LYSOSOMAL NEURAMINIDASE

A unique member of the sialidase superfamily

Erik Jacobus Bonten

The thesis cover shows the immunocytochemical localization of several natural neuraminidase mutants, over-expressed in deficient sialidosis fibroblasts (see Chapter 8).

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A unique member of the sialidase superfamily

Lysosomaal neuraminidase

Een uniek lid van de superfamilie der neuraminidases

Proefschrift

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Voor mijn ouders
Voor Jacqueline, Nathalie en Dominique

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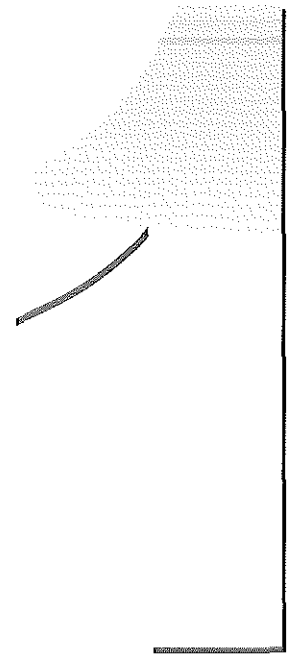
Scope of this thesis

Lysosomal neuraminidase initiates the hydrolysis of oligosaccharides, gangliosides, glycolipids and glycoproteins by removing their terminal sialic acid residues. The enzyme functions exclusively in a multi-enzyme complex, together with β-galactosidase and protective protein/cathepsin A (PPCA) and is dependent for its activity and stability on the latter protein. Mutations in the neuraminidase gene are the basis for the lysosomal storage disorder sialidosis, while mutations in the PPCA gene indirectly results in the impairment of neuraminidase and β-galactosidase activity, causing the lysosomal storage disorder galactosialidosis. The experimental work that is described in this thesis involves the isolation and characterization of the cDNA's encoding human and mouse lysosomal neuraminidase. Structural and functional analysis of this enzyme has focused on the interaction with PPCA in relation to its transport to the lysosomes and the mechanism of catalytic activation. These studies, in combination with the identification and functional analysis of a large number of novel mutations in sialidosis patients, have given new insights into the biochemical functions and properties of neuraminidase and provided reliable phenotype-genotype correlations. The results presented in this thesis may form the basis for understanding the structural characteristics of this unusual lysosomal protein and for the future development of strategies for therapy of both sialidosis and galactosialidosis patients.



Introduction

Introduction and history

Neuraminidases (neuraminidases EC 3.2.1.18) belong to a class of exoglycosyl hydrolases that release α-glycosidically linked terminal N-acylneuraminate (sialic acid) from a variety of glycoproteins, glycolipids and polysaccharides. The initial work in this field started with the observation that influenza virus particles were able to agglutinate chicken erythrocytes and that it was not possible to reagglutinate the red blood cells after they had been agglutinated by the virus once (1). Later it was shown that certain bacterial extracts could destroy receptor sites for influenza virus on the surface of human erythrocytes (2). The responsible enzyme was named receptor-destroying enzyme. The name 'sialidase' was proposed in 1956 by Heimer (3), and the name 'neuraminidase' by Gottschalk (4) after they showed that upon incubation of salivary mucin with bacterial or viral extracts, sialic acid was released. Both names have been used interchangeably ever since. The enzyme was first purified from the bacterium *Vibrio cholerae* and shown to be catalytically identical to the receptor-destroying enzyme (5). Since then, the worldwide public health burden of influenza has accounted for a wealth of research on viral neuraminidases, which extended to neuraminidases from other species. It is now known that these enzymes are common in viruses, bacteria, protozoa, mycoplasma, fungi, as well as in higher organisms, including all mammalian species, but absent in plants (reviewed in: (6-9)).

In contrast to the viral neuraminidases, the function of these enzymes in bacteria is less clear. Interestingly, in bacteria neuraminidases are found both in pathogenic and non-pathogenic species. However, they are not expressed in all bacteria, and even different strains of a single species may differ in neuraminidase expression (6,10). Moreover, many neuraminidase-containing bacteria are unable to synthesize sialic acid, whereas others, that do synthesize sialic acid, lack sialidases. Homologies of neuraminidases at the molecular level support the hypothesis of a common origin and hence of a neuraminidase superfamily.

Biological significance of sialic acid

The sialic acids are a group of 9-carbon carboxylated sugars (Figure 1), usually positioned at the termini of complex carbohydrates (Figure 2). They are structurally very diverse, with over 25 identified forms (Figure 1; (11-15)). In addition, they are target molecules in a variety of important biological events, mainly as ligands in recognitive interactions (14). The release of sialic acid from the oligosaccharide chain by neuraminidase is required for the hydrolysis of subsequent sugar residues by other glycosidases (Figure 2; (16). Sialic acid derivatives

have been found in higher organisms, from the echinoderms upwards and in some bacteria, viruses and protozoa (14).

The biosynthesis of sialic acids is a complex multiple-step process (14) starting from D-glucose, which is first converted to N-acetylglucosamine (GlcNAc). The sialic acid pathway reaches its first key step with the epimerisation of GlcNAc to N-acetylmannosamine (ManNAc). The biosynthetic route proceeds with the 6-phosphorylation of the amino sugar, using ATP as the phosphate donor. ManNAc-6-phosphate (ManNAc-6-P) is condensed with pyruvate to N-acetylneuraminic acid-9-phosphate (Neu5Ac-9-P). Dephosphorylation of the latter compound yields Neu5Ac, which needs to be activated to CMP-Neu5Ac for incorporation into complex carbohydrates. Sialyltransferases, which are responsible for the formation of sialoglycoconjugates, can process various Neu5Ac CMP-glycosides, but are highly specific for the carbohydrate acceptor structure and the type of glycosidic bond formed (17-19). Glycosidically bound Neu5Ac can undergo modifications of the OH groups, such as 8-O-methylation, 8-O-sulphation (20-22), 9-O-lactoylation, and 4, 7, 8 and 9 O-acetylation (Figure 1; Schauer, 1987; Diaz et al., 1989; Varki, 1992; (12, 23-26). Sometimes several of these substituents are present in one sialic acid molecule and no other natural sugar shows such a great variety. Moreover, this variation is often tissue and/or animal specific, some having only one form of sialic acid, while others have several kinds (14). For instance, significant differences in sialic acid types have been described between adult and fetal bovine tissues, suggesting their involvement in development (27).

In general, oligosaccharide chains of glycoconjugates are no longer believed to be inert structures, but rather structures that store biological information (28-30). The wide distribution of sialic acids in nature, their multiplicity, adult and embryonal tissue specificity and terminal position on carbohydrate chains, reflect their importance in a variety of cellular functions. Sialic acids can serve as recognition markers for several mammalian proteins, such as members of the selectin family (31-33) and the I-type lectins or sialoadhesin subgroup (12, 34-36). The enzymatic addition or removal of sialic acids can lead to significant differences in the biological properties of carbohydrates, resulting in altered recognition by sialic acid binding proteins and ultimately influencing the function or fate of cells. Numerous functions have been attributed to sialic acids (11, 14, 37-39). Because of their negative charge they are involved in cell adhesion, but also in cell repulsion, for instance between circulating blood cells (40). Furthermore, their negative charge is important for the maintenance of the viscoelastic properties of sialic acid rich compounds, such as mucins (41). They influence the properties of glycoproteins, including their conformation, enzyme activity and resistance to proteolytic degradation (11). N- and O-

R_1	R_2	R_3
H	N-Acetyl	Gal (3/4/6)
Acetyl (4,7,8,9)	N-Glycolyl	GalNAc (6)
Lactyl (9)	Amino	GlcNAc (4/6)
Methyl (8)	Hydroxyl	Neu5Ac (8/9)
Sulfate (8)		(absent in 2,6 and 2,7 anhydro compounds)
Phosphate (9)		
Anhydro (4,8 or 2,7)		
Neu5Ac (8,9)		
Fucose (4)		
Glucose (8)		
Galactose (4)		

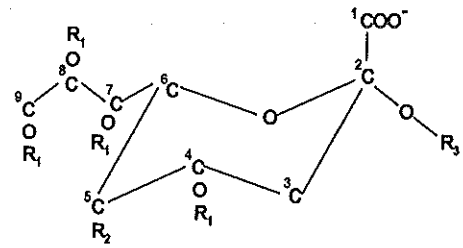


Figure1. Natural sialic acid compounds. The 9-carbon backbone common to all sialic acids is shown in chair formation. Substituents (R1,2,3) at different positions are indicated. Additional diversity is generated by various types of glycosidic linkage at positions 2, by generation of lactones at position 1, and by dehydro and anhydro forms. Adapted from (12).

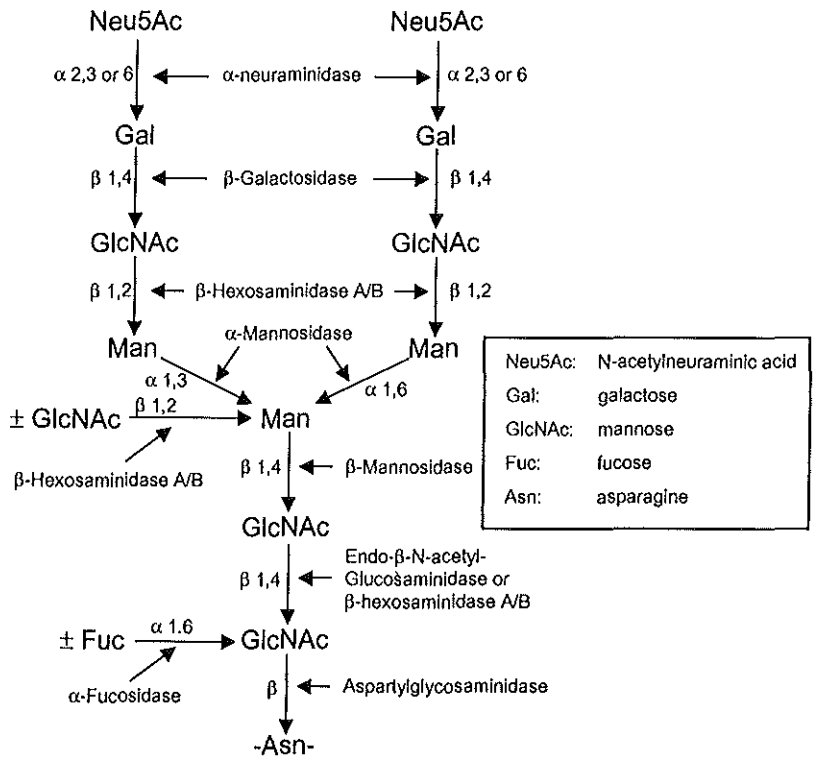


Figure 2. Composite complex type N-glycosidic oligosaccharide and the hydrolytic steps required for its degradation. Adapted from (16).

acetylated sialic acids are antigenic determinants. For instance, B-cells have O-acetylated sialic acids, whereas T-cells do not (42). Interestingly, the T-cells of patients with various malignancies have acquired O-acetylation (43). Sialic acids function in the suppression of

antigenic recognition and their loss has been implicated as one of the causes of autoimmune disease. In contrast, tumor cells appear to be protected from the immune system by highly sialylated cell surfaces (11). Sialic acids are also often present on the carbohydrate chains of receptors such as peptide hormones, toxins, viruses and mycoplasma species, where they apparently serve as essential recognition markers, for instance in the virus-receptor interaction. However, they can also function as biological masks, preventing ligand recognition of receptors (11).

In conclusion, sialic acids are involved in many important cellular mechanisms and pathways. Thus, neuraminidases, being responsible for the hydrolysis of sialic acid, are equally important participants for such processes.

Mammalian neuraminidases

Mammalian neuraminidases are expressed in all tissues and cell types (reviewed in: (8)) and they have been implicated in several critical and diverse metabolic processes, including the regulation of cell proliferation and cell differentiation (44-46), clearance of plasma proteins (47), membrane fusion and membrane fluidity (48-52), cell adhesion (53-55), catabolism of gangliosides and glycoproteins (56, 57), immunocyte function (58, 59), modification of receptors (60, 61) and the developmental modelling of myelin (57). The pivotal involvement of neuraminidases in these cellular mechanisms may account for the existence of four neuraminidases, differentially localized in (i) the cytosol, (ii) the plasma membrane, (iii) the lysosome and (iv) the lysosomal membrane. These enzymes have been characterized by their subcellular distribution, pH optimum, stability, kinetics, effect of ions, and substrate specificity (reviewed in: (8, 62, 63)). Recently cDNA's have been cloned that encode cytosolic, plasma membrane and lysosomal neuraminidases (64-69).

Cytosolic neuraminidase

The gene encoding cytosolic neuraminidase was the first mammalian enzyme to be cloned (64). The cDNA was isolated from a rat skeletal muscle cDNA library using degenerated oligonucleotide primers, derived from the partial amino acid sequence of the purified enzyme and encoded a protein of 379 amino acids. The amino acid sequence showed ~20% homology to the bacterial neuraminidases. A secreted form of neuraminidase, with similar properties and molecular weight (43 kDa) as the rat skeletal muscle enzyme, was purified from the medium of cultured chinese hamster ovary cells (CHO) (70). Its corresponding cDNA was isolated from a CHO cDNA library (65), encoding a protein nearly identical to the rat skeletal muscle cytosolic neuraminidase, which most likely represented the Chinese hamster homologue. This enzyme was secreted by an unknown

mechanism, since it lacked an N-terminal signal peptide known to be associated with compartmentalization in the secretory pathway.

The purified rat cytosolic neuraminidase was active *in vitro* towards sialo-oligosaccharides, sialoglycoproteins and gangliosides (in the presence of bile salts), except for submaxillary mucins, and both GM_1 and GM_2 gangliosides. The enzyme hydrolyzed α2-3 sialyl-linked substrates much faster than α2-6 and α2-8, with a pH optimum between 6 and 6.5 (71). Interestingly, this enzyme seemed to play a role in myotube differentiation, since its mRNA and enzyme activity were not detectable in cultured myoblasts, but became induced upon *in vitro* myotube differentiation (72). Moreover, the myotube formation was completely blocked by the addition of an antisense oligonucleotide primer complementary to the first 8 codons of the cytosolic neuraminidase. However, expression of cytosolic neuraminidase was not restricted to skeletal muscle, but was present in many other tissues, including kidney, brain, heart, stomach, intestine, testis, liver epithelium of cornea, blood vessels, Schwann cells and axons (8, 73, 74). The corresponding human and mouse genes encoding cytosolic neuraminidase have been cloned recently and shown to be ~80% identical to the other cytosolic neuraminidases (68, 69).

The exact function(s) of cytosolic neuraminidase in tissues and cell types other then muscle is unknown and no genetic disorders have as yet been associated with a deficiency of cytosolic neuraminidase.

