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Mild Feline Mucopolysaccharidosis Type VI

IDENTIFICATION OF AN N-ACETYLGLACTOSAMINE-4-SULFATASE MUTATION CAUSING INSTABILITY AND INCREASED SPECIFIC ACTIVITY

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The missense mutation, L476P, in the N-acetylglactosamine-4-sulfatase (4S) gene, has previously been shown to be associated with a severe feline mucopolysaccharidosis type VI (MPS VI) phenotype. The present study describes a second mutation, D520N, in the same MPS VI cat colony, which is inherited independently of L476P and is associated with a clinically mild MPS VI phenotype in D520N/L476P compound heterozygous cats. Biochemical and clinical assessment of L476P homozygous, D520N/L476P compound heterozygous, and D520N homozygous cats demonstrated that the entire range of clinical phenotypes, from severe MPS VI, to mild MPS VI, to normal, are clustered within a narrow range of residual 4S activity from 0.5% to 4.6% of normal levels. When overexpressed in CHO-KI cells, the secreted form of D520N 4S was inactivated in neutral pH conditions. In addition, intracellular D520N 4S protein was rapidly degraded and corresponded to 37%, 14.5%, and 0.67% of normal 4S protein levels in the microsomal, endosomal, and lysosomal compartments, respectively. However, the specific activity of lysosomal D520N 4S was elevated 22.5-fold when compared with wild-type 4S. These results suggest that the D520N mutation causes a rapid degradation of 4S protein. The effect of this is partially ameliorated as a result of a significant elevation in the specific activity of mutant D520N 4S reaching the lysosomal compartment.

The mucopolysaccharidoses (MPS)† are a group of lysosomal storage disorders that involve the deficiency of specific enzymes required for the degradation of glycosaminoglycans (GAG). MPS VI is characterized by a deficiency of N-acetyl-galactosamine 4-sulfatase (4S), which leads to the lysosomal accumulation and urinary excretion of the GAG dermanan sulfate (DS) (1). The severe MPS VI phenotype is characterized by growth retardation, coarse facial features, joint stiffness, corneal clouding, skeletal deformities, and organ and soft tissue involvement. As a result of DS storage in the heart valve and lung, the normal function of these organs is often compromised, leading to early death in affected individuals. The central nervous system does not appear to be affected, even in individuals with clinically severe MPS VI (2).

Patients with MPS VI are diagnosed by elevated levels of DS in their urine and a substantial reduction or lack of 4S activity in cells such as peripheral blood leukocytes and cultured skin fibroblasts. The clinical phenotype of MPS VI patients ranges from severe to relatively mild and has generally been shown to correlate with urinary DS and residual 4S activity levels (2). Generally, patients manifest symptomology associated with MPS VI when their 4S protein and activity levels are 5% or less of normal controls (3).

Currently, there is no safe and effective treatment available for MPS patients other than surgical intervention to alleviate symptoms whenever possible. MPS VI is a good candidate for both enzyme and gene replacement therapy protocols for a number of reasons. First, the complication of targeting recombinant enzyme across the blood-brain barrier is negated by the lack of central nervous system pathology in MPS VI patients. Second, circulating levels of recombinant enzyme can be efficiently targeted to the lysosomal compartment of MPS VI cells as a result of mannose 6-phosphate (M6P) receptor-mediated endocytosis of 4S (4).

The M6P moieties on lysosomal enzymes serve as high affinity ligands for binding to M6P receptors in the trans-Golgi network. Binding with M6P receptors permits lysosomal enzymes to be segregated from proteins destined for secretion or transport to other intracellular compartments. The ligand-receptor complex is then transported from the trans-Golgi network via a clathrin-coated vesicle to an acidified endosomal compartment, where the M6P receptor and lysosomal enzyme dissociate. Further processing of the enzyme occurs in the lysosome. The M6P receptor is either recycled from the endosome back to the Golgi for further targeting of endogenous de novo synthesized lysosomal enzymes or transported to the plasma membrane, where it functions to internalize exogenous lysosomal enzymes (4).

It is imperative that, where possible, enzyme and gene replacement therapy protocols for MPS disorders be extensively evaluated in large animal models prior to clinical application. A naturally occurring feline model for a severe form of MPS VI has previously been described (5), and a colony has been established in Adelaide to study the progression of the disease and evaluate the efficacy of therapy protocols. The intravenous administration of recombinant human 4S (rh4S) to severely affected MPS VI cats has been shown to dramatically reduce disease progression in a dose-dependent manner (6, 7). However, three cats placed on enzyme replacement therapy dis-
played significant antibody titers to the human enzyme, which may have reduced the availability and subsequent efficacy of 4S (8, 9). When using animal models to evaluate therapy protocols, it may be advantageous to use species-specific material in an attempt to avoid the complication of immunological responses to foreign proteins. As an initial step toward species-specific enzyme and gene replacement therapy in the MPS VI cat, the feline 4S (f4S) cDNA has been isolated and recombinant enzyme expressed and characterized (10).

