© 2000 Oxford University Press Human Molecular Genetics, 2000, Vol. 9, No. 18 2715–2725

Novel mutations in lysosomal neuraminidase identify functional domains and determine clinical severity in sialidosis

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Received 24 July 2000; Revised and Accepted 1 September 2000

Lysosomal neuraminidase is the key enzyme for the intralysosomal catabolism of sialylated glycoconjugates and is deficient in two neurodegenerative lysosomal disorders, sialidosis and galactosialidosis. Here we report the identification of eight novel mutations in the neuraminidase gene of 11 sialidosis patients with various degrees of disease penetrance. Comparison of the primary structure of human neuraminidase with the primary and tertiary structures of bacterial sialidases indicated that most of the single amino acid substitutions occurred in functional motifs or conserved residues. On the basis of the subcellular distribution and residual catalytic activity of the mutant neuraminidases we assigned the mutant proteins to three groups: (i) catalytically inactive and not lysosomal; (ii) catalytically inactive, but localized in lysosome; and (iii) catalytically active and lysosomal. In general, there was a close correlation between the residual activity of the mutant enzymes and the clinical severity of disease. Patients with the severe infantile type II disease had mutations from group I, whereas patients with a mild form of type I disease had at least one mutation from group III. Mutations from the second group were mainly found in juvenile type II patients with intermediate clinical severity. Overall, our findings explain the clinical heterogeneity observed in sialidosis and may help in the assignment of existing or new allelic combinations to specific phenotypes.

INTRODUCTION

Neuraminidases or sialidases are exoglycosidases that catalyze the cleavage of α-glycosidically linked terminal *N*-acetyl neuraminic acid from sialylated glycoconjugates (1). They are widely spread in nature, occurring in viruses, bacteria, fungi, protozoa, birds and mammals (2–8). Together, the neuraminidases form a family of hydrolases that share a conserved active site and similar sequence motifs (9–11). Three types of neuraminidase are found in mammals and are defined as lysosomal, plasma membrane and cytosolic on the basis of their biochemical properties and subcellular distribution (3–8,12–14). Lysosomal *N*-acetyl-α-neuraminidase has significant primary structure characteristics of other mammalian and microbial sialidases with similar substrate specificity. However, unlike other members of this family, lysosomal neuraminidase requires the carboxypeptidase protective protein/cathepsin A (PPCA) for intracellular transport and lysosomal activation (15). The enzyme is only catalytically active when it is bound to PPCA and is a component of a high molecular weight, multi-protein complex containing PPCA, β-galactosidase and *N*-acetylgalactosamine-6-sulfate sulfatase (16–21). A primary or secondary deficiency of lysosomal neuraminidase is associated with two neurodegenerative disorders of lysosomal metabolism, sialidosis and galactosialidosis (GS).

Sialidosis is an autosomal recessive disease caused by lesions in the lysosomal neuraminidase gene on chromosome 6p21 (12,14). Distinct clinical phenotypes are recognized, varying in the onset and severity of the symptoms. Type I sialidosis, which is also referred to as the cherry-red spot/myoclonus syndrome, is a relatively mild disease that occurs in the second decade of life and results in progressive loss of vision associated with nystagmus, ataxia and grand mal-seizures but not dysmorphic features (22). Type II sialidosis is the severe form of the disease characterized by the presence of abnormal somatic features,

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including coarse facies and dysostosis multiplex. On the basis of the age at onset of the symptoms, type II sialidosis is divided into three subtypes: (i) congenital or hydropic (*in utero*); (ii) infantile (0–12 months); and (iii) juvenile (2–20 years) (reviewed in ref. 22). The congenital form is associated with either hydrops fetalis and stillbirth or neonatal ascites and death at an early age; features include facial edema, inguinal hernias, hepatosplenomegaly, stippling of the epiphyses and periosteal cloaking. Type II patients with longer survival develop a progressive mucopolysaccharidosis-like phenotype; signs include coarse facies, visceromegaly, dysostosis multiplex, vertebral deformities, mental retardation and cherry-red spot/ myoclonus (22–25).

A primary defect of PPCA causes the lysosomal disorder GS, which presents with clinical signs strikingly similar to those of sialidosis (reviewed in ref. 26). The absence or impairment of PPCA leads to the secondary, combined deficiency of β-D-galactosidase and neuraminidase. Residual neuraminidase activity in patients with sialidosis or GS is typically <1% of normal levels. As for sialidosis patients, GS patients are diagnosed with either an early infantile, late infantile or a juvenile/ adult form of the disease, based on age at onset and severity of clinical manifestations. The early infantile form of GS is clinically very similar to the congenital type II form of sialidosis; both are characterized by visceromegaly, hydrops fetalis, ascites and early death. The late infantile/childhood forms of GS and sialidosis are also similar, with the exception of milder neurological involvement in the GS patients. At the other end of the spectrum, similarities also exist between patients with adult type I sialidosis and juvenile/adult GS, both characterized by the absence of visceromegaly. In contrast, juvenile/adult GS patients have dysmorphic features but milder neurological involvement (26). The biochemical and clinical similarities between sialidosis and GS suggest an important role for neuraminidase in the pathogenesis of these diseases.

Several mutations have been identified in the neuraminidase genes of unrelated patients with sialidosis (12,14,27). The mutations analyzed to date include point mutations, single nucleotide deletions and small insertions (12,14,27). Those sialidosis patients who were also tested for the presence of the neuraminidase mRNA were found to have normal amounts of transcript (12,14).

Here we report the identification of eight novel and five previously identified mutations in eleven patients with sialidosis (nine unrelated and two siblings). Collectively these patients exhibit the full range of severity of the disease. Expression of the mutant enzymes in sialidosis fibroblasts enabled us to classify the mutations according to the level of functional neuraminidase that they support and to identify functional domains or amino acid residues in the protein. From these studies we found a correlation between specific combinations of mutant alleles and the severity of the disease.

RESULTS

Clinical phenotypes

We have studied eleven patients with sialidosis (nine unrelated and two siblings) of different ethnic origins and with heterogeneous clinical presentations. The type I form of the disease was diagnosed in six patients (patients 1, 2, 4–7); the type II juvenile phenotype, in one (patient 3); the type II infantile form in three (patients 8–10); and the type II congenital/hydropic form in one (patient 11). Clinical reports have been published only for patients 9 and 10 (28–30); either the other patients were newly diagnosed or their cases had never been reported in the literature. Table 1 is a summary of the main clinical features, sex, ethnicity and biochemical data of these patients. The patients with the mildest form of sialidosis (patients 5–7) had slowly progressing disease. The two siblings, patients 5 and 6, presented with identical symptoms, but only in patient 5 had the disease affected the eyes (Table 1). Despite the relatively mild clinical manifestations, serious central nervous system (CNS) disorders, such as ataxia and epilepsy, developed in these patients, whereas patients with a mild form of GS have no relevant signs of neurological disorders (31). The other patients with type I or type II sialidosis experienced severe CNS symptoms, including epilepsy, ataxia, dysmetria, hypotonia, deafness and mental retardation.

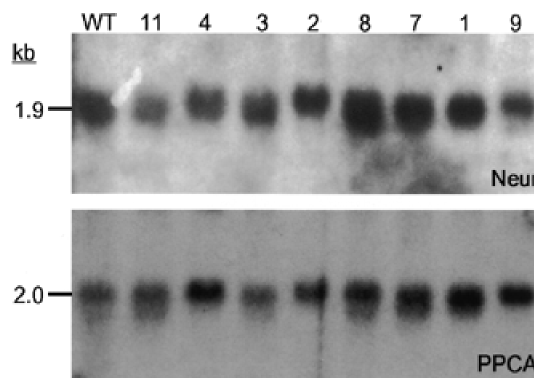

Figure 1. Neuraminidase mRNA expression. Radiolabeled full-length neuraminidase cDNA (top) and PPCA cDNA (bottom) were used to probe a northern blot containing RNA (10 μg) isolated from the cultured fibroblasts of a healthy person (WT), four patients with type I sialidosis (patients 1, 2, 4 and 7) and four patients with type II sialidosis (patients 3, 8, 9 and 11).

