Two site-directed mutations abrogate enzyme activity but have different effects on the conformation and cellular content of the *N*-acetylgalactosamine 4-sulphatase protein

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The sulphatase family of enzymes have regions of sequence similarity, but relatively little is known about either the structure–function relationships of sulphatases, or the role of highly conserved amino acids. The sequence of amino acids CTPSR at position 91–95 of 4-sulphatase has been shown to be highly conserved in all of the sequenced sulphatase enzymes. The cysteine at amino acid 91 of 4-sulphatase was selected for mutation analysis due to its potential role in either the active site, substrate-binding site or part of a key structural domain of 4-sulphatase and due to the absence of naturally occurring mutations in this residue in mucopolysaccharidosis type VI (MPS VI) patients. Two mutations, C91S and C91T, altering amino acid 91 of 4-sulphatase were generated and expressed in Chinese hamster ovary cells. Biochemical analysis of protein from a C91S cell line demonstrated no detectable 4-sulphatase enzyme activity but a relatively normal level of 4-sulphatase polypeptide (180% of the wild-type control protein level). Epitope detection, using a panel of ten monoclonal antibodies, demonstrated that the C91S polypeptide had a similar immunoreactivity to wild-type 4-sulphatase, suggesting that the C91S substitution does not induce a major structural change in the protein. Reduced catalytic activity associated with normal levels of 4-sulphatase protein have not been observed in any of the MPS VI patients tested and all show evidence of structural modification of 4-sulphatase protein with the same panel of antibodies [Brooks, McCourt, Gibson, Ashton, Shutter and Hopwood (1991) Am. J. Hum. Genet. **48**, 710–719]. The loss of enzyme activity without a detectable protein conformation change suggests that Cys-91 may be a critical residue in the catalytic process. In contrast, analysis of protein from a C91T cell line revealed low levels of catalytically inactive 4-sulphatase polypeptide (0.37% of the wild-type control protein level) which had missing or masked epitopes, suggesting an altered protein structure or conformation. Subcellular fractionation studies of the C91T cell line demonstrated a high proportion of 4-sulphatase polypeptide content in organelles characteristic of microsomes. The aberrant intracellular localization and the reduced cellular content of 4-sulphatase polypeptide was consistent with the observed structural modification leading to retention and degradation of the protein within an early vacuolar compartment.

INTRODUCTION

The lysosomal enzyme *N*-acetylgalactosamine 4-sulphatase (4-sulphatase; EC 3.1.6.12) is involved in the sequential degradation of the glycosaminoglycans dermatan sulphate and chondroitin sulphate. A deficiency in the activity of 4-sulphatase in humans results in the glycosaminoglycan storage disease mucopolysaccharidosis type VI (MPS VI) or Maroteaux–Lamy syndrome [1,2]. A failure to hydrolyse the 4-sulphate ester from the *N*-acetylgalactosamine sugar residue at the non-reducing terminus of dermatan sulphate and chondroitin sulphate results in the intracellular accumulation and urinary excretion of partially degraded glycosaminoglycans and clinical onset of MPS VI [3].

4-Sulphatase has been purified to homogeneity from human liver and has been shown to exist as a 57 kDa monomer, which can be dissociated into 43 kDa and 13 kDa polypeptides under sulphydryl-reducing conditions [4]. The molecular size of mature 4-sulphatase was further characterized as 43 kDa and 8 kDa [5] and more recently has been defined as being composed of 43 kDa, 8 kDa and 7 kDa components [6]. Biosynthetic studies demonstrate that 4-sulphatase is synthesized as a 64–66 kDa precursor molecule and is proteolytically processed to a 57 kDa mature form in fibroblast cells [5,7]. Monoclonal antibodies have been generated against different epitopes on the 4-sulphatase polypeptide and used to develop a specific and sensitive immunoquantification assay for 4-sulphatase protein [8]. 4-Sulphatase protein levels and residual enzyme activities have been determined on samples derived from MPS VI patients and show evidence of modifications in 4-sulphatase protein structure and enzyme kinetics [8,9]. These studies also indicate that enzyme catalytic capacity may be correlated with clinical severity in most patients.

