Expression and Characterization of Wild Type and Mutant Recombinant Human Sulfamidase

IMPLICATIONS FOR SANFILIPPO (MUCOPOLYSACCHARIDOSIS IIIA) SYNDROME*

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Kelly J. Perkins, Sharon Byers, Gouri Yogalingam, Birgit Weber, and John J. Hopwood†

From the Lysosomal Disorders Research Unit, Department of Chemical Pathology, Women's and Children's Hospital,
72 King William Rd., North Adelaide, South Australia 5006, Australia

Mucopolysaccharidosis IIIA (MPS-IIIA) is an autosomal recessive lysosomal storage disorder caused by the deficiency of sulfamidase (NS; EC 3.10.1.1), resulting in defective degradation and storage of heparan sulfate. This paper reports the production and characterization of monoclonal and polyclonal antibodies against recombinant human sulfamidase (rhNS) to quantitate and characterize normal and mutant sulfamidase produced from the wild type NS expression vector. Glycosylation and phosphorylation studies of immunoprecipitated rhNS show that all five potential glycosylation sites are utilized, with three high mannose/hybrid oligosaccharides and two simpler chains, with at least one functional mannose 6-phosphate group. An NS quantification system was developed to determine the effect of the three most common and severe patient mutations: S66W (Italy), R74C (Poland), and R245H (The Netherlands). The quantity and specific activity of expressed mutant rhNS was significantly lower than expressed normal rhNS, with 0.3, 0.2, and 0.05% of normal rhNS produced and 15, 17, and 83% of normal specific activity for S66W, R74C, and R245H observed, respectively. The recent structural elucidation of N-acetylgalactosamine-4-sulfatase was utilized to postulate the effect on the structure-function relationship of NS. The characterization of normal and mutated rhNS has relevance for efficient diagnosis and therapeutic developments for MPS-IIIA patients.

Sulfamidase (NS)† is a lysosomal enzyme involved in the lysosomal degradation of heparan sulfate (HS). The autosomal recessive disorder known as mucopolysaccharidosis type IIIA (MPS-IIIA) is caused by a deficiency of sulfamidase, leading to patients developing a clinical phenotype of Sanfilippo syndrome. This disorder is one of four MPS-III subtypes (A, B, C, and D) that are a subset of a group of disorders known as the mucopolysaccharidoses, caused by a deficiency in one of 10 exoenzymes necessary for the lysosomal degradation of keratan, heparan, and dermatan sulfates. The incidence of the combined MPS-III subtypes has been estimated at 1 in 24,000 births (1) and 1 in 66,000 in Australia (2), with MPS-IIIA the most common subtype in Northern Europe and Australia. Unlike other sulfatases, which catalyze the hydrolysis of O-linked sulfate groups, NS catalyzes the hydrolysis of the N-linked sulfate group from the nonreducing terminal glucosamine residues of HS (the only N-sulfated glycosaminoglycan). Two other enzymes are involved exclusively in the degradation of HS, with functional deficiencies of these causing two other MPS-III subtypes: MPS-IIIB (α-N-acetylgalactosaminidase deficiency) and MPS-IIIC (acyl-CoA-glucosaminide N-acetyltransferase deficiency). The last MPS-III subtype, MPS-IIID, is defined by a deficiency of N-acetylgalactosaminide-6-sulfatase, which degrades both HS and keratan sulfate. In patients with this disorder, HS is the only glycosaminoglycan stored, since an alternative pathway exists for the degradation of keratan sulfate (3).

Since a common enzymatic link between the MPS-III subtypes is present, a common biochemical disease phenotype (elevated levels of excreted HS in urine and HS storage) and clinical presentation results. This is normally characterized by severe and progressive mental deterioration (4). The similarity in presentation means that diagnostic analysis of the combined MPS-III subtypes depends on enzymatic analysis, which is further complicated by minimal skeletal involvement and false negative tests, by some methods, for heparan sulfaturia, leading to a delayed diagnosis (4). Symptoms that typify MPS-IIIA syndrome may begin after a short period of apparently normal development. Subsequent patterns of neurodegeneration develop that include rapid loss of social skills with aggressive behavior and hyperactivity, disturbed sleep patterns, hirsutism, coarse facies, and diarrhea. In profoundly affected patients, delayed speech development is often noticed at 2 years of age. As in other MPS conditions, considerable variation in the onset and severity of the clinical phenotype are observed.