The neuraminidase activities of the fibroblast lysates were compared within one experiment to allow for the reliable calculation of residual enzyme activity of each patient. The differences in residual activity among patients were small, ranging from 0.5 to 1.5% of the normal values and did not reflect the broad spectrum of clinical manifestations. These findings indicate that even small variations in residual catalytic activity can greatly influence disease severity and progression (Table 1).

Biochemical characteristics of the sialidosis patients

All patients included in this study expressed neuraminidase mRNA as determined by northern blot analysis (Fig. 1, top) (12,14). To compare the quantity and quality of the different RNA samples, the northern blot was also hybridized with a PPCA cDNA probe (Fig. 1, bottom). The presence of the 1.9 kb neuraminidase and 2.0 kb PPCA mRNAs was detected in all the patient samples.

Table 1. Clinical features of patients with sialidosis

	1 (female)	2 (male)	3 (female)	4 (female)	5 and 6[a] (female/ siblings)	7 (female)	8 (male)	9 (female)[b]	10 (female)[c]	11 (female)
Clinical type	I	I	II juvenile	I	I	I	II infantile	II infantile	II infantile	II congenital/ hydropic
Neur. act.[d]	0.71	0.56	0.76	0.49	0.65	1.02	0.50	0.87	0.42	0.98
% normal	1.0	0.82	1.1	0.72	1.0	1.5	0.72	1.3	0.62	1.4
Origin	African-American	African-American	Italian	Greek	German	Dutch	Hispanic-American	Unknown (Caucasian)	Unknown (Caucasian)	Italian
Onset	11 years	8 years	12 years	10 years	17 and 15 years	13 years	At birth	6 months	At birth	Gestational age of 20 weeks
Presentation	Muscle weakness, atrophy, seizures	Muscle aches	Generalized seizures	Seizures, myoclonus	Cerebellar atrophy	Gait disturbance, stumbling and falling	Coarse facies/ hepato-splenomegaly	Strabismaus, nystagmus	Coarse facies/ hepato-splenomegaly	Hydrops fetalis, joint contractures
Growth	Normal	Normal	Normal	Normal	Normal	Normal		Short stature	Normal	Weight/length under 3rd percentile
Skeleton	Normal	Normal	Dysostosis multiplex: skull, vertebral bodies, hips	Coarse facies, mild dysmorphic features, nystagmus, wheelchair age 16 years	Normal	Normal	Coarse facies; craniosynostosis	Coarse facies; dolichocephalic skull	Coarse facies, dysostosis multiplex	Dysostosis multiplex
Liver/spleen	Normal	Normal	Normal	Normal	Normal	Normal	Hepato-splenomegaly		Hepato-splenomegaly	Hepato-splenomegaly
Heart	Normal	Murmur	ECG aspecific alterations of repolarization; ultrasound normal		Normal	Normal			Cardiomegaly	Cardiomyopathy
Nervous system	Seizures, ataxia, slurred speech, dysmetria, myoclonus	Moderate developmental delay in language	Ataxia, spasticity, psychomotor delay, microcephaly, neurosensorial deafness, dysmetria, myoclonus	Hearing loss, myoclonus, mild hypotonia, cerebellar signs, dysarthria, dysphagia, borderline IQ, EEG abnormalities	Ataxia, dysarthria, myoclonic epilepsy, dydiadochokinesia	Ataxia, pyramidal signs, dysarthria, myoclonus, painful axonal polyneuropathy, epilepsy, EEG abnormalities, normal IQ	Developmental delay, orbital hypoplasia	Mental retardation, hearing loss, hypotonia	Mild mental retardation, hearing loss, hypotonia	Severe psychomotor retardation, hydrocephalus
Eyes	Cherry-red spots	Cherry-red spots, visual acuity, diminution	Cherry-red spots	Cherry-red spots, ocular lens opacities, impaired vision	Cherry-red spots (5) and normal (6)	Bilateral cataracts, prolonged latencies on visual evoked potentials, intolerance for light	Normal	Macular cherry-red spots	Normal	No corneal opacity
Course	Wheelchair and slowly progressing at 20 years	Otherwise normal health at 8 years	Slowly progressing at 28 years	Wheelchair age 16 years, dead at 24 years	Alive at 33 and 32 years; slowly progressing	Slowly progressing; death at 44 years	Progressing at 4 months	Wheelchair at 9 years, dead at 30 years	Progressing at 24 months	Exitus at 18 months

[a]Bonten *et al.* (12).
[b]Bakker *et al.* (30).
[c]Kelly and Graetz (28) and Kelly *et al.* (29).
[d]Neuraminidase activity (nmol/h/mg).

