Mucopolysaccharidosis Type VI (Maroteaux−Lamy Syndrome): A Y210C Mutation Causes either Altered Protein Handling or Altered Protein Function of *N*-Acetylgalactosamine 4-Sulfatase at Multiple Points in the Vacuolar Network†

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ABSTRACT: The lysosomal hydrolase *N*-acetylgalactosamine 4-sulfatase (4-sulfatase) is required for the degradation of the glycosaminoglycan substrates dermatan and chondroitin sulfate. A 4-sulfatase deficiency results in the accumulation of undegraded substrate and causes the severe lysosomal storage disorder mucopolysaccharidosis type VI (MPS VI) or Maroteaux−Lamy syndrome. A wide variation in clinical severity is observed between MPS VI patients and reflects the number of different 4-sulfatase mutations that can cause the disorder. The most common 4-sulfatase mutation, Y210C, was detected in ∼10% of MPS VI patients and has been associated with an attenuated clinical phenotype when compared to the archetypical form of MPS VI. To define the molecular defect caused by this mutation, Y210C 4-sulfatase was expressed in Chinese hamster ovary (CHO-K1) cells for protein and cell biological analysis. Biosynthetic studies revealed that Y210C 4-sulfatase was synthesized at a comparable molecular size and amount to wild-type 4-sulfatase, but there was evidence of delayed processing, traffic, and stability of the mutant protein. Thirty-three percent of the intracellular Y210C 4-sulfatase remained as a precursor form, for at least 8 h post labeling and was not processed to the mature lysosomal form. However, unlike other 4-sulfatase mutations causing MPS VI, a significant amount of Y210C 4-sulfatase escaped the endoplasmic reticulum and was either secreted from the expression cells or underwent delayed intracellular traffic. Sixty-seven percent of the intracellular Y210C 4-sulfatase was processed to the mature form (43, 8, and 7 kDa molecular mass forms) by a proteolytic processing step known to occur in endosomes−lysosomes. Treatment of Y210C CHO-K1 cells with the protein stabilizer glycerol resulted in increased amounts of Y210C 4-sulfatase in endosomes, which was eventually trafficked to the lysosome after a long, 24 h chase time. This demonstrated delayed traffic of Y210C 4-sulfatase to the lysosomal compartment. The endosomal Y210C 4-sulfatase had a low specific activity, suggesting that the mutant protein also had problems with stability. Treatment of Y210C CHO-K1 cells with the protease inhibitor ALLM resulted in an increased amount of mature Y210C 4-sulfatase localized in lysosomes, but this protein had a very low level of activity. This indicated that the mutant protein was being inactivated and degraded at an enhanced rate in the lysosomal compartment. Biochemical analysis of Y210C 4-sulfatase revealed a normal pH optimum for the mutant protein but demonstrated a reduced enzyme activity with time, also consistent with a protein stability problem. This study indicated that multiple subcellular and biochemical processes can contribute to the biogenesis of mutant protein and may in turn influence the clinical phenotype of a patient. In MPS VI patients with a Y210C allele, the composite effect of different stages of intracellular processing/handling and environment has been shown to cause a reduced level of Y210C 4-sulfatase protein and activity, resulting in an attenuated clinical phenotype.

Mucopolysaccharidosis type VI (MPS VI)[1] or Maroteaux−Lamy syndrome is a severe glycosaminoglycan storage disorder with an autosomal recessive mode of inheritance (*1*) and an incidence of 1:248000 live births (*2*). MPS VI is caused by a deficiency of the lysosomal acid hydrolase *N*-acetylgalactosamine 4-sulfatase (4-sulfatase; EC 3.1.6.12), which is required as an essential part of the sequential degradation of the glycosaminoglycans, dermatan sulfate and chondroitin sulfate. Failure to degrade these glycosaminoglycans results in accumulation of the undegraded or partially de-

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[1] Abbreviations: ALLM, *N*-acetyl-L-leucyl-L-leucinylmethioninal; ALLN, *N*-acetyl-L-leucyl-L-leucinylnorleucinal; CFTR, cystic fibrosis transmembrane conductance regulator; CHO, Chinese hamster ovary; ER, endoplasmic reticulum; MPS I, mucopolysaccharidosis type I; MPS VI, mucopolysaccharidosis type VI; NP40, Nonidet P40; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; 4-sulfatase, *N*-acetylgalactosamine 4-sulfatase; TfR, transferrin receptor.

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graded substrate(s) in the lysosomes of affected cells and a clinical phenotype characteristic of lysosomal storage disorders.

The phenotype of MPS VI patients is represented by a clinical spectrum, which ranges from nearly normal, in a few cases, to the classic Maroteaux−Lamy syndrome. The severe MPS VI clinical phenotype is characterized by growth retardation, dysostosis multiplex, coarse facial features, restricted joint mobility, hepatosplenomegally, cardiac complications, and corneal clouding, but unlike some other storage disorders, mental development is normal. Many MPS VI patients appear nearly normal at birth. However, the onset of clinical symptoms in the severe form of MPS VI is rapid, and death normally occurs before or during the second decade of life.

The spectrum of clinical presentations in MPS VI patients reflects the number of different mutations that can cause the disorder (e.g., *3*−*6*). For another lysosomal storage disorder, MPS I (α-L-iduronidase deficiency, Hurler syndrome), there are two common mutations, Q70X and W402X, which account for about 60% of disease alleles in northern European populations (*7*). In contrast, the most common mutations in an MPS VI patient study ($n = 50$ MPS VI patients), were Y210C, R95Q, and H393P and accounted for approximately 20% of disease alleles in Europeans (*5*). The most common MPS VI mutation in the latter study was the missense mutation Y210C, representing ∼10% of MPS VI disease alleles.

MPS VI patient fibroblasts have <5% of the 4-sulfatase protein levels observed in normal controls, with the most severely affected individuals having no detectable enzyme activity in fibroblast cell extracts (*8*). In MPS VI, the 4-sulfatase mutations mainly result in conformationally altered protein, which is recognized as incorrectly folded by a quality control process within the endoplasmic reticulum (ER; *3*, *8*−*11*). This ER retention of the mutant protein is probably orchestrated by molecular chaperones and results in subsequent degradation, which may be mediated by a proteasome-related degradation system. For mutations that alter protein structure but do not have a dramatic consequence for enzyme activity, the ER quality control system will contribute directly to the onset of patient pathophysiology by removing the mutant but partially functional protein (*10*).

Lysosomal storage disorder patient clinical phenotype represents a dynamic balance between substrate accumulation and the small amount of residual protein and enzyme activity which reaches the endosome−lysosome system (*12*). 4-Sulfatase mutations that result in an increase in enzyme catalytic capacity have been reported (*3*), and mutations that cause altered enzyme targeting in the vacuolar network have been implicated from protein processing studies in MPS VI patient fibroblasts (*13*). It is postulated that any process involved in the folding, modification, targeting, and traffic of lysosomal proteins (i.e., lysosomal biogenesis) may influence the final level of functional protein within a patient's cells, which in turn will directly influence the patient's clinical phenotype.