NS has been purified from a number of human tissues as a 56-kDa polypeptide (5). The predicted molecular mass of NS is 54,679 Da, and the sequence contains five potential N-glycosylation sites at positions 41, 142, 151, 264, and 413, with the first known to be functional through N-terminal amino acid sequencing (6). The recombinant human precursor (62-kDa) and mature (56-kDa) forms expressed in Chinese hamster ovary (CHO) cells have molecular masses within this range (7). Although the glycosylation and phosphorylation properties of NS have not been previously investigated, uptake and correction studies of rhNS on MPS-IIIA fibroblasts (7) have indicated that this process is dependent on the presence of phosphorylated mannose residues.

Both the genomic and cDNA sequences encoding NS have been cloned and characterized (6, 8), with the gene localized to...
Expression and Characterization of Sulfamidase Mutations

17q25.3 (6). Detailed mutational analysis of the NS gene (9–12) has identified a large number of genetic defects present in MPS-IIIA patients, with considerable genotypic heterogeneity. However, certain mutations show a definite prevalence in different geographical regions. For example, R245H accounts for 56% of mutant alleles in MPS-IIIA patients in The Netherlands (13), with S66W showing prevalence in Italy with 33% of alleles (11); R74C alone accounts for more than 50% of alleles in MPS-IIIA patients in Poland (10); and delC1091 accounts for almost half of the mutations in Spanish MPS-IIIA patients (12). Despite an increased effort to identify mutations in the NS gene, interpretation of clinical phenotype remains difficult, with identical genotypes often manifesting within a broad clinical spectrum (11). These previous studies have relied on clinical analysis of patient data in order to determine the effect of identified mutations on the activity of the wild type enzyme. Thus far, no studies have directly investigated the effect of MPS-IIIA mutations through direct expression of mutated rhNS. In this study, we have been able to provide a more detailed study of the maturation process of rhNS through the isolation of monoclonal and polyclonal antibodies. Further, the introduction of the three most common clinically severe mutations, S66W, R74C, and R245H, into the wild type rhNS expression vector has been used to determine their effect on the synthesis of NS, specific activity, and presence of carbohydrate moieties.

This study aims to provide a more complete understanding of the previously described properties of rhNS (7) in order to understand the effect of severe mutations present in MPS-IIIA patients. Structural data, together with the functional data obtained from the mutational analysis, will contribute greatly toward understanding of the enzymatic mechanism of the sulfatase family in general and the specificities of sulfamidase. The effect of mutations on rhNS will have implications for patient diagnosis and prognosis and provide a valuable insight for the eventual development of a therapy.

EXPERIMENTAL PROCEDURES

Monoclonal Antibody Preparation and Characterization—Preparations of rhNS from a high expressing CHO system (7) were column-purified and used to raise monoclonal and polyclonal antibodies. A standard immunization and fusion protocol was used to generate monoclonal antibodies (14), with products screened for antibody activity against purified rhNS through enzyme-linked immunosorbent assay with a peroxidase-labeled sheep anti-mouse Ig antibody detection system (Bio-Rad). Briefly, 1 μg of rhNS was added to 10 mM PBS, pH 7, in a total volume of 150 μl, added to individual wells of a polyclonal antibody serum (Costar, Cambridge, MA), and incubated at 37°C for 1 h. Each well was aspirated, and the remaining reactive sites were blocked by adding buffer A (150 mM PBS, 5% (w/v) bovine serum albumin) for an additional 1 h at 37°C. The wells were then aspirated, washed three times with wash buffer (10 mM PBS, pH 7), and incubated with 150 μl of the NS sample to be quantified. After a 1-h incubation at 37°C, the wells were washed, incubated with one of a panel of murine monoclonal antibodies, and aspirated, washed three times with wash buffer (10 mM PBS, pH 7), and incubated with 150 μl of the antiserum for 1 h at room temperature with blocking solution containing 15% (v/v) bovine immunoglobulin antibody (Silenus Laboratories). The membrane was washed with Tris-buffered saline (20 mM Tris/HCl, pH 7, 0.25 mM NaCl) and developed in 20 ml of 4-chloro-1-napthol color development reagent as described by the manufacturer (Bio-Rad) or, alternatively, using an ECL development kit (Amersham Pharmacia Biotech) using the supplied protocol.