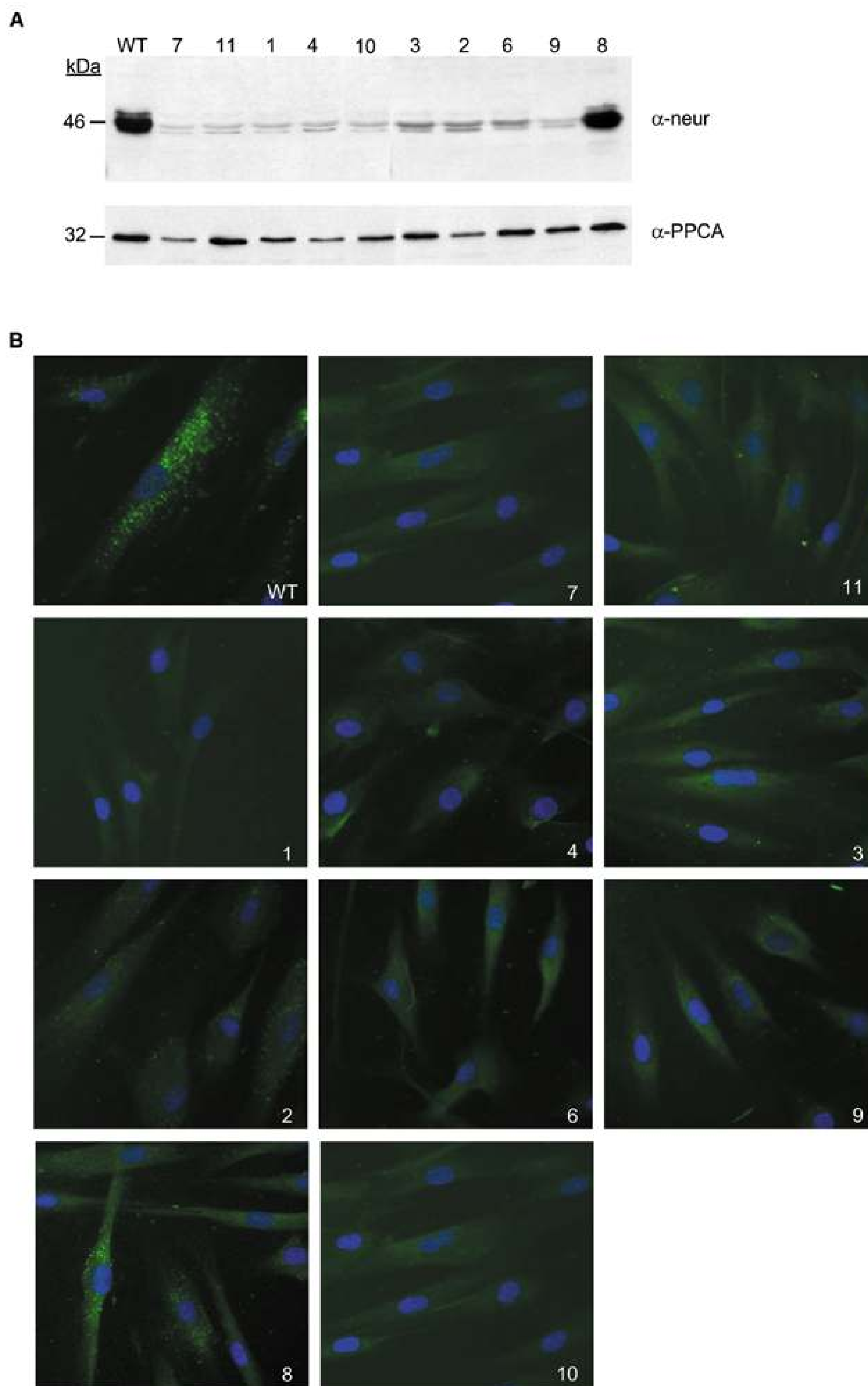

Figure 2. Detection of neuraminidase in fibroblasts of patients. (**A**) Affinity-purified anti-neur (top) and anti-PPCA antibodies (bottom) were used to analyze western blots containing protein (5 µg) from fibroblast lysates of a healthy person (WT), five patients with type I sialidosis (patients 1, 2, 4, 6 and 7) and five patients with type II sialidosis (patients 3, 8–11). (**B**) Immunocytochemical localization of neuraminidase in fibroblasts from a healthy person (WT) and from 10 patients as indicated. Affinity-purified anti-neur antibodies and FITC-conjugated secondary antibodies were used. The nuclei were stained with DAPI. The magnification is 400×.

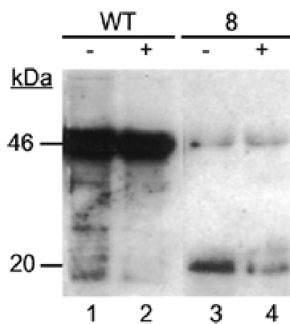

Figure 3. *In vitro* stability of neuraminidase from fibroblasts of patient 8. Fibroblast lysates (5 µg of protein) of a healthy person (WT) and of patient 8 were incubated for 15 min at 37°C in the absence (–) or presence (+) of protease inhibitors. After incubation, the samples were subjected to SDS–PAGE (12.5% acrylamide gel), subsequently transferred to a PVDF membrane and incubated with affinity-purified anti-neur antibodies.

Western blots of total homogenates of the cultured fibroblasts were analyzed with affinity-purified anti-neuraminidase (anti-neur) antibodies (12). The size of neuraminidase detected in the patients' fibroblasts (46 kDa) was the same as that in wild-type fibroblasts; however, the amount of neuraminidase in the samples of all patients except one was markedly less than that in wild-type fibroblasts (Fig. 2A). Only patient 8 had a normal amount of the 46 kDa neuraminidase (Fig. 2A, top, lane 11). When the same blot was analyzed with an anti-PPCA antibody that recognizes the 32 kDa subunit of the enzyme, similar quantities of fully processed PPCA were detected in wild-type fibroblasts and in fibroblasts of all patients (Fig. 2A, bottom).

To further ascertain whether the different neuraminidase variants were localized to aberrant subcellular regions, we used anti-neur antibodies to perform immunocytochemical analysis of the patient's fibroblasts. The wild-type fibroblasts displayed a punctate staining pattern that is characteristic of lysosomes (Fig. 2B, WT). In contrast, this punctate staining was not detected in any of the patients, with the exception of patient 8 (Fig. 2B). In this patient's cells, the protein appeared to be distributed in lysosomes in a pattern similar to that of wild-type fibroblasts, but the signal intensity was somewhat lower than in the wild-type fibroblasts (Fig. 2B). A normal subcellular distribution of PPCA was detected in all patient samples (data not shown). These results indicated that the biochemical phenotype of patients in this study, with the exception of that of patient 8, fit the profile of sialidosis.

The normal quantity of neuraminidase detected by western blot analysis and the punctate immunostaining in fibroblasts raised the possibility that patient 8 was affected by another lysosomal disorder. We excluded GS since the patient's fibroblasts had normal levels of cathepsin A and β-galactosidase activities and showed a normal punctate lysosomal localization of PPCA (data not shown). Moreover, we have shown earlier that in the absence of a functional PPCA neuraminidase is not transported to the lysosomes (15). We analyzed the *in vitro* stability of neuraminidase in this patient's fibroblasts. We incubated homogenates of fibroblasts, both of the patient and a normal individual, at 37°C for 15 min, in the absence and presence of protease inhibitors. This allows for the partial *in vitro* degradation of neuraminidase by endogenous proteases that are present in the cell lysates. After incubation, total proteins were subjected to western blot analysis with anti-neur antibodies. The 46 kDa wild-type neuraminidase was almost unaffected after the incubation at 37°C (Fig. 3, lanes 1 and 2) and only minor degradation was observed in the sample without the protease inhibitors (Fig. 3, lane 1). In contrast, only a small amount of the 46 kDa neuraminidase was present in the samples of patient 8 (Fig. 3, lanes 3 and 4). A degradation product of ~20 kDa was detected in the presence and absence of protease inhibitors. In conclusion, the mutant neuraminidase in this patient is apparently competent for transport to the lysosomes but is more susceptible than the wild-type protein to (*in vitro*) proteolytic degradation (Fig. 2). The protease(s) responsible for the degradation of neuraminidase in this experiment may not be lysosomal, since under physiological conditions neuraminidase stability does not seem to be affected. Nevertheless, the increased *in vitro* instability of neuraminidase in combination with the absence of catalytic activity in fibroblasts of patient 8 does imply a defect in the neuraminidase gene.

Molecular analysis of neuraminidase from the patients

The cDNA and genomic DNA from the 11 patients were analyzed as described earlier (12). We identified 13 mutations, 8 of which were novel. A compendium of the mutant alleles from all patients is presented in Table 2. Most of the mutant alleles contained point mutations which resulted in single amino acid substitutions. Heterozygous mutations occurred in seven patients and homozygous mutations occurred in four (Table 2). Three of the mutations were shared by more than one patient (Arg294Ser, Gly227Arg and Phe260Tyr). Patients 1 and 2, who shared a C→T transition at nucleotide 878, are African-Americans. A G→A transition at nucleotide 677 was present in patients 3 and 4, both of Mediterranean origin. Despite having the same mutation, patients 3 and 4 were diagnosed at the onset of the disease as type II and type I sialidosis, respectively. The same mutation was also present in two unrelated type II sialidosis patients from the USA and Mexico, both of Caucasian origin (27). The presence of this mutation in four patients with European ancestry suggests that this may be a founder allele that originated in Europe. Future molecular analysis of additional patients may establish the existence of more founder mutations and their migration into different populations. Genomic DNA analysis by PCR from patient 11 showed the presence of a second mutation, a C→T transition at nucleotide 836, resulting in a premature stop codon. Because this mutation was not detected in the patient's mRNA we can infer that the mutation caused instability of the neuraminidase mRNA transcribed from this allele.

Primary structure analysis of the mutant neuraminidases

We compared the predicted amino acid sequences of the mutant neuraminidases with those of wild-type mammalian and bacterial sialidases (Fig. 4A). In particular, Tyr370, which is replaced by Cys in patient 8, is fully conserved in all sialidases. It is noteworthy that the three-dimensional (3D) structures of the active sites of bacterial and viral neuraminidases have identified the tyrosine, corresponding to Tyr370 in

the human enzyme, as one of the five active site residues (9,11,32,33). The presence of a normal amount of correctly localized enzyme in fibroblasts of patient 8 that is nonetheless catalytically inactive suggests that the mutated Tyr370 is one of the active site residues of lysosomal neuraminidase.

Several mutations that are located within or near Asp-box motifs are also conserved in other sialidases (Fig. 4A: Ser182Gly, Leu231His, Ala298Val and Thr301stop) (27), with the exception of Arg294, which is adjacent to the Asp box IV but is not conserved. The mutation Leu91Arg, identified earlier in a patient with infantile type II sialidosis, involves a residue that is part of a 9 amino acid domain that is fully conserved among sialidases (Fig. 4A) (12).