The isolation of the f4S cDNA has also permitted detailed molecular analysis of mutations within this MPS VI cat colony. We have previously identified a missense mutation, L476P, which causes the clinically severe form of feline MPS VI characteristic of the colony, and we have developed a rapid PCR-based screening method to detect this mutation. L476P homozygous cats contain very low levels of 4S activity in blood leukocytes and display symptoms and disease progression identical to the severe form of human MPS VI. However, a number of colony cats that had no clinical indications of MPS VI also had very low or, in some instances, undetectable 4S activity levels in their blood leukocytes despite being genotyped as normal with respect to the L476P mutation. These observations suggested the presence of a second mutation (10). This study describes the identification and molecular characterization of this second mutation as the missense mutation, D520N. A PCR-based screening method enabled the identification of L476P homozygous cats, D520N/L476P compound heterozygous cats, and D520N homozygous cats in the colony. Clinical assessments of these cats have previously demonstrated that the three genotypes are associated with a severe MPS VI phenotype, mild MPS VI phenotype, and normal phenotype, respectively (10, 11). In this study, we determined the Asp520Asn mutation by restriction analysis of PCR products using oligonucleotides f4SP-31 and f4S-11 (see Table I of Ref. 10) and 2 units of Taq DNA polymerase (Boehringer Mannheim), gel-purified, and cloned into the normal f4S cDNA (10) digested with the same restriction endonucleases. Nucleotides 1148–1608 of 24 individual clones were PCR-amplified using primers 4SP-31 and f4S-11. Blunt-end restriction analysis of each PCR product was used to identify f4S clones containing the D520N mutation. Mutant f4S clones were sequenced with a fmol sequencing kit (Promega), and a clone that contained the D520N mutation and no other changes was then excised from pBluescript SK+ with EcoRI and cloned into the mammalian expression vector pEFNeo (pEFNeoD520N) (12). Restriction analysis was used to identify recombinants containing the 4S construct in the correct orientation. The wild-type f4S cDNA, which has previously been cloned into pEFNeo (pEFNeo4S), was used as a positive control (10).

**EXPERIMENTAL PROCEDURES**

Mutation Analysis of the f4S cDNA—Prior to the identification of D520N, cats were only screened for the L476P mutation using a PCR-based screening method (10). Cat 237 was identified to be homozygous for the L476P allele at position 476 (Leu-476) via mutation analysis, based on screening method (10). Cat 237 was identified to be homozygous for the normal 4S allele at position 476 (Leu-476) via mutation analysis, and then genotyped as normal with respect to the L476P mutation and thus classified as the wild-type genotype, mild MPS VI genotype, and normal genotype, respectively. The three genotypes are associated with a severe MPS VI phenotype, a mild MPS VI phenotype, and a normal phenotype (10, 11). A PCR-based screening method enabled the identification of L476P homozygous cats, D520N/L476P compound heterozygous cats, and D520N homozygous cats in the colony. Clinical assessments of these cats have previously demonstrated that the three genotypes are associated with a severe MPS VI phenotype, a mild MPS VI phenotype, and a normal phenotype, respectively (10, 11). In this study, we determined the Asp520Asn mutation by restriction analysis of PCR products using oligonucleotides f4SP-31 and f4S-11 (see Table I of Ref. 10) and 2 units of Taq DNA polymerase (Boehringer Mannheim), gel-purified, and cloned into the normal f4S cDNA (10) digested with the same restriction endonucleases. Nucleotides 1148–1608 of 24 individual clones were PCR-amplified using primers 4SP-31 and f4S-11. Restriction analysis of each PCR product was used to identify f4S clones containing the D520N mutation. Mutant f4S clones were sequenced with a fmol sequencing kit (Promega), and a clone that contained the D520N mutation and no other changes was then excised from pBluescript SK+ with EcoRI and cloned into the mammalian expression vector pEFNeo (pEFNeoD520N) (12). Restriction analysis was used to identify recombinants containing the 4S construct in the correct orientation. The wild-type f4S cDNA, which has previously been cloned into pEFNeo (pEFNeo4S), was used as a positive control (10).

**Construction of CHO-KI Cells—**Large scale plasmid preparations of pEFNeo4S and pEFNeoD520N (25 μg) were electroporated into Chinese hamster ovary (CHO-KI) cells as described previously (13). Transfected cells were selected with 0.5 mg/ml Geneticin (G418) and maintained in Ham's F-12 medium supplemented with 10% (v/v) FCS (CSL Ltd.).

**Percoll Granular Fractionation**—G418-resistant mass cultures of CHO-KI cells, expressing wild-type 4S and D520N 4S (CHO4S and CHOΔD520N, respectively) were grown to confluence in 75-cm² flasks. Two 75-cm² flasks of CHO4S and CHOΔD520N cells were metabolically labeled with 100 μCi/ml EXPRESS35S35S protein labeling mix (NEN Life Science Products) for 45 min. The labeling medium was removed and replaced with Ham's F-12 supplemented with 10% FCS. After 4 h, cells were harvested by treatment with trypsin-versene (Life Technologies, Inc.), recovered by centrifugation, washed three times with phosphate-buffered saline (PBS) by centrifugation/resuspension, and finally resuspended in 3 ml of 0.25 M sucrose, 10 mM HEPES, pH 7. Intracellular organelles were released by six exposures to hypobaric shock (14). The lysate was centrifuged at 200 × g for 5 min to remove nuclei. The supernatant was then layered on top of 17 ml of 0.25 M sucrose, 10 mM HEPES, 18% (v/v) Percoll (Amersham Pharmacia Biotech) and centrifuged at 50,000 × g for 1 h (15). One-mL fractions were collected from the Percoll gradient and assayed for total protein (Bio-Rad protein assay), acid phosphatase (a marker for endosomes and lysosomes) (16), the lysosomal enzyme β-hexosaminidase (17), and total arylsulfatase using 4-methylumbelliferyl sulfate (4MUS) (18). Fractions corresponding to the microsomal, endosomal, and lysosomal compartments were pooled. Each pool was further fractionated by five microcentrifugations, and each pooled fraction was solubilized in 0.5% Nonidet P-40 (PBS containing 1% sodium deoxycholate, 0.2% w/v SDS, 1% w/v Nonidet P-40) and used for immunoprecipitation (see below).