A Y210C 4-sulfatase mutation was previously described in five MPS VI patients (compound heterozygotes) and appeared to be associated with an attenuated clinical phenotype (*5*). Here we have examined the biosynthesis, processing, traffic, and biochemistry of the common 4-sulfatase mutation, Y210C, in Chinese hamster ovary (CHO-K1) expression cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Harvesting. CHO-K1 cells were grown in 75 cm^2 culture flasks in Ham's F12 medium containing L-glutamine (300 mg/mL), penicillin (100 IU/mL), streptomycin (100 mg/mL), and 10% fetal calf serum (Life Technologies, Melbourne, Australia). CHO-K1 cells were treated with either 50 μM ALLN (*N*-acetyl-L-leucyl-L-leucinylnorleucinal; Sigma-Aldrich Pty Ltd., Castle Hill, New South Wales, Australia), 50 μM ALLM (*N*-acetyl-L-leucyl-L-leucinylmethioninal; Sigma-Aldrich Pty Ltd., Castle Hill, New South Wales, Australia), or 7.5% glycerol by direct addition of these chemical reagents to the cell culture medium. CHO-K1 cells were harvested by removing the cell culture medium and washing the cell monolayer twice with Dulbecco's modified phosphate-buffered saline, and the cells were detached by trypsin digestion and vigorous agitation as previously described (*14*). The cells were then either prepared 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 and then centrifuged at 5000*g* for 5 min to remove the residual cell debris. The resulting supernatants (cell extracts) were used either for immunoquantification or in an enzyme immunobinding assay.

Monoclonal Antibodies. The monoclonal antibodies 4-S 4.1.1, 4-S F66, and 4-S F58.3 were produced as previously described (*8*).

Northern Blot Analysis. Northern blot analysis of CHO-K1, Y210, and Y210C RNA was carried out essentially as previously described (*4*). Total RNA was isolated from each cell line using the RNeasy Mini Kit (Qiagen). Six micrograms of total RNA was electrophoresed through a 1.2% agarose denaturing gel containing 0.7% formaldehyde in 20 mM morpholinopropane (MOPS), pH 6.8, and then transferred to GeneScreen Plus (NEN) and dried. The membrane was prehybridized in ULTRAhyb (Ambion) for 1 h at 68 °C. The cDNA probe (pBS.NXBAE.4S.6) was labeled with [^{32}P]dCTP and added to the prehybridization mixture and incubated overnight at 68 °C. The membrane was then washed twice in 2 × SSC (0.15 M NaCl, 15 mM $Na_3C_6H_5O_7 \cdot 2H_2O$, pH 7.0, with 0.1% DEPC) containing 0.1% SDS, at 68 °C for 5 min, and twice in 0.1 × SSC containing 0.1% SDS, at 68 °C for 15 min. The blot was then exposed to Hyperfilm CL (Amersham Pharmacia Biotech) for 24 h. The membrane was stripped before being rehybridized with a GAPDH control probe.

Immunobinding Assay for the Capture of Recombinant Human 4-Sulfatase (rh-4-Sulfatase) Activity. An immune capture method was used to specifically bind rh-4-sulfatase protein (removing endogenous CHO 4-sulfatase activity), followed by a 4-methylumbelliferyl sulfate assay to determine 4-sulfatase enzyme activity. The procedure used was as previously described (*15*), 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 the monoclonal antibody (in this case as either 4-S F66 or 4-S

F58.3 hybridoma culture supernatants). Enzyme activities of either antibody-bound or free 4-sulfatase were determined using the fluorogenic substrate 4-methylumbelliferyl sulfate as previously described (*15*).

Biosynthetic Labeling of CHO-K1 Cells. Wild-type (Y210) and mutant (Y210C) cell lines were labeled with [^{35}S]cysteine and [^{35}S]methionine protein labeling mix (1175 Ci/mmol; NEN Research Products, Dupont, Melbourne, Victoria, Australia). Cells from a confluent 75 cm^2 culture flask (Costar, New York) were preincubated in 5 mL of cysteine- and methionine-free Dulbecco's modified Eagle medium (Life Technologies, Melbourne, Australia) containing 10% (v/v) dialyzed fetal calf serum for 60 min and then labeled by adding 0.3 mCi of [^{35}S]cysteine and [^{35}S]methionine mix for 5 min−2 h at 37 °C. Cells were washed three times with 5 mL of phosphate-buffered saline (PBS) and then either harvested or chased by adding 5 mL of fresh Ham's F-12 medium (Life Technologies, Melbourne, Australia) without label. Harvested cell pellets were resuspended in 100 μL of lysis buffer [0.02 M Tris-HCl, pH 7.0, 0.15 M NaCl, 4 mM EDTA, and 1% (v/v) Nonidet P40 (NP40), plus the protease inhibitors 0.2 mM phenylmethanesulfonyl fluoride, 1 μM pepstatin A, and 1 μM leupeptin, which were purchased from Sigma] at 4 °C. Cells were frozen and thawed six times and then centrifuged (12000*g* for 5 min at 4 °C), and the supernatant was collected as cell lysate.

Immunoprecipitation. All procedures were carried out at 4 °C. Preimmune rabbit sera were coupled to protein A−Sepharose (Amrad, Victoria, Australia) by mixing 1 mL of sera with 1 mL of packed protein A−Sepharose overnight on a rotator, then recovered by centrifugation (800*g* for 1 min), and washed three times with 10 mL of 0.02 M Tris-HCl, pH 7.0, containing 0.25 M NaCl. Radiolabeled cell lysates were precleared by mixing with 100 μL of preimmune rabbit sera−protein A−Sepharose overnight. The precleared lysates were recovered by centrifugation (800*g* for 1 min) and then mixed with 5 μL of polyclonal anti-4-sulfatase antibody for immunoprecipitation (4 h). Antibody−antigen complexes were immobilized on 100 μL of protein A−Sepharose by mixing overnight, then washed 10 times with 10 mL of 0.02 M Tris-HCl, pH 7.0, 0.15 M NaCl, 4 mM EDTA, and 0.5% (v/v) NP40, and washed a further three times with 10 mL of 0.02 M Tris-HCl, pH 7.0, containing 0.25 M NaCl. The radiolabeled proteins were dissociated from the polyclonal antibody−Sepharose by boiling in reducing SDS−polyacrylamide gel electrophoresis (SDS−PAGE) loading buffer for 5 min, then separated by 10% SDS−PAGE, and, after incubating with the fluor Amplify (Amersham International, Amersham, Bucks, U.K.), visualized by autoradiography.

Granular Fractionation. Ten 75 cm^2 culture flasks of CHO-K1 cell lines (Y210, Y210C) were grown to confluency and then harvested as described above. The cells were then washed twice (10 mL/flask of cells) in phosphate-buffered saline containing 1% (v/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 10 mM Hepes, pH 7.0/0.25 M sucrose/1 mM EDTA/0.2 mM PMSF/1 μM pepstatin A/1 μM leupeptin, and subcellular fractions were prepared as described previously (*16*). Whole granular fractions were used for the Percoll subcellular fractionations to avoid any loss of rh-4-sulfatase protein and activity.