Immunquantification of Wild Type and Mutant Sulfamidase—A sandwich ELISA was developed to quantitate wild type and mutant NS levels in CHO cell constructs and cultured skin fibroblasts. Ten μg of total IgG isolated from rabbit rhNS serum was added to 10 mM PBS, pH 7, in a total volume of 150 μl and added to individual wells of a polyclonal antibody serum (Costar, Cambridge, MA) and incubated at 37°C for 1 h. Each well was aspirated, and the remaining reactive sites were blocked by adding buffer A (150 mM of 10 mM PBS, pH 7, 1% (w/v) bovine serum albumin) for an additional 1 h at 37°C. The wells were then aspirated, washed three times with wash buffer (10 mM PBS, pH 7), and incubated with 150 μl of the NS sample to be quantified. After a 1-h incubation at 37°C, the wells were washed, incubated with one of a panel of murine rhNS monoclonal antibodies at a concentration of 1 μg/well diluted in buffer A and incubated overnight with an affinity-purified horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin second antibody (1:1000 dilution (v/v) in buffer A) (Silenus Laboratories) for 1 h at 37°C. The antibody was removed, and the wells were washed three times with 150 μl of wash buffer. This was aspirated, and 100 μl of peroxidase substrate (ABTS substrate kit; Bio-Rad) was added to each well. After 5–10 min, color development was quantified by measuring absorbance at 414 nm on an automated enzyme-linked immunosorbent assay ELISA reader (TiterTek Multiscan; Flow Laboratories). All results were extrapolated through a standard curve and were expressed as ng of NS/mg of cell protein (Fig. 1).

Construction of the Mutant Sulfamidase Expression Vectors—Sulfamidase mutants S66W, R74C, and R245H were introduced using single-stranded oligonucleotide-mediated mutagenesis (17), into the expression vector pcDNA3 (Invitrogen) containing the full-length sulfamidase cDNA (pcDNA3s2; previously identified (6) as pcDNA3SFL8). The antisense oligonucleotides 5′-GCCGTTGCTGTGAGGCCAAGGTT-3′ (66W) and 5′-GAAGGTCTCGCGACACGTGGGAAG-3′ (R245H) were used to construct the appropriate vector. Positive clones were identified by autoradiography. The missense mutation R245H was introduced into the wild type vector using a polymerase chain reaction mutation genesis approach (QuickChange™ site-directed mutagenesis kit; Stratagen) with the mutant forward (5′-CACCACCTGGGCCACCTATGGACCAAGG-3′) and reverse (5′-CTTTTGCCATGTGGCACAAGTGGCAGTTGGAAGG-3′) CDNA sequence. All mutants were confirmed through automated polymerase chain reaction sequencing using methods outlined in the Taq Dye Deoxy™ Terminator Cycle FS Sequencing Kit (Applied Biosystems). Large scale plasmid preparations of mutant and wild type plasmids were column-purified using a commercially available kit (Qiagen), using the instructions provided.

Electroporation of CHO.K1 Cells—Large scale plasmid preparations of pcDNA3s2 (wild type), pcDNA3s2.66W, pcDNA3s2.74C, and pcDNA3s2.245H (25 μg) were electrooporated into CHO cells as described previously (18). Transfected cells were selected with 0.5 mg/ml

Polypeptide injection. The polyclonal antisera had a titer of 1:1,500,000 as shown by ELISA, as described below. An IgG fraction was prepared by precipitation with octanoic acid and concentrated to 12 μg/ml. Through immunoblotting and immunoprecipitation experiments, the rhNS polyclonal was shown to recognize both mature and precur-
Expression and Characterization of Sulfamidase Mutations

37195

Geneticin (G418) and maintained in Ham’s F-12 medium supplemented with 10% (v/v) FCS (CSL Ltd.).

Determination of Sulfamidase Expression—G418-resistant mass cultures of CHO-K1 cells expressing wild type rhNS (pCDNA3.n2), S66W-ns, R74C-ns, and R245H-ns were grown to confluency in two 75-cm² flasks. The sulfamidase activity of the normal and mutant expression constructs was measured in mass cultures of G418-resistant cells. The three cell lines were subcultured and grown synchronously before assay when the cells had reached a quiescent stage of growth, 4 days post-confluence. The expression of the mutant cell lines was calculated as a percentage of normal in three separate experiments using the tritiated tetrascarhide substrate (19) and immunquantification of sulfamidase (in pg/mg or ng/mg of rhNS/cell protein).