**Stability of Intracellular and Secreted 35S-Labeled D520N 4S—**G418-resistant mass cultures of CHO4S and CHOΔD520N were grown to confluence in 25-cm² tissue culture flasks and then metabolically labeled with 100 μCi/ml EXPRESS35S35S protein labeling mix for 1 h. Labeling medium was removed, and cells were rinsed in serum-free medium and then chased with 6 ml of Ham's F-12 supplemented with 10% FCS and 5 mM M6P to prevent re-uptake of secreted precursor 4S. After 4, 10, 24, and 48 h, the chased medium in one flask was collected, clarified and centrifuged (1000 × g, 5 min, 4 °C). At each time point, cells from one flask in each group were also harvested with trypsin-versene, washed, and resuspended in 5 ml of 1× solubilization buffer (PBS containing 2% w/v sodium deoxycholate, 0.2% w/v SDS, 1% w/v Nonidet P-40) and used for immunoprecipitation (see below).

**Western Blot Analysis—**Thirty microliters of each pooled endosomal and lysosomal fraction were immunoprecipitated using a rabbit polyclonal antibody raised against r4S (131,000 kDa) (131,000 kDa) and Pansorb cells (Calbiochem; 75 μM) and analyzed via SDS-PAGE and autoradiography as described previously (10).

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Percoll and subjected to Western blot analysis as described previously (19) using a rabbit polyclonal antibody raised against rf4S (titer 1/131,000) as the primary antibody.

**Immunooquantification of Wild-type 4S and D520N 4S—**The human 4S monoclonal antibodies F56.3 and F22.1 were used to quantify wild-type 4S and D520N 4S secreted from CHO-KI cells using a sandwich ELISA as described in Ref. 20. Rabbit and murine polyclonal orf4S sera were raised against immunopurified rf4S (titer 1/131,000 and 1/800,000, respectively). The specificity of both rf4S polyclonal antibodies was demonstrated by immunoprecipitation of 4S present in CHOF4S and CHOD520N samples. A sandwich ELISA was used to quantify rf4S protein in CHOF4S and CHOD520N microsomal, endosomal, and lysosomal fractions isolated from Percoll gradients. Approximately 11 mg of total IgG was purified from 1.5 ml of rabbit orf4S serum using a 1-ml protein G column (Amersham Pharmacia Biotech). Total IgG isolated from rabbit orf4S serum was diluted in 0.1 M NaHCO3, pH 10, to a final concentration of 10 μg/ml and added to individual wells of a polystyrene plate (Costar, Cambridge, MA) and incubated at 4 °C overnight (100 μl/well). Each well was then aspirated, and the remaining reactive sites were blocked by adding 200 μl of buffer A (0.02 M Tris-HCl, pH 7.0, 0.25 M NaCl, 1% (w/v) ovalbumin) and incubating for 2 h at 22 °C. The wells were then aspirated, washed three times with 0.02 M Tris-HCl, pH 7.0, 0.25 M NaCl (buffer B), and then incubated with 100 μl of samples diluted in buffer A at 4 °C overnight. The wells were aspirated, washed three times with 200 μl of buffer B, and then incubated with 100 μl of the murine orf4S polyclonal serum diluted 1/10,000 in buffer A (Silenus Laboratories) for 1 h at 22 °C. The antibody was removed and the wells washed three times with 200 μl of buffer B. The wash buffer was aspirated, and 100 μl of peroxidase substrate (ABTS substrate kit, Bio-Rad) was added to each well. After 10–20 min, color development was quantified by measuring absorbance at 414 nm on an automated ELISA reader (Titertek Multiscan; Flow Laboratories). All results were extrapolated through a standard curve and were expressed as nanograms of 4S protein/mg of total cell protein. Analysis of conditioned media from CHO-KI cells expressing D520N 4S, before and after inactivation at pH 7, showed that the immunoquantification assay can detect both active and inactive D520N 4S with equal efficiency.

**Analysis of GAG Storage in Cultured Fibroblasts—**Previous experiments in our laboratory have demonstrated a direct correlation between the amount of intracellular lysosomal 4S and 35S-labeled storage material in normal human MPS VI fibroblasts (21). Analysis of GAG storage in cultured feline skin fibroblasts was determined using the method described by Harper et al. (21), with the following modifications. Triplicate cultures of normal, D520N homozygous, D520N/L476P compound heterozygous, and L476P homozygous skin fibroblasts were grown to confluence in 25-cm2 flasks. Growth medium was Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) FCS. Twenty-four-hour conditioned medium (Ham’s F-12 supplemented with 10% (v/v) heat-inactivated FCS) was harvested from confluent, G418-resistant mass cultures of CHOF4S and CHOD520N cells, clarified by centrifugation (2000 × g for 5 min) and used immediately. The pH of CHOF4S and CHOD520N medium at 4 °C was sequentially reduced from pH 7.2 down to pH 4.4 with 1M glacial acetic acid. After 30, 70, and 195, and 360 min at 37 °C, a 10-μl aliquot was removed and assayed for arylsulfatase activity using 4MUS (18). After 360 min, the pH of CHOF4S pH 7.2 and CHOD520N pH 7.2 samples was reduced to pH 5.5 with 0.1 M glacial acetic acid. Samples were then incubated for another 16–18 h at 37 °C to determine whether the reduction in D520N 4S activity was reversible. After 2, 4, and 16 h, a 10-μl aliquot was removed and assayed for arylsulfatase activity using 4MUS.

