

# Characterization of human lysosomal neuraminidase defines the molecular basis of the metabolic storage disorder sialidosis

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Neuraminidases (sialidases) have an essential role in the removal of terminal sialic acid residues from sialoglycoconjugates and are distributed widely in nature. The human lysosomal enzyme occurs in complex with  $\beta$ -galactosidase and protective protein/cathepsin A (PPCA), and is deficient in two genetic disorders: sialidosis, caused by a structural defect in the neuraminidase gene, and galactosialidosis, in which the loss of neuraminidase activity is secondary to a deficiency of PPCA. We identified a full-length cDNA clone in the dbEST data base, of which the predicted amino acid sequence has extensive homology to other mammalian and bacterial neuraminidases, including the F(Y)RIP domain and "Asp-boxes." In situ hybridization localized the human neuraminidase gene to chromosome band 6p21, a region known to contain the HLA locus. Transient expression of the cDNA in deficient human fibroblasts showed that the enzyme is compartmentalized in lysosomes and restored neuraminidase activity in a PPCA-dependent manner. The authenticity of the cDNA was verified by the identification of three independent mutations in the open reading frame of the mRNA from clinically distinct sialidosis patients. Coexpression of the mutant cDNAs with PPCA failed to generate neuraminidase activity, confirming the inactivating effect of the mutations. These results establish the molecular basis of sialidosis in these patients, and clearly identify the cDNA-encoded protein as lysosomal neuraminidase.

[Key Words: Neuraminidase; lysosome; sialidosis; galactosialidosis; protective protein/cathepsin A; mutations]

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Neuraminidases (sialidases) constitute a large, diverse family of hydrolytic enzymes known to occur in a variety of organisms, including viruses, bacteria, protozoa, and vertebrates (Miyagi et al. 1993; Roggentin et al. 1993; Warner et al. 1993; Colman 1994; Schenkman et al. 1994; Chou et al. 1996). The wide distribution of sialidases reflects their indispensable role in the catabolism of sialic acids from various sialoglycoconjugates, which, in turn, are required for important cellular processes (Corfield et al. 1992a; Saito and Yu 1995; Schauer et al. 1995; Reuter and Gabius 1996). Sialidases have been implicated both directly and indirectly in a number of human pathologic conditions, including infectious diseases and genetic disorders of metabolism. Accordingly, a wealth of information is available on bacterial, viral, and protozoan sialidases (Corfield 1992b; Roggentin et al. 1989, 1993). For instance, in pathogenic bacteria such as *Vibrio cholerae*, the neuraminidase is thought to act as a virulence factor by uncovering toxin binding sites (Galen

et al. 1992). The neuraminidase of influenza virus, on the other hand, is needed apparently for both virion entry into lung and intestinal mucosa and for virus budding from the infected host cell (Colman 1989, 1994). Comparison of the primary structures of microbial and viral sialidases has revealed that the nonviral enzymes have an overall sequence identity of ~35%, and that they all contain the so-called F(Y)RIP domain located amino-terminally from a series of "Asp boxes" [consensus sequence Ser/Thr-X-Asp-(X)-Gly-X-Thr-Trp/Phe], that appear two to five times depending on the protein (Roggentin et al. 1993; Warner et al. 1993). Crystal structure analysis has shown that the active site of these enzymes is located in a conserved six-bladed  $\beta$ -propeller domain of ~40 kD. The arginine of the FRIP motif is part of the active site, being located in the center of the propeller, whereas the Asp boxes are found on the periphery and seem to have a structural role (Gaskell et al. 1995).

In contrast to the microbial and viral enzymes, information on the mammalian neuraminidases is more limited. The apparent low abundance, labile nature, and in some instances membrane association of these enzymes

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are features that have made their biochemical and genetic characterization difficult. Three mammalian neuraminidases, which differ in substrate preference, pH optimum, and subcellular localization, are known to date. They are the cytosolic, plasma membrane, and lysosomal neuraminidases. One of the best characterized is the cytosolic enzyme, which has been purified to homogeneity from rat liver and skeletal muscle, and its cDNA and gene have been cloned (Miyagi et al. 1990a, 1993; Miyagi and Tsuiki 1985; Sato and Miyagi 1995). In addition, the cDNA encoding a soluble sialidase, originally purified from the culture medium of Chinese hamster ovary (CHO) cells, but probably of cytosolic origin, also has been isolated (Warner et al. 1993; Ferrari et al. 1994). These two enzymes are 88% homologous at the amino acid level and both contain the FRIP domain and Asp boxes (Ferrari et al. 1994). Cytosolic sialidase is active at pH 6.5 and is expressed highly in skeletal muscle where it may have a role in myoblast differentiation (Sato and Miyagi 1996).

The plasma membrane neuraminidase, which is specific for ganglioside substrates, has been partially purified from brain tissues (Tettamanti et al. 1972; Miyagi et al. 1990a), although low levels have been measured in other tissues (Lieser et al. 1989; Zeigler et al. 1989). In addition to its acidic pH optimum, which is also characteristic of the lysosomal neuraminidase, the plasma membrane enzyme seems to bear biochemical and immunological properties distinct from those of its lysosomal counterpart (Miyagi et al. 1990b; Schneider-Jakob and Cantz 1991), and appears unaffected in disorders associated with the lysosomal neuraminidase (Lieser et al. 1989; Zeigler et al. 1989; Schneider-Jakob and Cantz 1991). It is still unclear, however, whether the cytosolic and plasma membrane enzymes really represent discrete proteins or merely different forms of the same enzyme.

Lysosomal N-acetyl- $\alpha$ -neuraminidase initiates the hydrolysis of oligosaccharides, gangliosides, glycolipids, and glycoproteins by removing their terminal sialic acid residues. The human enzyme has a preference for  $\alpha$ -2 $\rightarrow$ 3 and  $\alpha$ -2 $\rightarrow$ 6 sialyl linkages and is thought to act primarily on oligosaccharide and glycopeptide substrates (Frisch and Neufeld 1979; Cantz 1982), but can hydrolyze gangliosides with the aid of detergents or the sphingolipid activator Sap B (Schneider-Jakob and Cantz 1991; Fingerhut et al. 1992). Biochemical characterization of lysosomal neuraminidase has been difficult because it is extremely labile on extraction and may be membrane-bound. Since the first report by Verheijen et al. (1982), several other studies have established that neuraminidase activity can be recovered in mammalian tissues as part of a large molecular mass complex that contains the glycosidase,  $\beta$ -galactosidase, and the carboxypeptidase protective protein/cathepsin A (PPCA). It is thought that by associating with PPCA, neuraminidase and  $\beta$ -galactosidase acquire their active and stable conformation in lysosomes (d'Azzo et al. 1995). Biochemical evidence for the existence of the three-enzyme complex comes primarily from copurification studies. In particular, the

three enzymes can be isolated together using either  $\beta$ -galactosidase or PPCA affinity matrices (Verheijen et al. 1985; Yamamoto and Nishimura 1987; Potier et al. 1990; Scheibe et al. 1990; Pshezhetsky and Potier 1994). Only a small percentage of  $\beta$ -galactosidase and PPCA activities are consistently found in the complex, which nevertheless contains all of the neuraminidase activity. These studies support the notion that lysosomal neuraminidase activity cannot be isolated separately from the complex, whereas the other two hydrolases can exist in alternative forms (Hoogveen et al. 1983; Hubbes et al. 1992; and references above). The small yield of neuraminidase activity recovered after different purification procedures has led to inconsistent assignment of a molecular weight to the enzyme (Verheijen et al. 1987; van der Horst et al. 1989; Warner et al. 1990).