The isolation and sequencing of a full-length cDNA for 4-sulphatase has enabled the investigation of the molecular basis of mutations causing MPS VI [10,11]. Wicker et al. [12], Jin et al. [13], Litjens et al. [14], Isbrandt et al. [15] and Arlt et al. [16] have recently reported mutations which provide molecular evidence for clinical heterogeneity in MPS VI patients. The aim of these studies is to correlate the genetic defect with both the biochemical abnormality and the resultant clinical severity of the disease. Other sulphatases have been sequenced and indicate that selected regions of sequence are highly conserved between different sulphatases. Possible reasons for this sequence similarity include conservation of either critical structural domains, or of functional domains which might involve substrate binding and or elements involved in hydrolysis of sulphate ester bonds. In MPS VI patients, mutations involving these critical residues may be expected to result in a severe clinical presentation. The investigation of conserved amino acids should provide information

Abbreviations used: CHO, Chinese hamster ovary; MPS VI, mucopolysaccharidosis type VI; 4-sulphatase, *N*-acetylgalactosamine 4-sulphatase.

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defining the molecular effect of specific mutations, the role of specific amino acids within different sulphatases and potentially identify markers appropriate for the diagnosis and prognosis of patients.

In this paper we describe two site-directed mutations within a region of sequence which is highly conserved for all known sulphatases (e.g. see [17]). In human 4-sulphatase the cysteine at position 91 was changed to either a serine or a threonine and the recombinant proteins were expressed in Chinese hamster ovary (CHO) cells. Cell lines containing C91, C91S and C91T recombinant 4-sulphatase have been characterized for residual enzyme activity, cellular content of 4-sulphatase protein, 4-sulphatase protein structure using a panel of conformation-sensitive monoclonal antibodies and for the subcellular distribution of 4-sulphatase protein. The aim of this study was to attempt to define the structural constraints and possible functional role of the Cys-91 amino acid in 4-sulphatase.

MATERIALS AND METHODS

Materials

Poly(vinyl chloride) plates (96-well) and 75 cm^2 culture flasks were obtained from Costar (Cambridge, MA, U.S.A.). Peroxidase-labelled sheep anti-(mouse immunoglobulin) and sheep anti-(mouse immunoglobulin) reagents were purchased from Silenus Laboratories (Melbourne, Victoria, Australia). ABTS [2,2′-azinobis-(3-ethylbenzthiazolinesulphonic acid)] substrate kit and the muta-gene phagemid *in vitro* mutagenesis kit (pTZ-DNA) were from Bio-Rad Laboratories (Richmond, VA, U.S.A.) and were used according to the manufacturers' instructions. Ham's F12 medium and fetal calf serum were from Gibco Laboratories (Grand Island, NY, U.S.A.). Dulbecco's modified phosphate-buffered saline, penicillin, streptomycin and L-glutamine were obtained from Commonwealth Serum Laboratories (Melbourne, VIC, Australia). Centricon-100 microconcentrator columns were from Amicon (Danvers, MA, U.S.A.).

Cell culture and harvesting

CHO cells were grown in 75 cm^2 culture flasks in Ham's F12 medium containing L-glutamine (300 μg/ml), penicillin (100 IU/ml), streptomycin (100 μg/ml) and 10% (v/v) fetal calf serum. CHO cells were harvested by removing the cell culture medium, washing the cell monolayer twice with Dulbecco's modified phosphate-buffered saline, and the cells detached by trypsin digestion and vigorous agitation as described in [18]. The cells were then prepared either for organelle fractionation as described below or resuspended in 0.02 M Tris/HCl, pH 7.0, containing 0.5 M NaCl (100 μl/flask of cells) to prepare cell extracts. For cell extraction the latter suspension was freeze–thawed six times in rapid succession, then centrifuged at 5000 *g* for 5 min to remove the cell debris. The resulting supernatants were used either for immunoquantification or in an enzyme-immunobinding assay.

Primer sequences

The DNA sequence position of each primer is numbered as previously described [10]. 4SP5A [nucleotide (nt) 1–21, sense], 5′-ATGGGTCCGCGCGGCGCGGCG-3′, 4SP17 [nt 229–247, sense] 5′-GCCGGCGGGGTGCTCCTGG-3′; 4SP11 [nt 377–358, antisense] 5′-TCATCCAGAGGAACACAGCT-3′. Oligonucleotides were labelled at their 5′ ends with [γ-^{32}P]ATP and polynucleotide kinase as previously described [19].

DNA amplification

The PCR was carried out essentially as described in [20] using genomic DNA as the template. A 0.5 μl aliquot, from a 50 μl genomic DNA sample, was mixed with 50 pmol of each PCR primer (4SP5A and 4SP11) in a total volume of 100 μl containing Buffer A [50 mM KCl, 10 mM Tris/HCl, pH 8.4, 2.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 10% (v/v) DMSO], 2.5 units *Taq* polymerase (Cetus) and 0.4 mM each of dATP, dCTP, dGTP and dTTP. The reactions were subjected to a 7 min denaturation step at 95 °C and then 40 cycles of denaturation at 94 °C for 45 s, annealing at 58 °C for 45 s, and DNA polymerization at 72 °C for 1 min using a Perkin–Elmer Cetus DNA thermal cycler, followed by a final extension at 72 °C for 3 min. Control PCR reactions, in which no genomic DNA template was added, were included during each set of PCR reactions. The required PCR product (377 bp) was purified away from unincorporated primers using Centricon-100 microconcentrator columns.