**Mutant Normal**

![Table](image)

**Deterioration of L476P and D520N—**Cats were screened for the L476P and D520N alleles by restriction enzyme analysis of PCR products encoding the 4S cDNA, amplified from normal and normal first strand cDNA and directly sequenced. The G → A base substitution at nucleotide 1558 in the mutant sequence is indicated by an arrow.

CHOF4S and CHOD520N medium was clarified by centrifugation, assayed directly with 4MUS, and diluted in growth medium to a final 4S activity of 7 nmol/min/ml. Triplicate cultures of confluent L476P homozygous fibroblasts in 25-cm2 flasks were metabolically labeled with 15 μCi/ml Na235SO4, as described above. Cells were then harvested by treatment with trypsin-versene and transferred to new 25-cm2 flasks containing growth medium supplemented with 7 nmol/min/ml wild-type 4S activity or D520N 4S activity (derived from CHOF4S or CHOD520N conditioned medium, respectively) in the presence or absence of 5 μM M6P. After 48 h, cell lysates were prepared and assayed for total protein, 35S radioactivity, β-hexosaminidase, and 4S activity toward 4MUS.

**Immunoquantification of Wild-type 4S Activity in Neutral and Acidic pH Conditions—**Twenty-four-hour conditioned medium (Ham’s F-12 supplemented with 10% (v/v) heat-inactivated FCS) was harvested from confluent, G418-resistant mass cultures of CHOF4S and CHOD520N cells, clarified by centrifugation (2000 × g for 5 min) at 4 °C and used immediately. The pH of CHOF4S and CHOD520N medium at 4 °C was sequentially reduced from pH 7.2 down to pH 4.4 with 1M glacial acetic acid. At various pH levels, a 300-μl aliquot was removed and kept at 4 °C. Samples were then incubated at 37 °C for 360 min. After 30, 70, 195, and 360 min at 37 °C, a 10-μl aliquot was removed and assayed for arylsulfatase activity using 4MUS (18). After 360 min, the pH of CHOF4S pH 7.2 and CHOD520N pH 7.2 samples was reduced to pH 5.5 with 0.1 M glacial acetic acid. Samples were then incubated for another 16–18 h at 37 °C to determine whether the reduction in D520N 4S activity was reversible. After 2, 4, and 16 h, a 10-μl aliquot was removed and assayed for arylsulfatase activity using 4MUS.

**Densitometric Analysis—**Autoradiographs were scanned with a Molecular Dynamics personal densitometer and images analyzed with Molecular Dynamics ImageQuant software.

**RESULTS**

**Identification of D520N—**A colony cat (cat 237), with no detectable 4S activity and a normal L476 genotype, was used in the present study. PCR products encoding the 4S cDNA were amplified from cat 237 fibroblast first strand cDNA, sequenced directly and compared with the normal 4S cDNA (10). At nucleotide 1558 of the published sequence (20), a G → A base substitution was identified in the 4S sequence from cat 237, which resulted in a change at codon 520 from GAC (aspartic acid) to AAC (asparagine) (D520N, Fig. 1). No other changes were identified, suggesting that D520N is likely to be the cause of the reduced 4S activity in cat 237.

**Detection of L476P and D520N—**Cats were screened for the L476P and D520N alleles by restriction enzyme analysis of PCR products encoding the 4S cDNA, as described under “Experimental Procedures.”

**Fig. 2.** A representative family tree of the genotypes of five cats determined by restriction enzyme analysis with HaeIII and BstI. Identification of L476P and D520N with this PCR-based screening method has enabled cats within the colony to be divided into six groups based on their genotypes: D520N heterozygous, D520N homozygous, L476P heterozygous, L476P homozygous, L476P/L476P compound heterozygous, and L476P/L476P homozygous.
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**Fig. 2. Identification of cat genotypes by HaeIII and BslI restriction analysis.** A, pedigree chart of a family within the MPS VI cat colony. Females and males are represented by round and square symbols, respectively. D520N homozygous cats are represented as gray symbols. LA76P homozygous cats are represented as black symbols. Compound heterozygous carriers for D520N and L476P are represented as half-gray/half-black symbols. B, identification of D520N. PCR products encoding f4S cDNA nucleotides 1353–1649 were amplified from cat blood samples and digested with BslI to demonstrate the G → A base substitution that causes D520N. The G → A base substitution at position 1558 results in the destruction of a BslI restriction site. Two restriction fragments (203 and 93 bp) were generated from BslI digestion of L476P homozygous PCR products (lane 2). Heterozygous PCR products were partially digested with BslI (lanes 1, 3, and 5). D520N homozygous cats only displayed the 296-bp undigested PCR product following BslI restriction (lane 4). C, identification of L476P. PCR products were digested with HaeIII to demonstrate the T → C base substitution that causes L476P. HaeIII (lanes 1, 3, and 5). D520N homozygous PCR products only displayed the 245- and 51-bp fragments following HaeIII restriction (lane 4).

**Endocytosis of Wild-type 4S and D520N 4S by MPS VI Fibroblasts**—An uptake experiment was done in order to characterize the endocytosis and lysosomal targeting of the mutant D520N 4S enzyme secreted from CHO-K1 cells. LA76P homozygous MPS VI fibroblasts were used in this study because of their low levels of 4S activity. D520N 4S and wild-type 4S enzyme used in the uptake experiment were derived from CHOD520N and CHOF4S conditioned media and contained approximately 32.5 and 44.6 nmol/min/ml arylsulfatase activity, respectively. The CHOD520N and CHOF4S conditioned media were diluted in growth medium to a final arylsulfatase activity of 7 nmol/min/ml. MPS VI (LA76P homozygous) feline fibroblasts were metabolically labeled with Na$_{35}$SO$_4$, 24 h and then incubated with equal amounts of D520N 4S or wild-type 4S activity (7 nmol/min/ml) over 48 h in the presence or absence of 5 mM M6P.