Pulse-Chase Labeling—G418-resistant mass cultures of CHO cell lines expressing normal, S66W, and R74C sulfamidase were grown to confluency in 25-cm² tissue culture flasks. The cells were preincubated for 1 h in 2 ml of Eagle’s modified minimal essential media supplemented with 10% (v/v) (w/v) FCS, and then labeled with 0.020 mCi/ml EXPRE35S protein labeling mix for 30 min. Labeling medium was removed, and cells were rinsed in serum-free medium and then chased in 3 ml of Ham’s F-12 medium supplemented with 10% (v/v) FCS. After 0, 0.5, 2, 4, 8, and 24 h, the chased medium was collected and clarified by centrifugation (1000 g, 5 min, 4 °C). At each time point, cell lysates were harvested with trypsin-verseine (Life Technologies, Inc.), washed, and resuspended in 1 ml of solubilization buffer (PBS containing 1% (v/v) sodium deoxycholate, 0.1% (w/v) SDS, 0.5% (v/v) Nonidet P-40) and used for immunoprecipitation. Overnight labeling of CHO cells for the purpose of glycosylation studies was performed as above, except 0.020 mCi/ml EXPRE35S was used for labeling purposes.

Immunoprecipitation—Growth media and solubilized cell lysates were immunoprecipitated using the rabbit polyclonal raised against rhNS (1:1, 500,000 titer) and Pansorbin cells (Calbiochem; 50 μl) and analyzed via SDS-polyacrylamide gel electrophoresis and autoradiography as described previously (20).

Metabolic Labeling and Purification of [35S]Sulfamidase—Cells were grown to confluency in a T25 tissue culture flask, washed three times with PBS, and then starved for 1 h in Dulbecco’s modified Eagle’s medium without FCS. These cells were labeled with 2 mCi of labeled phosphate (135P)orthophosphate (>3000 Ci/mmol) in dilute hydrochloric acid, pH 2–3, Amersham Pharmacia Biotech) in 3 ml of Dulbecco’s modified Eagle’s medium without PO4 and with 10% (v/v) FCS for 1 h at 37 °C. The medium used in the labeling phase was removed and replaced with chase medium (F-12 plus 10% (v/v) FCS) for 4 h. The medium was collected, and the cells were harvested with 10% (w/v) trypsin. The cell pellet was resuspended in 1 ml of solubilization buffer (PBS containing 1% (v/v) sodium deoxycholate, 0.1% (w/v) SDS, 0.5% (v/v) Nonidet P-40) and used for immunoprecipitation and glycosylation studies.

Glycosidase Digestion Using Endoglucosaminidase H (Endo H) and Peptide:N-Glycosidase F (PNGase F)—Pansorbin pellets of immunoprecipitated proteins were resuspended and boiled for 5 min in 40 μl of 50 mM Tris/HC1, pH 6.8, containing 1% (w/v) SDS and 5% (v/v) β-mercaptoethanol. After centrifugation, 20 μl was used in both Endo H and PNGase F (New England Biolabs) digestions. The reaction mixture consisted of 0.5% (w/v) SDS, 10% β-mercaptoethanol, 50 mM sodium citrate (with 1% (v/v) Nonidet P-40 for PNGase F) with 1000 units of Endo H or PNGase F. Incubations were overnight at 37 °C.

RESULTS

Characterization of rhNS Monoclonal and Polyclonal Antibodies—Eleven clones containing antibodies against column-purified rhNS were detected by ELISA, selected, and isotyped. Western blot analysis of the characterized panel of monoclonal antibodies indicated that none bound denatured rhNS. However, all of the monoclonal antibodies recognize structural epitopes, since they bind seminative sulfamidase in ELISA, which renders them useful in immunoprecipitation experiments and, in the case of the IgG2a 43bNS-23.B2, to couple to a matrix in an affinity column to improve purification yields of rhNS. ELISA-based experiments, in combination with the polyclonal antibody raised against rhNS, were utilized to determine an antibody that could be used to quantify rhNS. The three most suitable candidates, rhNS-13.C1, rhNS-22.D5, and rhNS-23.B2 (an IgG1, a column-purified IgM, and an IgG2a, respectively), were identified using quantification studies through a developed sandwich ELISA protocol with the polyclonal antibody raised against rhNS. The rhNS polyclonal antibody was raised and characterized through immunoblotting experiments to be specific to both mature and precursor forms of rhNS (Fig. 2) and was deemed suitable for use in immunoprecipitation and quantification experiments.