Figure 4B shows a schematic representation of the neuraminidase protein and the relative position of the mutations (21 in total) identified to date by us and other investigators. Approximately 50% of all mutations either affect conserved residues within conserved domains or are located within or near an Asp-box motif (Fig. 4). This finding strongly suggests that Asp motifs are essential for neuraminidase function and that mutations within or near such domains are detrimental to the activity of the protein.

Mutant neuraminidase proteins in sialidosis fibroblasts

The individual mutations were engineered in the full-length wild-type neuraminidase cDNA and the resulting mutant cDNAs were then subcloned into the mammalian expression plasmid pSCTOP (12). It is well established that wild-type neuraminidase must bind to PPCA to be transported to the lysosomes and to become catalytically active (15). To create an optimal intracellular environment for the determination of the localization and residual activity of the mutant proteins, we transfected the mutant neuraminidase and wild-type PPCA expression plasmids into deficient fibroblasts. These fibroblasts were derived from a type II sialidosis patient with <1% residual neuraminidase activity and absence of immunofluorescent staining with anti-neur antibodies (12). An expression construct containing the *LacZ* gene was used as an internal

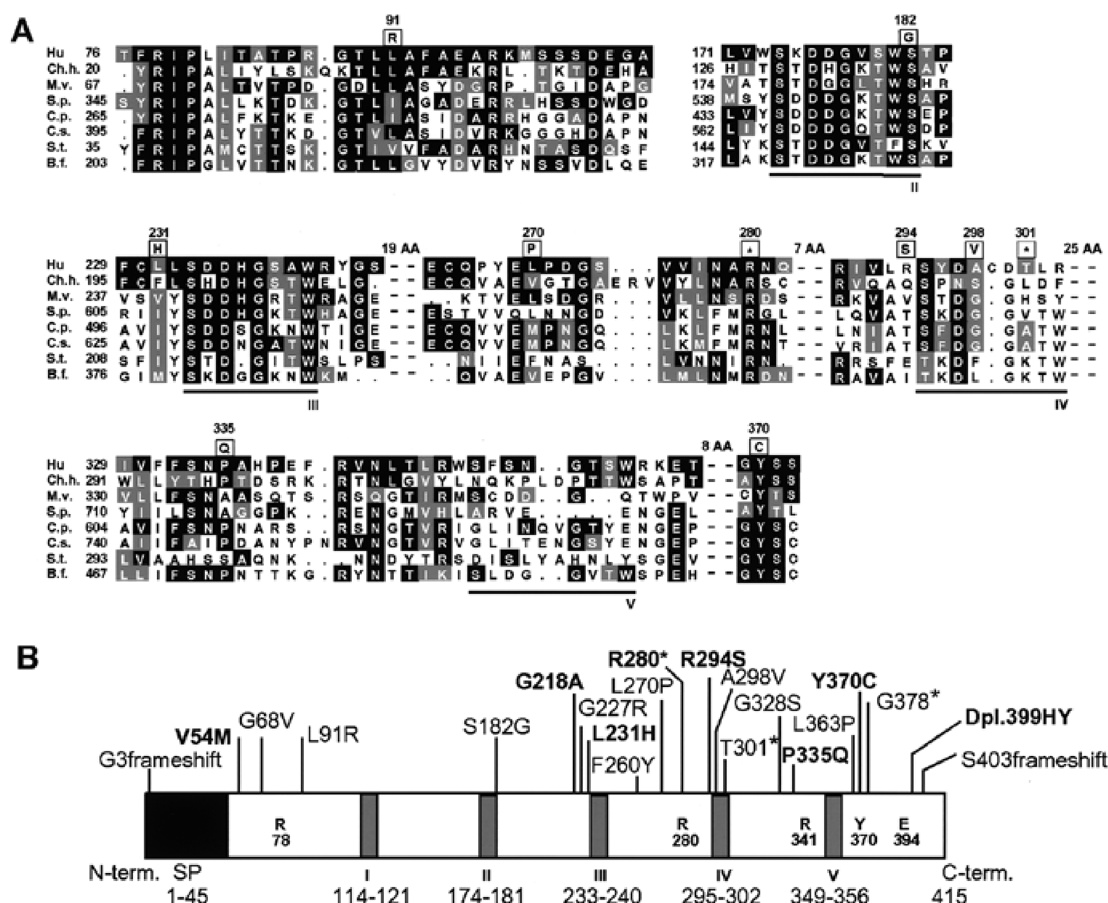

Figure 4. (**A**) Conserved amino acid residues within the sialidase superfamily that are mutated in sialidosis. Selected regions of human lysosomal neuraminidase (Hu) are compared with those of other mammalian and bacterial members of the sialidase superfamily: Chinese hamster cytosolic (Ch.h), *Micromonospora viridifaciens* (M.v.), *Streptococcus pneumoniae* (S.p.), *Clostridium perfringens* (C.p.), *Clostridium septicum* (C.p.), *Salmonella typhimurium* (S.t.) and *Bacteroides fragilis* (B.f.). Mutated residues in lysosomal neuraminidase of sialidosis patients are indicated above the aligned sequences. Regions without significant homology are indicated (–). (**B**) Schematic representation of neuraminidase mutations within the primary structure of neuraminidase. Conserved Asp-box motifs are numbered I–V, and amino acid positions are indicated. The five strictly conserved active site residues are indicated in bold, as are the eight novel mutations. The signal peptide (SP) is indicated by a black box.