The results show that MPS VI (LA76P homozygous) fibroblasts contained very low levels of arylsulfatase activity and in this experiment showed an 8-fold elevated level of $^{35}$S-labeled GAG storage when compared with normal fibroblasts (Table II). The level of intracellular $^{35}$S-labeled GAG storage material in MPS VI fibroblasts was reduced to below normal levels following endocytosis of either wild-type 4S or D520N 4S (Table II). These results suggest that the mutant D520N 4S enzyme can correct the $^{35}$S-GAG storage phenotype in MPS VI fibroblasts. Although equal amounts of D520N 4S activity and wild-type 4S activity were placed on the MPS VI fibroblasts, the amount of intracellular D520N 4S activity resulting from endocytosis appeared to be significantly lower for the D520N enzyme in comparison to wild-type 4S. The activity of the lysosomal enzyme β-hexosaminidase was unaffected by the endocytosis of D520N 4S or wild-type 4S.

The presence of 5 mM M6P significantly reduced the level of endocytosed D520N 4S and wild-type 4S enzyme activity, suggesting that both enzymes are predominantly endocytosed via the M6P receptor pathway (Table II). However, even in the presence of 5 mM M6P, there is a small amount of enzyme uptake that results in arylsulfatase activity of approximately 15% of normal, which is sufficient to efficiently clear the $^{35}$S-labeled storage product in these cells (Table II). In all of the MPS VI cells exposed to enzyme, storage is reduced to levels below that seen in the normal control. We believe this represents intrinsic differences in the cell lines used rather than a significant phenomenon.

The mannose 6-phosphorylation of mutant D520N 4S was confirmed in a $^{32}$P-labeling experiment. G418-resistant mass cultures of CHOF4S and CHOD520N were metabolically labeled with Na$_{32}$PO$_4$, immunoprecipitated, and analyzed via SDS-PAGE and autoradiography. The results suggested that the D520N mutation does not measurably affect the specific phosphorylation of intracellular or secreted 4S protein (results not shown).

**Subcellular Localization of D520N 4S—** Microsomal, endosomal, and lysosomal fractions were isolated from EXPRE$^{35}$S-labeled CHO-K1 cells overexpressing D520N 4S and wild-type 4S by Percoll gradient fractionation. The amounts of total protein across each gradient were similar (data not shown). The data in Fig. 3 (A and B) demonstrates the enrichment of endosomes and lysosomes at the middle and bottom of each Percoll gradient, respectively. The top of each Percoll gradient (fractions 1–3) contained a small amount of β-hexosaminidase and a significant amount of acid phosphatase activity consistent with some lysis of organelles prior to their separation (Fig. 3, A and B).
TABLE I

Analysis of 35S-labeled GAG storage and 4S activity in skin fibroblasts

Normal, D520N homozygous, D520N/L476P compound heterozygous, and L476P homozygous fibroblasts were isolated and cultured from skin biopsies. D520N homozygous cats have no clinical manifestations of MPS VI. D520N/L476P compound heterozygous cats display varying degrees of degenerative joint disease (11). L476P homozygous cats have a clinically severe form of MPS VI, which has previously been characterized (5). One skin biopsy was analyzed from a normal cat. For each MPS VI genotype, skin biopsies were obtained from three individual cats. Triplicate cultures of each primary fibroblast line were metabolically labeled with Na235SO4 for 24 h. Cells were then washed, incubated with growth medium for another 48 h, harvested, and assayed for total cell protein, 35S radioactivity (cpm/mg total cell protein), and 4S activity using the trisaccharide fluorogenic substrate, 4MUS. Results are expressed as the mean ± standard error. WT = wild type.

| Allele 1 | Allele 2 | Clinical phenotype | Skin biopsies analyzed | 4S activity | 35S Radioactivity |
|----------|----------|--------------------|------------------------|-------------|------------------|
| WT       | WT       | Normal             | 1                      | 99.00 ± 1.3 (n = 3) | 1038 ± 85 (n = 3) |
| D520N    | D520N    | Normal             | 3                      | 4.60 ± 0.9 (n = 8)  | 2198 ± 136 (n = 8) |
| D520N    | L476P    | Mild               | 3                      | 3.10 ± 1.3 (n = 9)  | 3126 ± 398 (n = 9) |
| L476P    | L476P    | Severe             | 3                      | 0.46 ± 0.29 (n = 9) | 11462 ± 780 (n = 9) |

The intracellular trafficking of newly synthesized (45-min pulse label and 4-h chase) 35S-labeled D520N 4S and 35S-labeled wild-type 4S through the microsomal, endosomal, and lysosomal compartments appeared to be similar. However, approximately 60% of the newly synthesized lysosomal D520N 4S protein was detected as a 64-kDa precursor polypeptide, whereas the majority (90%) of lysosomal wild-type 4S from CHO-KI cells was detected as a 44-kDa mature form (Fig. 4). These observations suggest that the rate of maturation of D520N 4S in the lysosome was significantly reduced in comparison to wild-type 4S. The small amount of organelle lysis observed in this experiment may explain the presence of mature 4S in the microsomal fractions. Both 35S-labeled D520N 4S and 35S-labeled wild-type 4S precursor protein were secreted into the culture medium in approximately equal amounts (Fig. 4). The 46-kDa polypeptide in the endosomal fraction is occasionally observed in analyses of 4S processing; however, its significance is unclear.2

Table III illustrates the enrichment of arylsulfatase activity in the lysosomal compartment of CHO-KI cells expressing wild-type 4S. As would be expected, the majority of the activity (203 nmol/min/mg, approximately three quarters) is found in the lysosomal compartment. In comparison, expression of D520N 4S resulted in a substantially reduced level of arylsulfatase activity in the lysosomal compartment that was equivalent to 17% (35 nmol/min/mg) of the level in cells expressing wild-type 4S. Lesser reductions in the relative levels of arylsulfatase activity were seen in the microsomal (33% of wild-type) and endosomal (52% of wild-type) compartments of CHO-KI cells expressing D520N 4S (Table III).