Our interest in human lysosomal neuraminidase stems from its direct involvement in two genetically distinct inborn errors of metabolism: sialidosis, which is caused by structural lesions in the lysosomal neuraminidase locus (Thomas and Beaudet 1995), and galactosialidosis, a combined deficiency of neuraminidase and  $\beta$ -galactosidase (Wenger et al. 1978; Andria et al. 1981; d'Azzo et al. 1995) caused by the absence of PPCA (d'Azzo et al. 1982). Sialidosis and galactosialidosis patients accumulate sialylated oligosaccharides and glycopeptides in tissues and excrete abnormal quantities of these compounds in urine and body fluids (van Pelt et al. 1988a,b,c). Different clinical forms of sialidosis are distinguished according to the age of onset and the severity of the symptoms (Thomas and Beaudet 1995). Type I is a mild form of the disease, corresponding to the cherry-red-spot-myoclonus syndrome. Symptoms appear in the second decade of life and are restricted to myoclonus and progressive impaired vision. Type II sialidosis has onset at birth or early infancy and is associated with progressive neurologic deterioration and mental retardation. Residual neuraminidase activity, measured in patients' fibroblasts and leukocytes, varies from 0% to 10% of control values (Thomas and Beaudet 1995). The gene defect in a type II sialidosis patient was mapped by Mueller et al. (1986) to chromosome 10. However, analysis of a female patient with infantile sialidosis type II and congenital adrenal hyperplasia, caused by 21-hydroxylase deficiency, suggested that the neuraminidase gene could be linked to the HLA locus, which is on chromosome 6 (Oohira et al. 1985; Harada et al. 1987).

Here we report the isolation and characterization of a human cDNA that was identified through its homology with other known sialidases. Expression of the cDNA in COS-1 cells and in patient fibroblasts confirmed the lysosomal nature of the encoded protein. Further, the increase in neuraminidase activity was strictly dependent on the presence of PPCA, an absolute requirement for physiologic enzyme activity. Our cDNA mapped to chromosome band 6p21, known to contain the HLA locus. In addition, we identified independent mutations in the mRNA of a type I and a type II sialidosis patient, which were shown to inactivate the enzyme. Taken together, these data provide compelling evidence that this

cDNA encodes human lysosomal neuraminidase, and they define the molecular basis of sialidosis.

## Results

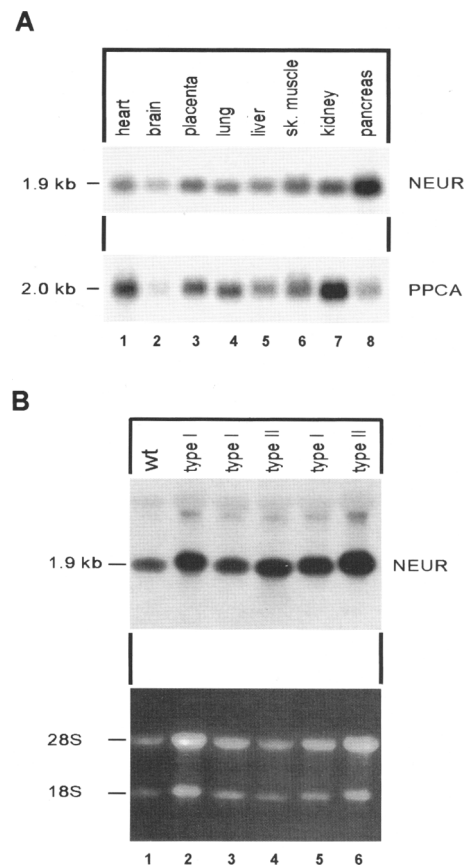
### *Expression of human neuraminidase mRNA and chromosomal localization*

Given the degree of similarity among sialidases from different species, we reasoned that if cDNAs representing the human lysosomal neuraminidase were present as expressed sequence tags (ESTs) in the dbEST computer data base (Boguski 1995), the enzyme might be cloned by screening the data base with the text string "neuraminidase or sialidase" by using an input device located on the World Wide Web (see also Materials and Methods). Therefore, cDNA sequence documents would be returned by virtue of attached protein mapping data containing the word neuraminidase or sialidase. Following this strategy, we found a putative neuraminidase cDNA clone (neur cDNA) of 1894 nucleotides, that showed a favorable alignment at the amino acid level to several bacterial sialidases and included a potential ATG translation initiation codon and a canonical polyadenylation signal. Hybridization of a Northern blot containing multiple human tissue poly(A)<sup>+</sup> RNAs with this cDNA revealed a single transcript of ~1.9 kb in all tissues, indicating that the acquired cDNA was full-length (Fig. 1A). The neur transcript appeared to be most abundant in pancreas and was expressed at relatively low levels in brain. Reprobing the Northern blot with PPCA cDNA showed remarkably similar expression patterns for the two mRNAs, with the exception of pancreas, where neur expression was clearly higher than that of PPCA, and vice versa for kidney. However, Northern blot analysis of five type I and type II sialidosis patients did not reveal any irregularities or abnormalities in the 1.9-kb transcript (Fig. 1B, upper panel). The only differences in intensity of the hybridizing bands were attributable to variations in the amount of RNA applied to the gel (Fig. 1B, lower panel).

In situ hybridization of metaphase chromosome spreads with either the 1.8-kb cDNA or a 3.5-kb genomic PCR product localized the neuraminidase gene to chromosome band 6p21 (Fig. 2), a chromosomal region known to contain the HLA locus. This confirmed previous observations that suggested an association between sialidosis and the HLA locus (Oohira et al. 1985).

### *Neur cDNA encodes a protein with sequence homology to bacterial and mammalian sialidases*

The sequence of the 1.9-kb cDNA showed an open reading frame (ORF) of 1245 nucleotides encoding a protein of 415 amino acids (Fig. 3A). The first 45 residues of the amino terminus have typical characteristics of a signal sequence (von Heijne 1986): a positively charged amino-terminal region (residues 1–18), a central hydrophobic core (residues 19–38), and a more polar carboxy-terminal domain (residues 39–45). Ser43, Ser45, and Trp44 con-

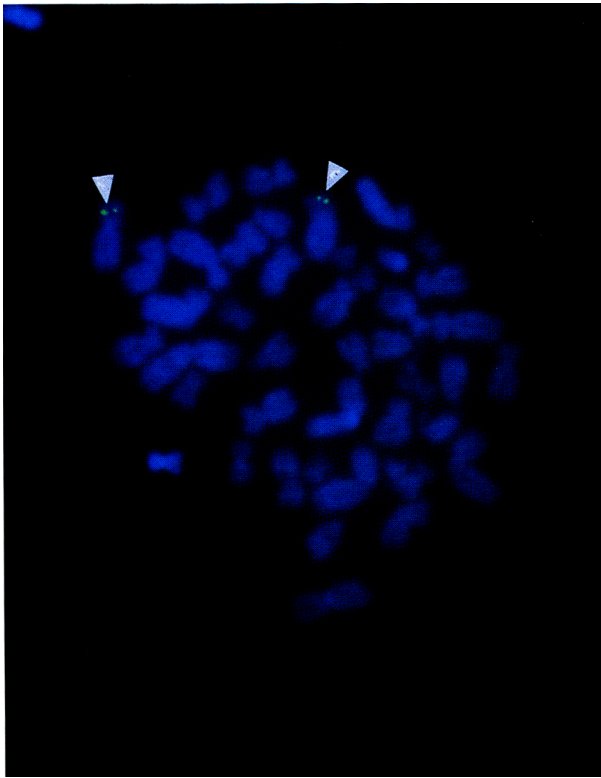


**Figure 1.** Neuraminidase mRNA expression. (A) Sequential hybridization of a multitissue Northern blot with the full-length neuraminidase cDNA (NEUR) and with the protective protein/cathepsin A (PPCA) cDNA. The size of the two transcripts was calculated on the basis of RNA markers. Exposure time for both hybridizations was 24 hr. (B) Northern blot (upper panel) containing RNA (~10 µg) isolated from the cultured fibroblasts of a normal individual (wt), three type I sialidosis patients, and two type II sialidosis patients, hybridized with the full-length 1.9-kb neuraminidase cDNA. The exposure time was 3 days. The lower panel shows the ethidium bromide stained RNA gel for comparison of RNA quantities.

