA sequences encoding NS have been cloned and characterized [4,5]. The gene localization is 17q25.3 [4]. NS is one of nine known exoenzyme activities involved in the degradation of the sulphated glycosaminoglycan (GAG), heparan sulphate (HS). Unlike the other sulphatases involved in the degradation of GAG which catalyse the hydrolysis of O-linked sulphate groups, NS catalyses the hydrolysis of an N-linked sulphate group from the non-reducing terminal glucosaminide residues of HS (HS is the only GAG that contains N-sulphated residues). Two other enzymes are involved exclusively in the degradation of HS, and functional deficiencies of these designate two other MPS III/Sanfilippo subtypes, namely B (α-*N*-acetylglucosaminidase deficiency) and C (acetyl-CoA-glucosaminide *N*-acetyltransferase deficiency). The fourth MPS III/Sanfilippo subtype, type D, is defined by a deficiency of *N*-acetylglucosaminide 6-sulphatase which is involved in the degradation of both HS and keratan sulphate. In patients with MPS IIID, HS is the only GAG stored, as an alternative pathway exists for the degradation of keratan sulphate [6].

The enzymic link between the MPS III subtypes results in both a common biochemical disease phenotype (storage and urinary excretion of elevated levels of HS) and a common clinical presentation characterized by severe and progressive mental deterioration [6]. Diagnostic analysis of the MPS III subtypes therefore depends on enzymic analysis.

The predominance of neurological symptoms in these patients is consistent with the relative abundance of HS in the central nervous system in comparison with other tissues. The somatic manifestations of the disease, which include hepatosplenomegaly, skeletal pathology and joint stiffness, are relatively less severe and are found mainly in older patients. In general, clinical onset of the severe form of the disease becomes evident after the age of 2 with the development of hyperactivity, behavioural disturbance and sleep disorder. Before this, development appears to be relatively normal. As the disease progresses, neurological problems such as hyperactivity, aggressive behaviour, sleep disturbance and delayed development, particularly with regards to speech, become pronounced. Other clinical symptoms of note include hirsutism with strikingly coarse hair and diarrhoea. Owing to the difficulty of early or presymptomatic diagnosis of the Sanfilippo syndromes and the degree of neurological involvement, treatment of these disorders is extremely challenging. The successful application of any therapy will depend on the development of screening programmes which can identify affected individuals before significant central nervous system damage occurs [7]. At present, the only therapy that may offer some alleviation of the Sanfilippo syndromes is bone marrow transplantation although, as this has only been applied after diagnosis of clinical symptoms, it is hard to evaluate its effect on the progression of the disease. Bone marrow transplantation also carries significant associated risk of morbidity and mortality.

The cloning of cDNA sequences encoding NS [4] has allowed several other approaches to therapy to be considered including enzyme-replacement therapy and gene therapy [8]. Both these approaches to treatment are complicated by the major site of

Abbreviations used: MPS, mucopolysaccharidosis; DHFR, dihydrofolate reductase; MTX, methotrexate; CHO, Chinese hamster ovary; NS, sulphamidase; rh, recombinant human; GAG, glycosaminoglycan; HS, heparan sulphate; M6P, mannose 6-phosphate; PS, penicillin/streptomycin; FCS, foetal calf serum; 4MUS, 4-methylumbelliferyl sulphate; BME, basal medium Eagle's; α-MEM, α-minimal essential medium; COON'S/DMEM, COON'S/Dulbecco's modified Eagle's medium.

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pathology being separated from the main body of the circulation by the blood–brain barrier. However, in the case of enzyme-replacement therapy there is reason to suppose that it may be possible to overcome this barrier to treatment using a variety of approaches [9–11].

As a first step towards enzyme-replacement therapy for MPS IIIA we have expressed recombinant human NS (rhNS) in Chinese hamster ovary (CHO) cells using an expression vector containing a methotrexate (MTX)-resistant dihydrofolate reductase (DHFR) cassette as a dominant selectable marker. Primary clones gave an expression level of less than 1 mg of rhNS/l of culture medium. After gene amplification with MTX, levels of approx. 15 mg/l of culture medium were obtained. NS expressed by gene-amplified and unamplified CHO cells was purified and characterized and both were found to be similar to the mature form isolated from liver with respect to native molecular mass, subunit size and kinetic parameters [12]. NS was endocytosed by MPS IIIA skin fibroblasts via the mannose 6-phosphate (M6P) receptor-mediated pathway and was targeted correctly to lysosomes where it was effective in abolishing the storage defect.