The human 4S monoclonal antibodies F58.3 and F22.1, which have previously been shown to cross-react with f4S (20), did not cross-react with D520N 4S in a sandwich ELISA. A sandwich ELISA with a murine and rabbit polyclonal antibody raised against f4S was therefore used to determine 4S protein levels in CHOD520N and CHO4F4S microsomal, endosomal, and lysosomal fractions. Immunquantification of 4S protein levels demonstrated a rapid degradation of D520N 4S mutant protein during synthesis in the endoplasmic reticulum and transport to the endosome and lysosome. 4S protein levels in CHOD520N microsomal, endosomal, and lysosomal enriched fractions were 38%, 15%, and 0.76% of wild-type 4S protein levels, respectively (Table III). Therefore, while there is almost complete degradation of D520N 4S in the lysosome, there is a corresponding 22.5-fold increase in specific activity that results in a much less severe reduction in the level of D520N 4S activity in the lysosomal compartment. (Table III). A smaller increase in D520N 4S specific activity was also evident in the endosomal compartment (3.6-fold increase when compared with endosomal wild-type 4S specific activity, Table III). The specific activity of D520N 4S in the microsomal compartment (58 nmol/min/µg) was partially reduced when compared with the specific activity of microsomal wild-type 4S (66 nmol/min/µg) (Table III).

The stabilization of Mature D520N 4S—To test the effect of the D520N missense mutation on 4S stability, G418-resistant mass cultures of CHO4F4S and CHOD520N were metabolically pulse-labeled with EXPRE35S-35S protein labeling mix. Cells were then chased for varying lengths of time in the presence of 5 mM M6P (to prevent re-uptake of 35S-labeled secreted precursor 4S). Immunoprecipitation showed that the intracellular and secreted forms of 35S-labeled wild-type 4S are stable over a 2-day chase period (Fig. 5, A and B). The stability of 35S-labeled precursor and mature D520N 4S secreted into the medium was not affected over a 2-day chase period (Fig. 5B). In contrast, the mature form of intracellular 35S-labeled D520N 4S protein was degraded within 4–10 h (Fig. 5A).

2. D. Brooks, personal communication.
pected, the lysosomal enriched fraction isolated from CHOf4S cells contained a large amount of mature wild-type 4S protein. In contrast, no mature D520N 4S was detected in the lysosomal enriched fraction isolated from CHOD520N cells, confirming the instability of mature D520N 4S in the lysosome (Fig. 6).

**Inactivation of D520N 4S in Neutral pH Conditions**—Conditioned medium from CHOD520N cells was incubated at 37 °C in various pH conditions. D520N 4S was rapidly inactivated with time upon incubation at 37 °C, pH 7.2. The rate of D520N 4S inactivation was reduced with increasingly acidic conditions (Fig. 7). ELISA demonstrated no difference in D520N 4S protein levels in CHOD520N conditioned medium samples following a 24-h incubation at pH 7.2 or pH 5.9. These observations suggest that the D520N mutation rapidly inactivates 4S secreted from CHO-KI cells in neutral pH conditions but does not result in the degradation of the secreted enzyme. The inactivation of D520N 4S in neutral pH conditions was not reversible. Following complete D520N 4S inactivation in neutral pH conditioned medium, no mature D520N 4S was detected in the lysosomal enriched fraction isolated from CHOD520N cells, confirming the instability of mature D520N 4S in the lysosome (Fig. 6).
growth medium for 4, 10, 24, and 48 h in the presence of 5 mM M6P to

Thirty microliters of each endosomal (\textit{A}) and growth medium (\textit{B}) D520N 4S and wild-type 4S in CHOD520N and CHO4S cells. CHOD520N and CHO4S cells were grown to confluence in 25-cm² tissue-culture flasks and metabolically labeled with EXPRET²S²S labeling mix for 1 h. The labeling medium was then removed, and cells were chased in growth medium for 4, 10, 24, and 48 h in the presence of 5 mM M6P to prevent uptake of ³⁵S-labeled secreted precursor 4S. At each time point, labeled cells (\textit{A}) and growth medium (\textit{B}) were harvested, immunoprecipitated, and analyzed via SDS-PAGE and autoradiography. The molecular sizes are indicated with arrows and were calculated by comparison with C-14 methylated standards (200, 97, 69, 46, 30, and 14 kDa; Amersham Pharmacia Biotech) run alongside the samples (data not shown).