form to the rules for amino acids at positions –1, –3 (small and uncharged), and –2 (large, bulky, or charged) with respect to signal sequence cleavage sites (von Heijne 1986). The protein also contains a FRIP domain, as well as three conserved and two degenerated Asp boxes. There are three potential Asn-linked glycosylation sites, at positions 185, 343, and 352, the last of which lies in the middle of Asp box V. The predicted molecular mass of the neuraminidase protein is 45.467 kD, which reduces to 40.435 kD after removal of the signal sequence. Assuming that glycosylation occurs at all three sites, with the consequent addition of ~6 kD, the estimated size of the protein would be 45 kD, which assigns the human enzyme to the low molecular mass group of sialidases (Crennell et al. 1996).

The human neuraminidase shares extensive homology





**Figure 2.** Chromosomal localization of human neuraminidase. Normal metaphase chromosomes were hybridized with a 3.5-kb neuraminidase genomic fragment, labeled with digoxigenin dUTP, and stained with antidigoxigenin antibodies. The white arrowheads indicate the hybridization signals on the chromatids of both copies of chromosome 6.

with other members of the sialidase superfamily, including bacteria, rodents, protozoa, and influenza virus. The rodent cytosolic neuraminidase and six bacterial sialidases appeared to be the most closely related to the human enzyme. Because of the variation in sizes among the different sialidases used in the alignment, the entire human sequence, excluding the signal peptide, was compared with only the fully overlapping regions of the other enzymes (Fig. 3B). The F(Y)RIP domain occurs in all eight neuraminidases. Interestingly, the extent of homology among the five Asp boxes identified in the different proteins gradually decreases from the first (most amino-terminal) to the fifth (most carboxy-terminal). It is worth noting that the rodent cytosolic neuraminidase lacks the first and most conserved Asp box, which may indicate that this motif confers biochemical specificity to the enzyme. The number of residues between the F(Y)RIP domain and the first Asp box is highly conserved among all low molecular mass neuraminidases, and the human lysosomal protein shares this feature. The extent of homology, including identical and conserved residues, lies between 32% and 38%, with the *Micromonospora viridifaciens* and the *Clostridium perfringens* sialidases being the most homologous, and the *Salmonella typhimu-*

*rium* the least (Roggentin et al. 1993). It is surprising that the human neuraminidase is overall more homologous to most of the bacterial sialidases than it is to the cytosolic enzyme from Chinese hamster and rat.

The similar expression patterns of the neur and PPCA mRNAs, together with the neuraminidase primary structure data, strongly suggest that the isolated cDNA encodes a mammalian neuraminidase that is clearly distinct from the cytosolic enzyme.

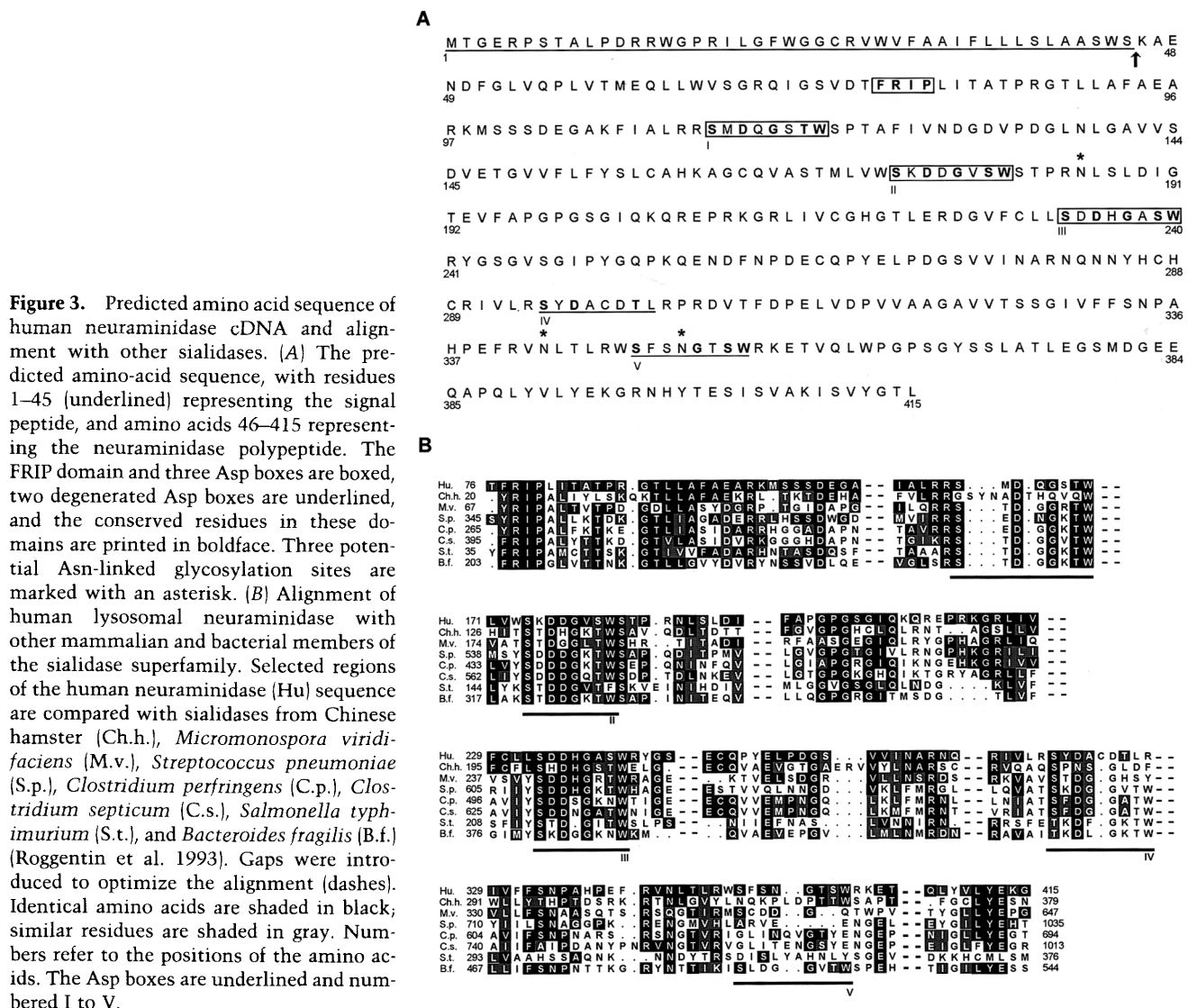
#### Subcellular localization and enzymatic activation

To assess the lysosomal nature of the protein encoded by the cDNA, we determined its intracellular distribution, catalytic properties, and, most important, dependence on PPCA and/or  $\beta$ -galactosidase for enzymatic activation. In single transfected cells, overexpression of the neur cDNA gave rise to a protein with a clear lysosome-like distribution, as evidenced by the punctated staining pattern (Fig. 4A; N). This pattern was analogous to that observed in cells overexpressing the PPCA cDNA and probed with the anti-PPCA antibody  $\alpha$ -BV32 (Fig. 4A; P). Surprisingly, in a significant number of neuraminidase-expressing cells, square crystal-like structures were recognized by the  $\alpha$ -neur antibody in the perinuclear region. These structures were present either alone (Fig. 4A; N, upper right) or in combination with lysosomal staining (Fig. 4A; N, upper left). The size and total number of crystals varied (Fig. 4, N, cf. upper left with upper right) and appeared to be inversely proportional to the amount of lysosomal staining.