Plasma membrane neuraminidase

A neuraminidase localized in the plasma membrane, specific for the hydrolysis of hydrophobic sialyl compounds, including gangliosides, has been characterized in various tissues and cell types, including liver, thyroid gland, skeletal muscle, myelin, lymphocytes, neuroblastoma cells, fibroblasts, erythrocytes, and the brain (8, 57, 75-77). The latter is one of the main sites for this enzyme, where it has been found in synaptisomal plasma membranes and myelin (72, 78-81). Plasma membrane ganglioside-neuraminidase is responsible for the growth control and differentiation of cultured human neuroblastoma cells (81-83). Inhibitors of ganglioside-neuraminidase, added to the culture medium of these cells, led to a complete release of contact inhibition of growth, loss of neuron-specific enolase differentiation marker and neurofilaments, and a decrease of cyclic AMP. A ganglioside neuraminidase present in mouse synaptic plasma membranes displayed a decreased activity with age (80), which correlated with age-related changes in ganglioside composition in these membranes (48). Thus far the functions of these age related changes are unknown.

Unlike catabolic reactions involving soluble enzymes and hydrophilic substrates, the interaction of ganglioside-neuraminidase with membranes and lipid substrates shows

unique kinetic features. Gangliosides are mainly located on the outer surface of the plasma membrane and are assumed to be involved in diverse biological functions, including receptor activity for hormones, toxins, bacteria and viruses, as well as modulation of membrane-bound enzyme activities, cell-growth, differentiation and cell-adhesion (84, 85). Ganglioside substrates within membranes gain access to the enzyme through lateral diffusion (86, 87). Hence, membrane-bound neuraminidase activity can be enhanced by membrane-fluidizing reagents such as Triton X-100, which enhances enzyme-substrate access, resulting in enzyme activity (78, 81, 82, 88, 89).

Differences in size, pH optimum, substrate specificity, inhibitors and activators between plasma membrane neuraminidases isolated from various tissues and cell types, suggested the existence of more than one species of this enzyme (78, 81, 90, 91). Whereas most plasma membrane neuraminidases were released by extraction with non-ionic detergents, a plasma membrane neuraminidase from pig brain was released by treatment with phosphatidylinositol phospholipase C (PIPL), indicating that this enzyme is anchored by means of a glycosyl phospatidylinositol anchor (GPI) (79). However, only 28% of the plasma membrane neuraminidase activity could be released by PIPL treatment, which also indicated the possible existence of a second plasma membrane neuraminidases in pig brain.

Recently a neuraminidase was purified from bovine brain to near homogeneity (89). The cDNA encoding this enzyme was cloned from a bovine brain cDNA library, using oligonucleotide primers that were based on partial amino acid sequences derived from the purified protein (66). The corresponding human gene was cloned from a human brain cDNA library and 83% identical to the bovine gene (67). This neuraminidase was expressed mainly in the brain and muscle, as indicated by Northern blot hybridization, which revealed a mRNA transcript of ~ 7.5 kb. The bovine protein of 428 amino acids had sequence homology to the rat and Chinese hamster cytosolic neuraminidase (~38%), the human and mouse lysosomal neuraminidase (~19%) and the bacterial neuraminidases (~19%). Curiously, the protein had no N-terminal signal sequence, but a putative trans-membrane domain, which was oddly located in the central part of the protein, dividing the catalytic domain at two sites of the plasma membrane. Analysis of the membrane topology indicated that this protein was an atypical type I membrane protein, with an extracellular N-terminus and a cytoplasmic C-terminus. Although the localization of the protein in the plasma membrane was confirmed by Percoll density gradient centrifugation, as well as immunofluorescence staining of transfected COS-7 cells, it appears to be unlikely that this enzyme is active in a membrane anchored state. The authors speculated that the enzyme binds gangliosides at the cell surface with its extracellular domain. The enzyme-substrate

complex may then be internalized in endosomal vesicles where it assumes the correct conformation to become catalytically active. The authors showed that the overexpressed enzyme was active at acidic pH, in the presence of Triton X-100, towards gangliosides other then GM_1 and GM_2, but was inactive with sialoglycoproteins, oligosaccharides and the synthetic 4MU-Neu5Ac substrate. The restricted expression patterns of this neuraminidase in brain and muscle and the presence of ATTTA motifs in the 3' untranslated region, which are thought to be involved in the regulation of gene expression during cell growth and differentiation, indicate that this neuraminidase has a specialized function (66).

A deficiency of ganglioside neuraminidase was suggested to be the cause of the rare autosomal metabolic storage disease mucolipidosis IV (MLIV) (92, 93). This was supported by the accumulation of gangliosides and mucopolysaccharides within membranous cytoplasmic bodies in patient's skin fibroblasts, Schwann cells, vessel walls, smooth muscle fibers and sweat glands (94, 95). Despite an early onset of the disease and severe mental retardation, the progression of the clinical symptoms is very slow during the first three decades of life. The patients have a deficiency in neuraminidase activity towards GD_{1a} and GD_{1b} gangliosides, which is caused by the accumulation of sulfated glycosaminoglycans, that have a strong inhibitory effect on the neuraminidase activity (95-97). Interestingly, cells of the patients also appeared to have a defect in the endocytosis process of membranous components, resulting in excessive transport of sulfated glycosaminoglycans into lysosomes, rather then recycling them to the plasma membrane (98). In MLIV cells none of the lysosomal hydrolases were found to be abnormal (98), however, the transport of lipids from the lysosomes was impaired (99). These data suggest that the primary genetic defect responsible for MLIV involves a receptor and/or transporter that in turn causes the secondary inhibition/inactivation of ganglioside-neuraminidase.

Recently the gene responsible for MLIV gene has been mapped to chromosome 19p13.2-13.3 by linkage analysis with fifteen markers in thirteen MLIV-families (100). The authors constructed haplotypes in twenty-six Ashkenazi Jewish families and demonstrated the existence of two founder alleles that probably carry MLIV-causing structural defects in this population. The localization of MLIV to chromosome 19 should result in the cloning of the disease causing gene in the near future.

Lysosomal neuraminidase

Lysosomal neuraminidase is present in virtually all vertebrate tissues and cell types and has been purified and characterized from many sources, including placenta, mammary gland, brain, kidney, liver, testis, thyroid, salivary gland, leukocytes, lymphocytes, macrophages and fibroblasts (reviewed in: (8)). The cloning of the lysosomal neuraminidase

gene has been hampered by substantial loss of the enzyme activity during purification procedures (8, 101-105).

As was the case for plasma membrane neuraminidase, also for lysosomes the potential existence of two neuraminidases has been reported (106). A neuraminidase from purified rat liver lysosomes was released into the supernatant by hypotonic shock and was active towards sialyllactose and fetuin. The lysosomal enzyme that was purified from bovine testis and human placenta was released by similar methods, which indicated that this neuraminidase had an intra-lysosomal origin (101, 103, 104, 107). On the other hand, a neuraminidase active towards submaxillary mucin and gangliosides was insoluble. These results argued for the existence of a soluble intra-lysosomal neuraminidase mainly active towards oligosaccharides and glycoproteins and a lysosomal membrane-bound neuraminidase that is specific for gangliosides (108). Using antibodies that were raised against neuraminidases that were purified from rat plasma membrane, lysosomes, and cytosol, it was shown that lysosomes from rat liver actually contained three neuraminidases. One enzyme was present in the lysosomal lumen (I) and two were found in the lysosomal membrane (II and III), one of which was identical to the plasma membrane ganglioside-neuraminidase (78, 109). Both lysosomal membrane neuraminidases II and III had a molecular weight of ~ 70 kDa (gel filtration) and a pH optimum near 5. However, neuraminidase III hydrolysed almost exclusively gangliosides, whereas neuraminidase II hydrolyzed preferentially gangliosides but also oligosaccharides, glycoproteins and 4MU-Neu5Ac. The existence of a lysosomal-membrane ganglioside-specific neuraminidase has been confirmed by others (8, 90, 110).

Different molecular weights have been reported for purified lysosomal neuraminidase, which may be dependent on the animal species and/or tissue. A 48.5-kDa form was purified from human leukocytes (111), a 56-60-kDa form from rat liver (108), a 55-kDa species from bovine testis (112), 61- and 66-kDa forms from human placenta (105, 113) and a 70-kDa neuraminidase from human liver and rat brain (78, 109).

The lysosomal neuraminidase that is active towards glycoproteins and oligosaccharides is only found in a high molecular weight multi-enzyme complex of >1000 kDa. Lysosomal neuraminidase activity is known to be particularly unstable during enzyme purifications, which is probably caused by its dissociation from the multi-enzyme complex. The multi-enzyme complex can be purified from different mammalian/avian tissues and species, including human, bovine, mouse, porcine, chicken, and rat. There is variation in the reported sizes of the multi-enzyme complex, which may be due to either species or tissue specific differences of the complex, or because of diversity in the applied purification methods. The purification of lysosomal neuraminidase usually requires tissue

homogenization, the isolation of a lysosomal/mitochondrial fraction and a hypotonic shock. The purification procedure may include one or more of the following chromatography techniques: Concanavalin A-sepharose (glycoprotein affinity), p-aminophenylthiogalactoside-CH-sepharose (β-galactosidase affinity), sucrose-density gradient, gel filtration, ion exchange and the use of antibodies (8). Neuraminidase that was isolated from the supernatant of homogenized human placenta could be 'rescued' by concentration and acidification of the preparation, resulting in a four fold increase in catalytic activity for every two fold of concentration (102). The stabilized neuraminidase was associated with β-galactosidase, but also with lysosomal protective protein/cathepsin A (PPCA). This neuraminidase was the intra-lysosomal form, mainly active towards oligosaccharides and 4MU-Neu5Ac synthetic substrate. However, most of the lysosomal neuraminidase remained in the lysosomal membrane fraction and could only be released with detergents, which suggested that this was a membrane bound enzyme (114, 115). There are two main forms of the lysosomal multi-enzyme complex, a >1000-kDa form which consists of neuraminidase and relatively small amounts of PPCA and β-galactosidase and a second complex of 600-700 kDa containing the latter two proteins (101, 102, 105, 107, 116-120). Figure 3 illustrates a classic gel filtration profile illustrating the different forms of the three enzymes when purified from cultured bovine kidney cells (121). PPCA and β-galactosidase activities were recovered in a >1000-kDa neuraminidase-containing complex (figure 3, complex I), in a lower molecular weight complex of ~740 kDa (figure 3, complex II), and as free dimeric forms of ~100 kDa and 160 kDa respectively. Neuraminidase activity was completely dependent on the presence and association with PPCA in the complex, although the cathepsin A activity in the >1000 kDa complex was very low. This suggests that only a small amount of PPCA is required to maintain neuraminidase activity. The co-precipitation of neuraminidase, β-galactosidase and Cathepsin A activities from complex I and the co-precipitation of β-galactosidase and PPCA from complex II, using PPCA specific antibodies, confirmed the association of the enzymes in the two complexes (E. Bonten, unpublished data). There may exist equilibrium between the two multi-enzyme forms and the free forms of β-galactosidase and PPCA. This hypothesis was substantiated by *in vitro* experiments in which 'free' PPCA was separated from the neuraminidase complex, which resulted in instability and substantial loss of neuraminidase activity (90%). However, the latter was restored when the fraction that contained the PPCA was added to the inactivated neuraminidase (E. Bonten, unpublished data). This indicated that the presence of 'free' PPCA and/or other soluble factors was required to keep neuraminidase active.

N-acetylgalactosamine-6-sulfatase (GALNS) was identified as an component of the multi-enzyme complex (122). GALNS activity was found to range between 6 and 40% of the normal value in the fibroblasts of galactosialidosis patients, whose excretion of keratan sulfate in their urine was comparable to that of patients with GALNS deficiency. It is possible that the multi-enzyme complex *in vivo* may contain several other hydrolases in addition to the already identified ones. The composition of the multi-enzyme complex may depend on the presence of specific substrates, thus creating a highly flexible and efficient hydrolytic system.

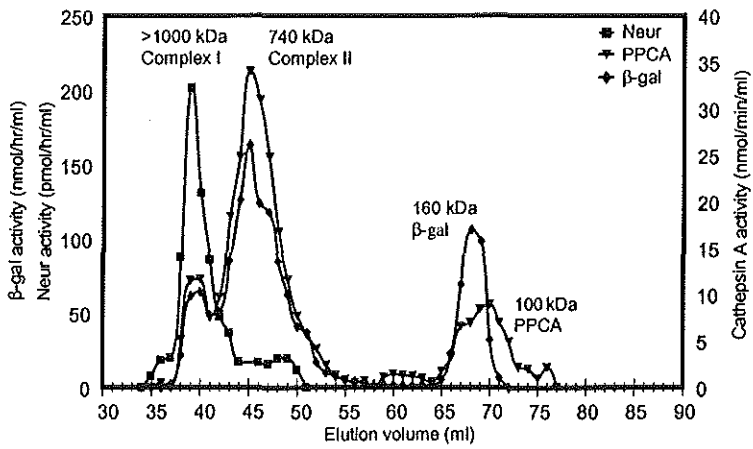


Figure 3. Elution profile of neuraminidase (Neur), β-galactosidase (β-gal), and Cathepsin A (PPCA) activities from MDBK cell lysate (50 mM Na-Acetate pH 5.0/150 mM NaCl/0.4% NP40), separated on a Sephacryl S300HR column (FPLC). The sizes of the complex forms I and II, and the free forms of the enzymes are indicated, and calculated using the elution profiles of standard molecular weight markers.

Deficiencies of lysosomal neuramimidase

Sialidosis

Sialidosis is an autosomal recessive disorder, caused by structural lesions in the lysosomal neuraminidase gene. The clinical manifestations of patients with this lysosomal storage disorder have given insight into some of the functions of this enzyme. Type I sialidosis is a mild form of the disease, with variable age of onset, but usually in the second decade of life. It is characterized by the cherry-red spot-myoclonus phenotype and progressive impaired vision, but absence of dysmorphic features, (reviewed in: (16)). Nystagmus, ataxia and grand mal seizures were also reported in these patients. Type II sialidosis is a severe form of the disease, which can be subdivided into three subtypes: (I) congenital or hydropic (in utero), (II) infantile (0-12 months) and (III) juvenile (2-20 years) (16). All type II patients eventually develop a progressive mucopolysaccharidosis-like

phenotype, including coarse facies, visceromegaly, dysostosis multiplex, vertebral deformities and severe mental retardation (16, 124-126). Cherry-red spots and myoclonus have been reported for older children and survival to the second decade for infantile patients. Congenital patients demonstrate hydrops fetalis and/or neonatal ascites with stillbirth or death at an early age; features may include facial edema, inguinal hernias, hepatosplenomegaly, stippling of the epiphyses and periosteal cloaking (123). Sialidosis patients have residual lysosomal neuraminidase activity, ranging from 0 to 10% of control values, with type I patients having the highest activities. Obligate carriers have intermediate enzyme values, but are phenotypically normal (16, 126). Vacuolated lymphocytes and bone marrow foam cells are prominent in type II, but absent in type I sialidosis. Vacuolation was further observed in Kuppfer cells, nerves, tissue fibroblasts, mysenteric plexus neurons and brain biopsy material (16). Electron microscopy analysis described the presence of membrane bound vacuoles, electron-dense bodies, floccular material, lamellar inclusions and lipofuscin (16). The concentration of the oligosaccharides in the urine of patients correlated with the severity of the clinical phenotype (127). Most compounds excreted in the urine of sialidosis patients contained N-acetylglucosamine at the reducing ends and N-acetylneuraminic acid at the non-reducing termini (127-130). The neuraminic acids are linked to galactose via either α2-6 (70%), or α2-3-linkage (30%) and likely originated from carbohydrate side chains that had been cleaved from glycoproteins by an endo-N-acetyl-glucosaminidase (16). In the organs of a sialidosis patient, obtained at autopsy, the water-soluble bound sialic acid was increased between 10- and 17-fold in visceral organs, but surprisingly only about 2-fold in the brain when compared to normal controls. Lipid-bound sialic acid was also increased up to 8-fold in visceral organs due to elevated amounts of gangliosides GM_3, GD_3 and probably GM_4 and LM_1, whereas the brain showed no deviation from controls. An alteration of the neutral glycolipid pattern was also observed (131). The results indicated an impaired catabolism of gangliosides in sialidosis in addition to that of sialo-oligosaccharides and sialoglycoproteins. This is in line with evidence that lysosomal neuraminidase, under specific conditions, can be also active towards gangliosides (90, 91, 110).