MATERIALS AND METHODS

Materials

Enzymes for manipulation of DNA were purchased from Boehringer-Mannheim (Dulwich, SA, Australia). CM-Sepharose, Superose 12 and high- and low-molecular-mass standard kits were bought from Pharmacia (North Ryde, NSW, Australia). Amicon (Danvers, MA, U.S.A.) supplied the ultrafiltration stirred cell (model 8200) and the Diaflo ultrafiltration member YM10. Dialysis membrane (10–12 kDa cut-off) was from Union Carbide Corp (Chicago, IL, U.S.A.). Dithiothreitol, 4-methylumbelliferyl sulphate (4MUS), MTX and M6P were purchased from Sigma (St. Louis, MO, U.S.A.). Ham's F12, basal medium Eagle's (BME) and α-minimal essential medium (α-MEM) (without nucleosides) were from Gibco (Glen Waverly, Vic, Australia) and Dulbecco's modified PBS, foetal calf serum (FCS), COON'S/Dulbecco's modified Eagle's medium (COON'S/DMEM), penicillin/streptomycin sulphate (PS) and trypsin were from CSL Ltd. (Parkville, Vic, Australia). Dialysed FCS was dialysed against four changes of more than 10 vol. of PBS.

DNA manipulation and construction of recombinant plasmid

All DNA preparation, modification and cloning procedures were carried out using standard techniques [13]. The 1.8 kb NS cDNA clone pUNSFL8 [4] contains the entire coding sequence (nucleotides 1–1802) of NS. pFR400 [14] contains a cDNA sequence encoding an MTX-resistant form of DHFR. pEhGH contains an expression cassette consisting of the human elongation factor-1α gene promoter and human growth hormone polyadenylation sequence cloned into pBluescript (Stratagene).

CHO cell culture and transfection

CHO-K1 cells were cultured in α-MEM (without nucleosides) containing 10% (v/v) FCS and antibiotics (penicillin, 50 units/ml, and streptomycin sulphate, 50 μg/ml; PS) and electroporated using a cell porator as described in [15]. Stable transfectants were selected using α-MEM (without nucleosides), 10% (v/v) dialysed FCS and 200 or 400 nM MTX.

For gene amplification, culture conditions were as for selection of transfectants but the MTX concentration was doubled approximately every 2 weeks up to a final concentration of 25 μM. Gene-amplified clones were maintained in COON'S/DMEM containing 10% (v/v) FCS and PS unless otherwise stated. For large-scale production of recombinant enzyme, two-layer cell factories (NUNC; 1200 cm^2) were inoculated with either the clone expressing the highest level of NS activity (clone NS-26) or the unamplified clone (13-200) in COON'S/DMEM/10% (v/v) FCS/PS. Once confluent, the cells were rinsed with PBS and the medium replaced with serum-free COON'S/DMEM with antibiotics, and this was exchanged every 4 days.

Media samples were clarified by centrifugation (2000 ***g***; 3 min; 4 °C) and the supernatant assayed using a mixture of radiolabelled tetrasaccharides derived from HS as previously described [8].

Purification and characterization of rhNS

rhNS was purified from conditioned medium by a one-column procedure. The medium was clarified by centrifugation as described above, concentrated in an Amicon ultrafiltration stirred-cell and dialysed against 50 mM sodium acetate, pH 5.0 (buffer A). The dialysed medium was centrifuged [2800 ***g*** (4000 rev./min); 10 min; 4 °C] before application to a CM-Sepharose column (2.0 cm × 1.0 cm) equilibrated in buffer A. After loading, the column was washed with buffer A and the rhNS was then eluted with buffer A containing increasing concentrations of NaCl ranging from 30 to 150 mM with a final elution of the column with 1 M NaCl in buffer A. Fractions containing NS activity were analysed by SDS/PAGE (12% acrylamide) by the method of Laemmli [16] to determine purity and subunit size. Those fractions that corresponded to pure enzyme were pooled and concentrated and used to determine the pH optimum of NS activity and kinetic parameters (K_m, V_{max}). Assays to produce data for Lineweaver–Burk plots were carried out using the radiolabelled substrate mixture (concentration range 6.8–34.2 μM) and the fluorogenic substrate 4MUS within the concentration range 0.5–5 mM [17]. Blank values for each substrate concentration were deducted from values obtained in the presence of enzyme. The results from six to eight substrate concentrations were used to generate Lineweaver–Burk plots. Regression analysis gave a correlation coefficient higher than 0.98 for all plots. K_m was determined as the negative reciprocal of the x-intercept, and V_{max} as the reciprocal of the y-intercept. The native size of rhNS was estimated from FPLC as described previously [18], except that a Superose 12 column (30 cm × 1 cm) was used instead of a TSK G3000SW column.