Apparently, the protein aggregates when produced in large amounts at the site of synthesis in the endoplasmic reticulum. When the neur cDNA was coexpressed with the PPCA (Fig. 4A; N/P) or  $\beta$ -gal cDNAs, the intracellular distribution of neuraminidase, in both lysosome-like structures and crystals, was comparable to that observed in single-transfected cells. However, the crystals were recognized only by the  $\alpha$ -neur antibodies and not by anti-PPCA or anti- $\beta$ -gal antibodies (data not shown), indicating that they were devoid of PPCA and  $\beta$ -gal. From these results we infer that neuraminidase, when overexpressed in COS-1 cells, is independent of PPCA for its lysosomal-like compartmentalization, unless it is able to use the endogenous simian PPCA.

On Western blots prepared with lysates of COS-1 cells transfected with neur cDNA alone or together with the PPCA cDNA, the  $\alpha$ -neur antibodies recognized two major bands of 46 and 44 kD and some smaller, minor forms (Fig. 4B, lanes 2 and 4). These molecular weights closely conform with the predicted size for the glycosylated protein. After deglycosylation with *N*-glycosidase F, a single band of 40 kD stained with the antibodies, indicating that the neuraminidase polypeptide occurs in at least two differentially glycosylated forms (Fig. 4B, lane 6).

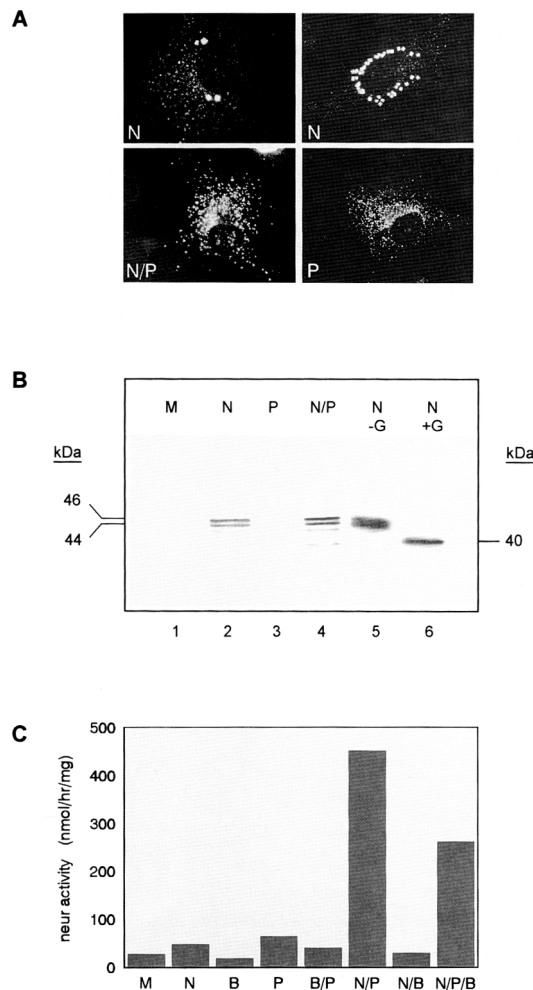
Cell homogenates from transfected COS-1 cells were assayed for neuraminidase activity using the artificial substrate 2'-(4-methylumbelliferyl)- $\alpha$ -D-*N*-acetylneuraminic acid at pH 4.3, which is optimal for detecting lysosomal neuraminidase. As seen in Figure 4C, cells



expressing either neuraminidase (N) or PPCA (P) had 1.5–2.0 times higher neuraminidase activity than mock (M) or  $\beta$ -gal (B) transfected cells. However, in cells cotransfected with neuraminidase and PPCA (N/P), the activity was 16-fold higher than in mock transfected cells (M). This increase was less pronounced, although still substantial (ninefold), in cells expressing all three enzymes together (N/P/B). No change in activity was observed in cells cotransfected with the neur and  $\beta$ -galactosidase cDNAs (N/B). These data strongly support the notion that the presence of PPCA, but not  $\beta$ -galactosidase, is essential for neuraminidase activity. Although the kinetics and mode of association of the three enzyme complex is unknown, the relatively low neuraminidase activity in cells transfected with the  $\beta$ -gal cDNA (N/B and N/P/B) could reflect competition between neuraminidase and  $\beta$ -galactosidase for binding sites on PPCA. Taken together, these results provide compelling evidence that the isolated cDNA encodes human lysosomal neuraminidase.

#### PPCA-dependent correction of neuraminidase activity in deficient fibroblasts

Cultured skin fibroblasts from one of two siblings with type I juvenile sialidosis and from a type II neonatal case were selected to ascertain whether the neur cDNA could correct their enzyme deficiencies. In addition, cells from an mRNA-negative galactosialidosis patient were used to establish the PPCA-dependent activation of the enzyme on a PPCA null background. To optimize expression in human cells, we subcloned the neur and PPCA cDNAs into the expression vector pSC-TOP, which contains the strong cytomegalovirus promoter (see Materials and Methods). Cells electroporated with either the neur cDNA construct, the PPCA cDNA construct, or both were tested for neuraminidase subcellular localization and enzymatic activity. Immunofluorescent staining of transfected cells with  $\alpha$ -neur antibodies is shown in Figure 5A. The endogenous neuraminidase in mock-transfected control fibroblasts (WT/M) displayed a typi-



**Figure 4.** Immunocytochemical localization and neuraminidase activity in transiently transfected COS-1 cells. (A) Immunocytochemical localization of neuraminidase in COS-1 cells, transfected with the neur cDNA clone (N), or with both the neur and PPCA cDNAs (N/P), using affinity-purified  $\alpha$ -neur antiserum. In addition, cells were transfected with the PPCA cDNA alone and processed for immunofluorescence with  $\alpha$ -32 antiserum (P). Magnification, 400 $\times$ . (B) Western blots prepared with equal amounts of protein (5  $\mu$ g) from COS-1 cell lysates, transfected with vector (M), neur cDNA (N), PPCA cDNA (P), or both neur and PPCA cDNAs (N/P). Aliquots of lysates from neuraminidase-overexpressing COS-1 cells were incubated either without (N, -G) or with N-glycosidase F (N, +G). The blots were incubated with affinity purified  $\alpha$ -neur antiserum. (C) COS-1 cells were transfected with vector alone (M), neur cDNA (N), PPCA cDNA (P),  $\beta$ -galactosidase cDNA (B), or a combination of these, as indicated. Seventy-two hours post-transfection, cells were harvested and assayed for acidic neuraminidase activity.

cal punctated lysosomal pattern. In contrast, the endogenous neuraminidase in mock-transfected sialidosis (S/M) and galactosialidosis (GS/M) cells was below the level of detection. The punctated pattern was restored when sialidosis fibroblasts were transfected with neur cDNA (S/N) or cotransfected with neur and PPCA

cDNAs (data not shown). We also reestablished the lysosomal localization of neuraminidase in PPCA-deficient cells by transfecting them with PPCA cDNA (GS/P). Overexpression of the neur cDNA alone in galactosialidosis cells (GS/N) created a lysosomal staining pattern, despite the absence of PPCA. These data prove that overexpressed neuraminidase does not require PPCA to reach a lysosome-like compartment.