Galactosialidosis

Patients with the lysosomal storage disorder galactosialidosis were shown to have a combined β-galactosidase and neuraminidase deficiency, secondary to the deficiency of the 'protective protein' (102, 132-136). There is a broad overlap in the clinical phenotypes of sialidosis and galactosialidosis patients, indicating an important role for neuraminidase also in the pathogenesis of galactosialidosis.

Galjart and co-workers cloned the cDNA encoding the 'protective protein' and found it to be homologous to two yeast carboxypeptidases, carboxypeptidase Y (CPY) and the KEX1 gene product (137, 138), as well as to the wheat serine carboxypeptidase CPW (139). The 'protective protein' is identical to the previously identified lysosomal serine-carboxypeptidase cathepsin A and is now named 'protective protein/cathepsin A' (PPCA) (140). It also functions as an aminocylamidase (140-142) and a deamidase *in vitro* active towards a selected number of bioactive peptides including substance P, oxytocin, angiotensin I and endothelin I (143, 144). Fibroblasts from galactosialidosis patients were shown to have less than 1% activity towards a specific synthetic substrate for cathepsin A (Z-Phe-Ala) and they were also impaired in lysosomal neuraminidase and β-galactosidase activities, whereas obligate carriers had about 50% activity compared to controls (140, 145). The two functions of PPCA, (I) protecting β-galactosidase and neuraminidase against intralysosomal degradation and (II) as serine carboxypeptidase/deamidase, were proven to be distinct, since it was shown that the catalytic activity was not required for its protective function towards the two glycosidases (140). However, it is yet unknown whether the cathepsin A deficiency in galactosialidosis patients also contributes to the disease phenotype.

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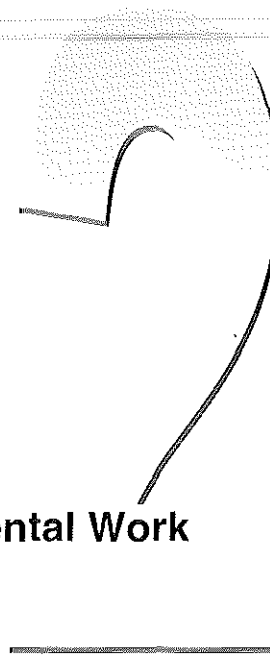

Introduction to the Experimental Work

Introduction to the experimental work

Lysosomal neuraminidase can be regarded as a unique member of the sialidase superfamily, since it is the only sialidase that is deficient in a human metabolic storage disorder, sialidosis.

The identification of lysosomal neuraminidase has been hampered by its apparent lability during purifications. This is reflected by many contradictory reports describing the characterization of the purified enzyme (molecular weights between 40 and 70 kDa), eventhough the purification methods were appropriate (Chapter 1 for references). Another factor that has complicated the identification of lysosomal neuraminidase is the existence of at least three distinct mammalian forms, characterized by different pH optimum, substrate specificity and subcellular localization. Of these enzymes the cytosolic neuraminidase was the first one to be cloned in 1993 by Miyagi and co-workers, who in 1999 were also responsible for the cloning of the plasma membrane neuraminidase (Chapter 1, pages 15-17). Until now, many questions about lysosomal neuraminidase and the gene-mutations underlying sialidosis had remained unanswered. We had no information about the primary structure of this enzyme and its potential homology to other sialidases. Furthermore, we could not explain its strict dependency on the protective protein/cathepsin A, since most microbial neuraminidases are generally stable and active, without the need of auxilary proteins. Last, but not least, we did not have information about the mode of interaction between these two enzymes. In conclusion, a thorough understanding of the characteristics of lysosomal neuraminidase and the identification and characterization of the responsible molecular defects in sialidosis patients could help us to address the potential therapeutic strategies for affected children.

In recent years the availability of rapidly expanding DNA databases, including the 'Expressed Sequence Tag' database (dBEST), has become a powerful tool to identify new cDNA's and genes that are expected to share homology at the DNA or protein level with other known genes or proteins. As discussed in Chapters 3 and 4 we were able to identify and clone the human lysosomal neuraminidase cDNA from the dBEST database through its sequence similarity to bacterial neuraminidases, and subsequently we also cloned the mouse neuraminidase cDNA. We identified and characterized mutations in the lysosomal neuraminidase gene that are responsible for sialidosis in humans and the neuraminidase deficiency in the SM/J mouse strain (Chapter 3 and 4). We identified mutations in a large group of sialidosis patients that cover the near complete clinical spectrum of the disease and established genotype-phenotype correlations (chapter 8). We analyzed the functional and intracellular behavior of different mutant proteins by over-expression experiments in

sialidosis fibroblasts (Chapter 8). Overall, the data showed that the level of residual activity and lysosomal localization of the mutant enzyme directly correlated with the severity of the patients' symptoms. In turn, the mildest type I patients had a small amount of functional neuraminidase in the lysosomes, while the severe type II patients completely lacked functional enzyme. We also demonstrated that the mere ability of a mutant enzyme to reach the lysosomes was not always sufficient to give a mild phenotype. One of the sialidosis patients had a mutation that involved one of the five active site residues of neuraminidase (Y370C). Despite normal amounts of correctly localized neuraminidase, the enzyme was inactive, and, as a result, the patient had the severe type II phenotype (Chapter 8).

Another important aspect presented in this thesis addresses the contribution of protective protein/cathepsin A to the transport, stability and catalytic activity of lysosomal neuraminidase (Chapters 5-7). We found that the turnover and posttranslational modifications of neuraminidase were unaffected by the presence of PPCA. However association with PPCA was required for the transport of neuraminidase to lysosomes, since it lacked a functional mannose-6-phosphate recognition marker (Chapter 6). We utilized the baculovirus expression system to characterize the mechanisms of catalytic activation of both PPCA and neuraminidase. We showed that through the excision of a linker peptide PPCA became a two-chain protein that was held together by disulfide bridges and was catalytically active (Chapter 6). In contrast, lysosomal neuraminidase did not require proteolytic processing to become catalytically active. PPCA associated with neuraminidase and promoted oligomerization of the latter enzyme, which, in turn, became fully active. The association with PPCA was pH independent, however, oligomerization and activation of neuraminidase could only occur at acidic pH (Chapter 7). These findings correlate with the ability of PPCA to associate with neuraminidase in an early biosynthetic compartment and promote transport of neuraminidase to lysosomes (Chapter 5). The acidic environment triggers the PPCA-mediated oligomerization and activation of neuraminidase (Chapter 7).

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

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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 β-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 β-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-α-neuraminidase initiates the hydrolysis of oligosaccharides, gangliosides, glycolipids, and glycoproteins by removing their terminal sialic acid residues. The human enzyme has a preference for α–2→3 and α 2→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, β-galactosidase, and the carboxypeptidase protective protein/cathepsin A (PPCA). It is thought that by associating with PPCA, neuraminidase and β-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 β-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 β-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 (Hoogeveen 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 β-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)$^+$ 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-

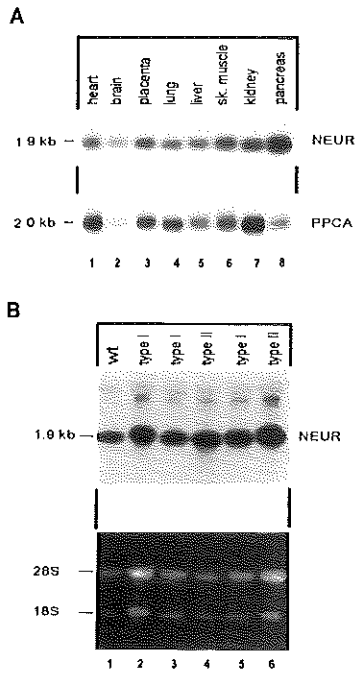

Figure 1. Neuraminidase mRNA expression. (*A*) Sequential hybridization of a multitissue Northern blot with the full-length neuraminidase cDNA (NEUR) and with the protective protein/cathepsin A (PPCA) cDNA. The size of the two transcripts was calculated on the basis of RNA markers. Exposure time for 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

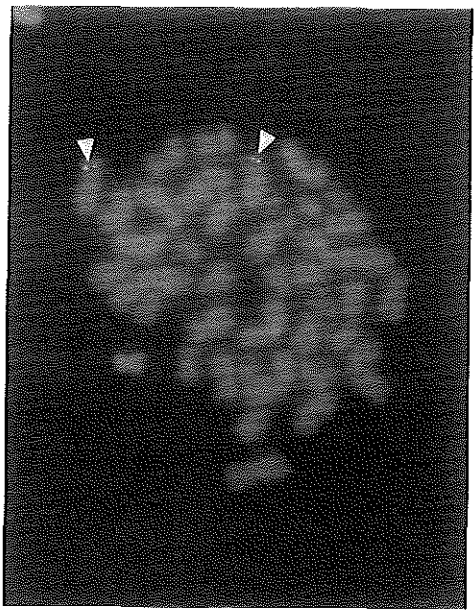

Figure 2. Chromosomal localization of human neuraminidase. Normal metaphase chromosomes were hybridized with a 3.5-kb neuraminidase genomic fragment, labeled with digoxigenin dUTP, and stained with antidigoxigenin antibodies. The white arrowheads indicate the hybridization signals on the chromatids of 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 typhimurium* 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 β-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 α-BV32 (Fig. 4A; P). Surprisingly, in a significant number of neuraminidase-expressing cells, square crystal-like structures were recognized by the α-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 β-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 α-neur antibodies and not by anti-PPCA or anti-β-gal antibodies (data not shown), indicating that they were devoid of PPCA and β-gal. From these results we infer that neuraminidase, when overexpressed in COS-1 cells, is independent of PPCA for its lysosomal-like compartimentalization, 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 α-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)-α-D-*N*-acetylneuraminic acid at pH 4.3, which is optimal for detecting lysosomal neuraminidase. As seen in Figure 4C, cells

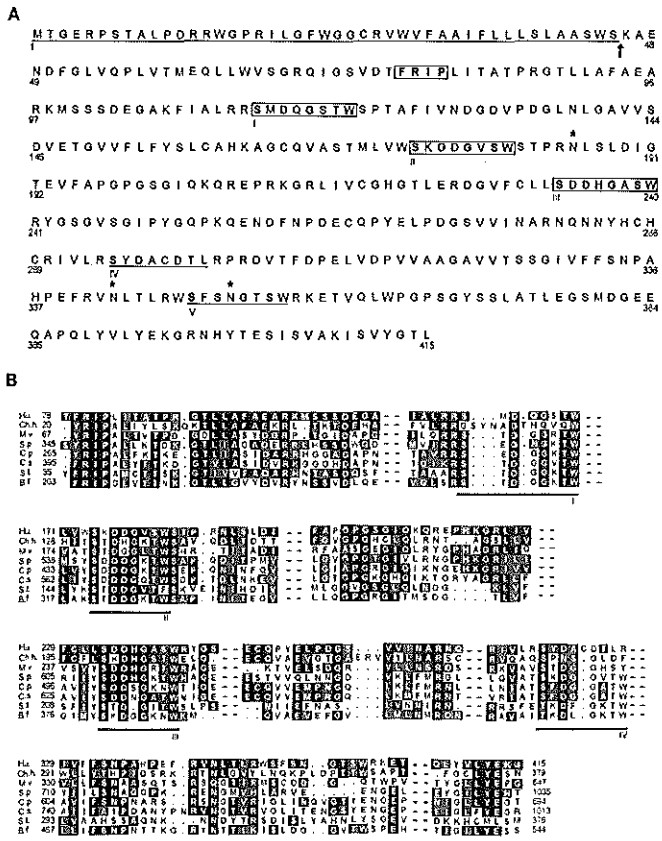

Figure 3. Predicted amino acid sequence of human neuraminidase cDNA and alignment with other sialidases. (*A*) The predicted amino-acid sequence, with residues 1–45 (underlined) representing the signal peptide, and amino acids 46–415 representing the neuraminidase polypeptide. The FRIP domain and three Asp boxes are boxed, two degenerated Asp boxes are underlined, and the conserved residues in these domains are printed in boldface. Three potential Asn-linked glycosylation sites are marked with an asterisk. (*B*) Alignment of human lysosomal neuraminidase with other mammalian and bacterial members of the sialidase superfamily. Selected regions of the human neuraminidase (Hu) sequence are compared with sialidases from Chinese hamster (Ch.h.), *Micromonospora viridifaciens* (M.v.), *Streptococcus pneumoniae* (S.p.), *Clostridium perfringens* (C.p.), *Clostridium septicum* (C.s.), *Salmonella typhimurium* (S.t.), and *Bacteroides fragilis* (B.f.) (Roggentin et al. 1993). Gaps were introduced to optimize the alignment (dashes). Identical amino acids are shaded in black; similar residues are shaded in gray. Numbers refer to the positions of the amino acids. The Asp boxes are underlined and numbered I to V.

expressing either neuraminidase (N) or PPCA (P) had 1.5–2.0 times higher neuraminidase activity than mock (M) or β-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 β-galactosidase cDNAs (N/B). These data strongly support the notion that the presence of PPCA, but not β-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 β-gal cDNA (N/B and N/P/B) could reflect competition between neuraminidase and β-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 α-neur antibodies is shown in Figure 5A. The endogenous neuraminidase in mock-transfected control fibroblasts (WT/M) displayed a typi-