Direct DNA sequencing of PCR products

The procedure was modified from that of Murray [21] such that the PCR product (0.5–1.0 pmol) was mixed with 3 pmol of ^{32}P end-labelled sequencing primer (4SP11 or 4SP17) in Buffer A, 2.5 units of *Taq* polymerase and 7.5 mM each of dATP, dCTP, dTTP and dGTP or 7-deaza-dGTP. This reaction mixture was divided into four termination reactions each containing either 0.25 mM ddATP, 0.05 mM ddCTP, 0.025 mM ddGTP or 0.5 mM ddTTP which were subjected to 15 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and DNA polymerization at 72 °C for 30 s. A 5 μl aliquot was removed from each termination reaction, 4 μl of formamide loading dye was added and the reactions were heat denatured for 3 min at 100 °C then loaded on to a 6% sequencing gel.

Oligonucleotide-directed *in vitro* mutagenesis of 4-sulphatase cDNA

4-Sulphatase cDNA [10] was digested with *Eco*RV and the resultant 535 bp N-terminal fragment was subcloned into a Bio-Rad pTZ mutagenesis vector using the manufacturer's instructions. The construct was transformed into a dUTPase- and uracil-*N*-glycosylase-deficient *Escherichia coli* strain which was then infected with helper phage. DNA was extracted from the isolated phagemids then annealed with specific mutagenic oligonucleotides. To introduce the threonine and serine mutations for the wild-type cysteine at amino acid position 91 the following oligonucleotides were used: T = (5′-ACGCAGCCGCTGACG-ACGCCGTCGCGG-3′), S = (5′-ACGCAGCCGCTGTCGA-CGCCGTCGCGG-3′). Using the oligonucleotides as primers complementary DNA strands were first synthesized then ligated to the oligonucleotides to form double-stranded cDNA. The double-stranded wild-type/mutant heteroduplex molecule was then transformed into the uracil-*N*-glycosylase-positive host strain which inactivates the parental wild-type strand (containing uracil) and preferentially replicates the mutant strand.

Confirmation of mutant 4-sulphatase sequence and *in vitro* expression

The authenticity of the introduced mutations and the integrity of the other 4-sulphatase fragments were confirmed by subcloning and sequencing the entire 535 bp of the modified DNA. 4-Sulphatase cDNA was then reconstituted with the mutated N-

terminal 4-sulphatase sequences and the full-length cDNA constructs were cloned into the pRSVN expression vector and introduced into CHO cells by electroporation as previously described [22]. Transformed cells were selected for by G418 resistance, and cultured as described below. The C91S and C91T mutations and the wild-type C91 sequence were confirmed in the resultant 4-sulphatase-transformed CHO cell lines by PCR sequence analysis as described above. Genomic DNA was extracted and prepared as described previously [23]. The genomic DNA from each cell line (one 75 cm^2 flask at confluency) was resuspended in 50 μl of 10 mM Tris/HCl, pH 8.0, with 1 mM EDTA.

Organelle fractionation

Ten 75 cm^2 culture flasks of CHO cell lines (C91, C91S, C91T) were grown to confluency then harvested as described above. The cells were then washed twice (10 ml/flask of cells) in phosphate-buffered saline containing 1% (w/v) fetal calf serum and recovered by centrifugation at 200 *g* for 10 min at 4 °C. The cells were then pooled and resuspended in 2.5 ml of 0.25 M sucrose and subcellular fractions prepared as described previously [24]. Post-nuclear supernatants were separated into crude organelle fractions by differential centrifugation (Beckman L5-75B ultracentrifuge with Ti70 rotor), using 6000 *g* to produce a granular fraction, 23000 *g* to produce another organelle fraction, and 100000 *g* to recover microsomes. Percoll gradient fractionation of post-nuclear supernatants was as previously described [24].

Monoclonal antibodies

The monoclonal antibodies 4-S 4.1.1, 4-S 5.2, 4-S 17.1, 4-S 22.1, 4-S 33.1 and 4-S 58.3 were produced as previously described [8]. The antibodies 4-S 15.1, 4-S 16.1, 4-S 59.2 and 4-S 66 were raised and characterized against immunopurified human liver 4-sulphatase according to methods described previously [4,25].