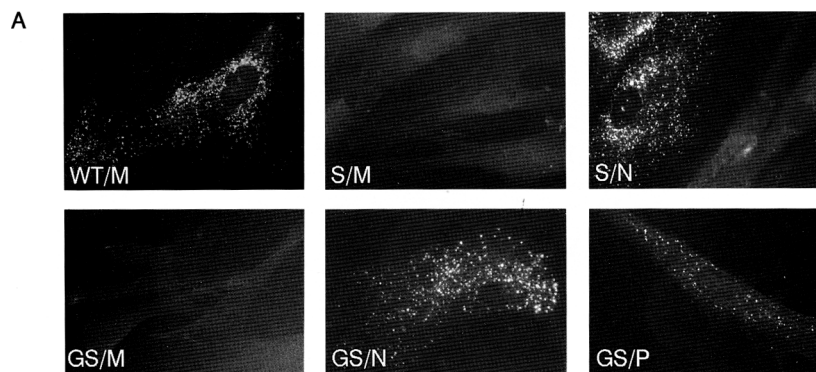
We next tested neuraminidase activity in these transfected fibroblasts (Fig. 5B). Because electroporation efficiency varied among the different cell strains, the relative enzyme activities were compared only within transfections of the same strain. Transfections with the neur cDNA alone (N) raised the endogenous neuraminidase activity slightly in wild-type cells (WT), but generated enzyme activity in fibroblasts from sialidosis patients (S type I and II), demonstrating that both types of sialidosis result from a primary defect in the lysosomal neuraminidase. Despite the apparent lysosomal distribution of neuraminidase in neur-transfected galactosialidosis cells (see above), no increase in activity was measured (GS, N), again demonstrating that neuraminidase is inactive without PPCA. Transfections with PPCA alone (P) did not alter neuraminidase activity in wild-type cells, failed to correct the two sialidosis strains, and only slightly induced activity in galactosialidosis cells. The largest increase in neuraminidase activity was measured when the neur and PPCA cDNAs were coexpressed (N/P).

#### *Lysosomal neuraminidase is mutated in type I and type II sialidosis patients*

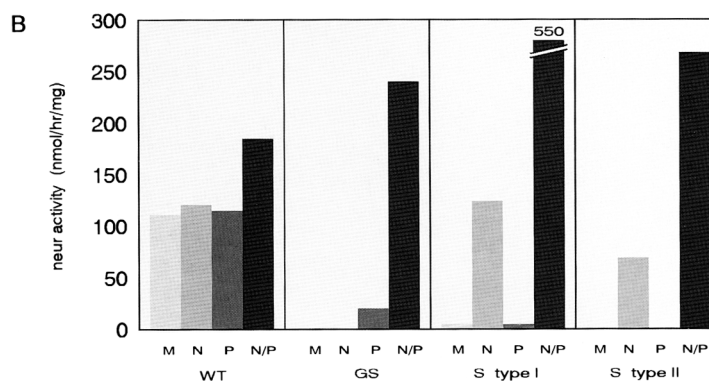
Because we were unable to detect any cross-reactive material in either the sialidosis or the galactosialidosis fibroblasts with immunofluorescence, we tried to immunoprecipitate the protein from radiolabeled deficient cells (Fig. 6). In normal fibroblasts, the  $\alpha$ -neur antibodies recognized a polypeptide of  $\sim$ 45 kD, that resolved on SDS-polyacrylamide gels as a broad heterogeneous band that probably represented different glycosylated forms of the enzyme (Fig. 6, lane 1). Cells from both type I sialidosis siblings, the type II sialidosis patient, and the galactosialidosis patient also contained the neuraminidase polypeptide but a much smaller amount (Fig. 6, lanes 2–5). In addition, the type II sialidosis cells contained a 53-kD product in an equimolar ratio with the 45-kD species (Fig. 6, lane 3). Because the sialidosis patients had apparently normal amounts of neuraminidase mRNA, the severely reduced quantities of protein recovered from these fibroblasts could be attributed to decreased stability of the mutant enzyme.

We then searched for mutations in the neur gene of these patients by direct sequencing of reverse transcriptase (RT)-PCR-synthesized cDNAs. As indicated in Figure 7A (left panel), both siblings with type I sialidosis were heterozygous for a G to T transversion at nucleotide 1258 of their neur cDNA, which introduced a premature TAG termination codon at amino acid 377. The mutant protein would then have a carboxy-terminal truncation of 38 amino acids. The type II sialidosis pa-

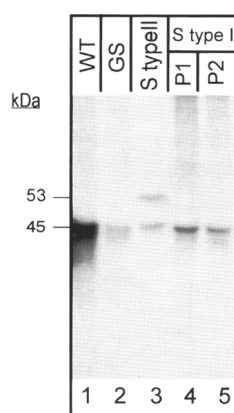




**Figure 5.** Correction of neuraminidase deficiency in sialidosis fibroblasts. (A) Immunocytochemical localization of neuraminidase in fibroblasts of a normal individual (WT), a sialidosis type I patient (S), and a galactosialidosis patient (GS), transfected with vector (M), neur cDNA (N), and PPCA cDNA (P). Cells were stained with affinity-purified  $\alpha$ -neur antiserum. Magnification, 400 $\times$ . (B) Neuraminidase activities in fibroblast cell lysates from a normal individual (WT), a galactosialidosis patient (GS), a sialidosis type I patient (S type I), and a sialidosis type II patient (S type II), transfected as described in Fig. 4C.



tient had one allele carrying a T to G transversion at nucleotide 401 and the other allele bearing a single-base deletion at nucleotide 1337 (Fig. 7A, right panel). The point mutation gave rise to the amino acid substitution Leu91Arg, whereas the base deletion caused a frameshift



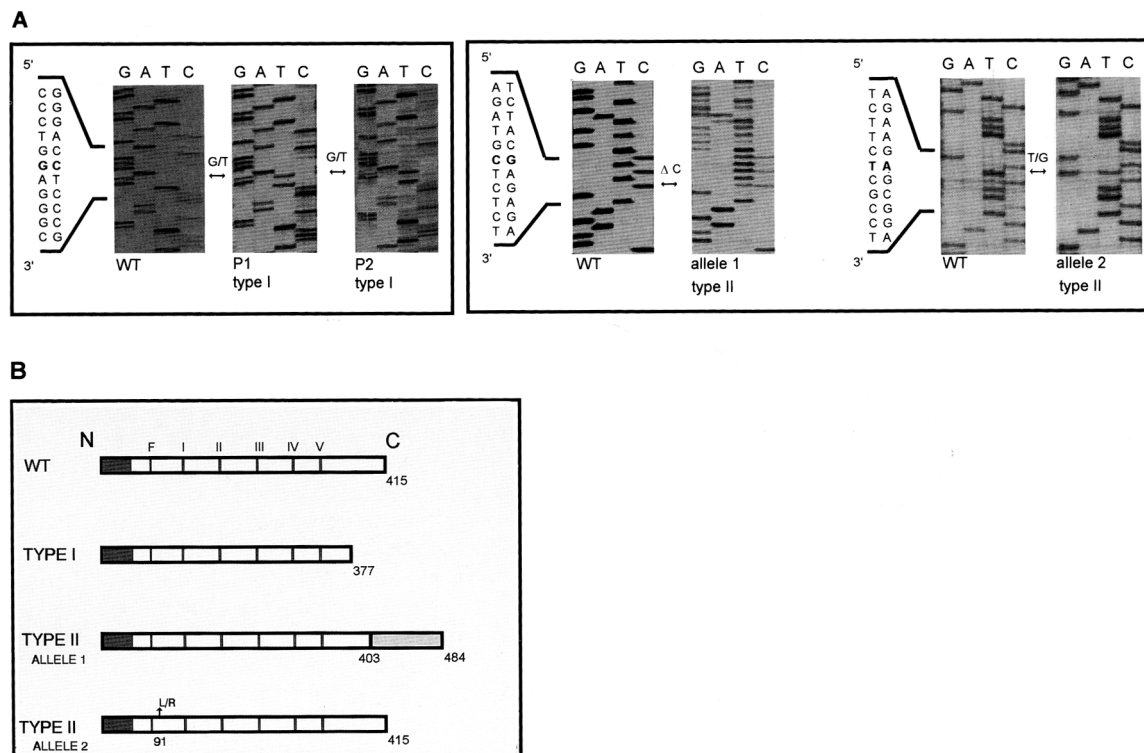
**Figure 6.** Immunoprecipitation of neuraminidase from sialidosis fibroblasts. Cultured fibroblasts from a normal individual (WT), an E.I galactosialidosis patient (GS), a type II sialidosis patient (S type II), and two siblings with type I sialidosis (S type I, P1, and P2) were labeled metabolically. The radiolabeled proteins were immunoprecipitated with  $\alpha$ -neur antibodies, and resolved by SDS-PAGE through a 12.5% gel. Estimated molecular masses are indicated left.