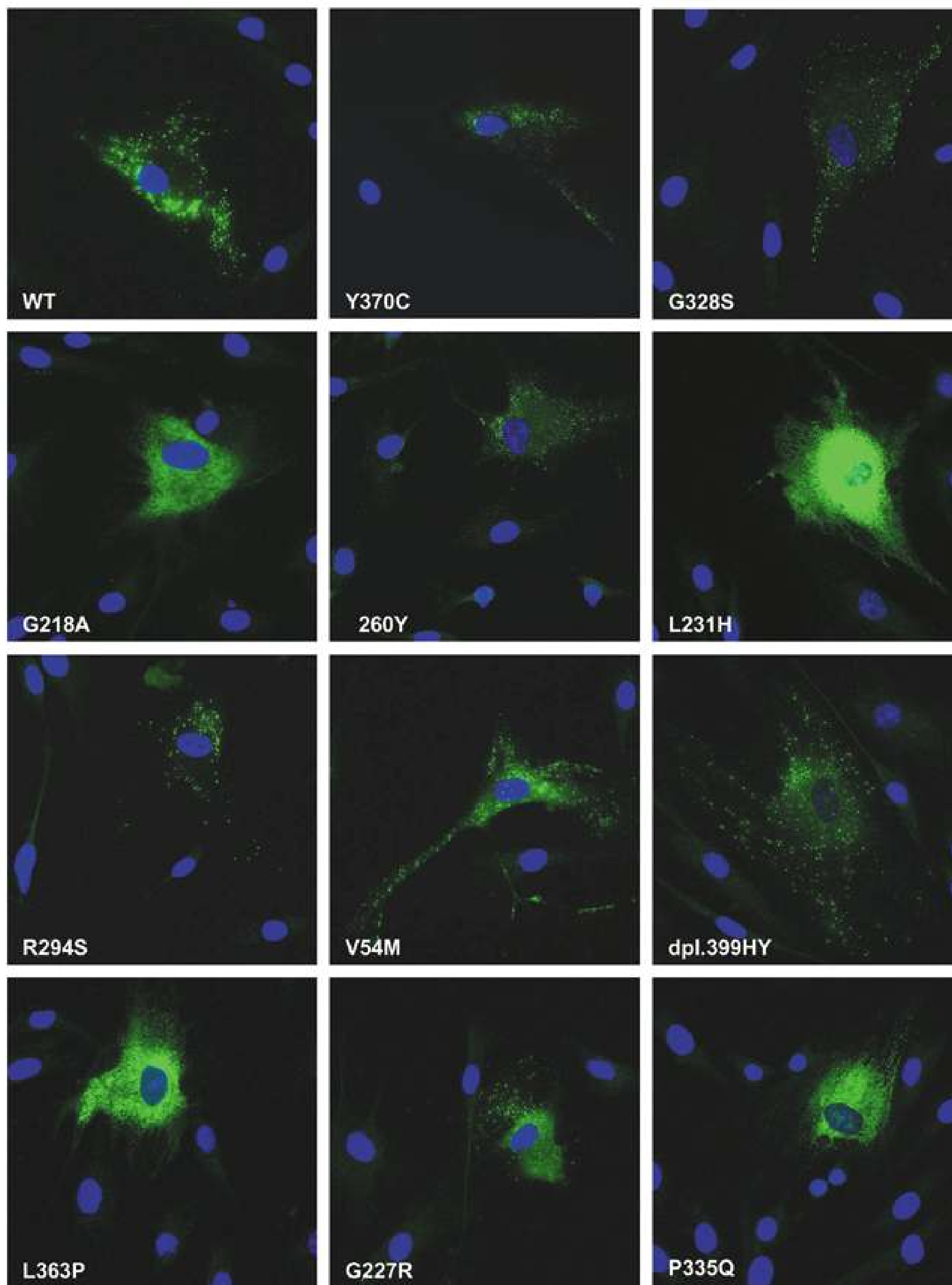

Figure 5. Immunocytochemical localization of neuraminidase overexpressed in sialidosis fibroblasts. Sialidosis fibroblasts were either transfected with the wild-type PPCA and neuraminidase cDNA expression constructs (WT) or with the wild-type PPCA and mutant neuraminidase cDNA expression plasmids as indicated. Cells were seeded on slides and incubated with affinity-purified anti-neur antibodies and FITC-conjugated secondary antibodies. The nuclei were stained with DAPI. The magnification is 400×.

control for transfection efficiency (Materials and Methods). Three days after electroporation, the cells were subjected to immunocytochemical analysis using affinity-purified anti-neur antibodies (12). No punctate staining was observed in four sets of cells expressing mutant proteins (Fig. 5; Leu363Pro, Pro335Gln, Gly218Ala and Leu231His), a finding indicating that these mutant proteins were unable to reach the lysosomes. This staining pattern was indicative of an endoplasmic reticulum (ER) and Golgi localization, probably due to protein misfolding (31). Surprisingly, most of the mutant proteins were apparently partially transported to the lysosomes, because the expressing cells showed, besides ER and Golgi

Table 2. Neuraminidase mutations in sialidosis patients

Patient	Clinical phenotype	Nucleotide mutation	Exon	Amino acid change
1	I	878C→T	5	Arg294Ser
		690T→A	4	Leu231His
2	I	878C→T	5	Arg294Ser
		654G→A	4	Gly218Ala
3	II juvenile	677G→A[a]	4	Gly227Arg[a]
		677G→A	4	Gly227Arg
4	I	677G→A	4	Gly227Arg
		677G→A	4	Gly227Arg
5 and 6	I	159G→A	2	Val54Met
		1127G→T[b]	6	Gly378stop[b]
7	I	980G→A[a]	5	Gly328Ser[a]
		Dpl1196ACCACT	6	Dpl399HisTyr
8	II infantile	1107A→G	6	Tyr370Cys
		1107A→G	6	Tyr370Cys
9	II infantile	777T→A[c]	4	Phe260Tyr[c]
		777T→A	4	Phe260Tyr
10	II infantile	777T→A	4	Phe260Tyr
		1086T→C[c]	6	Leu363pro[c]
11	II congenital/ hydropic	836C→T[d]	5	Arg280stop
		1002C→A	5	Pro335Gln

[a]Lukong *et al*. (27).
[b]Bonten *et al*. (12).
[c]Pshezetsky *et al*. (14).
[d]Mutation only detected in genomic DNA.

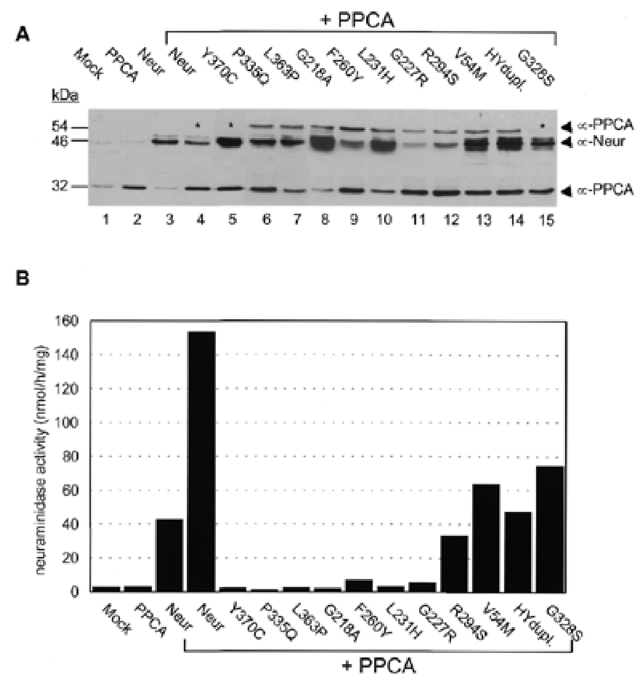

Figure 6. Western blot and catalytic analysis of mutant neuraminidase overexpressed in sialidosis fibroblasts. Sialidosis fibroblasts were transfected with wild-type PPCA and neuraminidase cDNA expression constructs or with wild-type PPCA and mutant neuraminidase cDNA expression plasmids. (**A**) Western blots containing lysates (5 µg of protein) of fibroblasts transfected with the indicated mutant plasmids were incubated with affinity-purified anti-neur and anti-PPCA antibodies. An asterisk indicates the absence of 54 kDa PPCA precursor in lanes 4, 5 and 15. (**B**) Neuraminidase activity in the indicated cell lysates, measured 4 days after transfection. Activities are expressed as released nmol sialic acid per mg of protein per h and normalized for the transfection efficiency (nmol/h/mg).

staining (data not shown), also some punctate staining (Fig. 5; Tyr370Cys, Phe260Tyr, Gly227Arg, Arg294Ser, Val54Met, dpl.399HisTyr and Gly328Ser).

All neuraminidase variants appeared to have the same molecular weight as the wild-type protein, but the amounts of the mutant proteins differed, probably because of variations in stability (Fig. 6A). The blots were subsequently analyzed with anti-PPCA antibodies; all samples contained uniform amounts of the 32 kDa PPCA subunit (Fig. 6A). However, it was surprising that in all mutants, with the exception of Gly328Ser and Tyr370Cys, we could still detect the 54 kDa PPCA precursor (Fig. 6A, lanes 6–14). This precursor is usually not detected by western blot analysis when the PPCA cDNA is expressed either alone or with wild-type neuraminidase, because the precursor is rapidly and efficiently processed into the two-chain mature enzyme (Fig. 6A, lanes 2 and 4). The fact that the PPCA precursor was still detected when co-expressed with neuraminidase mutants (Fig. 6A, lanes 6–14) suggested that it was retained in part in a prelysosomal compartment, probably because of association with the mutant neuraminidase proteins.