Enzyme Assays. To determine the efficacy of granular fractionation, the subcellular fractions were assayed for either β-hexosaminidase, acid phosphatase as previously described (*16*, *17*), or 4-sulfatase activity (as above).

Immunoquantification of 4-Sulfatase Protein. 4-Sulfatase polypeptide levels were measured by immunoquantification assay. This assay used a monospecific polyclonal antibody to capture the 4-sulfatase protein and a panel of conformation-sensitive 4-sulfatase monoclonal antibodies and a peroxidase-labeled secondary antibody to detect and quantify the bound protein (*8*). This assay was specific for human 4-sulfatase and did not detect endogenous CHO 4-sulfatase.

Immunoblotting. Organelle fractions were electrophoresed on a 10% SDS−PAGE gel and the separated proteins then transferred to PVDF membrane (Bio-Rad Laboratories, Richmond, CA) by electroblotting for 1 h at 0.5 A in a Hoefer electrophoresis unit. The transfer membrane was incubated for 2 h in blocking buffer [1% (w/v) milk, 1% (w/v) ovalbumin, 1 M glycine]. After being washed three times with PBS, the transfer membrane was incubated overnight at 4 °C with polyclonal antibodies (anti-TFR and anti-Rab5, from Affinity Bioreagents, New Jersey; anti-Rab7, a gift from Marino Zerial, EMBL, Germany) at a 1/250 dilution in PBS containing 1% (w/v) ovalbumin. The transfer membrane was then washed three times with PBS and incubated for 1 h with a 1/1000 dilution of horseradish peroxidase conjugated sheep anti-rabbit immunoglobulin (Silenus Laboratories, Melbourne, Victoria, Australia) in PBS containing 1% (w/v) ovalbumin. Antibody reactivity was detected by enhanced chemiluminescence according to the manufacturer's instructions (NEN Research Products, Dupont, Melbourne, Victoria, Australia).

Molecular Modeling of the Y210C 4-Sulfatase Mutation. The crystal structure of 4-sulfatase (*18*) and the molecular modeling program MOLMOL [MOLMOL 2.6, a program for display and analysis of macromolecular structures (*19*)] were used to define the molecular location and predict the effects of the Y210C mutation.

RESULTS

Charaterization of the Y210C Mutant Protein in Cultured Skin Fibroblasts from MPS VI Patients. The immunodetectable 4-sulfatase protein and 4-sulfatase enzyme activity in cultured skin fibroblast extracts from patients with a Y210C mutant allele (Table 1) were consistent with an attenuated clinical phenotype and the older age at diagnosis observed in these patients. The 4-sulfatase protein in cultured skin fibroblasts from MPS VI patients with a Y210C mutation was between 1.1% and 2.1% of the mean for normal controls (111 ng/mg, $n = 30$), while the 4-sulfatase activity was between 3.1% and 7.3% of the mean level for normal controls (26 pmol min^{-1} mg^{-1}, $n = 30$). This indicated a slightly higher specific activity for the 4-sulfatase protein detected in these MPS VI patients (0.44−0.91 pmol min^{-1} ng^{-1} of 4-sulfatase), when compared to normal human controls (0.23 pmol min^{-1} mg^{-1} of 4-sulfatase; calculated on the means for activity and protein, with a range between 0.19 and 0.39 pmol min^{-1} ng^{-1} of 4-sulfatase).

Expression of Y210C 4-Sulfatase. In Y210C 4-sulfatase CHO-K1 expression cells, 1.6% of 4-sulfatase protein and 1.7% of 4-sulfatase activity were detected when compared

Table 1: 4-Sulfatase Protein and Activity Levels in Cell Extracts from Normal Control and MPS VI Patient Fibroblasts

patient	age at diagnosis (years)	mutations	4-sulfatase protein[a] (ng/mg)	4-sulfatase activity[b] (pmol min^{-1} mg^{-1})
SF 2984	6.7	Y201C/H393P	1.2	1.1
SF 912	13	Y210C/R95Q	1.8	0.8
SF 913	16	Y210C/R95Q	1.5	1.2
SF 51-1	15	Y210C/?	1.6	NA
SF 2724	17.5	Y210C/?	2.3	1.9
normal			30−200	11.8−39.2
($n = 30$)			111*	26*
MPS VI			ND −4.5	ND −1.9
($n = 50$)				

[a] 4-Sulfatase protein was determined by immunoquantification and expressed as ng of 4-sulfatase/mg of total cell extract. [b] 4-Sulfatase activity was determined using a radiolabeled trisaccharide substrate (*17*) and expressed as pmol of 4-sulfatase per min per mg of total cell extract. ? refers to unknown genotype for the second patient allele.

Table 2: 4-Sulfatase Activity in Cell Extracts and Cell Culture Medium from Y210 and Y210C Expression Cell Lines

CHO-K1 cell line	4-sulfatase protein in cells (μg/mg)	4-sulfatase activity[a] in cells (nmol min^{-1} mg^{-1})	4-sulfatase activity in medium (nmol min^{-1} mL^{-1})
Y210	190 (100)	1060 (100)	45 (100)
Y210C	3 (1.6)	18 (1.7)	0.65 (1.4)

[a] 4-Sulfatase activity was determined by an immunobinding assay (to specifically assay rh-4-sulfatase by purifying away endogenous CHO-4-sulfatase) using 4-MUS substrate, and results were expressed as nmol of 4-sulfatase per min per mg of total cell extract. Values in parentheses are percentages expressed relative to the normal control Y210 value.

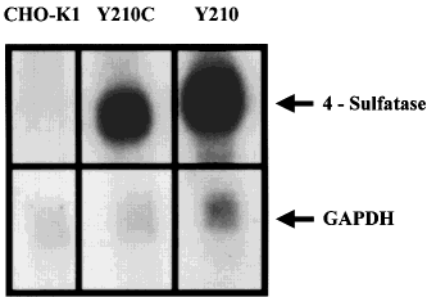

FIGURE 1: Northern analysis of Y210 and Y210C 4-sulfatase mRNA from CHO-K1 expression cells. Lane 1 contained RNA from CHO-K1 cells, lane 2 contained RNA from Y210C CHO-K1 cells, and lane 3 contained RNA from control Y210 CHO-K1 cells. The top of the figure shows the level of 4-sulfatase mRNA detected for each cell line and was contrasted with the level of GAPDH mRNA detected, as shown at the bottom of the figure. When expressed relative to GAPDH, there was a similar amount of 4-sulfatase mRNA detected in Y210C 4-sulfatase CHO-K1 cells when compared to wild-type Y210 4-sulfatase CHO-K1 cells.

to the Y210 4-sulfatase control cell line (Table 2). A similar ratio of medium to cellular 4-sulfatase activity was observed for Y210C 4-sulfatase (ratio 0.036) compared to Y210 4-sulfatase (ratio 0.043), indicating a similar percentage of 4-sulfatase enzyme secretion in both cell lines.