at amino acid 403 that extended the protein by 69 amino acids, which explained the presence of the 53-kD protein in the patient's fibroblasts (Fig. 6, lane 3).

Site-directed mutagenized cDNAs with either the type I mutation (premature stop) or the type II mutation (longer protein) were expressed alone or together with the PPCA cDNA in deficient fibroblasts and COS-1 cells. Western blot analysis of transfected cell lysates confirmed that the cDNA-encoded proteins had abnormal molecular masses: 53 kD for the type II mutation, and 41 kD for the type I mutation (Fig. 8). Both mutant proteins aberrantly localized to the perinuclear region but no lysosomal staining or neuraminidase activity was noted, regardless of whether PPCA was present (data not shown and Table 1). These data confirm that these clinically relevant mutations produce nonfunctional neuraminidase.

## Discussion

The comprehensive characterization of lysosomal neuraminidase has eluded investigators for many years because of the protein's apparent lability during purification procedures and its presumed membrane-bound character. Although these features have hampered the molecular cloning of this enzyme by conventional methods, interest in this important component of the lysosomal system has persisted for several reasons. The enzyme has a pivotal role in the intralysosomal degrada-



**Figure 7.** Mutations in the lysosomal neuraminidase gene of type I and type II sialidosis patients. (A) Partial nucleotide sequence of the neuraminidase cDNA from sialidosis patients. Total RNA was isolated from the fibroblasts of a normal individual (WT), the two siblings with type I sialidosis (P1 and P2, *left*) and the patient with type II sialidosis (*right*). This RNA was connected to cDNA by RT-PCR and the cDNA was directly sequenced through asymmetric PCR. In the *left* panel a G to T transversion at nucleotide 1258 in the neuraminidase cDNA is indicated (P1 and P2, *left* panel). This transversion creates a premature stop codon. In the type II sialidosis patient, a deletion of a G at position 1337 in the cDNA (*right* panel, allele 1, antisense sequence is shown) causes a frame shift that results in a longer ORF. The same patient has a T to G transversion at position 401, which causes an amino acid substitution at position 91 in the protein (*right* panel, allele 2). (B) Schematic representation of the type I and type II mutant neuraminidase polypeptides. Shown are the normal protein (wt), with amino-terminal signal peptide (gray shaded), and the conserved and degenerated Asp boxes (numbered I to V). The type I sialidosis mutation gives rise to a truncated polypeptide of 377 amino acids (type I). Allele 1 of the type II sialidosis patient (type II, allele 1), yields a longer protein of 484 amino acids that has a unique stretch of amino acids at the carboxyl terminus (shaded in gray). The second allele of the type II sialidosis patient (type II, allele 2) has a Leu to Arg amino-acid substitution at position 91 (L/R).

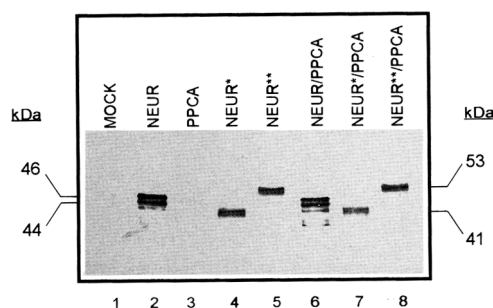
tion of sialoglycoconjugates catalyzing the release of terminal sialic acids, which, in turn, triggers further degradation of the sugar moiety. If this pivotal role is disrupted, the defective enzyme contributes to two lysosomal storage disorders: sialidosis and galactosialidosis. The former is caused by structural defects in neuraminidase itself, whereas the latter results from a primary deficiency of PPCA, a pleiotropic serine carboxypeptidase that is essential for neuraminidase activity (d'Azzo et al. 1995). In fact, neuraminidase activity is strictly dependent on the enzyme being part of a three-enzyme complex that includes PPCA and  $\beta$ -galactosidase.

In our effort to isolate the neur cDNA, we took advantage of the growing number of random, uncharacterized human cDNA sequences that are deposited daily in the dbEST data base. This "computer cloning" approach allowed us to identify >30 overlapping neuraminidase cDNA clones, many of which are royalty-free and available through the Integrated Molecular Analysis of Ge-

nomes and their Expression (IMAGE) Consortium. In principle, this system could be used to identify other human proteins of known function that have resisted conventional molecular cloning. Only two criteria must be met: The cDNA clones representing the protein must be present in the dbEST data base, and the protein must have some sequence homology to known proteins with a similar function in other organisms. In addition, care must be taken to ensure that the cDNAs do encode human mRNAs and are not derived from contaminating organisms.

Our neur cDNA clone recognizes an mRNA of ~1.9 kb, that is ubiquitously but differentially expressed in human tissues. By using this cDNA to localize the human neuraminidase gene to chromosomal band 6p21, in a region known to contain the HLA locus, we were able to not only establish that we had the correct cDNA, but also verify two other reports that mapped the neur gene to the 6p21 region (Oohira et al. 1985; Harada et al.





**Figure 8.** Western blot analysis of mutant neuraminidase from sialidosis patients. Western blot prepared with equal amounts of COS-1 cell lysates, transfected with vector (MOCK), neur cDNA (NEUR), PPCA cDNA (PPCA), or cotransfected with both the neur and PPCA cDNAs (NEUR/PPCA). In addition, two neuraminidase mutants type I Asp-377-stop (NEUR\*), and type II Ser403-frame shift (NEUR\*\*) were either expressed alone or co-expressed with PPCA. The Western blot was incubated with affinity-purified  $\alpha$ -neur antibodies. Estimated molecular masses of the wild-type proteins are indicated at *left* and of the aberrant polypeptides at the *right*.

1987). Interestingly, the murine Neu-1 locus, which seems to be responsible for the partial deficiency of neuraminidase in inbred SM/J mice, maps to chromosome 17, near the major histocompatibility complex H2, which is syntenic to the human 6p region (Womack et al. 1981).