FIG. 5. Stability of intracellular (\textit{A}) and secreted (\textit{B}) D520N 4S and wild-type 4S in CHOD520N and CHO4S cells. CHOD520N and CHO4S cells were grown to confluence in 25-cm² tissue-culture flasks and metabolically labeled with EXPRET²S²S labeling mix for 1 h. The labeling medium was then removed, and cells were chased in growth medium for 4, 10, 24, and 48 h in the presence of 5 mM M6P to prevent uptake of ³⁵S-labeled secreted precursor 4S. At each time point, labeled cells (\textit{A}) and growth medium (\textit{B}) were harvested, immunoprecipitated, and analyzed via SDS-PAGE and autoradiography. The molecular sizes are indicated with arrows and were calculated by comparison with C-14 methylated standards (200, 97, 69, 46, 30, and 14 kDa; Amersham Pharmacia Biotech) run alongside the samples (data not shown).

FIG. 6. Western blot analysis of endosomal and lysosomal D520N 4S and wild-type 4S. Thirty microliters of each endosomal (\textit{E}) and lysosomal (\textit{L}) fraction isolated from CHOD520N and CHO4S cells was separated on an SDS-PAGE gel, transferred onto nitrocellulose, and subjected to Western blot analysis. The primary antibody was a rabbit anti-4S polyclonal antibody. The secondary antibody was a sheep anti-rabbit peroxidase-conjugated antibody. The molecular sizes are indicated with arrows and were calculated by comparison with prestained SDS-PAGE standards (208, 115, 79.5, 49.5, 35, 28, 20.4, and 7 kDa; Bio-Rad).

analysis of these cats suggests that the entire range of MPS VI phenotypes from severe to mild are clustered within a narrow range of residual 4S activity in cultured skin fibroblasts from 0.5% to 4.6% of normal levels. L476P homozygous cats, which display a clinically severe MPS VI phenotype, contain very low levels of 4S activity in cultured skin fibroblasts (Table I), excrete substantially elevated levels of DS in their urine, show extensive lysosomal vacuolation in a large number of tissues including chondrocytes, and display severe degenerative joint disease (5, 8). Despite the presence of residual 4S activity (approximately 3.1% of normal 4S activity levels in cultured skin fibroblasts, Table I), adult D520N/L476P compound heterozygous cats contain marginally elevated levels of DS in their urine and increased lysosomal vacuolation in most chondrocytes, which is associated with variable degrees of degenerative joint disease. Lysosomal vacuolation was not observed in any other tissues (11). D520N homozygous cats, which contain higher levels of residual 4S activity (approximately 4.6% of normal 4S activity levels in cultured skin fibroblasts, Table I), also contain marginally elevated levels of urinary DS and lysosomal vacuolation in some chondrocytes. The small increase in residual 4S activity (from 3.1% to 4.6% of normal levels) appears to be sufficient to prevent the occurrence of degenerative joint disease in D520N homozygous cats (11).

Although D520N homozygous and D520N/L476P compound heterozygous cats contain low 4S activity, their normal growth and outward appearance (11) suggest that this residual 4S activity is sufficient to metabolize the majority of natural substrate in vivo. This is encouraging for gene and enzyme replacement therapy protocols for severely affected MPS patients, for whom only a small amount of recombinant enzyme may be required to prevent the majority of somatic symptoms.

Characterization of D520N/L476P compound heterozygous cats was complicated by the presence of two mutant 4S alleles, which together cause a mild biochemical and clinical MPS VI phenotype. To determine the effect of each mutation on 4S synthesis, subcellular distribution, and specific activity, both mutant alleles were engineered into the wild-type 4S cDNA and expressed. Expression of L476P 4S in CHO-KI cells has previously shown that this mutant of 4S is synthesized as an inactive precursor polypeptide at reduced levels and is not cleaved to the mature form (10). In this study, the D520N 4S mutant was overexpressed in CHO-KI cells to permit detailed molecular characterization.

Recently, the crystal structure of human 4S has been determined (24). As the human and feline forms of 4S are 91%
identical at the amino acid level, the tertiary structure of f4S is likely to be similar to that of human 4S. Asp-518 (Asp-520 of the f4S sequence) is in close proximity to the postulated phosphotransferase binding domain of 4S (24). From this observation, we initially speculated that the D520N mutation may cause a functional disruption of the phosphotransferase binding domain, leading to reduced mannose 6-phosphorylation of 4S in the cis-Golgi, and thereby disrupt lysosomal targeting. However, uptake studies of D520N 4S and wild-type 4S (secreted from CHO-KI cells) into L476P homozygous fibroblasts demonstrated that both enzymes were efficiently endocytosed via the M6P receptor pathway (Table II). Metabolic labeling with Na232P3O confirmed that the specific phosphorylation of precursor D520N 4S is indistinguishable from wild-type 4S. Furthermore, immunoprecipitation of 35S-labeled CHOD520N organelles fractionated on Percoll gradients suggested that the D520N mutation has no significant effect on the lysosomal targeting of 4S (Fig. 4). Collectively, these results suggest that the mannose 6-phosphorylation and lysosomal targeting of 4S protein is not altered by the D520N mutation.

Two monoclonal antibodies (F58.3 and F22.1) raised against human 4S, and shown previously to cross-react with f4S (20), did not cross-react with D520N 4S in a sandwich ELISA. This suggests that the epitopes on f4S that are recognized by these antibodies are altered by the D520N amino acid substitution. Therefore, a sandwich ELISA was developed using a murine polyclonal r4S antibody and a rabbit polyclonal r4S antibody and used to immunoquantitate 4S protein levels in CHOD520N and CHOF4S cells. The lowest amount of mutant protein (0.76% of normal levels) was detected in the lysosomal compartment of CHOD520N cells (Table III). When compared with wild-type 4S, the specific activity of D520N 4S was elevated by 22.5-fold in the lysosomal compartment of CHO-KI cells (Table III). These observations suggest that the D520N 4S undergoes rapid degradation in the lysosome, but the consequences of this are partially ameliorated by the increased specific activity of the small amount 4S mutant protein in the lysosome. The instability of D520N 4S and the low level of expression prevented accurate immunoquantification of 4S protein levels in skin fibroblasts from D520N homozygous and D520N/L476P compound heterozygous cats.