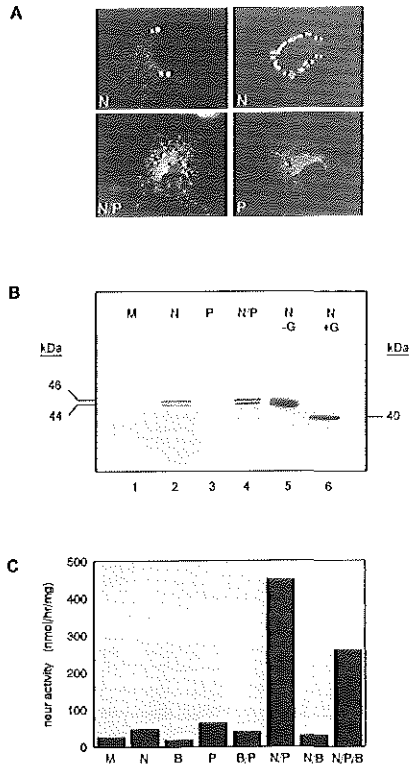

Figure 4. Immunocytochemical localization and neuraminidase activity in transiently transfected COS-1 cells. (*A*) Immunocytochemical localization of neuraminidase in COS-1 cells, transfected with the neur cDNA clone (N), or with both the neur and PPCA cDNAs (N/P), using affinity-purified α-neur antiserum. In addition, cells were transfected with the PPCA cDNA alone and processed for immunofluorescence with α-32 antiserum (P). Magnification, 400×. (*B*) Western blots prepared with equal amounts of protein (5 μg) 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 α-neur antiserum. (*C*) COS-1 cells were transfected with vector alone (M), neur cDNA (N), PPCA cDNA (P), β-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 α-neur antibodies recognized a polypeptide of ~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-

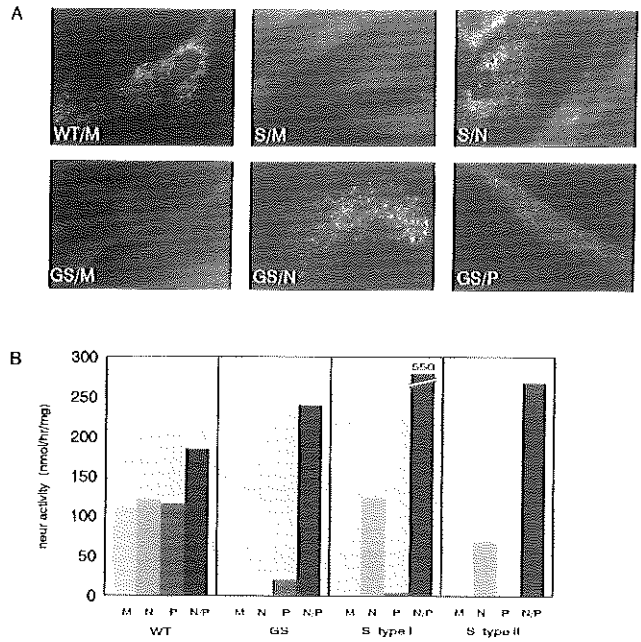

Figure 5. Correction of neuraminidase deficiency in sialidosis fibroblasts. (*A*) Immunocytochemical localization of neuraminidase in fibroblasts of a normal individual (WT), a sialidosis type I patient (S), and a galactosialidosis patient (GS), transfected with vector (M), neur cDNA (N), and PPCA cDNA (P). Cells were stained with affinity-purified α-neur antiserum. Magnification, 400×. (*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

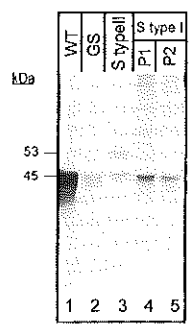

Figure 6. Immunoprecipitation of neuraminidase from sialidosis fibroblasts. Cultured fibroblasts from a normal individual (WT), an E.I galactosialidosis patient (GS), a type II sialidosis patient (S type II), and two siblings with type I sialidosis (S type I, P1, and P2) were labeled metabolically. The radiolabeled proteins were immunoprecipitated with α-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-

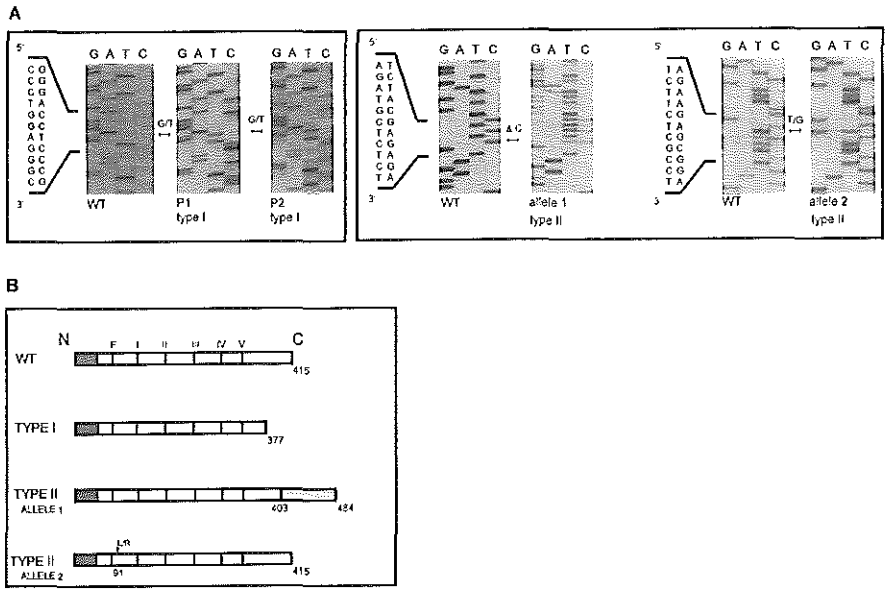

Figure 7. Mutations in the lysosomal neuraminidase gene of type I and type II sialidosis patients. (*A*) Partial nucleotide sequence of the neuraminidase cDNA from sialidosis patients. Total RNA was isolated from the fibroblasts of a normal individual (WT), the two siblings with type I sialidosis (P1 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 pleitropic 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 β-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 Genomes 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.

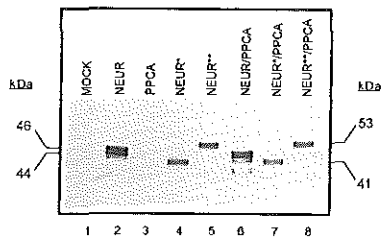

Figure 8. Western blot analysis of mutant neuraminidase from sialidosis patients. Western blot prepared with equal amounts of COS-1 cell lysates, transfected with vector (MOCK), neur cDNA (NEUR), PPCA cDNA (PPCA), or cotransfected with both the neur and PPCA cDNAs (NEUR/PPCA). In addition, two neuraminidase 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 α-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 β-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, α-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 occuring 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*

	Neuraminidase activity (nmol/hr per mg protein)	
cDNA construct	wild-type fibroblasts	sialidosis fibroblasts
mock	58 ± 8	0
PPCA	75 ± 9	0
neur	40 ± 5	30 ± 12
neur*	n.d.	0
neur**	n.d.	0
neur/PPCA	300 ± 21	108 ± 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 β-galactosidase is not directly involved in neuraminidase activation, a finding that supports earlier observations in PPCA-deficient knockout mice (Zhou et al. 1995), where β-galactosidase activity is reduced only in certain tissues, whereas neuraminidase deficiency parallels that of PPCA. Why then do PPCA, β-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 β-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). 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), and were compared with nonredundant peptide and nucleotide sequence data bases (PDB, SWISS-PROT, PIR, SPUpdate, GenPept, GPUpdate, GBUpdate, 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 μg) of human multitissue poly(A)$^+$ 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 μg) 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 β-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 μg) was then electroporated into ~1 × 10^6 cells, suspended in 500 μl of ISCOVE's medium, using a 0.4-cm electroporation cuvette in a BioRad Gene Pulser set at 0.320 kV, and 500 μF (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 (α-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 (α-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 μg of protein were resolved on SDS–polyacrylamide gels and transferred to Immobilon polyvinylidene difluoride membranes (Millipore). Western blots were incubated with affinity-purified α-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 chemiluminescent (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 μCi L-[4,5-^{3}H]-Leucine per dish for 20 hr. Proteins were precipitated with α-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 ~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 (~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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A point mutation in the *neu-1* locus causes the neuraminidase defect in the SM/J mouse

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Lysosomal neuraminidase (sialidase) occurs in a high molecular weight complex with the glycosidase β-galactosidase and the serine carboxypeptidase protective protein/cathepsin A (PPCA). Association of the enzyme with PPCA is crucial for its correct targeting and lysosomal activation. In man two genetically distinct storage disorders are associated with either a primary or a secondary deficiency of lysosomal neuraminidase: sialidosis and galactosialidosis. In the mouse the naturally occurring inbred strain SM/J presents with a number of phenotypic abnormalities that have been attributed to reduced neuraminidase activity. SM/J mice were originally characterized by their altered sialylation of several lysosomal glycoproteins. This defect was linked to a single gene, *neu-1*, on chromosome 17, which was mapped by linkage analysis to the H-2 locus. In addition, these mice have an altered immune response that has also been coupled to a deficiency of the Neu-1 neuraminidase. Here we report the identification in SM/J mice of a single amino acid substitution (L209I) in the Neu-1 protein which is responsible for the partial deficiency of lysosomal neuraminidase. We propose that the reduced activity is caused by the enzyme's altered affinity for its substrate, rather than a change in substrate specificity or turnover rate. The mutant enzyme is correctly compartmentalized in lysosomes and maintains the ability to associate with its activating protein, PPCA. We propose that it is this mutation that is responsible for the SM/J phenotype.

INTRODUCTION

Neuraminidases (sialidases) constitute a large and important family of hydrolytic enzymes that cleave the terminal sialic acid residues from a variety of sialoglycoconjugates [for a review see (10)]. This event influences many cellular processes, including cell–cell interaction/adhesion, protection from pathogens and antigen recognition (10–14). Some family members share certain characteristic features, including the F(Y)RIP domain in the N-terminal region of the protein, where the arginine is part of the active site, and two to five evenly spaced Asp boxes (S/T-X-D-X-G-X-T-W/F), which are located C-terminal of the F(Y)RIP sequence (15,16). The three-dimensional structure of bacterial and viral sialidases has shown that these enzymes have a common catalytic core of ~40 kDa with a characteristic six-bladed β-propeller fold (17,18). Human lysosomal *N*-acetyl-α-neuraminidase is deficient or defective in two distinct metabolic storage disorders: sialidosis, which is caused by structural lesions in the neuraminidase gene; and galactosialidosis, in which neuraminidase deficiency is secondary to a primary defect in the serine carboxypeptidase protective protein/cathepsin A (PPCA) (1,4). Recently we and others cloned the human neuraminidase cDNA and identified a number of independent mutations in the neuraminidase gene that we associated with different clinical variants of sialidosis (3,19,20). The neuraminidase locus maps to the HLA region on chromosome 6p21 (3,21,22).

In the mouse a partial deficiency of a neuraminidase was first identified in the naturally occurring strain SM/J (23). These inbred mice had already been selected in the early 1940s for their relative small body size following cross matings with seven different inbred strains. Later, biochemical analysis demonstrated abnormal sialylation of at least four lysosomal glycoproteins that showed an altered migration pattern on starch gel electrophoresis. This defect was corrected by treatment with bacterial sialidase [reviewed in (5)]. This hypersialylation was attributed to a reduction in activity of a liver-specific sialidase (5,24,25), although some reports suggested that other organs were also affected (23,26). The responsible gene was designated *neu-1* and mapped, by linkage analysis, to the histocompatibility locus on chromosome 17, in the region between H-2D and H-2E$_\alpha$ (6,7), which is syntenic to the human HLA locus on chromosome 6p21. Besides the abnormal sialylation of lysosomal glycoproteins, SM/J mice also have an impaired immune response, which is thought to result from the altered processing of sialic acids present on cell surface molecules of subpopulations of T cells (8,9,27–31). An important step in the development of an immune response is differentiation of activated naive T cells into either IFN-γ-producing (T_{h1}) or IL-4-producing (T_{h2}) cells [for a review see (32)]. Although SM/J mice can stimulate a T_{h1}-mediated immune response, they cannot stimulate the conversion of naive T cells into IL-4-producing T_{h2} lymphocytes. This altered response has been attributed to reduced activity of Neu-1 neuraminidase, which is thought to result in: (i) improper desialylation of surface antigens on T_{h2}-committed cells; (ii) reduction in early IL-4 production; and (iii) absence of IgG1 and IgE production by B cells after *in vivo* immunization of SM/J mice with pertussis toxin (31). T cell Neu-1 neuraminidase has

also been implicated in conversion of vitamin D_3 binding protein into macrophage activating factor (27). Together these data suggest an important role for Neu-1 neuraminidase in processing of selected sialoglycoconjugates at either the plasma membrane or within intracellular compartments.

In this paper we describe identification of a single amino acid substitution, L209I, in the *neu 1*-encoded lysosomal neuraminidase of SM/J mice. Analysis of the biochemical properties of this mutant enzyme demonstrates that its reduced neuraminidase activity is indeed caused by the presence of this mutation and not by improper compartmentalization of the protein, altered turnover or a lack of association with PPCA.

RESULTS

Isolation of the murine neuraminidase cDNA and expression pattern of *neu-1* in mouse tissues

Two murine neuraminidase cDNAs (1.8 and 2.4 kb) were isolated using the human cDNA as probe. Both contained the same open reading frame, but the 2.4 kb clone lacked the first two codons and had an extended 3′-untranslated region (UTR). The deduced amino acid sequence of the mouse protein is 91% similar to its human counterpart: the N-terminus begins with a conventional 39 amino acid signal sequence (33) and includes a FRIP domain as well as three conserved and two degenerated Asp boxes. The protein has four potential N-linked glycosylation sites; the first three are found at identical positions in the human neuraminidase, whereas the fourth, which is close to the C-terminus, is only present in the mouse sequence (Fig. 1). Northern blot analysis of multiple tissues, using probes spanning the cDNA (Fig. 1) demonstrated two major and two minor transcripts, which vary in length from 1.8 to 4.0 kb (Fig. 1). The 3′-UTR probe, unique for the 2.4 kb cDNA, recognized only the 2.4 and 4.0 kb transcripts, indicating that the four mRNAs use alternative 3′-UTRs. The hybridization results suggest that all four transcripts contain the same protein encoding sequence. The 1.8 and 2.4 kb mRNAs were the most abundant and displayed a differential pattern of expression which closely correlated with expression of PPCA, which forms a complex with the neuraminidase protein (34,35). The murine gene coding for the isolated cDNAs contains six coding exons (Table 1). The gene spanned a small region of 4 kb and was mapped, using the 1.8 kb cDNA insert as probe, to the H-2 region of chromosome 17 (data not shown).