Enzyme immunobinding assay

An immune capture method was used to specifically bind 4-sulphatase protein and followed by a 4-methylumbelliferyl sulphate assay to determine 4-sulphatase enzyme activity. The procedure used was as described previously [26], except that an affinity-purified sheep anti-(mouse immunoglobulin) antibody (1 μg/well in 0.1 M $NaHCO_3$, pH 8.5) was bound to each well of a 96-well poly(vinyl chloride) plate, before the addition of individual monoclonal antibodies (in this case as hybridoma culture supernatants). Enzyme activities of either antibody-bound or free 4-sulphatase were determined used the fluorogenic substrate 4-methylumbelliferyl sulphate as previously described ([26] and [4] respectively).

Immunoquantification

4-Sulphatase polypeptide levels were measured with an immunoquantification assay, using a monospecific polyclonal antibody to capture the protein and a panel of ten conformation-sensitive 4-sulphatase monoclonal antibodies and a peroxidase-labelled second antibody to detect and quantify the bound protein [8].

Immunopurification and SDS/PAGE

C91 and C91S polypeptides were immunopurified from the medium of expression cell cultures using a chromatography procedure as previously described [4], except that the monoclonal antibody used was 4-S58.3 [8]. Purified C91 and C91S polypeptides were electrophoresed and proteins visualized by Coomassie Blue staining as previously described [4].

RESULTS

Mutagenesis and expression of 4-sulphatase

T and S oligonucleotides were used to introduce the respective C91T and C91S mutations into 4-sulphatase cDNAs and the resultant sequences expressed in CHO cells. The mutated 4-sulphatase cDNAs were sequenced and shown to be identical to the normal 4-sulphatase cDNA gene except for the specifically introduced mutations (Table 1).

Effect of amino acid substitution on the 4-sulphatase polypeptide

The levels of 4-sulphatase polypeptide in the transfected CHO cell extracts C91 (wild type), C91S and C91T were determined using the monoclonal antibody 4-S 58.3, in an immunoquantification assay (Table 2). The C91S cell line had a higher 4-sulphatase polypeptide content than the control C91 cell line. In contrast, the C91T cell line only had 0.37% of the 4-sulphatase polypeptide content observed in the C91 control cell line. This may be an underestimate of the 4-sulphatase protein content in the C91T cell line as the epitope detected by 4-S 58.3 appeared to be modified in the C91T polypeptide (Table 3).

Specific 4-sulphatase epitopes were detected in cell extracts from the C91, C91S and C91T cell lines using a panel of ten monoclonal antibodies. C91S 4-sulphatase had an almost identical pattern of epitope reactivity to that of the control C91 4-sulphatase. That is, there were no missing or masked epitopes observed with the C91S 4-sulphatase polypeptide, suggesting that this protein had a normal protein conformation. Analysis of the 4-sulphatase produced from the C91T cell line, besides showing considerably reduced 4-sulphatase protein levels with all of the monoclonal antibodies tested, also demonstrated that the C91T 4-sulphatase had variable reactivity with different antibodies. Four monoclonal antibodies (4-S 4.1.1, 4-S 5.2, 4-S 15.1 and 4-S 17.1) failed to react with the C91T 4-sulphatase, while the others had reduced reactivity when compared with the 4-S 59.2 antibody (Table 3).

Enzyme activity analysis of mutant 4-sulphatase polypeptide

Sulphatase activities present in C91, C91S and C91T CHO cell extracts were determined using 4-methylumbelliferyl sulphate substrate which detects arylsulphatases A, B and C. The C91S and C91T cell lines had no detectable 4-sulphatase (arylsulphatase B) activity as they gave the same activity as the CHO cell line control (Table 2). In contrast, the C91 cell extract had a high level of sulphatase activity. A more sensitive and specific immunobinding assay was used to determine the level of 4-sulphatase activity in C91S and C91T cell extracts and demonstrated that the 4-sulphatase protein in these cell lines had no detectable sulphatase activity (Table 3). In contrast, the C91 control polypeptide demonstrated a high level of 4-sulphatase enzyme activity and the level of enzyme activity detected with individual antibodies was directly proportional to the epitope reactivity for the antibody (Table 3).

Intracellular distribution of mutant and control 4-sulphatases

Post-nuclear supernatants prepared from all three cell lines were subfractionated on 18% Percoll gradients and fractions were assayed for acid phosphatase (Figure 1a) and β-hexosaminidase activities (Figure 1b) and immunoquantified for 4-sulphatase protein (Figure 1c). The C91 (wild-type control) 4-sulphatase

Table 1 4-Sulphatase mutations

Sequence analysis was performed on each of the CHO cell expression lines to establish the correct amino acid substitution. The cell line C91 represents the wild-type 4-sulphatase sequence (see [10] or [11] for full-length sequence of 4-sulphatase).