Seven of the mutant neuraminidases failed to generate any detectable enzyme activity (Fig. 6B). This result was expected for Leu363Pro, Pro335Gln, Gly218Ala and Leu231His mutants because they did not localize to lysosomes. However, despite the punctate subcellular distribution of Tyr370Cys, Phe260Tyr and Gly227Arg (Fig. 6A), these proteins appeared to be catalytically inactive. Thus, these mutations probably affect the catalytic machinery of neuraminidase. In contrast, four of the mutant proteins (Arg294Ser, Val54Met, dpl399HisTyr and Gly328Ser) exhibited substantial residual neuraminidase activity (Fig. 6B) and thus could be considered mild mutations. These mutations may be selective for the mild clinical phenotypes in patients with type I sialidosis. It is noteworthy that the residual activities of the mutant enzymes were higher than the endogenous enzyme activities in the corresponding mutant fibroblasts (Table 1). This is likely an effect of the high expression levels in transfected cells, that may either activate 'ER stress-response' proteins, facilitating folding of normal as well as mutant proteins or promote oligomerization and complex formation of the mutant proteins (34). Response will retain the improperly folded proteins in the ER (34).

DISCUSSION

We have investigated the properties of neuraminidase mutants identified in eleven patients with sialidosis (nine unrelated

patients and two siblings). The phenotypes of these patients represent the complete spectrum of clinical severity. The number of cases is significant, given that the frequency of diagnosed sialidosis in the population (1 in ~4 million live births) is much lower than that of other lysosomal disorders (35). To date all of the patients for whom mutations in the neuraminidase gene have been identified expressed neuraminidase mRNA (12,14). This finding is in contrast with those reported for GS (26) and suggests that a complete absence or deficiency of lysosomal neuraminidase is lethal during development or at birth.

On the basis of their biochemical properties, the neuraminidase variants can be divided into three groups: (i) the mutant enzymes are catalytically inactive and do not localize to lysosomes; (ii) the variants reach the lysosomes but are catalytically inactive; and (iii) the enzymes have residual activity and localize to the lysosomes. Most importantly, we found a correlation between the impact of the individual mutations and the clinical severity of sialidosis. All four patients with type I sialidosis have at least one of the 'mild' amino acid substitutions from the third group; patient 7, who died at the age of 44 years, had two 'mild' mutations (dpl399HisTyr and Gly328Ser). In contrast, two patients with infantile type II disease (patients 9 and 10) had mutations belonging to the first or second group of catalytically inactive enzymes. Both unrelated patients with juvenile type II sialidosis (patients 3 and 4) were homozygous for the mutation Gly227Arg, which belongs to the second group. Because these mutant proteins localize to the lysosomes, they may still retain *in vivo* a low residual activity, which could account for the differences in clinical severity among the patients with type II disease. The Tyr370Cys mutation is associated with a very severe sialidosis phenotype. Thus, residual amounts of mutant enzyme in lysosomes are clearly not sufficient to cause a milder phenotype, unless part of the catalytic machinery is retained. It is conceivable that environmental factors, including diet, prophylactic therapies or genetic factors besides neuraminidase mutations, may influence the penetrance of the disease or phenotype. This is most evident in patients 3 and 4, who were diagnosed with type II and type I sialidosis, respectively, but are homozygous for the same mutation (Gly227Arg). Moreover, patient 3 has a higher residual neuraminidase activity (Table 1), milder symptoms and a longer lifespan. In addition, because sialidosis is a very rare disease with varying degrees of severity, there may be differences in the classification of patient's phenotypes at the time of diagnosis of the disease.

The amino acid substitution Tyr370Cys is of special interest, because it affects a residue that was shown in other sialidases to be one of the five active site residues (9,11,32,33). This, together with the fact that this renders the enzyme inactive, suggests that the catalytic machinery of lysosomal neuraminidase is similar to that of other sialidases (15), despite the dependence of the lysosomal enzyme on PPCA (12,15). Crystal structure analyses of several viral and bacterial sialidases revealed that all of them have a conserved six-bladed propeller fold and identical catalytic pocket. Although it is likely that the structure of lysosomal neuraminidase will be very similar to that of other sialidases, it is risky to make assumptions about the structural effects of the different neuraminidase mutations without the crystal structure of the mammalian enzyme (27). However, as mentioned earlier, the active sites of both viral and bacterial neuraminidases are strictly conserved. Therefore, we can predict the impact of the Tyr370 change on the 3D structure of the *Salmonella typhimurium* sialidase, which is the closest in primary structure to the lysosomal enzyme (9). In this structure, the tyrosine (Tyr342) approaches the sugar ring of the sialic acid and stabilizes the carbonium transition state intermediate (9). The substitution of Tyr342 with a Cys does not create steric clashes or loss of bonds with neighboring amino acids. Therefore, the sialic acid substrate could still enter the catalytic pocket. However, the smaller Cys residue is unable to interact with the sugar ring of the sialic acid and consequently, hydrolysis of the substrate cannot be achieved. This model would fit with our biochemical analysis that showed the synthesis of a normal amount of enzyme without catalytic activity.

The Asp-box motif is an eight amino acid domain [(S/W)xDxGx(S/T)(W/F)] that is repeated two to five times in all members of the sialidase superfamily, with the exception of the viral sialidases (3,10,36,37). The regions adjacent to the Asp-box motifs, although without an obvious consensus sequence, show a high degree of conservation. Asp-boxes are located at equivalent positions in the fold of the protein, far from the active site, with the aspartic acid residue exposed to the solvent. Their peripheral position indicates that they are not involved in the catalytic mechanism (9). However, they may have a function in maintaining the β-propeller fold of the enzyme and they may be involved in the initial recognition and binding of the substrate. Six of the mutations are either within, directly adjacent to or near an Asp-box motif. These mutations may affect the function of the Asp box, in that they may compromise the structural integrity of the domain or indirectly hamper the catalytic properties of the enzyme. The future determination of the crystal structure of lysosomal neuraminidase will provide the structural basis for these and other neuraminidase mutations.

The determination of the 3D structure of PPCA has already been of great value in understanding the mechanisms of maturation and catalytic activation of the enzyme and has given insight into the impact of specific mutations on the protein structure (38,39). In this report we show indirect evidence that the amino acid substitutions characterized did not prevent the binding of neuraminidase to PPCA. It is conceivable that the two proteins have multiple attachment sites for each other. Thus, the association between neuraminidase and PPCA will be abolished only if multiple amino acid residues are changed. In contrast with our findings, others have postulated that several neuraminidase mutations in sialidosis patients affect the binding with PPCA, which in turn promotes rapid intralysosomal degradation of the mutant proteins (27). A biochemical and structural understanding of the mode(s) of interaction between the two enzymes could clarify the differences and facilitate the design of novel therapeutic approaches for patients with GS or sialidosis.

MATERIALS AND METHODS

Cell culture

Human skin fibroblasts from a healthy person and from patients 7 and 9 were obtained from the European Cell Bank (Rotterdam, The Netherlands; Dr W.J. Kleijer). Sialidosis

fibroblasts GMO1718A (patient 10) were obtained from the NIGMS human genetic mutant cell repository (Camden, NJ). Fibroblasts of patients 1, 2 and 8 were obtained from Dr D. Wenger (Jefferson Medical College, Division of Medical Genetics, Philadelphia, PA). The other fibroblast strains were isolated in the laboratories of the clinicians who diagnosed the cases (3, 4, 6 and 11). The primary fibroblasts were maintained in Dulbecco's modified Eagle's medium, supplemented with antibiotics and 10% fetal bovine serum.

Northern blot analysis

Total RNA and poly(A)$^+$ RNA were isolated from the cultured fibroblasts by using Oligotex and RNAeasy mRNA purification kits (Qiagen, Valencia, CA). RNA was separated in a 1% agarose gel containing 0.66 M formaldehyde. After electrophoresis, the RNA was blotted onto a Zeta-Probe-GT membrane (BioRad, Hercules, CA) and hybridized in ExpressHyb (Clontech, Palo Alto, CA) at 68°C with the full-length human neuraminidase cDNA (12) or human PPCA cDNA (40). The blots were washed according to the manufacturer's protocol (Clontech) and exposed to X-ray film overnight.