Phenotypic characterization of SM/J mice and identification of the mutation in the *neu-1* gene

All four neuraminidase transcripts displayed similar patterns of expression in kidney, brain, liver and spleen RNA preparations from SM/J mice (Fig. 1). In addition, a single polypeptide was immunoprecipitated with anti-human neuraminidase antibodies (anti-neur) from radiolabeled lysates of SM/J fibroblasts; this immunoprecipitated protein was comparable in size with the normal murine protein (data not shown). Although we found no overt changes at the RNA or protein level, we did find that neuraminidase activity of SM/J Neu-1 differed from that of wild-type Neu-1. Using sodium 4-methyl-umbelliferyl-α-D-*N*-acetylneuraminate (4-MU-NANA) as substrate, SM/J Neu-1 activity was significantly reduced in lysosomal/mitochondrial extracts derived from several SM/J tissues and this partial deficiency was clearly not restricted to any one tissue (Fig. 2). SM/J neuraminidase activity in kidney and liver extracts was also lower than that of control values when assayed with either α-2,3- and α-2,6-sialyllactose (α-2,3- and α-2,6-NANA-lactose) as substrate, thus demonstrating that the defective enzyme did not show altered specificity for either of the two linkages (Fig. 2). However, using fibroblast extracts we could demonstrate that SM/J neuraminidase assayed with substrate concentrations ranging from 0.1 to 1.5 mM 4-MU-NANA had an ~3-fold lower V_{max} than the wild-type enzyme (Fig. 2). This suggests that the L209I substitution influences the kinetic properties of the mutant enzyme. Furthermore, the mutant mice displayed an abnormal pattern of urinary oligosaccharides (Fig. 2), indicative of oligosaccharidiuria, a condition commonly observed in galactosialidosis mice (2). Histological analysis of the SM/J mice showed evidence of storage products in specific cells, such as the Purkinje cells of the cerebellum and the glomerular epithelium, which appeared to accumulate over time (data not shown). Because these parameters are commonly used in biochemical diagnosis of sialidosis and galactosialidosis patients (1,4), it is clear that SM/J mice share similar phenotypic abnormalities with these two human diseases.

To identify the underlying genetic lesion responsible for these abnormalities we searched for a mutation(s) in the neuraminidase gene. Using RT-PCR on brain and liver RNA derived from SM/J mice of different ages and from different litters we amplified four overlapping fragments that span the entire neuraminidase cDNA (Fig. 3). Three mouse strains, BALB/c, 129/Sv and FVB, were used as controls. Sequence comparison identified seven nucleotide changes in the SM/J cDNA; four involved the wobble base of amino acid codons Lys93, Arg202, Thr295 and Ala316, two were present in the 3′-UTR and one was a C→A transversion at nt 625 within exon IV of the gene. This transversion resulted in the amino acid change Leu209 to Ile (L209I). Because exon IV is present in all four neuraminidase transcripts (Fig. 1), we inferred that this point mutation must be present in all of the mRNAs and in the corresponding protein.

Table 1. Sizes and locations of exons and introns and sequences at the exon/intron boundaries of the *neu-1* gene

Exons		cDNA position	Introns			Intron size	
Number	Size		5′ splice site		3′ splice site		
I	171	−30 to 141	AGCCTGgtgagc	.....	gcgcagGTGCAG	365	(1)
II	190	142 to 331	ACCAGGgtaaca	.....	ttctagGTAGCA	453	(2)
III	266	332 to 597	ATTCAGgtttca	.....	taacagAAACAG	1200	(3)
IV	183	598 to 780	TGCCAGgtcagg	.....	acgcagCCCTAC	97	(4)
V	221	781 to 1001	AGTTCCgtgagt	.....	tcctagGAGTGA	99	(5)
VI	1365	1002 to 2366					

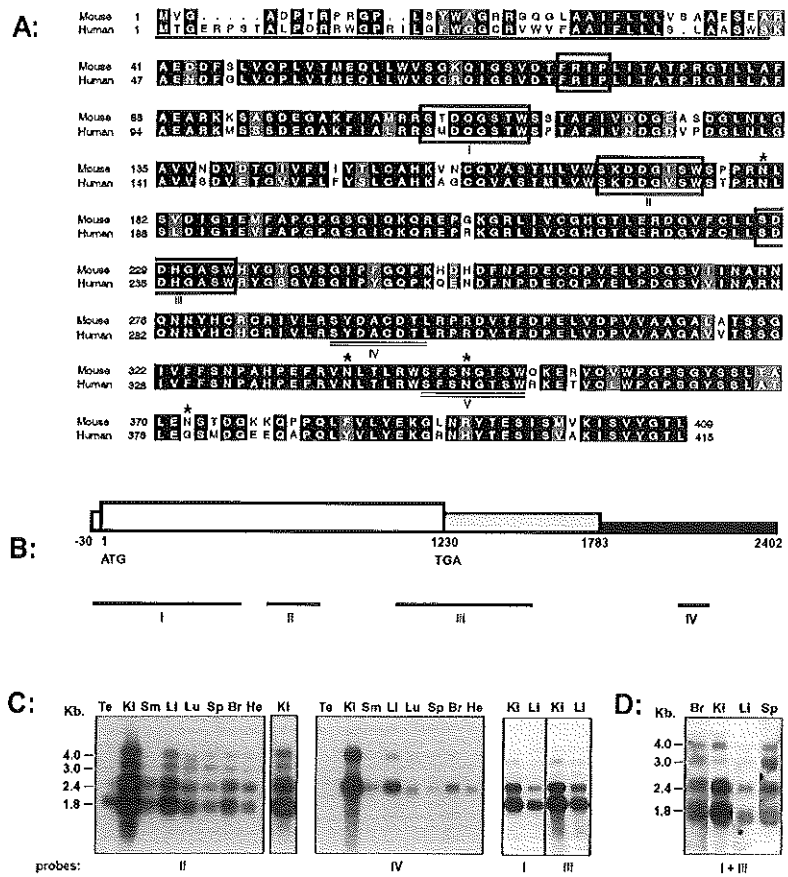

Figure 1. (A) Comparison of the amino acid sequences of mouse and human neuraminidases. Identical residues are shown in black and similar residues in gray. The signal sequence is underlined; the FRIP sequence and the conserved Asp boxes (I–III) are boxed; the degenerate Asp boxes (IV and V) are double underlined. The glycosylation sites are indicated by an asterisk above the sequence. (B) Linear representation of the two neuraminidase cDNAs: the coding region is indicated as an open box; the 5′-UTR, unique for the 1.8 kb clone, and the part of the 3′-UTR shared by both cDNA clones are indicated by gray boxes. The part of the 3′-UTR unique for the 2.4 kb clone is shown as a smaller black box. Numbers represent nucleotide positions. The different probes used to hybridize the northern blot are: I, nt –23 to 491; II, nt 601 to 780; III, nt 1049 to 1539; IV, nt 2072 to 2168. (C) Northern blot analysis using the probes outlined in (B) and indicated under each panel. Exposure times were 3 days for blots probed with II and IV, 16 h for blots probed with I and III. The blot hybridized with probe II was also exposed for 16 h to resolve the different transcripts in the kidney sample (shown as separate lane). Br, brain; He, heart; Ki, kidney; Li, liver; Lu, lung; Sm, smooth muscle; Sp, spleen; Te, testis. (D) Northern blot prepared with total RNA from SM/J-derived tissues and hybridized with a combination of probes I and III. Exposure time was 5 days.

Expression of L209I mutant neuraminidase in deficient fibroblasts

To assess the impact of the L209I change on biochemical properties of the normal enzyme we engineered this mutation into the normal 1.8 kb murine cDNA. The resulting mutant clone (Mo-smj) was completely sequenced to confirm correct introduction of the mutation. This mutant cDNA was transiently expressed in two human sialidosis type II fibroblasts. These cells were chosen because, unlike SM/J fibroblasts, they totally lack neuraminidase activity (3). Mo-smj cDNA only partially corrected the deficient fibroblasts, generating neuraminidase activity of between 40 and 65% of that of the wild-type murine (Mo-neur) enzyme. Given the strict dependence of neuraminidase on PPCA for full enzymatic activity (1,2), we also tested the effect of both mouse and human PPCA on SM/J neuraminidase. Co-transfection of the Mo-smj and Mo-neur cDNAs with either the mouse or human PPCA cDNA (Mo-ppca and Hu-ppca) resulted in a clear

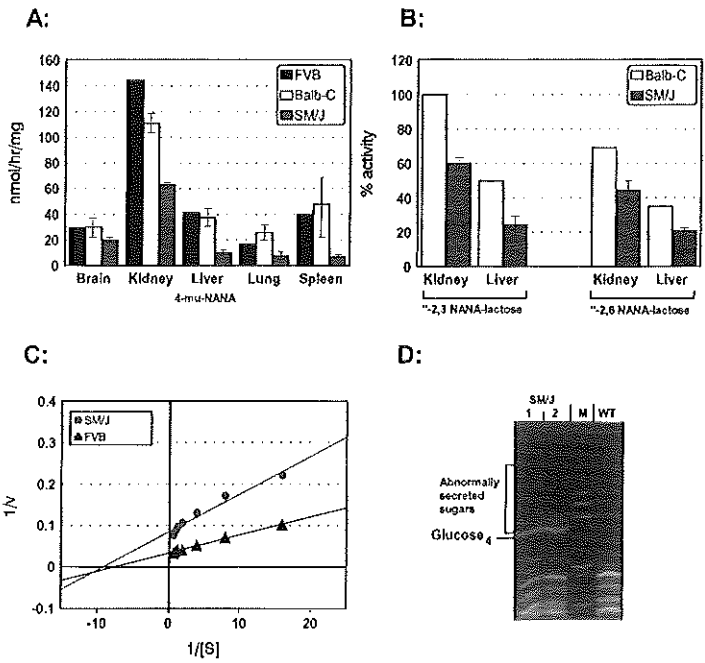

Figure 2. (A) Lysosomal/mitochondrial fractions of different mouse tissues assayed with the 4-MU-NANA substrate. Values shown represent the average of three independent experiments. (B) Lysosomal/mitochondrial fractions of different mouse tissues assayed with either α-2,3-NANA-lactose or α-2,6-NANA-lactose. Values given are the average of three independent experiments. Activities are expressed as a percentage of normal kidney neuraminidase activity assayed with α-2,3-NANA-lactose. Both control and SM/J mice were between 3 and 4 months old. (C) Lineweaver–Burke analysis showing dependence of the 4-MU-NANA substrate concentration on initial rate of neuraminidase activity. V_{max} for the SM/J neuraminidase is ~12 nmol/h/mg, while the wild-type enzyme has a V_{max} of ~30 nmol/h/mg. Activities were assayed as described in Materials and Methods; v is the velocity rate of the reaction in nmol/h/mg and S is the substrate concentration in mM. (D) Urine analysis of SM/J and control mice, displaying an abnormal pattern of oligosaccharides. SM/J 1 and 2 represent two independent urine samples from two different SM/J mice, M is the OLIGO ladder standard from Glyko Inc. and WT refers to the urine sample collected from a wild-type mouse.

increase in neuraminidase activity for both the normal and mutant protein (Fig. 3). The induced SM/J activity, however, remained lower than that of the wild-type. The L209I mutation was also introduced into the human neuraminidase cDNA (Hu-smj). Expression of this mutant clone alone or in combination with human or mouse PPCA again resulted in reduced neuraminidase activity (Fig. 3), unequivocally demonstrating that the L209I substitution is responsible for the enzyme defect. Immunofluorescence analysis of singly and doubly transfected cells showed that the presence of the mutation in either the mouse or the human neuraminidase molecule does not alter the subcellular distribution of the enzyme, which maintained a typical punctate lysosomal staining (Fig. 3). The lysosomal localization of the mutant enzyme was confirmed using Percoll density gradients with transfected COS-1 cells (data not shown). Co-expression of either mouse or human PPCA clearly enhanced the lysosomal signal, further indicating that PPCA has a stabilizing effect on the mutant protein.

Biosynthesis of the L209I mutant in COS-1 cells and its association with the PPCA precursor

The increase in SM/J neuraminidase activity in cells co-expressing mutant Neu-1 neuraminidase and PPCA suggested that interaction between the two proteins was not affected by the L209I mutation. We tested this assumption by overexpressing the Smj-neu1 and the PPCA cDNAs in COS-1 cells and then immunoprecipitating radiolabeled proteins with anti-neur or anti-PPCA antibodies (Fig. 4). Although the anti-neur antibodies recognized the murine protein with lower affinity than they did the human protein, more SM/J protein than wild-type mouse protein was immunoprecipitated from equally transfected cells (Fig. 4, lanes 2 and 3). Nevertheless, neuraminidase activity in singly transfected cells was again 50% of that of control values (data not shown). In co-transfected cells both the mouse and human PPCA precursors were co-precipitated with anti-neur antibodies, together with the SM/J polypeptide (Fig. 4, lanes 8

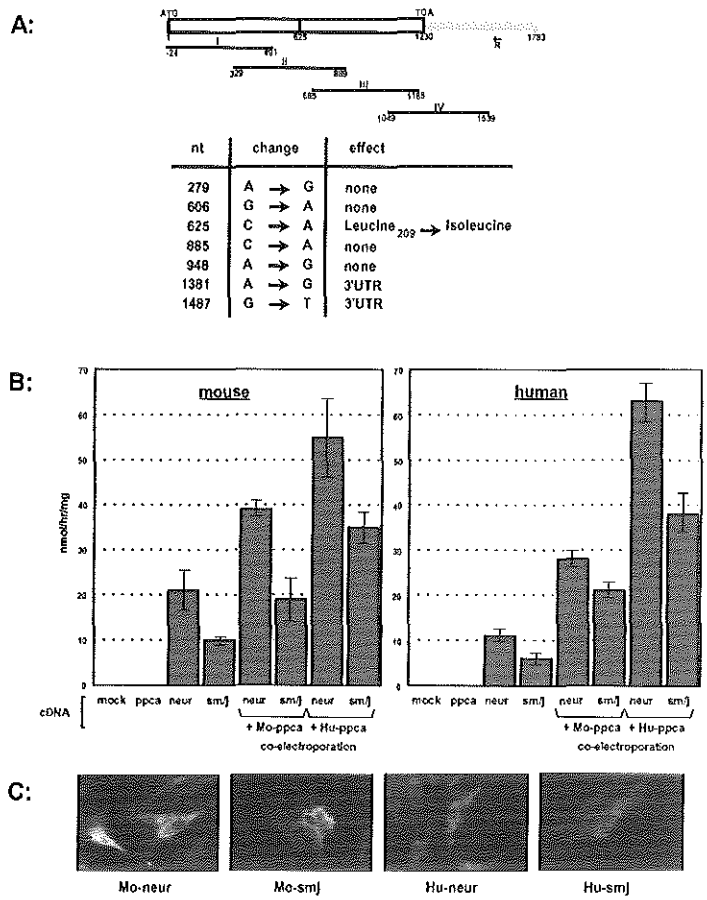

Figure 3. (A) Strategy used to screen for mutations in the *neu-1* cDNA. R indicates the primer used to reverse transcribe the mRNA; individual fragments were amplified using the gene-specific primers listed in Materials and Methods. The table represents the results obtained with this screening procedure. (B) Neuraminidase activity in cell lysates of electroporated GM01718 sialidosis type II fibroblasts using 4-MU-NANA as substrate. Values represent the average of four independent electroporations. The diagram to the left shows results obtained with the murine neuraminidase cDNAs, whereas the diagram to the right shows results with the human cDNA samples. The last four samples on the right of each panel represent the neuraminidase cDNAs co-electroporated with either mouse (Mo-ppca) or human (Hu-ppca) protective protein/cathepsin A cDNA. ppca, protective protein/cathepsin A cDNA; neur, wild-type 1.8 kb neuraminidase cDNA; smj, 1.8 kb neuraminidase cDNA containing the SM/J mutation. (C) Immunofluorescence with anti-human neuraminidase antibodies of fibroblasts electroporated with the mouse neuraminidase cDNA (Mo-neur), the SM/J cDNA (Mo-smj), human neuraminidase cDNA (Hu-neur) and human mutant cDNA (Hu-smj). Lysosome-like punctated staining was evidenced in the different electroporated fibroblasts.

and 11). The mutant protein, the wild-type mouse protein and human neuraminidase all co-precipitated the PPCA precursor equally efficiently (lanes 7, 9, 10 and 12). Therefore, the L209I substitution did not interfere with association between the mutant protein and either mouse or human PPCA, excluding the possibility that the SM/J mutation affects complex formation. Sequential immunoprecipitation of all samples with anti-PPCA antibodies explained the difference in the ability of the mouse and human PPCA to enhance neuraminidase activity (Fig. 3). The murine PPCA precursor in overexpressing cells was not as well processed to the mature two chain form as the human PPCA precursor (Fig. 4, lanes 7–9 and 10–12). This reduced level of processing could have led to a smaller pool of mature PPCA available for 'protection' of the lysosomal neuraminidase.