CHO cell line	Amino acid sequence (91–95)	Codon sequence at amino acid 91	Mutation at amino acid 91
C91	CTPSR	5′ TGC 3′	Wild type
C91S	STPSR	5′ TCG 3′	Cysteine to serine
C91T	TTPSR	5′ ACG 3′	Cysteine to threonine

Table 2 Determination of 4-sulphatase protein content and arylsulphatase activity in C91, C91S and C91T cell extracts and medium from cultured cells

Total cell protein levels were determined on extracts of the CHO expression cells and the control CHO cell line using a Lowry protein assay. 4-Sulphatase protein levels of these cell extracts were measured by immunoquantification using the monoclonal antibody 4-S 58.3 (N.B. the result for the C91T polypeptide may represent an underestimate due to modification of the epitope detected by 4-S 58.3 in this protein; see Table 3) and arylsulphatase levels were determined using the substrate 4-methylumbelliferyl sulphate as described in the Materials and methods section. Abbreviation: ND, not detected. (N.B. 4-S 58.3 does not react with CHO 4-sulphatase protein).

	Cell lines			
	CHO	C91	C91S	C91T
Cell extracts				
Total cell protein (mg/ml)	2.6	2.5	2.35	2.9
4-Sulphatase protein (μg/ml)	ND	17	30.9	0.063
Arylsulphatase activity (nmol/min per mg)	0.2	96.4	0.2	0.2
Medium				
4-Sulphatase protein (μg/ml)	0.008	3.6	0.06	0.06

polypeptide co-fractionated with both β-hexosaminidase and acid phosphatase activities associated with high-density organelles characteristic of lysosomes. The bimodal distribution of acid phosphatase in the Percoll fractionations (Figure 1a) was consistent with the observations of Peters et al. [27] where soluble acid phosphatase is associated with lysosomes (high-density organelles) and in endosomes and microsomes (low-density organelles) acid phosphatase is membrane-bound. Moreover, lysis of organelles from high-density fractions of Percoll gradients (lysosomes) results in the release of soluble acid phosphatase, whereas lysis of organelles from the low-density fractions results in acid phosphatase activity, which remains membrane associated (D. A. Brooks, D. A. Robertson, C. Bindloss, T. Litjens, D. S. Anson, C. Peters, C. P. Morris and J. J. Hopwood, unpublished work). The majority of the C91S 4-sulphatase polypeptide was detected in high-density organelles characteristic of lysosomes, although increased amounts of 4-sulphatase polypeptide were observed in lower-density fractions/organelles when compared with the C91 control. However, relatively little C91T 4-sulphatase polypeptide was detected in the high-density organelles (fractions 19–23 of the Percoll gradient) which are characteristic of lysosomes and containing soluble acid phosphatase and β-hexosaminidase. Most of the C91T polypeptide was detected in the lower-density fractions (fractions 10–16). Almost no 4-sulphatase polypeptide was observed in fractions 1–9, which represent the sample load, consistent with very few organelles being disrupted during the processing of the granular fraction. These patterns were reproduced in a duplicate Percoll gradient fractionation experiment (results not shown).

To investigate further the subcellular distribution of the mutant 4-sulphatase polypeptides, partially purified organelle fractions were prepared by ultracentrifugation. Most lysosomes containing C91 4-sulphatase polypeptide could be pelleted by centrifugation at 6000 *g*, as demonstrated by the enrichment of β-hexosaminidase activity (Figure 2). This fraction contained approx. 20% of both of the C91S and C91T polypeptides (Figure 2). The majority of C91S polypeptide sedimented in organelles at 23000 *g*, whereas most of the C91T polypeptide was detected in

Table 3 4-Sulphatase immunoquantification (epitope) and enzyme immunobinding assay analysis of C91, C91S and C91T expression cell extracts

Cell extracts were immunoquantified using ten monoclonal antibodies against different 4-sulphatase epitopes or assayed for 4-sulphatase activity in an enzyme immunobinding assay as described in the Materials and methods section. 'Epitope', represents the 4-sulphatase protein levels detected by individual monoclonal antibodies determined on cell extracts which were adjusted for total cell protein, then referenced as a percentage of the reactivity observed for the 4-S 59.2 antibody (i.e. 0 = no detectable reactivity; 100 = reactivity of the 4-S 59.2 epitope, which had the highest reactivity with the control C91, C91S and C91T 4-sulphatase polypeptides). 'Activity', represents the amount of 4-sulphatase activity bound by a monoclonal antibody in an immunobinding assay and again referenced to 100% for the 4-S 59.2 antibody. 'ND', represents either no detectable protein or no detectable 4-sulphatase activity.