Synthesis and Proteolytic Processing of Y210C 4-Sulfatase. Northern analysis demonstrated that a similar amount of 4-sulfatase mRNA was produced by Y210C compared to wild-type Y210 4-sulfatase CHO-K1 cells (Figure 1). Despite a reduced level of 4-sulfatase activity in Y210C CHO-K1

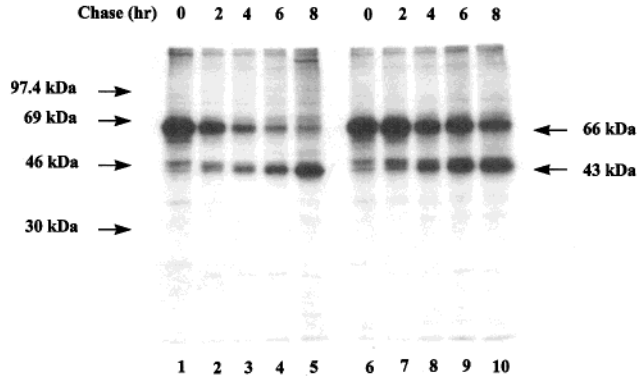

FIGURE 2: Synthesis and proteolytic processing of Y210 and Y210C 4-sulfatase. CHO-K1 cells expressing either wild-type Y210 4-sulfatase (normal Y210, lanes 1−5) or mutant Y210C 4-sulfatase (mutant Y210C, lanes 6−10) were pulse labeled with [^{35}S]-methionine and [^{35}S]cysteine for 10 min and then either harvested (Y210, lane 1, and Y210C, lane 6) or chased for 2 h intervals up to 8 h in DMEM culture medium minus the label (Y210, lanes 2−5, and Y210C, lanes 7−10). Cell lysates from two confluent flasks of either Y210 or Y210C CHO-K1 cells were immunoprecipitated with a specific polyclonal anti-4-sulfatase antibody and analyzed by SDS−PAGE and autoradiography. Precursor 4-sulfatase (66 kDa), mature 4-sulfatase (43 kDa subunit), and the molecular mass standards have been marked. [Note: The 8 and 7 kDa subunits of mature 4-sulfatase require specialized running conditions (*23*) to be visualized and separated from the running front (shown as a faint band at the bottom of the figure).] Scanning densitometry of autoradiographs revealed the following: at $t = 0$ (pulse label) 92.6% precursor and 7.4% mature form were detected for wild-type 4-sulfatase (lane 1) compared to 92.2% precursor and 7.8% mature form for Y210C 4-sulfatase (lane 6); at $t = 8$ h (chase time) 11.3% precursor and 88.7% mature form were detected for wild-type 4-sulfatase (lane 5) compared to 32.6% precursor and 67.4% mature form for Y210C 4-sulfatase (lane 10).

cells (Table 2), there also appeared to be a normal level of Y210C 4-sulfatase protein synthesis in a 10 min pulse label, compared to the Y210 control CHO-K1 cells (Figure 2, lanes 1 and 6). In pulse−chase experiments, at least some Y210C 4-sulfatase (67% of intracellular pool) was proteolytically processed to the mature lysosomal form (represented by the 43 kDa molecular form; Figure 2, lanes 7−10). However, approximately 33% of the intracellular Y210C 4-sulfatase was still present as a 66 kDa 4-sulfatase precursor, in an 8 h chase (Figure 2, lane 10), when compared to approximately 10% for the wild-type Y210 4-sulfatase precursor (Figure 2, lane 5).

Effect of Proteolytic Inhibitors and Glycerol on Y210C 4-Sulfatase Synthesis and Processing. Y210C cells were treated with either 50 μM ALLN (Figure 3, lanes 3 and 4), 50 μM ALLM (Figure 3, lanes 5 and 6), or 7.5% glycerol (Figure 3, lanes 9 and 10) for 2 h prior to metabolic labeling and then harvested for 4-sulfatase immunoprecipitation. Addition of 50 μM ALLN (Figure 3, lanes 3 and 4) did not appear to alter either the amount of Y210C 4-sulfatase synthesis or the processing of Y201C 4-sulfatase protein to the mature 43 kDa form, when compared to the untreated Y210C control (Figure 3, lanes 1 and 2). However, Y210C CHO-K1 cells treated with 50 μM ALLM (Figure 3, lanes 5 and 6) showed a 50% increase in the amount of 66 kDa precursor and 43 kDa mature 4-sulfatase compared to the appropriate untreated Y210C controls (Figure 3, lanes 1 and 2). For treatment with 7.5% glycerol there was a 100% increase in both the 66 kDa precursor and 43 kDa mature forms of

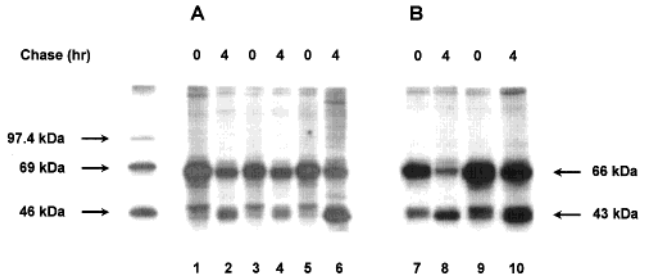

FIGURE 3: Effect of proteasome inhibitors and glycerol on Y210C 4-sulfatase synthesis and degradation. CHO-K1 cells expressing Y210C 4-sulfatase were preincubated for 2 h with either proteasome inhibitors (A) or glycerol (B). The effect of the proteasomal inhibitors ALLN (lanes 3 and 4) and ALLM (lanes 5 and 6) at 50 μM on Y210C 4-sulfatase synthesis and proteolytic processing was examined and compared to an untreated Y210C control (lanes 1 and 2). The effect of 7.5% glycerol (lanes 9 and 10) on Y210C 4-sulfatase synthesis and proteolytic processing was examined and compared to an untreated Y210C control (lanes 7 and 8). Cells were labeled with [^{35}S]methionine and [^{35}S]cysteine for 10 min and then chased for either 0 or 4 h in DMEM culture medium minus the label (inhibitors and glycerol were present in the chases). Cell lysates were immunoprecipitated with polyclonal anti-4-sulfatase and then analyzed by SDS−PAGE and autoradiography. Precursor 4-sulfatase (66 kDa), mature 4-sulfatase (43 kDa subunit), and the molecular mass standards have been marked. The level of immunoprecipitated Y210C protein in lanes 1−6 represents a single confluent 75 cm^2 flask of Y210C CHO-K1 cells, while that in lanes 7−10 represents two confluent flasks of Y210C CHO-K1 cells. Scanning densitometry of autoradiographs revealed the following: for wild-type Y210 4-sulfatase at $t = 0$ (pulse label), 91.4% precursor and 8.6% mature form (lane 1), and at $t = 4$ (chase time), 46.8% precursor and 53.2% mature (lane 2). For ALLM and glycerol treatment the percentages of precursor and mature form detected were almost identical compared to the Y210 control, but at $t = 4$ (chase time) there was either a 50% increase (ALLM) or 100% increase (glycerol) in the level of both Y210C 4-sulfatase precursor and mature form when compared to the Y210 4-sulfatase control.