Correction of human MPS IIIA fibroblasts

Fibroblasts from a clinically severe MPS IIIA patient (SF4210), which had undetectable levels of NS activity, and from two normal individuals (SF4353 and SF4427) were grown to confluence in 25 cm^2 flasks in BME containing 10% (v/v) FCS and PS. To deplete SO_4^{2-} pools before labelling, the above medium was removed, the cells were rinsed and re-fed with 3 ml/flask Ham's F12 containing 10% (v/v) FCS and penicillin only. After 4 h the medium was replaced with fresh Ham's F12 containing 10% (v/v) FCS and penicillin supplemented with $Na_2{}^{35}SO_4$ at 10 μCi/ml. After labelling for 48 h, the cells were rinsed with PBS and the medium changed to BME/10% (v/v) FCS/PS. rhNS (in the form of conditioned medium from the NS-26 cell line) was added to a final concentration of 320 or 3200 pmol/min in the presence or absence of M6P (5 mM final concentration). An equal volume of conditioned medium from CHO cells overexpressing feline *N*-acetylgalactosamine-4-sulphatase [17] which had undetectable levels of NS was added to triplicate

cultures of SF4210 cells. Cells were harvested 2 days later by trypsin treatment and washed three times with 0.15 M NaCl by centrifugation (750 ***g***; 5 min; 4 °C)/resuspension. Cell pellets were finally resuspended in 100 μl of 20 mM Tris/HCl/0.25 M NaCl, pH 7.0, the cells lysed by six cycles of freeze/thaw and the resulting cell lysates clarified by microcentrifugation (13000 ***g***; 5 min; 4 °C) before dialysis against 5 mM sodium acetate, pH 4.0. Cell lysates were then assayed for NS, β-hexosaminidase [19], iduronate 2-sulphatase [20], protein content [21] and radioactivity (^{35}S c.p.m.).

RESULTS AND DISCUSSION

Construction of NS expression vector

The expression vectors pFRNEE.1 and pFRNEE.2 were made by cloning a *Sal*I–*Bam*HI fragment containing an expression cassette consisting of the human elongation factor-1α gene promoter and polyadenylation signal from the human growth hormone gene from pEhGH into the *Sal*I site of pFR400 [14]. A polylinker containing *Eco*RI, *Eco*RV and *Not*I sites was then inserted into the *Eco*RI site between the promoter and polyadenylation sequences. pFRNEE.1 contains the polylinker in the orientation 5′ *Not*I–*Eco*RV–*Eco*RI 3′, and pFRNEE.2 contains the polylinker in the orientation 5′ *Eco*RI–*Eco*RV–*Not*I 3′. The NS cDNA coding sequence was excised from pUNSFL8 as an *Eco*RI fragment and cloned into the *Eco*RI site of the expression vector pFRNEE.2 (Figure 1). Recombinants of the correct orientation were identified by restriction enzyme analysis, and one such clone, designated pFRNEE.2NS, was used for all further experiments.