Monoclonal antibody	C91 epitope	C91 activity	C91S epitope	C91S activity	C91T epitope	C91T activity
4-S 4.1.1	61	72	63	ND	ND	ND
4-S 5.2	42	48	45	ND	ND	ND
4-S 15.1	46	40	57	ND	ND	ND
4-S 16.1	72	63	62	ND	24	ND
4-S 17.1	57	44	70	ND	ND	ND
4-S 22.1	100	100	100	ND	59	ND
4-S 33.1	61	75	60	ND	49	ND
4-S 58.3	81	91	91	ND	22	ND
4-S 59.2	100	100	100	ND	100	ND
4-S 66	72	67	79	ND	50	ND

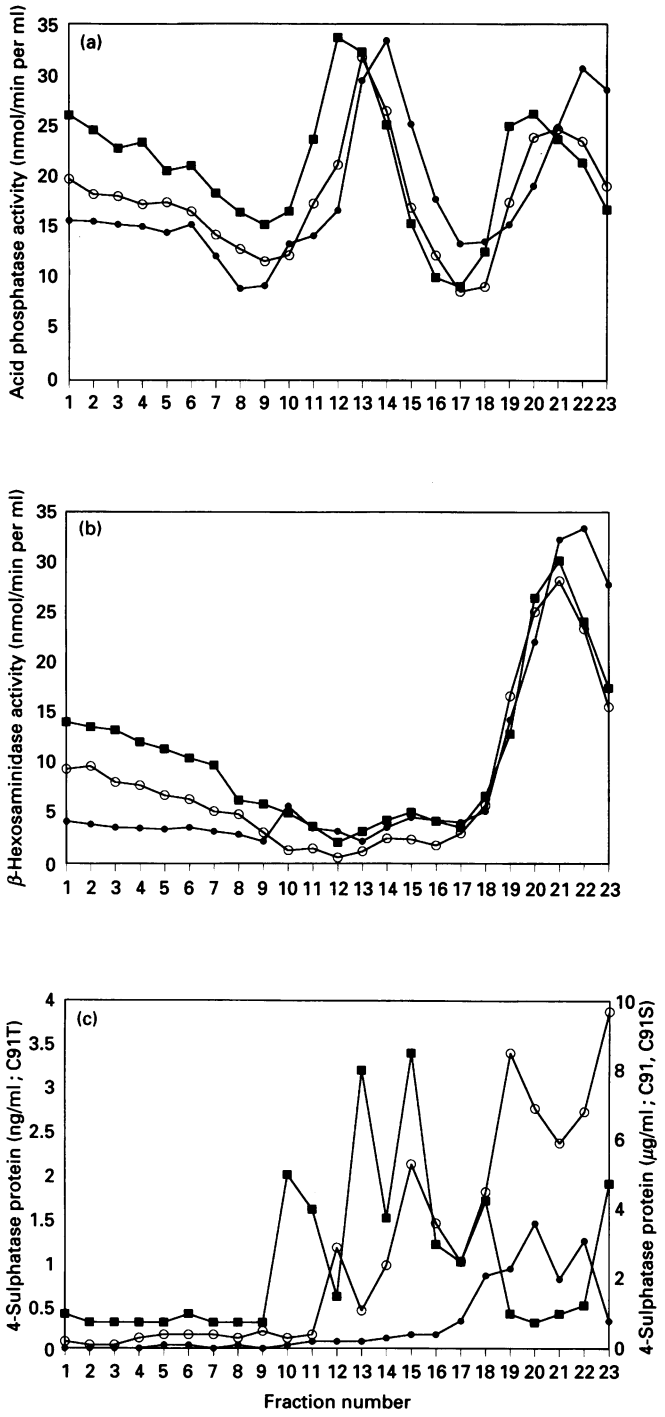

Figure 1 C91T and C91S polypeptides appear to be retained in an early vacuolar compartment when compared with wild-type C91 4-sulphatase polypeptide

Post-nuclear supernatant from C91 (●), C91S (○) and C91T (■) expression cells were subfractionated by centrifugation on 18% Percoll gradients. Acid phosphatase activity (**a**), β-hexosaminidase activity (**b**) and 4-sulphatase polypeptide levels (**c**) were determined as described in the Materials and methods section. β-Hexosaminidase activity fractionated with the C91 wild-type control 4-sulphatase polypeptide in high-density organelles, in fractions 19–23 of the gradients. The 4-sulphatase activity levels in fractionated C91 cells were coincident with the 4-sulphatase polypeptide levels (results not shown). Fractions 1–9 represent the sample load volume and therefore contain minimal organelles and 4-sulphatase protein. C91T polypeptide content is expressed in ng/ml, whereas C91 and C91S polypeptide content is expressed in μg/ml. The sensitivity of the immunoquantification assay used allows the detection of 10 pg of 4-sulphatase protein [8].