When compared with wild-type 4S, the specific activity of D520N 4S was elevated by 3.6-fold and 22.5-fold, respectively, in the increasingly acidified endosomal and lysosomal compartments of CHO-KI cells (Table III). These observations suggested that the specific activity of D520N 4S may be pH-sensitive. D520N 4S secreted from CHO-KI cells was rapidly inactivated at pH 7.2. With increasingly acidic conditions, secreted D520N 4S became less susceptible to inactivation (Fig. 7). These results provide further evidence for the sensitivity of D520N 4S to pH. This may result in a loss of specific activity in the pH neutral endoplasmic reticulum and Golgi compartments. However, only a small reduction in the specific activity of microsomal D520N 4S (58 nmol/min/μg) was observed, when compared with the specific activity of microsomal wild-type 4S (66 nmol/min/μg) (Table III). The extent of decreased specific activity would depend on the time for which D520N 4S resides in these pH neutral compartments.

The increased specific activity of lysosomal D520N 4S may be a result of the effect of acidic pH on the enzyme. In addition, the increased specific activity of lysosomal D520N 4S may be associated with the mature form of the mutant molecule. Metabolic labeling with EXPRE35S-35S protein labeling mix demonstrated that D520N 4S is targeted to the lysosomal compartment of CHO-KI cells and cleaved to a mature enzyme, which is rapidly degraded (Figs. 4 and 5). The 22.5-fold increase in D520N 4S specific activity may be associated with the highly unstable mature form of the mutant enzyme in the hydrolytic environment of the lysosomal compartment. This would be consistent with the observation that secreted D520N 4S enzyme, which is predominantly precursor form, has a normal specific activity at acidic pH (Fig. 7).

The studies of D520N 4S subcellular distribution in CHO-KI cells (Table III, Fig. 3) clearly demonstrate why D520N homozygous and D520N/L476P compound heterozygous cats develop a mild MPS VI phenotype despite the relatively high levels of 4S activity measured in fibroblast lysates (4.6% and 3.1% of normal levels, respectively; Table I). It is evident that only a small proportion of this activity, approximately 10%, will be in the correct cellular compartment; i.e. the lysosome. Therefore, the effective levels of 4S activity in D520N homozygous and D520N/L476P compound heterozygous animals will be approximately 0.5% and 0.3%, respectively.

A C-terminal extension mutation in the human 4S gene which is associated with an increased catalytic efficiency has previously been reported in a juvenile MPS VI patient (25). The mutation of a stop codon to a glutamate codon extended the open reading frame of the human 4S gene by 50 codons (*534Q). When overexpressed in LTK- cells, the majority of mutant *534Q enzyme was rapidly degraded in the trans-Golgi network. The small amount of *534Q mutant enzyme that was sorted to lysosomes was associated with an 8.6-fold increase in catalytic efficiency (25). Characterization of the *534Q mutation (25) and the D520N mutation in this study suggest that alterations of the C-terminal region of 4S can result in a higher susceptibility to proteolytic degradation but can also cause a significant increase in the specific activity of the molecule. These observations raise the possibility of engineering a derivative of 4S with an elevated specific activity, which could be used in gene and enzyme replacement therapies. However, to be effective, such a derivative would need to be resistant to proteolytic degradation.

In summary, we have identified a novel mutation, D520N, that causes a mild MPS VI phenotype in D520N/L476P compound heterozygous cats. Biochemical analysis of cultured skin fibroblasts from D520N/L476P compound heterozygous and D520N homozygous cats suggest that only small amounts of residual lysosomal 4S activity (probably less than 1% of normal lysosomal levels) are required to prevent the majority of somatic symptoms associated with severe feline MPS VI. However, significant chondrocyte vacuolization and degenerative joint disease were observed in D520N/L476P compound heterozygous cats (11). These observations suggest that the residual 4S enzyme activity present in these cats, while significant, is not sufficient to completely turn over the high levels of GAG present in the extracellular matrix of cartilage. Successful correction of chondrocytes by therapy strategies faces two problems: their higher requirement for 4S and the inaccessibility of these cells to circulating recombinant enzyme, as evident from enzyme replacement therapy studies (6). The mild feline MPS VI phenotype identified in this study will be useful for the evaluation of therapies directed to correction of lysosomal storage in chondrocytes.

The D520N mutation was engineered into the wild-type f4S cDNA. When expressed in CHO-KI cells, the secreted form of D520N 4S was susceptible to inactivation in neutral pH conditions. With increasingly acidic conditions, the mutant D520N 4S enzyme became more resistant to inactivation. The intracellular D520N 4S protein was rapidly degraded, with less than 1% of normal 4S protein levels reaching the acidified lysosomal compartment of CHO-KI cells. The small amount of D520N 4S that was sorted to the lysosomal compartment was associated
with a 22.5-fold increase in specific activity. The lack of severe MPS VI clinical symptoms in D520N homozygous and D520N/L476P compound heterozygous cats is therefore a result of a significant increase in the specific activity of the D520N 4S mutant enzyme reaching the lysosomal compartment. The subsequent residual levels of 4S activity in the lysosomes of these affected cats appear to be sufficient to metabolize the majority of GAG in vivo and thereby prevent severe MPS VI disease progression.

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