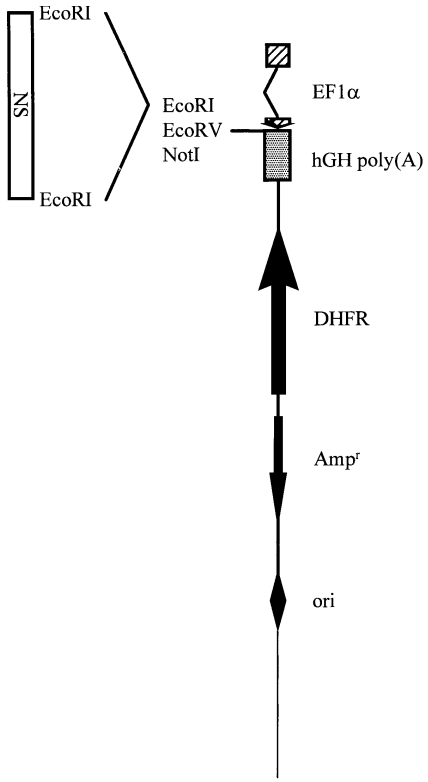

Figure 1 Construction of NS expression plasmid

The expression plasmid pFRNEE.2 is shown in a linear representation. The human elongation factor-1α gene promoter (EF1α) is shown as a cross-hatched box with the intronic sequence shown as an angled line. The human growth hormone polyadenylation sequence [hGHpoly(A)] is shown as a stippled box, the expression cassette for the MTX-resistant DHFR is shown as a large solid arrow, the ampicillin-resistance marker (Amp^r) as a small solid arrow and the pBR322 origin of replication (ori) as a solid lozenge. The sulphamidase cDNA sequence (NS) is shown as an open box and was inserted into the *Eco*RI site of pFRNEE.2.

Table 1 Comparison of expression levels of rhNS from gene-amplified and unamplified clones

CHO-K1 is an untransfected cell line. 13-200, 19-200 and 23-400 are unamplified clones. NS-26, NS-110, NS-142 and NS-172 are amplified clones. All cell lines were conditioned for 5 days as described in the Materials and methods section. The data from one of several assay series are shown; all gave similar results.

Cell line (parental clone)	NS activity (pmol/min per ml)
CHO-K1	18
13-200	249
19-200	218
23-400	697
NS-26 (23-400)	2344
NS-110 (23-400)	2831
NS-142 (13-200)	2670
NS-172 (13-200)	2165

Generation and amplification of clonal cell lines expressing NS

The pFRNEE.2NS construct was electroporated into CHO cells and stable clones selected using 200 or 400 nM MTX. For each selection condition, 24 clones (clones 1-200 to 24-200 and 1-400 to 24-400 respectively) were isolated and assessed for the level of NS secretion. Expression levels of between 7.74 and 132 pmol/min per ml and 7.43–167.2 pmol/min per ml were found for the 200 and 400 nM MTX selection conditions respectively. Based on this analysis the three highest-expressing clones, 13-200, 19-200 and 23-400, were exposed to increasing levels of MTX up to 25 μM as described in the Materials and methods section. After amplification, the cultures were maintained in 25 μM MTX for 6 weeks and then recloned by limiting dilution in microtitre plates. A total of 242 clones were screened for NS activity, demonstrating a range of activities from 248 to 1299, 104.6 to 733.9 and 140.7 to 1191 pmol/min per ml for clones derived from 13-200, 19-200 and 23-400 respectively. Of these, the four highest-expressing clones, NS-26 and NS-110 (parental clone 23-400) and NS-142 and NS-172 (parental clone 13-200) (Table 1) were used to determine the degree of amplification of expression in comparison with the original unamplified parental clones. After conditioning for 5 days in COON'S/DMEM/10% (v/v) FCS/PS, results indicated that, compared with unamplified cell lines, there was a 12-fold increase in the level of NS expression when the initial concentration of MTX was 200 nM and a 4-fold increase when the starting concentration was 400 nM (Table 1). Untransfected CHO-K1 cells secreted less than 1% of the maximal activity observed in gene-amplified clones. The gene-amplified clone that showed the highest level of expression most consistently was NS-26, and this was used for the production of rhNS for purification and characterization. In addition an unamplified clone, 13-200, was also used to produce rhNS to see if amplification resulted in any changes in the characteristics of the recombinant enzyme.