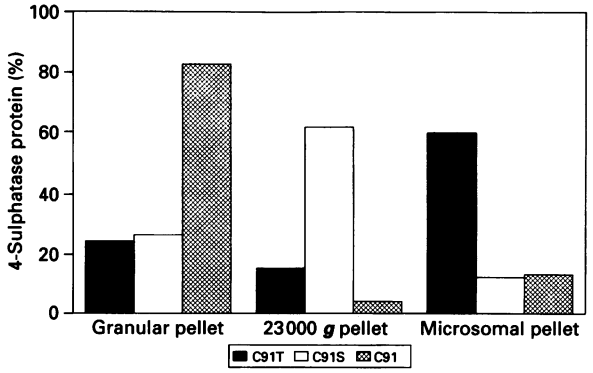

Figure 2 Subcellular distribution of normal and mutant 4-sulphatase polypeptides in partially purified organelle fractions from C91, C91S and C91T CHO expression cells

Post-nuclear supernatants were subfractionated into a granular fraction (6000 *g* organelle pellet) a 23000 *g* organelle pellet and a microsomal fraction (100000 *g* organelle pellet). Organelles were freeze–thawed six times in 0.01 M Hepes buffer, pH 7.0, containing 0.25 M sucrose then immunoquantified using the monoclonal antibody 4-S 58.3. The levels of β-hexosaminidase activity in the same granular fractions were 85% for C91, 81% for C91T and 83% for C91T, demonstrating enrichment of a lysosomal enzyme.

microsome-enriched organelles which sedimented at 100000 *g* (Figure 2).

Molecular size of the C91 and C91S polypeptides

The possibility that the cysteine at amino acid position 91 could potentially be involved in an inter-chain disulphide bond accounting for the effects of substituting this amino acid on protein conformation and/or enzyme activity was investigated by comparing the molecular masses of the C91 and C91S polypeptides under both reducing and non-reducing conditions. There was no detectable difference between the molecular masses of the C91 and C91S polypeptides under either reducing (43 kDa) or non-reducing (57 kDa) conditions by SDS/PAGE, implying that the cysteine at position 91 is not involved in an inter-chain disulphide linkage (results not shown).

DISCUSSION

The characterization of mutations in the 4-sulphatase gene will facilitate both the determination of the relationship between molecular mutations and their effect on clinical severity in MPS VI patients and the investigation of functionally important amino acid residues in the molecule. We have generated two mutations in the 4-sulphatase gene by substituting an amino acid residue in a region where amino acid sequence is highly conserved among a group of sulphatases. The sequence CTPSR is rigidly conserved in four different sulphatases including human 4-sulphatase (arylsulphatase B; [10,11]), human galactose 3-sulphatase (arylsulphatase A; [28]), human arylsulphatase C [29,30] and sea urchin arylsulphatase [31] and occurs with a single amino acid change in human iduronate-2-sulphatase (CAPSR; [17]) and human glucosamine 6-sulphatase (CCPSR; [32]). The high degree of conservation of this sequence strongly suggests that it plays a major structural or functional role in sulphatase enzymes and was therefore chosen for further investigation. The amino acid cysteine in the CTPSR sequence motif (amino acid 91; see Schuchman et al. [11] or Peters et al. [10] for full-length sequence) was targeted for substitution, as to date

there have been no mutations described in MPS VI patients which involve this residue. The cysteine was replaced with either a serine (C91S) or a threonine (C91T) residue, both representing relatively conservative substitutions, although the threonine is slightly larger in size.

Expression constructs for the C91, C91S and C91T cDNAs were transfected into CHO cells to investigate the effect of the C91 mutations on the 4-sulphatase polypeptide. Immunoquantification analysis of cell extracts from the C91S cell line revealed that the 4-sulphatase polypeptide content of these cells was equal to or greater than that observed for the wild-type C91 control cell line. Moreover, multiple antibody analysis demonstrated that the C91S polypeptide reacted with ten different 4-sulphatase conformation-sensitive monoclonal antibodies with a similar pattern and level of reactivity to that observed for 4-sulphatase polypeptide from the C91 control cell line. No epitopes were missing or masked, as has been observed for 4-sulphatase polypeptides from a panel of MPS VI fibroblast cell lines [8]. This implies that, at most, the C91S mutation only has a relatively minor effect on 4-sulphatase polypeptide structure or conformation. To fully substantiate this hypothesis would require X-ray crystallographic analysis of wild-type and mutant C91S polypeptides. However, on the basis of epitope reactivity and the failure of the mutant polypeptide to be recognized and degraded within the endoplasmic reticulum (as appears to be the case for the C91T mutation), it is suggested that there is no significant structural change in the C91S polypeptide.