Western blotting, immunocytochemistry and enzyme assays

For western blotting, fibroblasts grown to confluence in 85 mm Petri dishes were harvested by trypsinization and lysed in milli-Q water (Millipore, Bedford, MA). Fibroblast lysates (5 µg of protein) were subjected to SDS–PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). To assay the *in vitro* stability of neuraminidase, cell lysates (5 µg) were incubated at 37°C in phosphate-buffered saline in the presence or absence of a protease Inhibitor cocktail (complete protease inhibitors; Roche, Indianapolis, IN). The samples were subjected to SDS–PAGE and to western blotting. Western blots were incubated for 16 h with affinity-purified anti-neur antibodies (12) and with an affinity-purified anti-PPCA antibody that is specific for the 32 kDa mature subunit (anti-32) (12) as described earlier (41). After washing, the blots were incubated with peroxidase-conjugated anti-rabbit IgG secondary antibodies (Jackson Immuno-Research, West Grove, PA) and the bound antibodies were detected by using chemiluminescent substrate (Renaissance; DuPont NEN, Boston, MA).

For immunocytochemical analysis, fibroblasts were seeded onto Superfrost/Plus glass slides (Fisher, Houston, TX) and processed as described by Cullen (42) using anti-neur antibodies (16 h at room temperature) and FITC-conjugated secondary antibodies (Sigma-Aldrich, St Louis, MO). The nuclei were stained with 4′6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich), which was added to Vectashield mounting medium (Vector, Burlingame, CA).

For enzyme assays, fibroblasts were harvested as described above. Neuraminidase activity was assayed by using the synthetic 4-methylumbelliferyl-neuraminic acid (4MU) substrate (Sigma-Aldrich) as described (43). Total protein concentrations were measured with the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL).

PCR analysis

The amplification of mutant neuraminidase cDNAs was perfomed with PCR primers as previously described (12). Four overlapping cDNA fragments (~500 bp each) that encompassed the entire coding region of the neuraminidase gene were amplified by RT–PCR (Titan RT–PCR; Roche). The PCR products were purified with the Qiaquick Spin PCR purification kit (Qiagen) and subjected to automated direct sequencing by using internal neuraminidase-specific primers. Mutations were verified by PCR amplification of the corresponding exons and automated direct sequencing.

Transient expression of mutant neuraminidase cDNAs

To introduce neuraminidase mutations into the full-length cDNA, fragments (400–600 bp) containing the mutation of interest were excised from RT–PCR products by using two restriction endonucleases, each of which has only one recognition site in the neuraminidase cDNA (*Bsm*I, *Pst*I and *Sma*I). After electrophoresis in 1% agarose gels the DNA fragments were purified with Qiaex II (Qiagen). The mammalian expression plasmid pSCTOP (44), which contained the wild-type neuraminidase cDNA (12), was restricted at two of the three unique endonuclease sites to excise the corresponding wild-type fragment. The fragment carrying the mutation was then ligated into the plasmid. The constructs containing the full-length mutant neuraminidase cDNAs were then sequenced to ensure that the mutations had been correctly introduced.

Thirty micrograms of plasmid DNA (pSCTOP-neur, pSCTOP-neur mutants and pSCTOP-PPCA) was transfected by electroporation into fibroblasts as described (12). As an internal control for the electroporation efficiency, 3 µg of the plasmid CMV-LacZ was co-transfected. Three days after electroporation the cells were harvested and lysed in water. Neuraminidase activity was assayed as described above, and neutral β-galactosidase activity (10 mM Tris–HCl pH 7.5, 100 mM NaCl) was assayed by using the synthetic 4MU substrate (Sigma-Aldrich). To adjust for differences in transfection efficiencies, the neuraminidase values were normalized on the basis of neutral β-galactosidase activity calculated in each transfected sample. Superfrost/plus slides containing transfected fibroblasts were incubated with affinity-purified anti-neur and anti-PPCA antibodies and processed for immunocytochemical staining as described above. The absolute transfection efficiencies were determined by counting positively stained cells versus cells lacking staining. The transfection efficiencies ranged between 20 and 30%.

ACKNOWLEDGEMENTS

We thank Drs W. Kleijer, H.D. Bakker and D. Wenger for providing us with fibroblasts and sharing clinical information about the referred patients. We thank Charlette Hill for editing this manuscript. These studies were supported in part by National Institutes of Health grant RO1-GM60950, the Assisi foundation of Memphis, the Cancer Center Support Grant (CA 21765) and the American Lebanese Syrian Associated Charities (ALSAC) of St Jude Children's Research Hospital.