We had expected up to 100-fold amplification of sulphamidase expression as the MTX concentration was increased from 200 nM to 25 μM. However, initial selection in 400 nM MTX did not decrease the number of colonies obtained, suggesting that

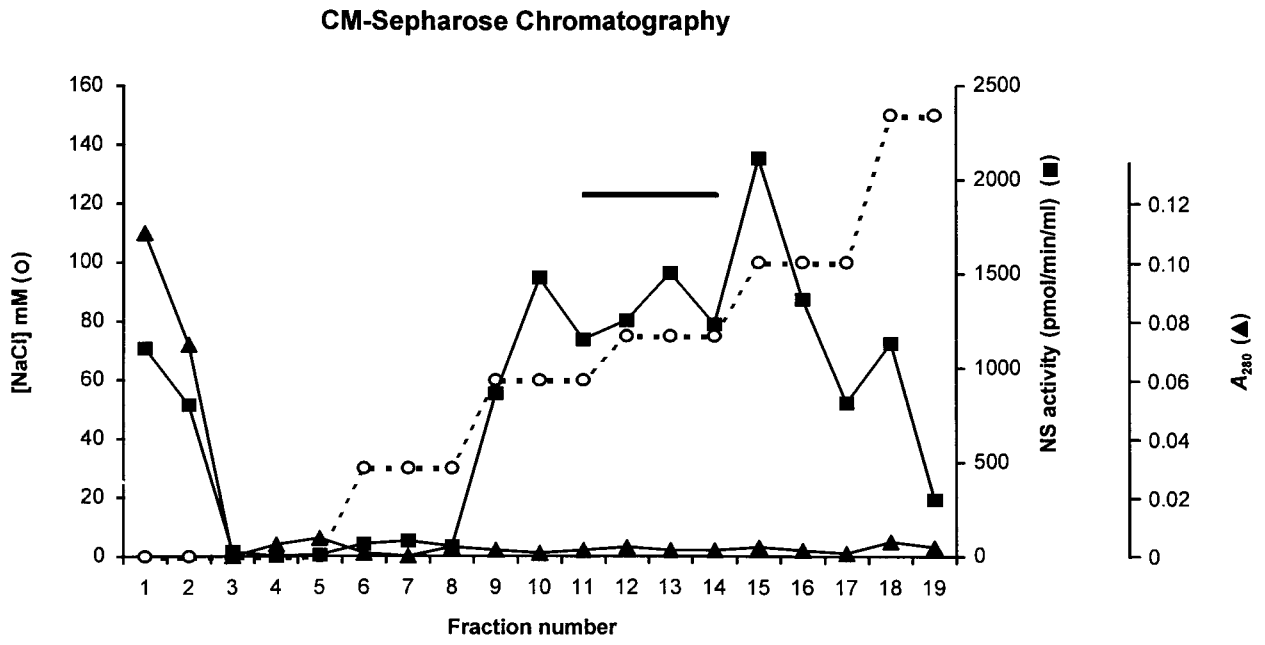

Figure 2 CM-Sepharose chromatography of rhNS

For experimental details see the Materials and methods section. Fractions indicated were assayed for NS activity (■), protein (▲) and NaCl (○). Fractions were pooled as indicated by the horizontal bar.

unamplified clones are resistant to levels of MTX higher than 400 nM. The amplification results suggest that clones may be initially resistant to more than 1 μM MTX, severely limiting the potential for amplification. Above 25 μM MTX, transport of MTX into the cell becomes the limiting factor preventing further gene amplification. Higher levels of amplification are possible if the normal DHFR sequence is used as a selectable marker, although this requires the use of DHFR-deficient CHO cells to facilitate the initial selection. There are two problems with this approach. First, DHFR-deficient CHO cells are less robust in culture than wild-type cells. Secondly, and more importantly, high levels of amplification often result in the generation of unstable cell lines that degenerate (in terms of expression levels) rapidly once selective pressure is removed [22,23]. We found no evidence for instability in the amplified cell lines described in this paper during long-term culture.

Purification and characterization of rhNS

Clone NS-26 expressed approx. 15 mg of rhNS/l of culture medium while that from the unamplified clone (13-200) was of the order of less than 1 mg/l of culture medium. rhNS was purified from conditioned medium from a gene-amplified clone (NS-26) and unamplified clone (13-200) by a one-step procedure on the cation-exchanger CM-Sepharose. After dialysis of the concentrated medium a precipitate was observed which was removed on centrifugation. This accounted for approx. 90% of the contaminating proteins. When this precipitate was re-dissolved in buffer, less than 5% NS activity was detected. The dialysis step also removed inhibitor(s) of NS, as the activity of NS after dialysis increased approximately threefold. In both the amplified and unamplified cases the rhNS bound to the ion-exchanger at pH 5.0 in the absence of NaCl, with most of the contaminating proteins in the flow-through (fraction 1, Figure 2). NS activity was eluted in a broad peak between 60 and 150 mM NaCl (fractions 9–19, Figure 2). Analysis of each NS-activity-containing fraction by SDS/PAGE indicated that fractions 11–14 had one major band at 62–64 kDa with a minor band at 56–58 kDa (Figure 3). N-Terminal amino acid sequence analysis (results not shown) indicated that both are NS polypeptides with the same *N*-terminal residues as the mature form of