Y210C 4-sulfatase (Figure 3, lanes 9 and 10) compared to the untreated Y210C controls (Figure 3, lanes 7 and 8).

Subcellular Localization of Y210C 4-Sulfatase. Percoll fractionation of subcellular organelles demonstrated that at least some Y210C 4-sulfatase protein and activity was reaching high-density organelles characteristic of endosomes−lysosomes (Figure 4). The Y210C protein (Figure 4B) was mainly coincident with both β-hexosaminidase activity and the soluble form of acid phosphatase (Figure 4A, fractions 13−20). Some Y210C 4-sulfatase protein and activity were also present in lower density organelles characteristic of endosomes. Fractions 9−13 contained Y210C 4-sulfatase protein (Figure 4B) which was coincident with the membrane-bound form of acid phosphatase (Figure 4A) and which was low in the amount of β-hexosaminidase (Figure 4A). This Y210C 4-sulfatase protein and activity were also coincident with other endosomal markers (Figure 4C), including the transferrin receptor (TfR, a recycling endosome marker), Rab5 (an early endosomal marker), and Rab7 (a late endosome marker). The majority of Y210C 4-sulfatase was detected in fractions that contained Rab7 and β-hexosaminidase, suggesting that it was reaching either late endosomal or lysosomal compartments. This was consistent with the processing of a proportion of Y210C 4-sulfatase protein to the 43 kDa mature form of 4-sulfatase (Figure 2, lane 10), which is a known endosome−lysosome proteolytic processing event. However, when compared to a wild-type Y210 4-sulfatase granular fractionation (Figure 5, fractions

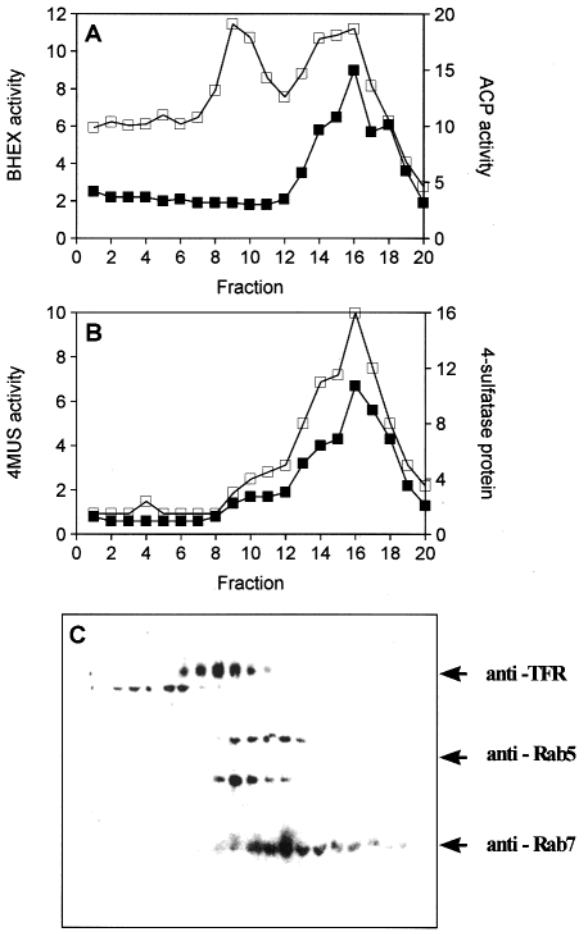

FIGURE 4: Subcellular localization of Y210C 4-sulfatase. Subcellular organelle preparations from CHO-K1 cells expressing Y210C 4-sulfatase were fractionated on an 18% Percoll gradient and collected into 20 × 1 mL fractions. Fractions were each assayed for β-hexosaminidase (A, BHEX, ■), acid phosphatase (A, ACP, □), and 4-sulfatase activity (B, 4MUS activity, ■) and 4-sulfatase protein and quantified by ELISA assay (B, 4-sulfatase protein, □). The enzyme activities (A, B) and 4-sulfatase protein (B) represent the average of duplicate determinations and were expressed as nmol min^{-1} mL^{-1} and ng/mL, respectively. In (C), the positions of the endosome−lysosome markers, transferrin receptor (anti-TFR), anti-Rab5, and anti-Rab7, were determined within the same gradient by immunoblotting each fraction (detection by ECL). The level of LAMP-1 protein was immunoquantified (*25*) and shown to mainly reside in fractions 14−20, indicating the location of organelles characteristic of lysosomes (data not shown).

17−20), the Y210C 4-sulfatase did not appear to be reaching the higher density fractions characteristic of lysosomes (Figure 4B).

Altered Level and Intracellular Distribution of Y210C 4-Sulfatase Following Treatment of CHO-K1 Expression Cells with ALLM and Glycerol. Subcellular fractionation of organelles from Y210C cells treated for 24 h with 50 μM ALLM showed an increase in the amount of 4-sulfatase protein reaching higher density organelles characteristic of lysosomes (Figure 6B). There was a similar pattern of distribution for 4-sulfatase activity in the ALLM-treated and untreated Y210C CHO-K1 cells, but the level of activity was lower in the high-density lysosomal fractions of the ALLM-treated Y210C CHO-K1 cells (Figure 6A,B). This was consistent with lysosomal inactivation and degradation of the mutant Y210C 4-sulfatase.

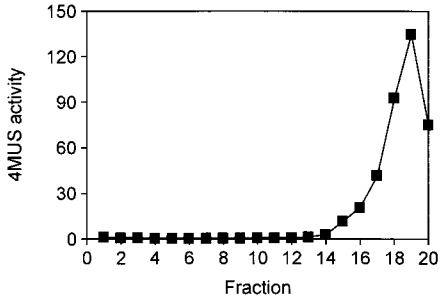

FIGURE 5: Subcellular localization of Y210 4-sulfatase, demonstrating normal lysosomal distribution. Subcellular organelle preparations from CHO-K1 cells expressing wild-type Y210 4-sulfatase were fractionated on an 18% Percoll gradient and collected into 20 × 1 mL fractions. Fractions were each assayed for β-hexosaminidase (coincident with 4-sulfatase activity, data not shown) and 4-sulfatase activity (4MUS activity, ■). 4-Sulfatase activity represented the average of duplicate determinations and was expressed as nmol min^{-1} mL^{-1}.