Turnover of the SM/J neuraminidase was apparently not influenced by the L209I substitution, as determined by pulse-

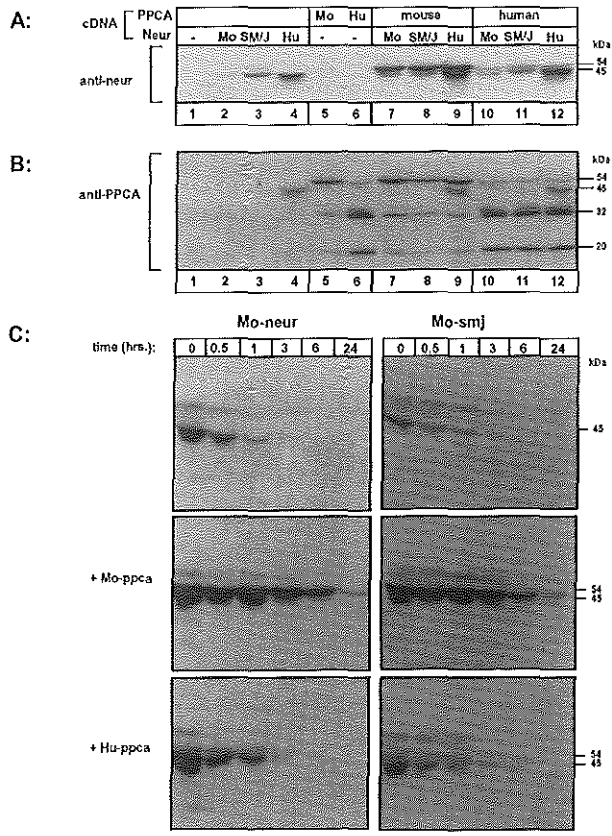

Figure 4. (A) Immunoprecipitation of radiolabeled cell lysates from transiently transfected COS-1 cells using anti-human neuraminidase antibodies. Cells were either singly or doubly transfected with the indicated cDNA clones and then labeled for 16 h with 50 μCi [^{3}H]-4,5-leucine before harvesting. Lane 1, mock-transfected cells. Mo, mouse; SM, SM/J; Hu, human. (B) Sequential immunoprecipitation of the same lysates as used in (A) using either anti-mouse (lanes 1–5 and 7–9) or anti-human (lanes 6 and 10–12) PPCA antibodies. In lanes 4, 9 and 12 small quantities of the 45 kDa neuraminidase protein are still visible, because the samples were not precleared prior to performing the second immunoprecipitation. (C) Pulse-chase analysis of transiently transfected COS-1 cells. Cells were labeled for 1 h with 50 μCi [^{3}H]-4,5-leucine and then chased for the indicated times with non-radioactive medium. Samples were then immunoprecipitated with the anti-human neuraminidase antibodies. (Top panel) Pulse-labeled COS-1 cells transfected with the mouse neuraminidase cDNA (Mo-neur) or the mutant cDNA (Mo-smj); (middle panel) COS-1 cells co-transfected with mouse neuraminidase and PPCA (Mo-ppca) cDNAs; (lower panel) COS-1 cells co-transfected with mouse neuraminidase and human PPCA (Hu-ppca) cDNAs. Molecular weights were calculated on the basis of protein standards.

chase labeling of transfected COS-1 cells (Fig. 4). Both the mutant and wild-type neuraminidase appeared to be stabilized upon co-expression of mouse PPCA, since immunoprecipitable material could still be detected at the 3–6 h time points (Fig. 4, middle panels). The stabilizing effect was less apparent, but still recognizable, when human PPCA was co-expressed with mutant or wild-type neuraminidase (Fig. 4, lower panels). These results clearly correlate with the observed increase in enzyme activity in cells co-expressing mutant or wild-type neuraminidase with PPCA.

DISCUSSION

Overall the results we present here provide strong evidence that the subtle L209I substitution is responsible for the altered neuraminidase activity in SM/J mice. Leu209 in the murine enzyme falls in an amino acid stretch that is highly conserved among the different sialidases (10,36). This residue coincides with Leu221 and Leu199 of the *Micromonospora viridifaciens* and *Salmonella typhimurium* sialidases respectively, which are located in the three-dimensional structure of these enzymes in the

vicinity of the active site (17,18). It is therefore conceivable that this amino acid substitution in the SM/J neuraminidase could affect substrate recognition, rate of substrate cleavage or release of the product, as evidenced by the altered V_{max} of the mutant protein. Although SM/J mice present with some of the biochemical abnormalities that are associated with the human lysosomal disorder sialidosis, the relatively high residual neuraminidase activity prevents the occurrence in young mice of excessive storage in their tissues. Older mice, on the other hand, eventually develop visible cellular changes, especially in the CNS. Therefore this animal model may be regarded as a mild form of sialidosis.

The residual SM/J activity varied slightly in different tissues. This could be attributed to the occurrence in some tissues, like brain, of neuraminidase 'isoenzymes' thought to be localized in the lysosomal (37,38) or plasma membrane (38–42) and the cytosol (16,38,43–45). However, the existence of various lysosomal neuraminidases is questionable, since in PPCA-deficient mice no residual neuraminidase activity is detected at acidic pH (2). The same holds true for human sialidosis patients with structural mutations in the neuraminidase gene that result in complete loss of neuraminidase activity (3).

Modification of sialic acid residues, which are present as terminal sugars on various types of sialoglycoconjugates, is essential for regulation of many cellular activities. The Neu-1 neuraminidase plays a key role in such modifications, for example in processing of cell surface molecules that are involved in modulating an immune response (9,27–31). T lymphocyte activation is normally accompanied by an increase in endogenous Neu-1 neuraminidase (30,31), which, in turn, results in hyposialylation of glycoproteins on the surface of activated T cells (9,46–49). These surface glycoproteins are required for T cell differentiation [for a review see (32)] and several of them are known to be internalized from the plasma membrane and subsequently re-exposed by a 'recycling' process. MHC class I and class II molecules and the T cell receptor are examples of such molecules (50–53). Therefore, it may be that processing of the sialic acid residues on these and other glycoproteins present on the surface of specific T cells is mediated intracellularly by lysosomal neuraminidase. If this enzyme is part of the main mechanism for sialic acid processing in T cells then the altered V_{max} value of SM/J neuraminidase would quite logically account for the abnormal sialylation of these molecules. Our data suggest that the mutant enzyme retains the capacity to recognize its substrate but that its rate of catalysis and/or release of product is impaired. The type of substrates that are cleaved by the enzyme may determine whether or not a certain cell type can compensate for a reduction in activity of mutant neuraminidase. Again, this is best exemplified in the T cell system, where the immune response in SM/J mice involves differentiation of naive T cells to T_{h1} but not to T_{h2} cells.

Interestingly, reduced neuraminidase activity has also been detected in rat strain KGH (54). The responsible gene, *neu-1*, was mapped to the RT1 locus (55), which is syntenic to the mouse H-2 and human HLA loci. It is unclear whether this defect results in the same phenotypic alterations identified in SM/J mice. It will be instructive to identify the molecular basis of the defective neuraminidase activity in this rat strain and to compare it with that found in SM/J mice. A second gene, *neu-2*, has also been described in both mouse and rat (54,56). However, the encoded enzyme is localized in the cytoplasm and does not cleave the fluorimetric substrate (57). Furthermore, linkage analysis demonstrated that the *neu-2* is not linked to the *neu-1* locus (54).

Once the three-dimensional structure of the lysosomal mammalian neuraminidase becomes available we could gain a better understanding of the impact of the L209I mutation on structure and function of the enzyme. Our findings on SM/J mice will hopefully facilitate further genetic and immunological studies on this animal model.

MATERIALS AND METHODS

Isolation of the mouse cDNA

A mouse BALB/c cDNA library was screened according to the manufacturer's instructions (Clontech). cDNA clones were sequenced with the Amersham thermocycler kit and subcloned into the mammalian expression vector pSCTOP (58).

RNA isolation and Northern blot analysis

RNA was prepared from SM/J mouse tissues by the LiCl/urea method as previously described (59). Total RNA was separated on 1% agarose gels that contained 0.66 M formaldehyde in MOPS buffer, was then blotted onto Zetaprobe membranes (BioRad) and was finally hybridized under standard conditions (60). The multiple tissue northern blot was purchased from Clontech and handled according to the manufacturer's instructions.

Mutation analysis and mutagenesis

Total RNA preparations from different SM/J mice and FVB controls were subjected to RT-PCR (3,61). The following primers were used in the reactions: 5′-CCCTAGGACACCGGGCCTTC-3′ (antisense, primer R); 5′-CCTGGACAGGGATCGCCG-3′ and 5′-GTAGAGGCCACCTGGCAG-3′ (fragment I); 5′-CGGACCAGGGTAGCACGTGG-3′ and 5′-GGGTGTCACAGGCGTCATAG-3′ (fragment II); 5′-GATGACCACGGTGCCTCC-3′ and 5′-GGTGTACCGGTTCAGGCC-3′ (fragment III); 5′-CCTGGCAGAAGGAGAGGG-3′ and 5′-CTGTTCATCTCTCCAGGG-3′ (fragment IV).

Amplified products were purified by phenol/chloroform extraction, on Centricon-100 columns (Amicon) and by ethanol precipitation. The purified products were directly sequenced with the fmol sequencing kit (Promega). The mutation was inserted into the wild-type cDNA by combining fragments II and III (Fig. 3) and using the *Stu*I restriction sites at positions 377 and 1168 to substitute the *Stu*I fragment for the wild-type fragment.

Cell culture, electroporation of fibroblasts and transfection of COS-1 cells

Human skin fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics and 10% fetal bovine serum (FBS). COS-1 cells were maintained in DMEM supplemented with 5% FBS. Fibroblasts were electroporated according to the manufacturer's instructions (BioRad) with the following modifications. Cells were harvested by trypsinization and washed once in Iscove's medium. They were then counted and 25 µg plasmid DNA were electroporated into 1×10^6 cells suspended in 500 µl Iscove's medium using a BioRad Gene Pulser set at 0.32 kV and 500 µF. Electroporated cells were seeded in 6-well plates for 14–18 h before the medium was changed. They were harvested 72 h later. Immunofluores-

cence of electroporated fibroblasts was performed as described previously (3). COS-1 cells were transfected with Qiagen's Superfect according to the manufacturer's instructions and harvested 72 h post-transfection. Transfection efficiency was checked by immunofluorescence and the total amount of synthesized neuraminidase protein was estimated by western blot analysis of total cell lysates. Comparable transfection efficiencies were obtained among samples within each experiment and similar levels of neuraminidase protein were synthesized.

Lysosomal/mitochondrial extract

Mice were sacrificed by cervical dislocation and their tissues immediately isolated and placed in ice-cold 10 mM HEPES, pH 7.4, 250 mM sucrose. After the tissues were washed several times in this buffer they were weighed and homogenized in a tight-fitting dounce (Kontes) in 4 vols HEPES-buffered sucrose. A lysosomal/mitochondrial extract was prepared according to the procedure described by Gieselmann (62). The resulting lysosomal/mitochondrial pellet was dissolved in HEPES-buffered sucrose and analyzed for enzyme activity.

Enzyme activities and urine analysis

Lysosomal/mitochondrial extracts and cell lysate from either transfected COS-1 cells or electroporated fibroblasts were assayed for neuraminidase activity using the artificial substrate 4-MU-NANA according to Galjaard (63). Protein concentrations were determined using the BCA kit from Pierce Chemical Co. Neuraminidase activity also was assayed with α-2,3- and α-2,6-NANA-lactose as substrates, according to the procedure described previously (64,65). Urine samples were collected and analyzed using a FACE® Urinary Carbohydrate Analysis kit purchased from Glyko Inc. following the manufacturer's instructions.

Immunoprecipitation

Transfected COS-1 cells were seeded in 6-well plates and labeled for 16 h with 50 μCi [^{3}H]-4,5-leucine. Radiolabeled proteins were immunoprecipitated with anti-neur antibodies, as described previously (66). For the pulse-chase experiment transfected cells were labeled with 50 μCi [^{3}H]-4,5-leucine for 1 h and then chased in fresh DMEM over different time periods (67).

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Transport of human lysosomal neuraminidase to mature lysosomes requires protective protein/cathepsin A

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Human lysosomal *N*-acetyl-α-neuraminidase is deficient in two lysosomal storage disorders, sialidosis, caused by structural mutations in the neuraminidase gene, and galactosialidosis, in which a primary defect of protective protein/cathepsin A (PPCA) leads to a combined deficiency of neuraminidase and β-D-galactosidase. These three glycoproteins can be isolated in a high molecular weight multi-enzyme complex, and the enzymatic activity of neuraminidase is contingent on its interaction with PPCA. To explain the unusual need of neuraminidase for an auxiliary protein, we examined, in transfected COS-1 cells, the effect of PPCA expression on post-translational modification, turnover and intracellular localization of neuraminidase. In pulse–chase studies, we show that the enzyme is synthesized as a 46 kDa glycoprotein, which is poorly phosphorylated, does not undergo major proteolytic processing and is secreted. Importantly, its half-life is not altered by the presence of PPCA. However, neuraminidase associates with the PPCA precursor shortly after synthesis, since the latter protein co-precipitates with neuraminidase using anti-neuraminidase antibodies. We further demonstrate by subcellular fractionation of transfected cells that neuraminidase segregates to mature lysosomes only when accompanied by wild-type PPCA, but not by transport-impaired PPCA mutants. These data suggest a novel role for PPCA in the activation of lysosomal neuraminidase, that of an intracellular transport protein.
Keywords: activation/lysosomes/neuraminidase/protective protein/transport

Introduction

Neuraminidases (sialidases) are exoglycosidases that catalyze the removal of terminal sialic acid residues, α-ketosidically linked to mono- or oligosaccharide chains of glycoconjugates. In mammals, three distinct neuraminidases have been identified in the cytoplasm, plasma membrane and lysosomes. These enzymes differ in their pH optimum, interaction with detergents, and stability (reviewed in Saito and Yu, 1995; Schauer *et al.*, 1995). Lysosomal neuraminidase preferentially cleaves terminal α(2,3)- and α(2,6)-linked sialic acid residues and has an acidic pH optimum. In man, deficiency of this enzyme is associated with two distinct genetic disorders of metabolism: sialidosis, caused by structural lesions in the neuraminidase gene (Thomas and Beaudet, 1995), and galactosialidosis (GS), in which neuraminidase deficiency is secondary to a primary defect in the serine carboxypeptidase protective protein/cathepsin A (PPCA) (d'Azzo *et al.*, 1982). For both diseases, early onset forms with severe CNS pathology and systemic organ involvement, as well as milder late onset variants, have been identified. The lack of PPCA has also been shown to hamper neuraminidase activity severely in the mouse model of GS (Zhou *et al.*, 1995).