Lysosomal neuraminidase shares significant homology with many members of the sialidase superfamily (Roggentin et al. 1993). Based on our observations, we can speculate that rodent sialidases are positioned evolutionarily between the bacterial/human neuraminidases, which contain the FRIP domain and Asp boxes, and the viral sialidases, which mostly lack Asp boxes. The exact function of the Asp box is unknown but they have been found in at least seven other unrelated proteins from plants, viruses, bacteria, and yeast (Rothe et al. 1991). The only characteristic these proteins share is their ability to bind carbohydrates. Because sialidases and their sialyl substrates are absent in plants and metazoans, the occurrence of Asp boxes in plant proteins suggests that these boxes do not contribute to sialic acid metabolism. In spite of the differences observed at the amino-acid level, the crystal structure of bacterial and viral sialidases indicates that the fold topology of these enzymes is identical and consist of the same six-bladed  $\beta$ -propeller around an axis that passes through the active site (Crennell et al. 1993; Gaskell et al. 1995). Several of the residues in the catalytic pocket of bacterial sialidases are conserved in the human enzyme, including the Arg in the FRIP domain. It is therefore very likely that human neuraminidase has a similar three-dimensional structure. The primary structure of neuraminidase does not reveal any obvious membrane targeting domain, besides the signal peptide, which suggests that this protein is unlikely to associate with the membrane. This finding is not in keeping with the insoluble nature of the enzyme.

By expressing the full-length neur cDNA in COS-1 cells, we confirmed the lysosomal localization of the protein and the generation of PPCA-dependent neuraminidase activity at an acidic pH optimum. Surprisingly, we found that a significant number of cells overexpressing neuraminidase accumulate crystal-like structures in their perinuclear regions, that stained only with anti-neur antibodies. Although this "crystallization" effect was most likely attributable to overexpression, it must reflect an intrinsic, unique property of the enzyme because crystals of this size of other overexpressed proteins have not been reported previously. Another lysosomal enzyme,  $\alpha$ -galactosidase, was shown to form crystalline structures when overexpressed in CHO cells; however, in this case, the crystals were only visible at the electron microscopy level (Ioannou et al. 1992). In vivo crystallization of proteins is a rare though naturally occurring event. It has been reported for crystallin proteins in the eye lens (Russell et al. 1987) and for insulin in pancreatic acinar cells (Kuliawat and Arvan 1992). In both of these reports, the crystals are relatively small. It may be that the insoluble nature of lysosomal neuraminidase is a direct result of this ability to crystallize or aggregate.

Our most compelling evidence that the cDNA-encoded protein is the lysosomal neuraminidase came from studies on patient fibroblasts. Overexpression of our neur cDNA in the sialidosis patients' fibroblasts restored neuraminidase localization and activity. We found that PPCA is not required for correct lysosomal localization of neuraminidase, but is indispensable for enzyme activation. Catalytically inactive PPCA mutants rescue neuraminidase activity in the galactosialidosis fibroblasts (data not shown; Galjart et al. 1991), which suggests that the carboxypeptidase activity of PPCA is not

**Table 1.** Transfection of sialidosis fibroblasts with mutant neuraminidase cDNA constructs

cDNA construct	Neuraminidase activity (nmol/hr per mg protein)	
	wild-type fibroblasts	sialidosis fibroblasts
mock	58 $\pm$ 8	0
PPCA	75 $\pm$ 9	0
neur	40 $\pm$ 5	30 $\pm$ 12
neur*	n.d.	0
neur**	n.d.	0
neur/PPCA	300 $\pm$ 21	108 $\pm$ 18
neur*/PPCA	n.d.	0
neur**/PPCA	n.d.	0

Sialidosis type II fibroblasts were electroporated with the sialidosis mutant pSCTOP cDNA constructs alone, type I Asp377-stop (neur\*), and type II Ser403-frameshift (neur\*\*), and with the mutant neuraminidase and wild-type PPCA constructs (neur\*/PPCA and neur\*\*/PPCA). Wild-type and sialidosis fibroblasts were also electroporated with the wild-type neuraminidase and PPCA cDNAs (neur and PPCA), and coelectroporated with both constructs (neur/PPCA). The mutant cDNAs were not expressed in the wild-type fibroblasts (n.d.).

required to activate neuraminidase. It is clear from these studies that  $\beta$ -galactosidase is not directly involved in neuraminidase activation, a finding that supports earlier observations in PPCA-deficient knockout mice (Zhou et al. 1995), where  $\beta$ -galactosidase activity is reduced only in certain tissues, whereas neuraminidase deficiency parallels that of PPCA. Why then do PPCA,  $\beta$ -galactosidase, and neuraminidase form a multienzyme complex? A possible explanation is that association between the different components could alter the active sites of the enzymes, influencing their substrate specificity and/or catalytic activity. By coupling catalytic activity to assembly, protein components can be regulated through coordinated activation or stoichiometry in the complex. Although the exact mode of neuraminidase activation remains unclear, it is conceivable that the inactive neuraminidase polypeptide associates with PPCA, which promotes a crucial conformational change that renders the enzyme substrate accessible. Alternatively, PPCA could present the inactive neuraminidase to a different processing enzyme, which then activates it.

The identification of mutations in the neur mRNA from type I and II sialidosis patients, that are directly linked to the inactivation of the enzyme, provided the ultimate proof that the disease is caused by genetic lesions in the neuraminidase gene. On the basis of the experimental data presented here, we cannot at this time correlate the genetic defect in sialidosis type I with their mild phenotype. It is likely that an as yet unknown mutation in the second allele from these patients produces an enzyme with residual activity. A comprehensive analysis of the mutations in these and other sialidosis patients and their effect on the protein will be the subject of future studies.

The availability of the lysosomal neuraminidase cDNA enables us to investigate the neuraminidase protein in depth, particularly its association with other components of the complex, such as PPCA. In addition, we should gain better insights into the mechanisms that regulate neuraminidase activation and inactivation. Elucidation of the three-dimensional structure of lysosomal neuraminidase, either alone or complexed with PPCA and  $\beta$ -galactosidase, would offer essential insights into the specific physiological properties of the individual enzymes. This information, coupled with mutation analyses from other sialidosis patients, will help to explain the structure and function relationships of the wild-type protein and the defective mutant enzymes. Finally, it will be particularly interesting to assess the relative contributions of the three neuraminidase enzymes to catabolism of sialic acid-containing compounds, both under normal conditions and in the diseased state.

## Materials and methods

### Cell culture

Human skin fibroblasts from a normal individual and patients with galactosialidosis or sialidosis are deposited in the European Cell Bank, Rotterdam, The Netherlands (Dr. W.T. Kleijer). Fibroblasts from two siblings with the type I form of sialidosis

were kindly provided by Dr. Beck (Klinikum der Johannes Gutenberg-Universität, Mainz, Germany), who diagnosed the disorder in these patients. Fibroblasts from the type II sialidosis patients were kindly sent to the Rotterdam cell bank by Drs. G. Parenti and P. Strisciuglio (Dipartimento di Pediatria, Università di Napoli, Italy). Primary fibroblasts and COS-1 cells (Gluzman 1981) were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with antibiotics and 10% or 5% fetal bovine serum, respectively.

### Screening of the EST data base

The dbEST data base (Boguski 1995) was searched with the text string: "neuraminidase or sialidase" using an input device located on the World Wide Web ([http://www3.ncbi.nlm.nih.gov/dbest\\_query.html](http://www3.ncbi.nlm.nih.gov/dbest_query.html)). Putative neuraminidase cDNA clones homologous to known sialidases were retrieved. Their nucleotide and amino acid sequences (translated in all six reading frames) were analyzed for actual homologies using the NCBI Blast e-mail server ([blast.ncbi.nlm.nih.gov](mailto:blast.ncbi.nlm.nih.gov)), and were compared with nonredundant peptide and nucleotide sequence data bases (PDB, SWISS-PROT, PIR, SPUUpdate, GenPept, GPUUpdate, GBUUpdate, GenBank, EMBL, EMBLUpdate). A dbEST cDNA clone with favorable alignment to bacterial neuraminidases, accession no. R13552 (IMAGE clone 26525) was acquired, royalty free, from the IMAGE Consortium, Huntsville, Alabama. This clone is henceforth referred to as neur cDNA.