While the C91S polypeptide appeared to be structurally similar to the wild-type control 4-sulphatase, enzyme activity studies revealed no detectable 4-sulphatase activity. This suggests that the cysteine at amino acid position 91 is crucial for 4-sulphatase activity and is potentially crucial for the enzyme activity of other sulphatases. The loss of enzyme activity did not appear to be due to destabilization of an inter-chain disulphide linkage, as there was no observed change in molecular mass for the C91S polypeptide compared with the C91 polypeptide, when analysed by SDS/PAGE under either reducing or non-reducing conditions. This is also consistent with the observation that there are no major differences between the epitopes detected for the C91 and C91S polypeptides. The possibility remains that the cysteine at amino acid position 91 is involved with an intra-chain disulphide bridge and results in only a minor change in mobility of the C91 polypeptide, which was not detected by SDS/PAGE.

In contrast to the C91S mutation, the C91T amino acid substitution had a marked effect on the 4-sulphatase polypeptide content in expression cells (0.37% of that detected in the wild-type C91 control cells). There was also evidence of missing or masked epitopes on the C91T polypeptide, with four of the ten monoclonal antibodies tested in an immunoquantification assay failing to react with the protein and other antibodies showing reduced reactivity. These results are consistent with a significant structural modification of the 4-sulphatase polypeptide, which may be due to the threonine at position 91 exerting a steric constraint. The structural constraint associated with the CTPSR region must be high as the only difference between a serine and threonine residue is an additional methyl group. Thus, these two apparently conservative amino acid substitutions appear to have markedly different structural effects on the 4-sulphatase protein. Moreover, there was no detectable 4-sulphatase activity observed for the residual C91T polypeptide, supporting the hypothesis that the cysteine at amino acid 91 of 4-sulphatase is either necessary for catalytic activity or directly involved in the sulphatase catalytic process.

Subcellular distribution studies of 4-sulphatase polypeptide in cells expressing the C91S and C91T mutations showed evidence of retention within early vacuolar compartments. The C91T polypeptide was detected mainly in low-density fractions containing either microsomes or endosomes, as marked by the presence of the membrane-bound form of acid phosphatase. Only very small amounts of the C91T polypeptide appeared to be correctly targeted to lysosomes marked by β-hexosaminidase and the soluble form of acid phosphatase. The reduced cellular level of C91T polypeptide, the altered cellular localization and the changes in epitope reactivity are consistent with previous reports of protein retention and degradation of incorrectly folded protein within the endoplasmic reticulum.

The subcellular distribution of the C91S polypeptide was more consistent with that observed for the wild-type C91 4-sulphatase, with the majority of the C91S polypeptide being detected in high-density organelles characteristic of lysosomes. Thus, the C91S polypeptide was mainly coincident with β-hexosaminidase activity, the soluble form of acid phosphatase and the 4-sulphatase polypeptide and activity observed in C91 cell fractionations. The observed normal conformation of the C91S polypeptide may allow it to bypass the recognition system which removes the C91T polypeptide. There was, however, evidence of some changes in intracellular distribution of the C91S polypeptide compared with the wild-type control 4-sulphatase, which may be a result of its increased cellular content. The altered subcellular distribution of 4-sulphatase polypeptide in the C91T cell line is unlikely to be due to stored polysaccharide causing a density change in organelles, as CHO cells have hamster 4-sulphatase which would prevent accumulation of storage products.

The effect of single amino acid substitutions on both protein folding and on protein processing is clearly dramatic. It may be proposed that mutations not directly affecting 4-sulphatase enzyme activity may still result in MPS VI due to the cellular recognition of incorrect folding or altered conformation resulting in removal or retention of protein within an early biosynthetic or protein processing compartment. Retention of 4-sulphatase protein, which is still catalytically active within an early vacuolar compartment, has recently been demonstrated for a mutation affecting the 4-sulphatase stop codon [16]. Again, this mutation caused protein conformation changes in the 4-sulphatase polypeptide which led to a substantial increase in specific activity of the protein but also resulted in degradation of most of the protein in an early vacuolar compartment. These observations have significant implications for pathology in MPS patients and patients with other genetic disorders. If the hypothesis of protein retention and degradation can be substantiated it will demonstrate that the primary defect may not necessarily affect the function of the mutated enzyme, but indicates that even apparently minor protein structural changes may have profound affects on removal of protein in the biosynthetic compartment.

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