The cDNA for human lysosomal neuraminidase was isolated recently (Bonten *et al.*, 1996; Milner *et al.*, 1997; Pshezhetsky *et al.*, 1997). It encodes a protein of ~45 kDa, with three potential N-linked glycosylation sites, and 32–38% sequence homology to several bacterial sialidases as well as to the cytosolic mammalian enzyme. These homologous sequences include the characteristic FRIP sequence, three conserved copies of an 'Asp box' [consensus sequence Ser/Thr-X-Asp-(X)-Gly-X-Thr-Trp/Phe (Roggentin *et al.*, 1993)] and two degenerated Asp boxes. Electroporation of the neuraminidase cDNA into sialidosis fibroblasts restores enzymatic activity (Bonten *et al.*, 1996). Furthermore, analysis of the neuraminidase cDNA from different sialidosis patients has identified six independent mutations in the gene (Bonten *et al.*, 1996; Pshezhetsky *et al.*, 1997), two of which were shown to render the protein non-functional (Bonten *et al.*, 1996). These studies have thus defined the molecular basis of sialidosis.

Mammalian lysosomal neuraminidase is unique among other sialidases in that it requires the serine carboxypeptidase PPCA for enzymatic activity (d'Azzo *et al.*, 1995; Thomas and Beaudet, 1995). Neuraminidase shares this feature with a third lysosomal enzyme, β-D-galactosidase (d'Azzo *et al.*, 1995; Suzuki *et al.*, 1995). The dependence of these glycosidases on the carboxypeptidase is evident in GS, where malfunctioning or absence of PPCA leads to the combined deficiency of neuraminidase and β-galactosidase (d'Azzo *et al.*, 1995). These three lysosomal enzymes can be co-purified in a high molecular weight complex with either β-galactosidase or PPCA affinity matrices (Verheijen *et al.*, 1985; Yamamoto and Nishimura, 1987; Pshezhetsky and Potier, 1994, 1996). Both neuraminidase and β-galactosidase activities in cultured GS fibroblasts are restored by the addition of exogenous PPCA precursor (54 kDa), which is internalized via the mannose-6-phosphate (M6P) receptor, routed to the lysosome and processed into its mature 32/20 kDa two-chain form (Galjart *et al.*, 1988; Zhou *et al.*, 1996).

How PPCA influences the generation and maintenance of neuraminidase and β-galactosidase activities is not yet clear. It is known that the half-life of mature β-galactosid-

ase is severely reduced in GS fibroblasts, and that treatment with the protease inhibitor leupeptin increases the amount and activity of β-galactosidase (d'Azzo *et al.*, 1982; van Diggelen *et al.*, 1982; Pshezhetsky and Potier, 1996). This implies that PPCA protects β-galactosidase against rapid proteolytic degradation. In contrast, the neuraminidase activity of GS cells is hardly affected by leupeptin treatment (d'Azzo *et al.*, 1982; Pshezhetsky and Potier, 1996), suggesting that this enzyme is influenced by PPCA in a different way. In this study, we have investigated whether neuraminidase requires the presence of PPCA for protection against intralysosomal degradation, for specific post-translational modifications like proteolytic processing and phosphorylation, or for its intracellular transport. We also compared the effect of transport-deficient PPCA variants on the intracellular behavior of neuraminidase. Our results offer a first explanation for the PPCA dependence of lysosomal neuraminidase activity.

Results

Neuraminidase associates with PPCA and β-galactosidase precursors and has a short half-life

To determine whether the PPCA-dependent activation of neuraminidase is accompanied by specific structural modifications of the enzyme en route to the lysosome, we transfected COS-1 cells with the neuraminidase cDNA either alone or in combination with the cDNA for PPCA and/or β-galactosidase. Transfected cells were pulse-labeled for 1 h and then chased for different time periods in medium containing cold leucine. The cells were then lysed in buffer at pH 7.4 and immunoprecipitated with anti-neuraminidase (anti-Neur) antibodies. As shown in Figure 1, neuraminidase was recovered from single and co-transfected cells in multiple forms, migrating on SDS–polyacrylamide gels either as a broad band or as discrete bands with molecular weights of ~44–46 kDa. These multiple forms represent different glycosylation states of the enzyme (Bonten *et al.*, 1996; Milner *et al.*, 1997). In both single and co-transfections, the size of the newly synthesized neuraminidase did not change during the chase periods, suggesting that post-translational processing of the enzyme was completed within 1 h and was not influenced by the presence of PPCA and/or β-galactosidase (Figure 1A–D). Furthermore, in all transfected cells, the neuraminidase levels began to decrease after 3 h of chase, and the enzyme was largely degraded 24 h after synthesis. Therefore, co-expression of PPCA or β-galactosidase did not grossly alter the half-life of neuraminidase. After immunoprecipitation with anti-Neur antibodies, lysates from double- or triple-transfected cells were subjected to a second round of immunoprecipitation with anti-PPCA and anti-β-galactosidase antisera (Figure 1E and F). PPCA and β-galactosidase precursors were converted slowly to their mature forms (32/20 and 64 kDa, respectively) that were stable for >24 h after synthesis (see also Morreau *et al.*, 1992; Zhou *et al.*, 1996). This clearly illustrates that the turnover of neuraminidase was more rapid than that of PPCA or β-galactosidase. From triple-transfected cells, both PPCA precursor and small amounts of its mature form were co-precipitated with β-galactosidase.

During the 1 h pulse labeling, both PPCA and β-galactosidase precursors were co-precipitated with neuraminidase from double- and triple-transfected cells (Figure 1B–D), indicating that association of these three proteins occurred shortly after their synthesis. The mature forms of PPCA and β-galactosidase did not co-precipitate with neuraminidase under the neutral immunoprecipitation conditions. However, by immunotitration with *Staphylococcus aureus*-bound anti-PPCA antibodies, up to 60% of neuraminidase activity was co-precipitated with cathepsin A activity from co-transfected cells lysed in buffer at pH 5.5. From these results, we could infer that the majority of enzymatically active neuraminidase remains associated with mature PPCA (Figure 2).

Neuraminidase is secreted into the extracellular space

We found overexpressed neuraminidase in the medium of transfected cells, and the time course of its secretion was independent of PPCA and β-galactosidase co-expression. The level of secreted enzyme was maximal after 3–6 h and had diminished at the 24 h time point (Figure 1A–D, lower panels). To compare the relative levels of secretion, the amount of neuraminidase immunoprecipitated from medium samples was quantitated by densitometry scanning and expressed as a percentage of the total amount immunoprecipitated from pulse-labeled cell lysates (Figure 1A and B, lower panels). The secretion of neuraminidase, quantitated in this fashion, was clearly reduced when co-expressed with PPCA, irrespective of β-galactosidase expression (Figure 1C and D, lower panels). This variation was not the result of differences in the rate of synthesis of neuraminidase, because the intracellular levels of neuraminidase were similar in all transfected cells (Figure 1A–D, upper panels). Thus, the intracellular routing of the enzyme was influenced by the concomitant overexpression of PPCA. Extracellular neuraminidase, on the other hand, did not associate with PPCA or β-galactosidase, since we could not co-precipitate the three proteins from the medium of triple-transfected cells (Figure 1D, lower panel); in turn, no neuraminidase activity was detected in concentrated medium samples from co-transfected cells, even though high activity was measured in the corresponding lysates. Moreover, secreted neuraminidase was not activated when we mixed concentrated media from PPCA- and neuraminidase-overexpressing cells, in an attempt to promote *in vitro* association of the two molecules (data not shown).

PPCA controls the intracellular routing of neuraminidase

By using immunofluorescence, we and others have reported that when neuraminidase is expressed in COS-1 cells it has either a punctate distribution or it is localized in the endoplasmic reticulum (ER)/Golgi region. Further, depending on the expression levels, the enzyme can also form small square crystals in the perinuclear area that stain strongly with anti-Neur antiserum (Bonten *et al.*, 1996; Milner *et al.*, 1997). Here, we analyzed whether the proportion of cell populations showing a different subcellular distribution of neuraminidase was contingent on the level of expressed PPCA. In cells transfected with the same amount of neuraminidase cDNA but increasing concentrations of PPCA cDNA, neuraminidase activity increased in parallel with the number of cells exhibiting punctate staining (Figure 3A and B). In contrast, cells

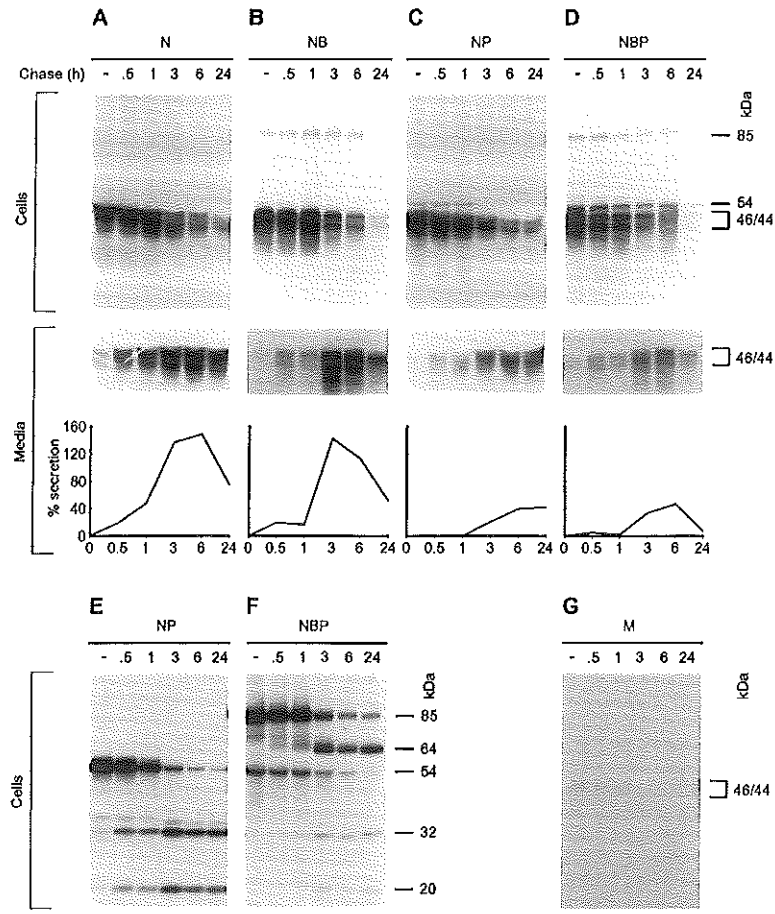

Fig. 1. Pulse–chase labeling. COS-1 cells were transfected with vector alone [denoted M in (G)] or with cDNAs encoding neuraminidase (N), β-galactosidase (B) or PPCA (P), in various combinations (A–F), labeled with [^{3}H]leucine for 1 h and chased with cold leucine for the time periods indicated. Cell lysates and medium samples were immunoprecipitated with anti-Neur antiserum (A–D and G), and selected lysates were used for a secondary round of immunoprecipitation with anti-PPCA antiserum (E) or anti-β-galactosidase antisera (F). Exposure was for 20 days (B and D upper panels, and E and F), 1 month (B and D, lower panels), 3 days (A and C, upper panels), 4 days (A and C, lower panels) or 14 days (G).

transfected with different amounts of PPCA cDNA and no neuraminidase cDNA displayed slightly higher endogenous neuraminidase activity, which remained constant irrespective of the amount of PPCA cDNA added (Figure 3A). This result demonstrates that PPCA affects the subcellular localization of neuraminidase, which in turn may influence its activation.

To analyze the subcellular distribution of neuraminidase further, we separated the organelles of transfected cells on self-generating Percoll density gradients (Figure 4). This procedure separates mature dense lysosomes from lighter membranes such as microsomes and early and late endosomes. β-Hexosaminidase and horseradish peroxidase (HRP) were used as lysosomal and endosomal markers, respectively. In cells transfected with the β-hexosaminidase cDNA (β-chain), enzyme activity peaked in both the dense bottom fractions (fractions 1 and 2) and in fraction 5 (Figure 4A). After a 4 min incubation of the cells with HRP, the internalized enzyme was detected only in fractions 4–6, confirming the presence of endosomal vesicles in this part of the gradient (Figure 4B). During the 3 h chase period that followed, however, the internalized enzyme shifted to the denser fractions (Figure 4B). In parallel experiments, we monitored the distribution and enzymatic activities of PPCA and neuraminidase. Neuraminidase expressed alone was detected on immunoblots with anti-Neur antibodies exclusively in the lighter fractions (Figure 4C). Its activity was marginal throughout the gradient (Figure 4C). In contrast, blots probed with anti-PPCA antibodies showed the PPCA precursor in both the light and dense fractions, while its mature two-chain form was confined to the bottom of the gradient (Figure

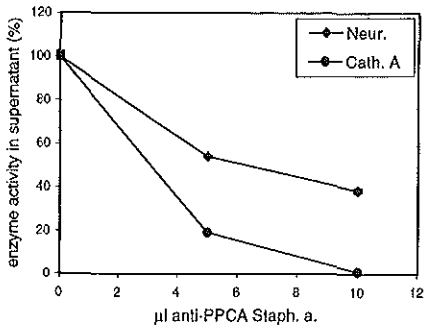

Fig. 2. Precipitation of neuraminidase activity from co-transfected COS-1 cells with anti-PPCA antibodies. Aliquots of lysates of COS-1 cells overexpressing both neuraminidase and PPCA were incubated under acidic conditions with increasing amounts of fixed *S.aureus* cells that had been pre-loaded with IgG from either pre-immune or anti-PPCA antiserum. After removal of the bacteria by centrifugation, neuraminidase and cathepsin A activities were assayed in the supernatants. The data are expressed as a percentage of the level measured in samples incubated with pre-immune IgG-*S.aureus*. Error bars indicate standard deviation for duplicate experiments.