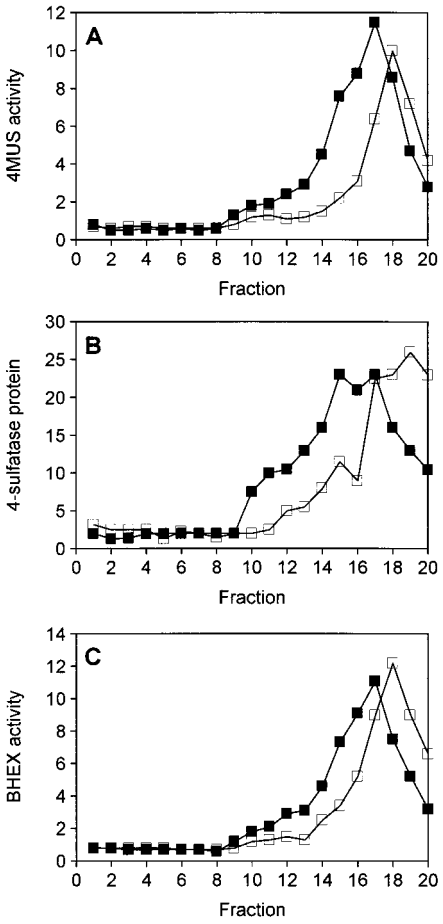

FIGURE 6: Subcellular localization of Y210C 4-sulfatase in the presence of the proteasome inhibitor ALLM. CHO-K1 cells expressing Y210C 4-sulfatase were treated either with (□) or without (■) 50 μM ALLM in DMEM culture medium for 18 h at 37 °C. Subcellular organelles were prepared and fractionated on an 18% Percoll gradient and collected as 20 × 1 mL fractions. 4-Sulfatase activity (A, 4MUS activity), immunoquantified 4-sulfatase protein (B, 4-sulfatase protein), and β-hexosaminidase activity (C, BHEX activity) were determined for each fraction. The enzyme activities (A, C) and 4-sulfatase protein (B) represent the average of duplicate determinations and were expressed as nmol min^{-1} mL^{-1} and ng/mL, respectively.

Glycerol treatment (7.5% in the culture medium) for 24 h markedly increased the amount of Y210C 4-sulfatase observed in Percoll granular fractions, particularly in fractions characteristic of late endosomes (Figure 7A,B, fractions 11−16). In the granular fractions from glycerol-treated Y210C cells, the increase in 4-sulfatase activity (Figure 7A) was not as high as expected when compared to the amount of 4-sulfatase protein (Figure 7B), again implying that there was significant inactivation of the Y210C 4-sulfatase.

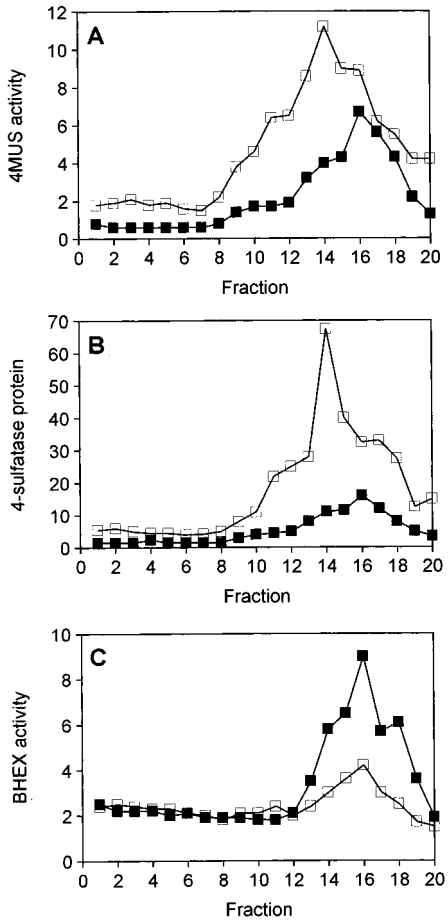

FIGURE 7: Subcellular localization of Y210C 4-sulfatase in cells treated with 7.5% glycerol. CHO-K1 cells expressing Y210C 4-sulfatase were treated either with (□) or without (■) DMEM culture medium containing 7.5% glycerol for 24 h at 37 °C. Subcellular organelles were prepared and fractionated on an 18% Percoll gradient and collected as 20 × 1 mL fractions. 4-Sulfatase activity (A, 4MUS activity), immunoquantified 4-sulfatase protein (B, 4-sulfatase protein), and β-hexosaminidase activity (C, BHEX activity) were determined for each fraction. The enzyme activities (A, C) and 4-sulfatase protein (B) represent the average of duplicate determinations and were expressed as nmol min^{-1} mL^{-1} and ng/mL, respectively.

Glycerol treatment (7.5% glycerol in cell culture medium) of Y210C CHO-K1 cells for 24 h was followed with a normal medium chase for 24 h to determine the effect on Y210C 4-sulfatase trafficking. Similar amounts of 4-sulfatase protein and activity were detected in the glycerol plus chase treated Y210C CHO-K1 cells when compared to the glycerol-treated Y210C CHO-K1 cells. However, for the glycerol plus chase treated Y210C CHO-K1 cells, the 4-sulfatase protein and activity were localized in higher density organelles when compared to the glycerol only treated Y210C CHO-K1 cells (Figure 7A,C). The ability to chase the glycerol-induced Y210C 4-sulfatase from a lower density "endosome-like" location (Figure 7) to a higher density "lysosome-like" location (Figure 8) was consistent

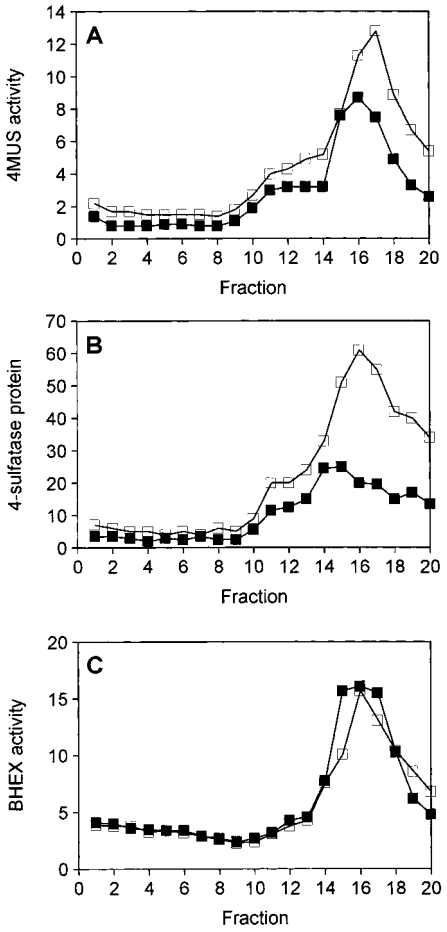

FIGURE 8: Effect of treating CHO-K1 cells with glycerol followed by incubation in normal medium on the subcellular localization and amount of Y210C 4-sulfatase. CHO-K1 cells expressing Y210C 4-sulfatase were treated either with (□) or without (■) DMEM culture medium containing 7.5% glycerol for 24 h at 37 °C and then incubated in only DMEM culture medium for 24 h (24 h chase). Subcellular organelles were prepared and fractionated on an 18% Percoll gradient and collected as 20 × 1 mL fractions. 4-Sulfatase activity (A, 4MUS activity), immunoquantified 4-sulfatase protein (B, 4-sulfatase protein), and β-hexosaminidase activity (C, BHEX activity) were determined for each fraction. The enzyme activities (A, C) and 4-sulfatase protein (B) represent the average of duplicate determinations and were expressed as nmol min^{-1} mL^{-1} and ng/mL, respectively.