Development of a Sulfamidase Immunounquantification Assay—To enable the quantification of normal and mutant rhNS and native NS from skin fibroblasts, a sandwich ELISA was developed using the polyclonal antibody and the three monoclonal antibodies (rhNS-13.C1, rhNS-23.B2, and rhNS-
which is thought to lie at the end of an protein levels of the C-terminally located mutant R245H, turally, would lend support to this proposal, especially since two mutants in amino acid sequence, and presumably struc-

mutant protein, probably through a structural alteration in the structure of the protein away from the active site when compared with pcDNA3ns2. Immunoprecipitation experiments showed that in cell lines containing the normal rhNS expression construct, chase experiments were performed in stably expressing CHO attempt to understand the processing pathway of rhNS, pulse-

EXPRE35S35S protein labeling mix. Cells were then chased for varying time periods of up to 24 h. Immunoprecipitation of the precursor rhNS into the medium was detected 30 min after labeling, where the amount increases for up to 8 h. A slight degradation was observed 1 day after labeling (Fig. 3). In addition, G418-resistant mass cultures of rhNS-S66W and rhNS-R74C were metabolically pulse-labeled with EXPRESS35S35S protein labeling mix. Cells were then chased for varying time periods of up to 24 h. Immunoprecipitation of the mutant cell homogenates indicated faint bands of mature enzyme produced from S66W and R74C. Since the resolution of mutant sulfamidase expressed from the CHO cell system was difficult to determine from these studies (being at least 100-fold less than normal rhNS), cell extract samples containing the normal expression vector and those containing mutant sequences were run on an SDS-polyacrylamide gel, with purified rhNS from the CHO expression system as a control. The samples were transferred onto a nitrocellulose membrane, the extra sites were blocked, the rhNS polyclonal antibody was added, and then the samples were washed, and the membrane was developed using ECL. Banding patterns corresponding to rhNS for both mutants were present that were not observed in the CHO control. The amount of protein produced from R245H was insufficient to enable clear viewing of banding patterns in pulse-chase studies.

Glycosylation and Phosphorylation of rhNS—In order to de-
terminate the extent and characteristics of glycosylation, labeled immunoprecipitated rhNS was deglycosylated with Endo H and PNGase F. Digestion with Endo H was shown to cause a

TABLE I
Total protein and specific activity of wild type rhNS and expression constructs S66W, R74C, and R245H

| Expression construct and antibody | rhNS protein | rhNS activity | Specific activity a |
|----------------------------------|--------------|---------------|--------------------|
|                                  | ng/mg cell protein | nmol/min/mg cell protein | nmol/min/mg rhNS |
| Wild type (pcDNA3ns2)            |              |               |                    |
| arhNS-22.D5                      | 7305 ± 119.5 | 6.6668 ± 0.89 | 895.88             |
| arhNS-23.B2                      | 6306 ± 477.9 | 5.50367 ± 0.85 | 7.78736 ± 0.41 | 862.63, 940.68 |
| S66W (pcDNA3ns2.66W)             |              |               |                    |
| arhNS-22.D5                      | 130 ± 13.81  | 0.01370 ± 0.29 (0.3%) | 105.38 | 12% |
| arhNS-23.B2                      | 34.85 ± 9.27 | 0.0165225 ± 0.00025 (0.3%) | 474.10 | 52% |
| arhNS-13.C1                      | 124 ± 32.92  | 0.0165225 ± 0.00025 (0.3%) | 126.19 | 15% |
| Overnight incubation              | 124 ± 32.92  | 0.011508 ± 0.00081 (0.21%) | 92.74 | 13% |
| arhNS-23.B2 from S66W (+/+ fibroblast b) | 0.63 | 0.00027 ± 0.041012 (−0.5%) | 423.95 | 48% |
| R74C (pcDNA3ns2.74C)             |              |               |                    |
| arhNS-22.D5                      | 118 ± 7.45   | 0.011246 ± 0.0122 (0.16%) | 95.3 | 10.64% |
| arhNS-23.B2                      | 33.93 ± 12.4 | 0.014525 ± 0.00024 (0.3%) | 428.57 | 47.27% |
| arhNS-13.C1                      | 165.95 ± 9.33| 0.014525 ± 0.00024 (0.3%) | 87.522 | 9.653% |
| Overnight incubation              | 153.48 ± 29.8| 0.005656 ± 0.00072 (0.15%) | 55.9 | 6.48% |
| R245H (pcDNA3ns2.245H)           |              |               |                    |
| arhNS-22.D5                      | 4.74 ± 1.83  | 0.003642 ± 0.0058 (0.05%) | 658.89 | 72.67% |
| arhNS-13.C1 and arhNS-22.D5      | 5.52 ± 1.54  | 0.003642 ± 0.0058 (0.05%) | 747.42 | 82.441% |
| Overnight incubition              | 4.78 ± 0.646 | 0.004778 ± 0.0000064 (0.07%) | 865.50 | 99.10% |
| CHO control                      | NA a          |               |                    |
|                                  | 0.099188 ± 28.46 | NA |                     |