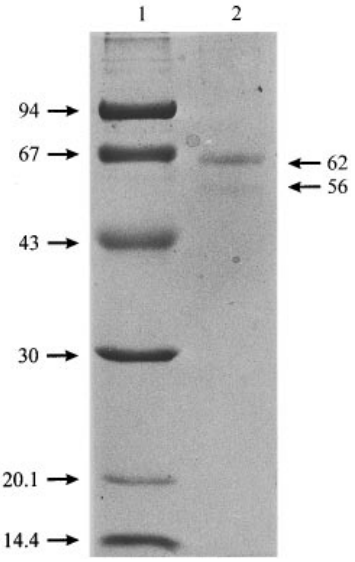

Figure 3 SDS/PAGE of rhNS

Lanes 1 and 2 are molecular-mass standards and purified rhNS respectively, electrophoresed under reducing conditions with dithiothreitol (30 mM). The gel was stained with Coomassie Brilliant Blue R. Molecular-mass standards in kDa and the two forms of rhNS with their respective molecular mass are indicated by arrows.

Table 2 Comparison of the kinetic properties of gene-amplified and unamplified rhNS with NS isolated from human liver

Kinetic properties were determined using the radiolabelled tetrasaccharide substrate. For details, see the Materials and methods section. n.d., Not determined.

	K_m (μM)	V_{max} (nmol/min per mg)	Specific activity (nmol/min per mg)	pH optimum
Gene-amplified rhNS (clone NS-26)	12.25	934	828	5.2
Unamplified rhNS (clone 13-200)	10.7	802	731	5.2
Human liver NS*	10.3	928	n.d.	5.6

* From Freeman and Hopwood [12].

Table 3 Catalytic properties of rhNS compared with other sulphatases using the fluorogenic substrate 4MUS

For details, see the Materials and methods section. rcGlc6S, Recombinant caprine *N*-acetylglucosaminide 6-sulphatase; rhIDS, recombinant human iduronate 2-sulphatase; rhGal6S, recombinant human *N*-acetylgalactosamine 6-sulphatase; rhGal4S, recombinant human *N*-acetylgalactosamine 4-sulphatase.

	K_m (mM)	V_{max} (nmol/min per mg)	pH optimum
rhNS	4.8	114	5.4
rcGlc6S*	5.8	10	5.2
rhIDS†	12.4	300	5.6
rhGal6S†	4.4	120	4.7
rhGal4S†	1.2	48485	5.6

* From Litjens et al. [24].
† From Bielicki et al. [25].

NS isolated from liver [4]. Therefore there is no processing of either polypeptide at the N-terminus after cleavage of the leader peptide. Thus the major (62–64 kDa) polypeptide is most likely to be the precursor form of rhNS. The minor one is analogous to NS purified from human liver, which also has an estimated molecular mass of 56 kDa [12]. The native molecular mass determined by FPLC was 115 kDa, which is in agreement with the native size of mature NS purified from human liver, indicating that it exists as a dimer [12]. The predicted molecular mass of the mature core protein as determined from the amino acid sequence obtained from the cDNA sequence is 54679 Da. There are five potential asparagine-linked glycosylation sites [4], at least one of which must be used to produce a functional M6P targeting signal. Amino acid sequencing indicates that Asp-41 is glycosylated [4], and we expect that, depending on the degree of complexity of the carbohydrate chains, all other sites may be occupied.

The yield of pure rhNS from this procedure was approx. 35%. Kinetic data (Table 2) for the purified gene-amplified and unamplified enzymes showed that they were virtually identical with regard to their K_m, V_{max} and pH optimum. Both the physical and kinetic data indicate that overexpression of NS by gene amplification has not altered either the physical or catalytic parameters of the enzyme. In accordance with observations made with other non-arylsulphatases, rhNS also binds the fluorogenic substrate 4MUS; however, its turnover rate is 425-fold lower than that of the arylsulphatase *N*-acetylgalactosamine 4-sulphatase (Table 3).