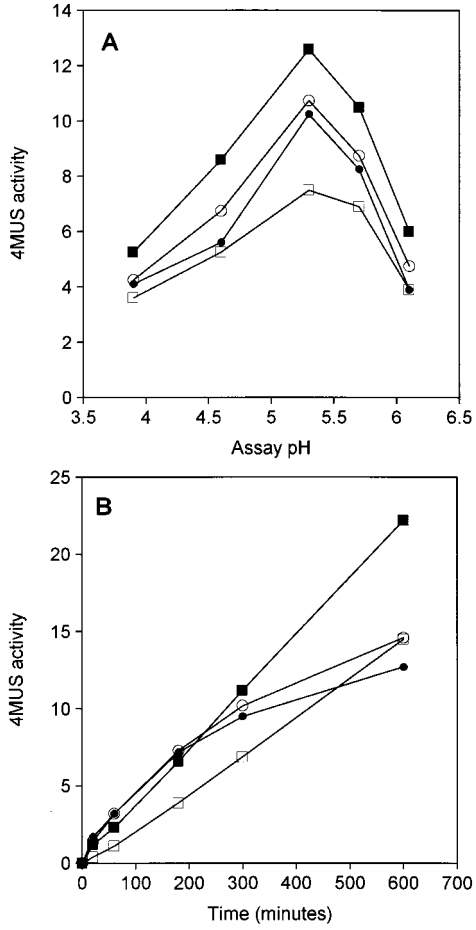

FIGURE 9: Comparison of Y210 and Y210C 4-sulfatase enzyme activities. Cell extracts and cell culture medium from either Y210 or Y210C CHO-K1 expression cell lines were diluted to give similar levels of substrate reactivity in assays designed to test either enzyme pH optima (A) or enzyme stability and substrate turnover with time (B). Y210 4-sulfatase from cell extracts was diluted 1/1600 (■), Y210 4-sulfatase from cell culture medium was diluted 1/50 (□), Y210C 4-sulfatase from cell extracts was diluted 1/80 (●), and Y210C 4-sulfatase from cell culture medium was assayed neat (○). The 4-sulfatase protein was immunocaptured with the monoclonal antibody 4-S F66 (specific for human 4-sulfatase) and then assayed for activity using the fluorometric substrate 4MUS. 4MUS activity was expressed as nmol min^{-1} mL^{-1}. The experiments in (A) and (B) are representative of a duplicate set of data performed using two different enzyme dilutions. (Note: only one set of dilutions has been presented, but both gave similar results.) The experiments in (B) (5 h assay) were done at pH 5.3 and at the same enzyme dilutions used in (A) [i.e., similar substrate reactivity at pH 5.3 in (A) compared to 300 min time point in (B)]. From the data above, the Y210C 4-sulfatase had an enzyme half-life of approximately 10 h compared to previous reports of 4 days for wild-type 4-sulfatase (*24*).

with a delay in the traffic of newly synthesized Y210C 4-sulfatase to the lysosomal compartment.

Reduced Stability of Y210C 4-Sulfatase. Both the Y210C and Y210 4-sulfatase, in either CHO-K1 cell extracts or cell culture medium, had a similar pH optimum of 5.3 (Figure 9A). However, the stability of Y210C 4-sulfatase with time of assay was reduced when compared to Y210 4-sulfatase (Figure 9B). This reduction in Y210C enzyme reactivity with time was observed in both cell extracts and cell culture medium samples, suggesting an inherent problem with enzyme stability, not just susceptibility to low pH. These observations, together with the detection of inactive Y210C in endosomal fractions (e.g., Figures 6 and 7), suggested that Y210C protein had a problem with stability.

Molecular Modeling of the Y210C 4-Sulfatase Mutation. Using the crystal structure of 4-sulfatase (*18*) and the molecular modeling program MOLMOL (*19*) the molecular location of the Y210C mutation was defined and the effects of the mutation predicted (Figure 10). The Y210 residue of 4-sulfatase was localized in a position that suggested it may be involved with intrapolypeptide chain interaction. The location of the Y210C mutation was distant from the known disulfide bonds in the 4-sulfatase molecule. Molecular modeling of the Y210C mutation predicted minor structural changes throughout the molecule, consistent with a destabilization effect on the 4-sulfatase protein (Figure 10).

DISCUSSION

This study describes the molecular characterization of the 4-sulfatase missense mutation Y210C, which represents the

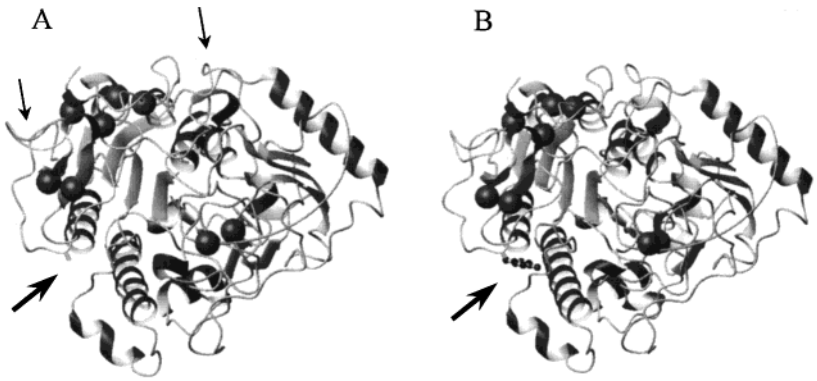

FIGURE 10: Molecular modeling of normal control Y210 and mutant Y210C 4-sulfatase protein. The crystal structure of 4-sulfatase (*19*) and the molecular modeling program MOLMOL (*20*) were used to visualize the location of the Y210 residue (B, thick arrow) on a molecular model of 4-sulfatase. The location of the Y210C mutation (A, thick arrow) and the predicted structural effects of this mutation [examples highlighted in (A) by thin arrows] are shown on a molecular model of 4-sulfatase. The proximity of the Y210C mutation to known 4-sulfatase disulfide bonds (shown as spheres on both models) can be visualized in the figure.

most common mutant allele found in human MPS VI patients (~10% of disease alleles). The Y210C mutation was detected in MPS VI patients who had an attenuated clinical phenotype (*5*) compared to the archetypical form of the disorder (*1*). The Y210C protein was shown here to be synthesized at normal levels in the ER but encountered protein folding/processing, trafficking, and stability problems. All of these factors potentially contribute to the molecular lesion underlying the MPS VI clinical phenotype resulting from this mutation.