a Values in the separate experiments are similar to the specific activity for rhNS (822 nmol/min/mg (Bielic et al. (1998))).

b Taken from the fibroblast experiments (K. Perkins, unpublished observations).

NA, not applicable.
decrease of the apparent molecular mass from the precursor 64 kDa into three distinct species with the molecular masses of 60, 58, and 56 kDa, whereas digestion with PNGase F indicates two additional species of approximately 54 and 52 kDa (Fig. 4A). Taking into account the decreased quantity of NS produced by the mutant cell lines, a similar glycosylation pattern was observed in the two expressed mutations, rhNS-S66W and rhNS-R74C (Fig. 4B). Small amounts of undegraded enzyme have therefore been properly processed through the endoplasmic reticulum to reside in the lysosome, suggesting that glycosylation (and most likely mannose 6-phosphorylation) are not altered by either mutation.

In addition, rhNS was shown to be phosphorylated by the incorporation of 33P into the immunoprecipitated protein (labeled band of 62 kDa present upon SDS-polyacrylamide gel electrophoresis). The label could be released by treatment with the PNGase F, indicating that phosphorylation is restricted to the carbohydrate moieties and does not occur on hydroxyamino acids. Endo H digestion released the 33P label in the immunoprecipitated labeled sulfamidase sample, indicating that the phosphate label resides in high mannose type oligosaccharide side chains (Fig. 5). This study therefore shows that all five potential glycosylation sites are utilized, with two simple chains and three high mannose/complex chains, with at least one mannose group being phosphorylated.

Expression of Sulfamidase Constructs in CHO Cells—Oligonucleotide-mediated mutagenesis was used to introduce two missense mutations, S66W and R74C, into pcDNA3ns2, an expression vector containing the entire cDNA sequence of sulfamidase (6). Numerous attempts to introduce R245H using this method failed, presumably due to the formation of secondary structures; thus, a polymerase chain reaction-based method was utilized. The transfected CHO cell lines were designated rhNS-N, rhNS-S66W, rhNS-R74C, and rhNS-R245H, respectively, and selected using G418. Resistant clones were expanded as mass cultures, and cell lysates expressing rhNS were assayed for total sulfamidase protein and specific activity using the radiolabeled tetrasaccharide substrate and developed NS immunoquantification system. In the case of the immunoquantification system, the specificity of the assay is achieved by the purification of recombinant human sulfamidase away from other proteins within the sample (including CHO and fetal bovine sulfatases), using one of the three selected rhNS monoclonal antibodies. In order to confirm whether the mutated constructs were responsible for substrate breakdown and not endogenously produced NS from CHO and/or natural degrada-
tion, an extended overnight assay at 60 °C (85 and 80% of total wild type breakdown with S66W and R74C, respectively, above CHO background) indicated that both mutations maintain at least a limited enzymatic capacity. The reduction of activity observed in both S66W and R74C is therefore unlikely to be an artifact of the rhNS22.5 monoclonal antibody used in the immunocapture.