REFERENCES

1. Reuter, G. and Gabius, H.-J. (1996) Review: sialic acids. *Biol. Chem. Hoppe Seyler*, **377**, 325–342.
2. Saito, M. and Yu, R.K. (1995) Biochemistry and function of sialidases. In Rosenberg, A. (ed.), *Biology of the Sialic Acids*. Plenum Press, New York, NY, pp. 261–313.
3. Miyagi, T., Konno, K., Emori, Y., Kawasaki, H., Suzuki, K., Yasui, A. and Tsuiki, S. (1993) Molecular cloning and expression of cDNA encoding rat skeletal muscle cytosolic sialidase. *J. Biol. Chem.*, **268**, 26435–26440.
4. Ferrari, J., Harris, R. and Warner, T.G. (1994) Cloning and expression of a soluble sialidase from Chinese hamster ovary cells: sequence alignment similarities to bacterial sialidases. *Glycobiology*, **4**, 367–373.
5. Miyagi, T., Wada, T., Iwamatsu, A., Hata, K., Yoshikawa, Y., Tokuyama, S. and Sawada, M. (1999) Molecular cloning and characterization of a plasma membrane-associated sialidase specific for gangliosides. *J. Biol. Chem.*, **274**, 5004–5011.
6. Wada, T., Yoshikawa, Y., Tokuyama, S., Kuwabara, M., Akita, H. and Miyagi, T. (1999) Cloning, expression and chromosomal mapping of a human ganglioside sialidase. *Biochem. Biophys. Res. Commun.*, **261**, 21–27.
7. Fronda, C.L., Zeng, G., Gao, L. and Yu, R.K. (1999) Molecular cloning and expression of mouse brain sialidase. *Biochem. Biophys. Res. Commun.*, **258**, 727–731.
8. Monti, E., Preti, A., Rossi, E., Ballabio, A. and Borsani, G. (1999) Cloning and characterization of NEU2, a human gene homologous to rodent soluble sialidases. *Genomics*, **57**, 137–143.
9. Crennell, S.J., Garman, E.F., Laver, W.G., Vimr, E.R. and Taylor, G.L. (1993) Crystal structure of a bacterial sialidase (from *Salmonella typhimurium* LT2) shows the same fold as an influenza virus neuraminidase. *Proc. Natl Acad. Sci. USA*, **90**, 9852–9856.
10. Vimr, E. (1994) Microbial sialidases: does bigger always mean better? *Trends Microbiol.*, **2**, 271–277.
11. Gaskell, A., Crennell, S. and Taylor, G. (1995) The three domains of a bacterial sialidase: a β-propeller, an immunoglobulin module and a galactose-binding jelly-roll. *Structure*, **3**, 1197–1205.
12. Bonten, E., van der Spoel, A., Fornerod, M., Grosveld, G. and d'Azzo, A. (1996) Characterization of human lysosomal neuraminidase defines the molecular basis of the metabolic storage disorder sialidosis. *Genes Dev.*, **10**, 3156–3169.
13. Rottier, R., Bonten, E. and d'Azzo, A. (1998) A point mutation in the *neu-1* locus causes the neuraminidase defect in the SM/J mouse. *Hum. Mol. Genet.*, **7**, 313–321.
14. Pshezhetsky, A., Richard, C., Michaud, L., Igdoura, S., Wang, S., Elsliger, M., Qu, J., Leclerc, D., Gravel, R., Dallaire, L. *et al.* (1997) Cloning, expression and chromosomal mapping of human lysosomal sialidase and characterization of mutations in sialidosis. *Nature Genet.*, **15**, 316–320.
15. van der Spoel, A., Bonten, E. and d'Azzo, A. (1998) Transport of human lysosomal neuraminidase to mature lysosomes requires protective protein/cathepsin A. *EMBO J.*, **17**, 1588–1597.
16. Verheijen, F., Brossmer, R. and Galjaard, H. (1982) Purification of acid beta-galactosidase and acid neuraminidase from bovine testis: evidence for an enzyme complex. *Biochem. Biophys. Res. Commun.*, **108**, 868–875.
17. Verheijen, F.W., Palmeri, S., Hoogeveen, A.T. and Galjaard, H. (1985) Human placental neuraminidase. Activation, stabilization and association with beta-galactosidase and its protective protein. *Eur. J. Biochem.*, **149**, 315–321.
18. Yamamoto, Y. and Nishimura, K. (1987) Copurification and separation of β-galactosidase and sialidase from porcine testis. *J. Biol. Chem.*, **19**, 435–442.
19. Pshezhetsky, A.V. and Potier, M. (1994) Direct affinity purification and supramolecular organization of human lysosomal cathepsin A. *Arch. Biochem. Biophys.*, **313**, 64–70.
20. Pshezhetsky, A. and Potier, M. (1996) Association on N-acetylgalactosamine-6-sulfate sulfatase with the multienzyme lysosomal complex of β-galactosidase, cathepsin A and neuraminidase. *J. Biol. Chem.*, **271**, 28359–28365.
21. Hiraiwa, M. and Kishimoto, Y. (1996) Saposins and sphingolipid metabolisms. *Seikagaku*, **68**, 464–474.
22. Thomas, G.H. and Beaudet, A.L. (1995) Disorders of glycoprotein degradation and structure: α-mannosidosis, β-mannosidosis, fucosidosis, sialidosis, aspartylglucosaminuria and carbohydrate-deficient glycoprotein syndrome. In Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. (eds), *The Metabolic and Molecular Bases of Inherited Disease*. McGraw Hill, New York, NY, pp. 2529–2561.
23. Lowden, J. and O'Brien, J. (1979) Sialidosis: a review of human neuraminidase deficiency. *Am. J. Hum. Genet.*, **31**, 1.
24. Matsuo, T., Egawa, I., Okada, S., Suhtsugui, M., Yamamoto, K. and Watanabe, M. (1983) Sialidosis type 2 in Japan. Clinical study in two siblings cases and review of literature. *J. Neurol. Sci.*, **58**, 45.
25. Young, I.D., Young, E.P., Mossman, J., Fielder, A.R. and Moore, J.R. (1987) Neuraminidase deficiency: case report and review of the phenotype. *J. Med. Genet.*, **24**, 283–290.
26. d'Azzo, A., Andria, G., Strisciuglio, P. and Galjaard, H. (1995) Galactosialidosis. In Scriver, C., Beaudet, A., Sly, W. and Valle, D. (eds), *The Metabolic and Molecular Bases of Inherited Disease*. McGraw-Hill, New York, NY, pp. 2825–2838.
27. Lukong, K.E., Elsliger, M., Chang, Y., Richard, C., Thomas, G., Carey, C., Tylki-Szymanska, A., Czartoryska, B., Buchholz, T., Criado, G.R. *et al.* (2000) Characterization of the sialidase molecular defects in sialidosis patients suggests the structural organization of the lysosomal multienzyme complex. *Hum. Mol. Genet.*, **9**, 1075–1085.
28. Kelly, T.E. and Graetz, G. (1977) Isolated acid neuraminidase deficiency: a distinct lysosomal storage disease. *Am. J. Med. Genet.*, **1**, 31–46.
29. Kelly, T.E., Bartoshesky, L., Harris, D.J., McCauley, R.G., Feingold, M. and Schott, G. (1981) Mucolipidosis I (acid neuraminidase deficiency). Three cases and delineation of the variability of the phenotype. *Am. J. Dis. Child*, **135**, 703–708.
30. Bakker, H.D., Abeling, N.G.G.M., Staalman, C.R. and van Gennip, A.H. (1998) Thirty-years follow-up of a patient with sialidosis. *J. Inherit. Metab. Dis.*, **21**, 116.
31. Zhou, X.-Y., van der Spoel, A., Rottier, R., Hale, G., Willemsen, R., Berry, G.T., Strisciuglio, P., Andria, G. and d'Azzo, A. (1996) Molecular and biochemical analysis of protective protein/cathepsin A mutations: correlation with clinical severity in galactosialidosis. *Hum. Mol. Genet.*, **5**, 1977–1987.
32. Crennell, S., Garman, E., Laver, G., Vimr, E. and Taylor, G. (1994) Crystal structure of *Vibrio cholerae* neuraminidase reveals dual lectin-like domains in addition to the catalytic domain. *Structure*, **2**, 535–544.
33. Crennell, S., Garman, E., Philippon, C., Vasella, A., Laver, W., Vimr, E. and Taylor, G. (1996) The structures of *Salmonella typhimurium* LT2 neuraminidase and its complexes with three inhibitors at high resolution. *J. Mol. Biol.*, **259**, 264–280.
34. Kaufman, R.J. (1999) Molecular chaperones and the heat shock response. *Biochim. Biophys. Acta*, **1423**, R13–R27.
35. Meikle, P.J., Clague, A.E. and Carey, W.F. (1999) Prevalence of lysosomal storage disorders. *J. Am. Med. Assoc.*, **281**, 249–254.
36. Roggentin, P., Rothe, B., Kaper, J.B., Galen, J., Lawrisuk, L., Vimr, E.R. and Schauer, R. (1989) Conserved sequences in bacterial and viral sialidases. *Glycoconj. J.*, **6**, 349–353.
37. Roggentin, P., Schauer, R., Hoyer, L. and Vimr, E. (1993) MicroReview: the sialidase superfamily and its spread by horizontal gene transfer. *Mol. Microbiol.*, **9**, 915–921.
38. Rudenko, G., Bonten, E., d'Azzo, A. and Hol, W.G.J. (1995) Three-dimensional structure of the human 'protective protein': structure of the precursor form suggests a complex activation mechanism. *Structure*, **3**, 1249–1259.
39. Rudenko, G., Bonten, E., Hol, W. and d'Azzo, A. (1998) The atomic model of the human protective protein/cathepsin A suggests a structural basis for galactosialidosis. *Proc. Natl Acad. Sci. USA*, **95**, 621–625.
40. Galjart, N.J., Gillemans, N., Harris, A., van der Horst, G.T.J., Verheijen, F.W., Galjaard, H. and d'Azzo, A. (1988) Expression of cDNA encoding the human 'protective protein' associated with lysosomal beta-galactosidase and neuraminidase: homology to yeast proteases. *Cell*, **54**, 755–764.
41. Bonten, E.J., Galjart, N.J., Willemsen, R., Usmany, M., Vlak, J.M. and d'Azzo, A. (1995) Lysosomal protective protein/cathepsin A: role of the 'linker' domain in catalytic activation. *J. Biol. Chem.*, **270**, 26441–26445.
42. Cullen, B.R. (1987) Use of eukaryotic expression technology in the functional analysis of cloned genes. *Methods Enzymol.*, **152**, 684–704.
43. Galjaard, H. (1980) *Genetic Metabolic Disease: Diagnosis and Prenatal Analysis.* Elsevier Science, Amsterdam, The Netherlands.
44. Fornerod, M., Boer, J., van Baal, S., Jaegle, M., von Lindern, M., Murti, K.G., Davis, D., Bonten, J., Buijs, A. and Grosveld, G. (1995) Relocation of the carboxyterminal part of CAN from the nuclear envelope to the nucleus as a result of leukemia-specific chromosome rearrangements. *Oncogene*, **10**, 1739–1749.