Stable expression of the 4-sulfatase mutation, Y210C, in CHO-K1 expression cells revealed normal mRNA production and a similar amount of mutant 4-sulfatase precursor synthesis, when compared to wild-type 4-sulfatase. The molecular size and amount of the Y210C precursor (66 kDa) indicated not only normal polypeptide synthesis but also appropriate N-glycosylation, which is known to occur in the lumen of the ER (*20*, *21*). Despite this normal level of Y210C 4-sulfatase protein synthesis and glycoprocessing, cell extracts from Y210C CHO-K1 cells only had 1.6% of the 4-sulfatase protein and 1.7% of the 4-sulfatase activity when compared to Y210 CHO-K1 cells. Moreover, MPS VI patients with a Y210C allele had reduced 4-sulfatase protein and activity in cell extracts from cultured skin fibroblasts (up to 2.1% and 7.3% of the mean for normal human controls, respectively). The high level of 4-sulfatase activity in MPS VI patients with a Y210C allele (up to 7.3% of normal controls) was surprising, as a patient with 5% of normal 4-sulfatase activity has been previously shown to have minimal clinical signs of MPS VI (*8*). Further cell biological and biochemical analysis was required to determine the fate of newly synthesized Y210C 4-sulfatase protein in the vacuolar network (i.e., ER, Golgi, endosomes, and lysosomes).

While wild-type precursor 4-sulfatase was trafficked and proteolytically processed to a mature lysosomal form, the Y210C precursor 4-sulfatase appeared to exhibit delayed processing/traffic. After a 10 min pulse radiolabel and 8 h chase at least 30% of the intracellular Y210C 4-sulfatase remained as a 66 kDa precursor, which was consistent with either ER retention or delayed traffic to the site of proteolytic processing (i.e., proteolytic processing to 43, 8, and 7 kDa forms, which is known to be an endosome−lysosome processing event). Treatment of Y210C cells with glycerol resulted in an increase in both precursor and mature Y210C 4-sulfatase in radiolabel pulse−chase studies. The increase in the 66 kDa precursor, together with the increase in the total amount of 4-sulfatase protein evident in granular fractions following glycerol treatment, suggested that the Y210C protein may have had problems with either folding or stability. Previous studies involving lysosomal storage disorders identified normal protein synthesis (*3*, *11*), but the mutant protein did not appear to fold correctly and was recognized by the ER quality control system and targeted for degradation (*10*). In MPS VI patients this is the most common scenario with normal synthesis of mutant protein, but low levels of mutant protein at steady state due to the destabilizing effect of the introduced mutation and the ensuing action of the ER quality control process. Glycerol has been previously reported to stabilize mutant protein, resulting in higher intracellular levels and even relatively normal traffic and function of the mutant protein (e.g., CFTR; *22*). Clearly, some Y210C protein was lost early in the vacuolar network, probably in the ER, and this process would therefore be expected to contribute to the pathogenic effect of the Y210C mutation.

However, unlike the mutant protein resulting from mutations in other MPS VI patients, a significant amount of Y210C 4-sulfatase was trafficked beyond the ER to distal elements of the vacuolar network. In a 10 min pulse label and 8 h chase experiment, 67% of the intracellular Y210C 4-sulfatase was detected as a 43 kDa subunit, indicating that it had reached the endosome−lysosome compartment (i.e., the site of proteolytic processing). At steady state, granular fractionation experiments (Figure 3B, fractions 8−14) revealed a pool of mutant 4-sulfatase resident in lower density organelles characteristic of endosomes. This was consistent with delayed transfer to the lysosome. The treatment of Y210C CHO-K1 cells with glycerol resulted in increased amounts of Y210C 4-sulfatase, with this protein detected mainly in vacuolar organelles characteristic of endosomes. Subsequent culture of glycerol-treated Y210C CHO-K1 cells demonstrated that the endosome pool could be chased over 24 h to higher density organelles. This was again consistent with a delay in traffic of newly synthesized Y210C 4-sulfatase to the lysosomal compartment. Delayed traffic and

processing would result in a reduced level of 4-sulfatase activity in the lysosomal compartment and could be expected to contribute to the molecular defect, as substrate turnover is dependent on the correct intracellular location and hydrolytic environment for optimum enzyme activity. Moreover, if 67% of the Y210C 4-sulfatase reached the lysosomal compartment and was active, then this would provide more than enough catalytic activity to prevent the onset of disease, as evident by a previous report of a patient with 5% of normal 4-sulfatase activity having almost no clinical signs of MPS VI (*8*).

The Y210C 4-sulfatase detected in organelles characteristic of endosomes had a reduced level of catalytic activity. Following glycerol treatment there was a 7-fold increase in Y210C 4-sulfatase protein within the gradient, but there was only a 3-fold increase in activity. This indicated at least some inactivation of Y210C 4-sulfatase prior to reaching the lysosomal compartment. In addition, Y210C present in both cell extract and culture medium showed inactivation with time. The inactivation of Y210C 4-sulfatase present in the culture medium strongly suggested an inherent instability in the fully folded Y210C 4-sulfatase protein. The Y210C 4-sulfatase activity appeared to have an enzyme half-life of approximately 10 h, compared to 4 days for wild-type enzyme. This was consistent with the residual Y210C 4-sulfatase activity detected in CHO-K1 cells at steady state.

Having reached the lysosomal milieu, Y210C 4-sulfatase appeared to be inactivated and degraded at an enhanced rate when compared to either endosomal Y210C 4-sulfatase or wild-type 4-sulfatase. In granular fractionation experiments there were relatively low levels of Y210C 4-sulfatase protein and activity in high-density fractions, when compared to wild-type Y210 4-sulfatase. However, treatment with the proteolytic inhibitor ALLM showed a higher level of Y210C protein in these fractions, demonstrating lysosomal degradation. This protein that had been prevented from proteolysis by ALLM treatment showed only a very low level of 4-sulfatase activity, showing that Y210C 4-sulfatase was being inactivated as well as degraded in the lysosomal compartment. This was again consistent with the residual Y210C 4-sulfatase protein and activity detected in CHO-K1 cells at steady state. Relative to the endosome pool of Y210C 4-sulfatase, the residual Y210C in the lysosome had a lower activity, suggesting that the inactivation process was more rapid in the degradative conditions of the lysosome.

Molecular modeling studies indicated that the Y210 residue was located between two juxtaposed regions of the 4-sulfatase protein and may normally have a role in stabilizing the protein. It was postulated that the Y210C mutation destabilized the 4-sulfatase protein, causing initial problems with folding but also resulting in unfolding/inactivation in the distal elements of the vacuolar network. This inherent instability, particularly in the lysosomal milieu, eventually resulted in the early degradation of the mutant protein in the lysosomal compartment.

The findings in this study highlighted the concept that multiple cell biological processes, involved in the biogenesis of lysosomal proteins, may contribute to the amount of residual 4-sulfatase in an MPS VI patient and therefore influence the patient's clinical phenotype. Together, a reduced rate of traffic, structural instability, loss of 4-sulfatase activity, and subsequent degradation would all significantly impact on the pathogenic effect of the Y210C mutation. The detection of reduced levels of mutant protein and activity at steady state were consistent with the Y210C mutation contributing to a very mild MPS VI clinical phenotype. However, most of the residual 4-sulfatase activity detected in Y210C CHO-K1 cells was not appropriately located in the lysosomal compartment, reflecting altered intracellular traffic/handling of the mutant protein. The reduced cellular capacity to degrade and clear storage product resulting from this mutation was consistent with the onset of an attenuated MPS VI clinical phenotype.

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