Studies of the expression of rhNS in cultured CHO cells (6.548 nmol/min/mg) yielded an approximate 10-fold increase in protein level compared with wild type NS in human fibroblasts (range of 0.64 nmol/min/mg). The two cell lines transfected with the mutant rhNS constructs, S66W and R74C, expressed much reduced rhNS protein quantities (0.3 and 0.2% for rhNS-S66W and rhNS-R74C, respectively) relative to the wild type rhNS construct, with R245H expressing approximately 0.05%. The specific activity of rhNS was comparable with values reported previously (7) for selector-amplified (889 nmol/min/mg) and unamplified rhNS (731 nmol/min/mg). In comparison, the specific activities of rhNS-S66W (105 nmol/min/mg) and unamplified rhNS (731 nmol/min/mg) were greatly reduced. However, the R245H exchange, although clearly affecting the amount of measurable protein, did not appear to significantly decrease specific activity (747 nmol/min/mg rhNS; 83% of normal). The reduced in vitro activities of the rhNS mutants S66W and R74C are therefore due to the double effect of reduction in synthesis and/or stability and a drastically reduced level of specific activity. Conversely, the nearly undetectable levels of either enzyme activity or protein in studies of R245H indicate the rapid degradation of this protein.

**DISCUSSION**

This study describes the production of monoclonal and polyclonal antibodies raised against purified rhNS in order to characterize three common and clinically severe patient mutations, S66W, R74C, and R245H. Through the development of an ELISA-based quantification assay with a panel of monoclonal antibodies, the quantity and specific activity of the mutants could be determined. As expected, both protein and specific activity levels are drastically decreased, culminating in defective HS degradation and the phenotype observed in MPS-IIIA patients.

Initial characterization of rhNS in the CHO cell expression system (7) indicated a major species of between 52 and 64 kDa with a minor band of 56–58 kDa. N-terminal amino acid sequencing indicated that both are NS polypeptides with the same N-terminal residues as the mature form of NS isolated from liver, therefore showing that there is no processing of the N terminus after leader peptide cleavage (7). Therefore, the major (62–64 kDa) polypeptide is most likely to be the precursor form of rhNS. The minor band is comparable with the mature form of NS purified from human liver (5), which also has a molecular mass of 56 kDa, similar to the predicted amino acid sequence from the cDNA of 54.67 kDa. This study further strengthens previous observations through immunoblotting experiments using the purified orhNS polyclonal antibody, which show the predominance of the precursor form of rhNS secreted into and purified from CHO cell medium (Fig. 2) (7).

**Glycosylation and Phosphorylation Properties of rhNS**—All soluble lysosomal enzymes are glycoproteins with N-linked oligosaccharide side chains. The particular importance of these side chains is that they harbor the mannos-6-phosphate recognition marker, which is essential for the correct intracellular sorting of these proteins. Lysosomal enzymes acquire these residues in the Golgi apparatus, where they are specifically recognized by a phosphotransferase, which transfers N-acetylglucosaminidase-1-phosphate to mannosyl residues in high mannos-6-phosphate (37198) oligosaccharide side chains. The structure common to all lysosomal enzymes leading to their specific recognition by the phosphotransferase is unknown, but it is known to be dependent on the correct three-dimensional structure of the lysosomal enzymes.

The appearance of three distinct species of Endo H-treated rhNS and a further two distinct bands present with PNGase F treatment indicates that all five potential glycosylated sites are used, with three high mannose (and potentially phosphorylated) or complex oligosaccharide chains present, with two additional sites with more simple tri- and/or tetracentenary complex-like and biantennary chains.

The presence of phosphorylated mannose residues supports previous experiments of uptake and correction of MPS-IIIA patient fibroblasts (7) using recombinant human sulfamidase.

In this study, rhNS was endocytosed via the mannose 6-phosphate receptor-mediated pathway and was targeted correctly to lysosomes, where it was effective in abolishing the storage effect. The addition of 5 mM mannan 6-phosphate inhibited the uptake of rhNS, indicating that the enzyme is targeted through the mannose-6-phosphate receptor.