Demonstration of correction of MPS IIIA fibroblasts

MPS IIIA fibroblasts (SF4210) have undetectable levels of NS activity and store approx. 10–20 times more ^{35}S-labelled storage products in the form of undegraded HS fragments than normal control fibroblasts (Table 4). On the addition of rhNS at 320 nmol/min to the medium, the level of NS activity is restored to normal and concomitantly storage is reduced to levels comparable with that observed in control cells. Addition of ten times

Table 4 Correction of the MPS IIIA defect by rhNS

Normal (SF4353, SF4427) and MPS IIIA (SF4210) fibroblasts were labelled with $Na_2{}^{35}SO_4$. MPS IIIA fibroblasts were incubated with either 320 (indicated by an asterisk) or 3200 (indicated by a dagger) pmol/min rhNS in the presence or absence of M6P as described in the Materials and methods section. Dialysed cell lysates were assayed for NS activity, β-hexosaminidase activity, iduronate 2-sulphatase activity and radioactivity. Individual results for three replicates are shown; the mean is given in parentheses. All results are normalized to total cell protein.

	NS activity (pmol/min per mg)	β-Hexosaminidase activity (nmol/min per mg)	Iduronate 2-sulphatase activity (pmol/min per mg)	$10^{-3} \times {}^{35}$S radioactivity (c.p.m./mg)
SF4353	647, 561, 673 (627)	81, 76, 94 (83)	123, 92, 143 (119)	13.9, 12.5, 11.8 (12.7)
SF4427	750, 400, 429 (526)	109, 54, 59 (74)	113, 65, 64 (80)	16.0, 36.8, 31.5 (28.1)
SF4210	None detected	82, 75, 59 (72)	168, 67, 61 (98)	341.1, 264.4, 268.6 (291)
SF4210 + NS*	565, 1013, 622 (733)	74, 83, 95 (84)	67, 97, 54 (73)	36.9, 17.9, 38.8 (31.2)
SF4210 + NS* + M6P	112, 108, 134 (118)	90, 55, 74 (73)	60, 91, 83 (78)	10.3, 25.6, 31.7 (22.5)
SF4210 + NS†	10109, 6690, 6709 (7836)	99, 65, 75 (79)	114, 68, 74 (85)	33.4, 35.2, 39.9 (36.2)
SF4210 + NS† + M6P	1456, 2168, 1479 (1701)	121, 141, 74 (112)	102, 118, 70 (97)	23.2, 19.7, 20.3 (21.1)

the concentration of enzyme results in a similar decrease in ^{35}S-labelled storage material and a 10.7-fold increase in the amount of enzyme endocytosed. M6P added to cells receiving 320 or 3200 nmol/min rhNS inhibits uptake of NS activity by 6.2-fold and 4.6-fold respectively, demonstrating that endocytosis of rhNS is mediated by the M6P receptor pathway. The observation that the labelled storage material is also eliminated in the presence of 320 nmol/min rhNS and 5 mM M6P indicates that the amount of enzyme that is endocytosed under these conditions (20–25% of the normal level) is sufficient to degrade stored HS. The uptake of rhNS has no effect on the activities of the two other lysosomal enzymes assayed, β-hexosaminidase and iduronate 2-sulphatase.

Conclusion

We have constructed CHO-derived cell lines that express rhNS at levels of up to 15 mg/l after co-amplification of the NS gene expression cassette with an MTX-resistant DHFR gene. Although the degree of amplification obtained is relatively small, the resulting cell lines appear to be phenotypically stable in the absence of selective pressure. The rhNS has similar physical and kinetic parameters to enzyme purified from human liver and is able to be efficiently endocytosed by MPS IIIA fibroblasts via the M6P receptor resulting in enzymic and phenotypic correction. The rhNS would therefore appear to be suitable for enzyme-replacement therapy, although this may require suitable modification of the enzyme to facilitate transcytosis across the blood–brain barrier, as the central nervous system is the major site of pathology in MPS IIIA. There are precedents to suggest that this may be feasible. Modification of other polypeptides with polyamines to enhance uptake in brain has been described [10]. Chemical coupling of enzyme to insulin fragment [9] and to a mouse anti-rat antibody to the transferrin receptor [11] have also demonstrated possible mechanisms for transport of macromolecules to the central nervous system.

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