### Northern blot analysis

A Northern blot (Clontech) containing equal amounts (2  $\mu$ g) of human multitissue poly(A)<sup>+</sup> RNA was hybridized with the 1.9-kb neur cDNA labeled according to Sambrook et al. (1989). The membrane was stripped according to the manufacturer's instructions and rehybridized with the 1.8-kb human PPCA cDNA (Hu54) (Galjart et al. 1988). Total RNA was isolated from control and sialidosis patients' fibroblasts using TRIzol reagent according to manufacturer's instructions (Life Technologies). RNA (~10  $\mu$ g) was separated on a 1% agarose gel containing 0.66 M formaldehyde. After electrophoresis, the RNA was blotted onto a Zeta-probe membrane (Bio-Rad) and hybridized with the neur cDNA probe. Standard hybridization and washing conditions were applied (Sambrook et al. 1989).

### cDNA sequencing

The 1.9-kb neuraminidase cDNA clone was subcloned into pBluescript II KS (Stratagene) using standard procedures (Sambrook et al. 1989) and sequenced using the fmol kit (Promega) on double-stranded DNA (Murphy and Kavanagh 1988). Nucleotide sequence data were analyzed using the Wisconsin package (version 8, Genetics computer group). Homology searches were carried out using the NCBI Blast e-mail server, as stated above. Alignment of protein primary structures was performed using the computer programs ClustalW and Boxshade (Hofmann and Baron, Bioinformatics group, Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland), with a gap penalty of 10.0 and a gap extension of 0.05.

### Chromosomal localization

A 3.5-kb PCR fragment was amplified from human genomic DNA, using 18-mer oligonucleotide primers, synthesized according to 5' (sense) and 3' (antisense) sequences in the neuraminidase cDNA. The 1.9-kb cDNA and the 3.5-kb genomic fragment were labeled separately by nick translation with

digoxigenin dUTP. The labeled probes were then combined with sheared human DNA and hybridized independently to normal metaphase chromosomes derived from PHA-stimulated peripheral blood lymphocytes as described (Morris et al. 1991). Specific hybridization signals were detected by incubating the hybridized slides in fluorescein-conjugated antidigoxigenin antibodies. The chromosomes were then stained with DAPI and analyzed. Fluorescence signals observed with these probes were specific to the middle of the short arm of a C group chromosome with DAPI-banded morphology consistent with chromosome 6. Based on the distance from the centromere of the hybridization signal relative to the entire length of the short arm of chromosome 6, we assigned the neur locus to band p21.

#### *Transfections and enzyme assays*

cDNAs encoding neuraminidase, PPCA (Galjart et al. 1988), and  $\beta$ -galactosidase (Morreau et al. 1989), subcloned into the expression vector pCD-X (Galjart et al. 1988; Okayama and Berg 1982), were transfected into COS-1 cells using calcium phosphate precipitation as described (Chen and Okayama 1987).

The cDNAs mentioned above were also subcloned into pSCTOP (Fornerod et al. 1995; Rusconi et al. 1990) and electroporated into primary fibroblasts according to the manufacturer's instructions (Bio-Rad), with the following modifications. Primary fibroblasts were trypsinized, resuspended in DMEM supplemented with 10% fetal calf serum, and washed once in ISCOVE's medium. Plasmid DNA (30  $\mu$ g) was then electroporated into  $\sim 1 \times 10^6$  cells, suspended in 500  $\mu$ l of ISCOVE's medium, using a 0.4-cm electroporation cuvette in a BioRad Gene Pulser set at 0.320 kV, and 500  $\mu$ F (time constant 11–13). The electroporated cells were then seeded into 50-mm Petri dishes and cultured for 16 hr, at which point the medium was changed.

Transfected COS-1 cells and primary fibroblasts were harvested by trypsinization 72 hr post-transfection and assayed for neuraminidase activity with the artificial 4-methylumbelliferyl substrate, according to Galjaard (1980). Total protein concentrations were quantitated with bicinchoninic acid (Smith et al. 1985) following the manufacturer's guidelines (Pierce, Chemical Co.).

#### *Immunofluorescence, Western blotting, and immunoprecipitation*

Antiserum was raised in rabbits against a bacterially produced GST-neuraminidase fusion protein that lacks neuraminidase amino-acid residues 1–50. This antiserum ( $\alpha$ -neur) was affinity-purified as described previously (Smith and Fisher 1984). The denatured 32-kD chain of PPCA, generated through its overexpression in insect cells (Bonten et al. 1995), was used to raise anti-PPCA antiserum ( $\alpha$ -32) in rabbits.

For indirect immunofluorescence, COS-1 cells and primary fibroblasts were seeded 48–72 hr post-transfection on Superfrost/Plus glass slides (Fisher). The next day, the cells were processed according to van Dongen et al. (1985), using the antisera mentioned above and FITC-conjugated antirabbit IgG antibodies (Sigma).

For Western blotting, COS-1 cells were harvested by trypsinization 72 hr post-transfection and lysed in milli-Q water (Millipore). Aliquots of cell lysates containing 5  $\mu$ g of protein were resolved on SDS-polyacrylamide gels and transferred to Immobilon polyvinylidene difluoride membranes (Millipore). Western blots were incubated with affinity-purified  $\alpha$ -neur antibodies as described Bonten et al. (1995), using either alkaline phosphatase- or horseradish peroxidase-conjugated antirabbit IgG secondary antibodies, with a colorimetric (Sigma) or chemilu-

minescent (Renaissance, DuPont NEN) substrate, respectively. Deglycosylation reactions were performed with recombinant N-glycosidase F (Boehringer Mannheim) according to the supplier's instructions.

For immunoprecipitation, fibroblasts were grown to confluence in 85-mm Petri dishes and labeled with 350  $\mu$ Ci L-[4,5- $^3$ H]-Leucine per dish for 20 hr. Proteins were precipitated with  $\alpha$ -neur as reported previously (Proia et al. 1984) and resolved by SDS-PAGE under denaturing and reducing conditions. Radioactive bands were visualized by fluorography of gels impregnated in Amplify (Amersham). Apparent molecular masses were calculated by comparison with marker proteins (Life Technologies).

#### *Mutation analysis*

For amplification of mutant cDNAs, four sets of 18-mer oligonucleotide primers were synthesized based on the wild-type cDNA sequence. Total RNA was isolated from control fibroblasts and the fibroblasts of sialidosis patients' by using TRIzol reagent according to the manufacturer's instructions (Life Technologies). Four overlapping cDNA fragments of  $\sim 500$  bp each encompassing the entire coding region of the neuraminidase cDNA, were synthesized by RT-PCR (Hermans et al. 1988). For direct cDNA sequence analysis, a portion of PCR-amplified cDNA was subsequently subjected to asymmetric PCR (Kadowaki et al. 1990), using a 1:100 ratio of sense:antisense or antisense:sense primer concentrations, for an additional 30 cycles. The PCR products were phenol/chloroform extracted, desalted on Centricon-100 units (Amicon), and precipitated with isopropanol. The single-stranded products were sequenced by the dideoxy-chain termination method (Sanger et al. 1977) using the Sequenase kit according to the manufacturer's instructions (USB).

#### *Transient expression of mutant neuraminidase cDNAs*

To introduce the mutations found in the neuraminidase of sialidosis patients, into the full-length cDNA, small fragments ( $\sim 400$  bp), containing the identified mutations, were excised from the RT-PCR products described above and subcloned into the pSCTOP-neuraminidase cDNA construct. The plasmids were then sequenced to ensure that the mutations had been correctly introduced. They were then transfected into COS-1 cells and primary sialidosis fibroblasts as described above.

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