**Correlation between Mutant Protein Expression and Activity with Patient Phenotype**

R74C—R74C is the most prevalent MPS-IIIA mutation in Poland and is the second most frequent in Germany (10) after R245H. However, the frequency of this mutation in the British population is considerably less, with one allele carrying the mutation in 17 patients (9). Sulfamidase, an N-sulfatase, shares homology with O-sulfatases and is comparable with N-acetylgalactosamine-4-sulfatase (4-S). The two motifs identified in prokaryotic and eukaryotic sulfatases that are conserved in the sulfatase family, C/S/TFSRASLLTG and GVTR-GIIGKK (Prosite Protein data base) are conserved in NS (amino acids 70–80 and 115–124) as well as 4-S (amino acids 91–101; to a lesser extent, 137–146). Recently reported crystallographic structural data for 4-S (21) showed that amino acid residue Arg245 is involved in formation and stabilization of the active site, and the alignment predicts that this residue is equivalent to Arg74 in sulfamidase. It has been postulated (9, 10) that the nonconservative replacement of this basic arginine by a small and nonpolar cysteine (R74C) would remove a stabilizing hydrogen bond, which appears to be critical for the hydrolysis of the sulfate ester at the nonreducing end of the substrate (21). The expression of R74C has shown a substantial decrease in both the amount of rhNS protein/mg of total cell protein (0.2%), most likely the result of increased degradation, and in specific activity (12%) compared with the wild type enzyme, presumably due to the impairment of normal function of the active site. This combination causes a significant decrease in HS turnover and results in a severe clinical phenotype.

S66W—S66W represents the most common alteration in the Italian MPS-IIIA population, accounting for 33% of total alleles (11). Interestingly, all six patients described (11) originate from Sardinia, and five are homozygous for S66W, suggesting that these subjects may have been derived from a common founder. The serine residue at position 66, unlike Arg245, is not conserved between sulfamidase and 4-S but is in close proximity to the CTPS motif and hence is positioned within the coordination sphere for the cysteine residue that is post-translationally modified in the active site of eukaryotic sulfatases (21). As predicted (9), the effect of replacing this serine by the larger tryptophan residue results in a drastically reduced level of functional NS (0.3%) and lowered specific activity (15%), which, like R74C, is most likely caused by distortion of the active site.
Phenotypically, patients homoalectic for this mutation show variable phenotypes from severe to intermediate, suggesting that the effect of S66W may be modified by environmental factors or as yet unidentified polymorphisms. The presence of the common polymorphism R456H is discounted in a study with all patients homozygous for S66W and showing variable clinical symptoms negative for R456H (11). Quantitative studies of S66W in patient fibroblasts\(^2\) show that there is a residual level of sulfamidase that has a similarly low specific activity, supporting the expression data presented in this paper.

**R245H**—The difficulty in the hybridization of the mutant oligonucleotide to identify this allele in allele-specific oligonucleotide analysis of patient DNA (9, 22) was thought to be the reason behind the inability to introduce the mutation into the sulfamidase cDNA using oligonucleotide-mediated mutagenesis, which uses a similar methodology. This was circumvented through the use of a polymerase chain reaction-mediated protocol, with the mutant sequence analyzed as per S66W and R74C. Like Ser\(^{66}\), Arg\(^{245}\) is not conserved among the sulfatases; however, the high incidence of the R245H mutation in MPS-IIIA patients makes this transition important in studies concerned with the relationship between genotype and phenotype. This change is also common in Germany (35% of alleles (10)) and is extremely frequent in The Netherlands (56.7% of alleles (12)), indicating that the origin of this mutation lies in Western Europe. Although the tertiary structure of two sulfatases, 4-S and sulfatidase (arylsulfatase A), have been determined (21, 23), it is difficult to surmise the effect of R245H, due to the lack of homology toward the C terminus. Through a sequence comparison with 4-S and subsequent structural superimposition, Arg\(^{245}\) is postulated to lie near the surface of the protein on \(\alpha\)-helix 7 of 4-S. Therefore, since the mutation does not lie within the coordination sphere, it was shown that the specific activity of the enzyme was not affected; however, the change probably results in increased degradation. The function of this region has not been elucidated for any of the sulfatases. Analysis of fibroblasts from R245H homozygotes\(^2\) indicates a NS protein level that is lower than both S66W and R74C, and in many cases indistinguishable from the ELISA background signal. The specific activity in R245H homozygotes with detectable levels of enzyme show that there is no significant drop in specific activity, which is observed in the expression studies. The almost null levels of protein observed in the stable expression of common mutants support the detrimental effects of these substitutions on the active site of sulfamidase, with their ability to result in distorted structure leading to degradation, and correlate to the severe phenotypes observed in patients with these mutations (9). Although the expression of the three most common mutations has provided an insight into the structure/function relationship in NS, correlation between patient genotype and phenotype remains extremely difficult and is complicated further by the relatively mild manifestation of MPS-IIIA through progressive mental retardation and not noticeable physical characteristics as exhibited in other MPS disorders.

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\(^2\) K. Perkins, unpublished observations.