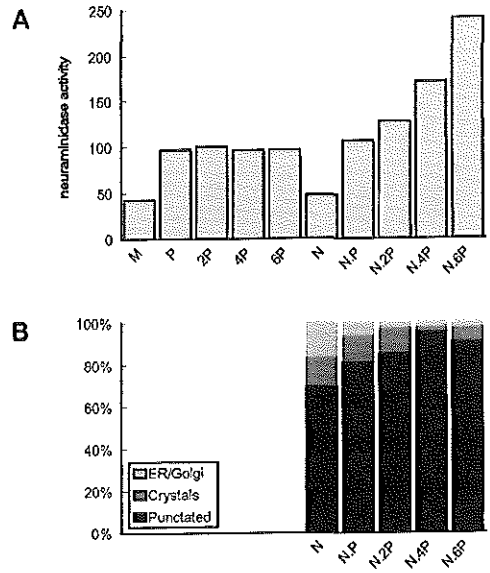

Fig. 3. Titration of neuraminidase. COS-1 cells were transfected with vector alone (M), or with increasing amounts of PPCA (P) cDNA (0.2, 0.4, 0.8 or 1.2 µg per 35 mm dish), alone or in combination with a fixed amount of neuraminidase (N) cDNA (0.2 µg per 35 mm dish). Cells were (A) assayed for neuraminidase activity or (B) processed for immunofluorescence using affinity-purified anti-Neur antiserum. Enzyme activity is expressed in nmol/mg/h. At least 70 cells were examined microscopically and scored for neuraminidase staining.

4D). In keeping with this finding, cathepsin A activity increased only in the fractions that contained the mature enzyme (Figure 4D). When neuraminidase cDNA was transfected into COS-1 cells together with PPCA cDNA, the two proteins co-distributed, and equal amounts of neuraminidase polypeptide were found in the light and dense fractions (Figure 4E and F). However, enzyme activity was detected primarily in the mature lysosomal fractions, with only a moderate increase in fraction 5 (Figure 4E and F). These results show that when the neuraminidase polypeptide is expressed alone it localizes in only light biosynthetic vesicles, but it reaches mature lysosomes when accompanied by PPCA. The generation of neuraminidase activity seems, therefore, to depend on the lysosomal localization of the enzyme. The interaction of neuraminidase with PPCA in an early biosynthetic compartment is sufficient merely for partial activation, which is fully unraveled in the lysosomal milieu.

Effect of PPCA mutants on neuraminidase compartmentalization

We next investigated the effect of different PPCA variants on the intracellular transport and activation of neuraminidase. Four mutants were selected, each carrying a single amino acid substitution that impairs its lysosomal compartmentalization to a different degree: PPCA-Leu208Pro (LP) and PPCA-Val104Met (VM) were originally identified in a severe early infantile case of GS. These mutant proteins are not transported out of the ER and persist as immature precursors in this compartment (Zhou *et al.*, 1996). In the patient with these mutations, neuraminidase activity is undetectable. In contrast, PPCA-Phe412Val (FV) and PPCA-Tyr221Asn (YN), which are associated with the mild, late-infantile phenotype, are partially transported to lysosomes, where PPCA-FV is degraded more rapidly than PPCA-YN (Zhou *et al.*, 1991, 1996; Shimmoto *et al.*, 1993). The presence of small amounts of mutant PPCA in lysosomes supports the finding of residual neuraminidase activity in patients with this phenotype. COS-1 cells were transfected with neuraminidase cDNA or co-transfected with neuraminidase cDNA and a cDNA encoding either wild-type PPCA or one of the PPCA mutants. Cells were then metabolically labeled for 16 h, lysed, and the cell lysates were immunoprecipitated sequentially with anti-Neur and anti-PPCA antisera. Figure 5 shows that all four mutant precursors co-precipitated with neuraminidase when treated with anti-Neur antiserum, demonstrating that the PPCA mutants associate with the enzyme as efficiently as the wild-type PPCA precursor does (lanes 5, 7, 9, 11 and 13). As observed previously (Zhou *et al.*, 1991, 1996), all of the PPCA mutants accumulated in the precursor form, except for PPCA-YN which was partially converted to the mature form (Figure 5, lanes 6, 8, 10, 12 and 14). The amount of PPCA precursor associated with neuraminidase was proportional to the level of unbound precursor that was not brought down by the anti-Neur antibodies but was precipitated with anti-PPCA antibodies (Figure 5, compare adjacent lanes).

Fractionation of the COS-1 cells co-expressing neuraminidase and PPCA-YN or PPCA-LP revealed that the mutant precursors localized in light organelles (Figure 6B and D). This method was not sensitive enough to detect the small amount of mature PPCA-YN that could be immunoprecipitated from radiolabeled cells (see Figure 5), which also explains why we saw no increase over background levels in cathepsin A activity throughout these gradients (data not shown). In both sets of co-transfected

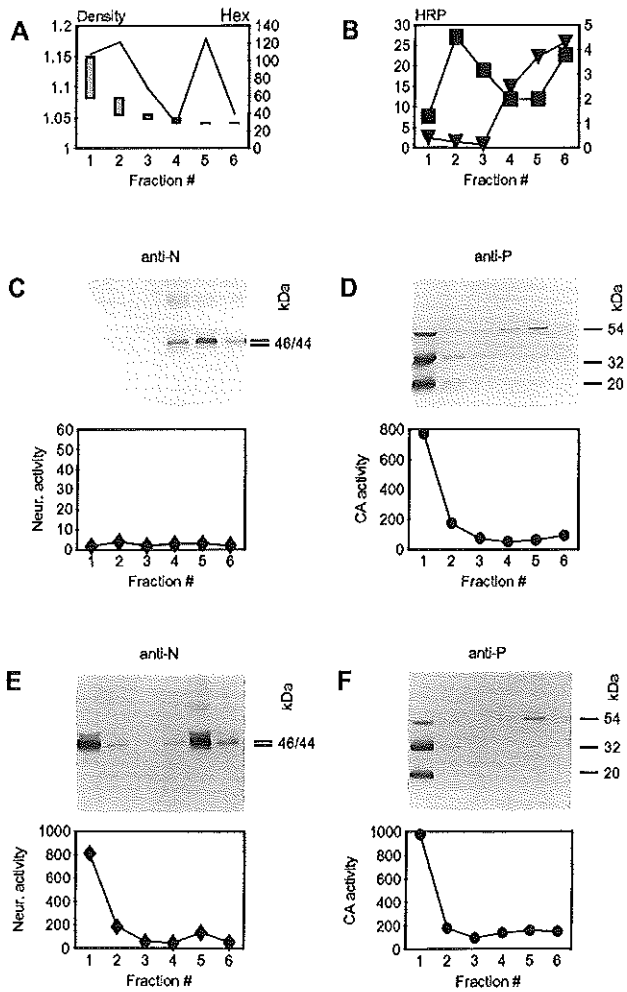

Fig. 4. Subcellular fractionation of COS-1 cells on density gradients. (A) Density distribution of Percoll gradients, as determined with density marker beads (Pharmacia), and profile of β-hexosaminidase activity (nmol/h/ml) in COS-1 cells overexpressing β-hexosaminidase β-chain. (B) After pulse-labeling with HRP, COS-1 cells were either stored on ice (▼) or chased for 3 h in HRP-free medium (■). Cells were then fractionated and the resulting gradient fractions assayed for HRP content (μg/ml). Alternatively, COS-1 cells, transfected with (C) neuraminidase cDNA, (D) PPCA cDNA or (E and F) co-transfected with both cDNAs were homogenized and loaded onto Percoll gradients. Fractions were analyzed for enzyme activities and used in Western blots, which were probed with anti-Neur (anti-N) or anti-PPCA (anti-P) antisera. Neuraminidase activity (◆) is expressed in nmol/h/ml and cathepsin A activity (●) in nmol/min/ml. Blots were developed with colorimetric substrates.

cells, most of the neuraminidase was found in light organelles (fractions 4, 5 and 6) (Figure 6A and C); however, a small portion of the enzyme was detected in the denser fractions (fractions 2 and 3) from cells co-expressing PPCA-YN (Figure 6A). Consequently, only these cells displayed a low level of neuraminidase activity in both dense and light organelles (Figure 6A). These data further support the concept that neuraminidase must interact with transport-competent PPCA for its lysosomal localization and activation.

Neuraminidase is poorly phosphorylated

Soluble lysosomal enzymes are segregated to lysosomes via the interaction of their M6P recognition marker with the M6P receptor (Hille-Rehfeld, 1995; Sabatini and Adesnik, 1995). We therefore investigated whether neuraminidase acquires an M6P recognition marker when it interacts with PPCA. COS-1 cells, transfected with the neuraminidase cDNA, the PPCA cDNA or both cDNAs, were metabolically labeled with [^{32}P]orthophosphate. Cell lysates and media were immunoprecipitated with either

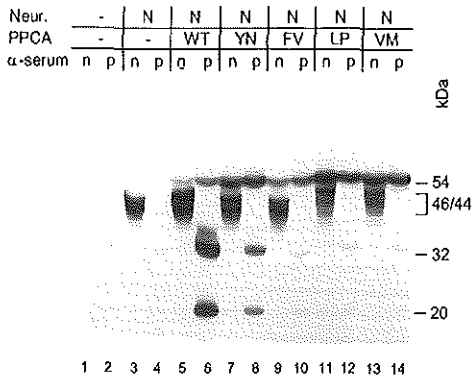

Fig. 5. Co-immunoprecipitation of PPCA mutants with neuraminidase. COS-1 cells, transfected with the neuraminidase cDNA alone, or in combination with a cDNA encoding wild-type PPCA, PPCA-YN, PPCA-FV, PPCA-LP or PPCA-VM, were metabolically labeled with [^{3}H]leucine and used for sequential immunoprecipitations with anti-Neur (n) and anti-PPCA antiserum (p). Exposure was for 1 day.

anti-Neur or anti-PPCA antibodies. Figure 7 shows that in all of the transfected cells the level of phosphorylation of neuraminidase (lanes 5, 7, 13 and 15) was considerably lower than that of PPCA (lanes 3 and 11), to the extent that the neuraminidase signal from cell lysates barely exceeded background levels even though both proteins were synthesized in comparable amounts in similarly transfected cells (see Figure 1). A clearer 44/46 kDa band could be resolved when the neuraminidase was immunoprecipitated from samples of medium because of the higher signal/noise ratio, which allowed a longer exposure time of this autoradiograph (Figure 7, lanes 13 and 15). We know that neuraminidase was phosphorylated on one or more of its N-linked oligosaccharide chains because the phosphate label was lost after the cells were cultured in the presence of tunicamycin and after treatment with *N*-glycosidase F (data not shown). The phosphorylation of neuraminidase was not influenced by PPCA.

Addition of the M6P marker to lysosomal enzymes is a two-step process: first, an *N*-acetylglucosamine-1-phosphate residue is bound to the N-linked high-mannose oligosaccharide through the formation of a phosphodiester bond; second, the terminal *N*-acetylglucosamine is removed by *N*-acetylglucosamine-1-phosphodiester α-*N*-acetylglucosaminidase, leaving the M6P exposed (reviewed in von Figura and Hasilik, 1986; Sabatini and Adesnik, 1995). Because the efficiency of lysosomal transport depends on how accessible the M6P marker is to its receptor, we examined the extent to which the mannose-bound phosphate of neuraminidase is exposed. We incubated immunoprecipitated neuraminidase with calf intestinal alkaline phosphatase (CIP), which removes only terminal, monoester-bound phosphate from N-linked oligosaccharides (Isidoro *et al.*, 1991). After CIP treatment, the PPCA was almost completely dephosphorylated (Figure 7, lanes 4 and 12), whereas the neuraminidase retained a substantial proportion of its phosphate label (Figure 7, lanes 14 and 16). Furthermore, we found that neuraminidase from the culture medium of transfected

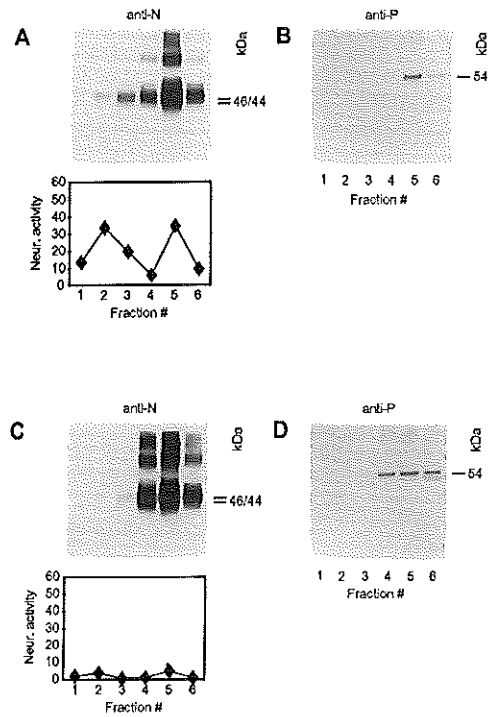

Fig. 6. Density fractionation of COS-1 cells expressing neuraminidase and PPCA mutants. Following co-transfection of COS-1 cells with neuraminidase cDNA and either (A and B) PPCA-YN or (C and D) PPCA-LP cDNA, cellular organelles were separated on Percoll gradients. Fractions were used to assay for neuraminidase activity (◆), expressed in nmol/h/ml, and to prepare Western blots, which were probed with anti-PPCA antiserum (anti-P) or affinity-purified anti-Neur antiserum (anti-N). For increased sensitivity, blots probed with anti-Neur antiserum were developed with chemiluminescent substrates.

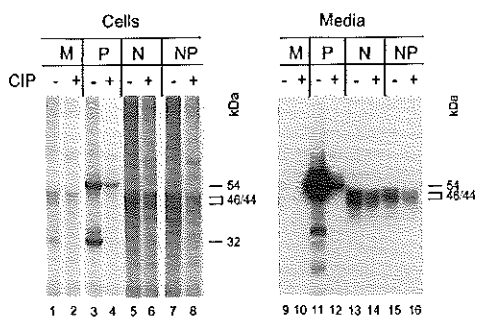

Fig. 7. Phosphorylation of neuraminidase. COS-1 cells were transfected as outlined in the legend to Figure 1, and metabolically labeled with [^{32}P]orthophosphate. Cell lysates and media were used for immunoprecipitation with anti-PPCA antiserum (lanes 3, 4, 11 and 12) or anti-Neur antiserum (all other lanes). The recovered proteins were divided into two aliquots and incubated with or without alkaline phosphatase (CIP). Signals are stronger in the medium samples because of a longer exposure time (4 days) compared with cell lysates (16 h).

COS-1 cells could not be internalized by deficient sialidosis fibroblasts (data not shown).

Discussion

Human lysosomal neuraminidase is the only member of the sialidase superfamily that needs another protein, PPCA, to be catalytically active, although influenza virus neuraminidase and some of the bacterial sialidases depend on Ca^{2+} for optimal activity (reviewed in Saito and Yu, 1995). To explain this unusual requirement of lysosomal neuraminidase, we have studied the various biochemical properties of this enzyme in relation to PPCA. We show that most of the neuraminidase activity, generated through co-expression of neuraminidase and PPCA, is measured in mature dense lysosomes, with only low levels detected in light organelles, although equal amounts of the neuraminidase polypeptide are present in these two compartments. This apparent discrepancy indicates that it is the subcellular location of neuraminidase that determines, to a large extent, its enzymatic activity. Moreover, in the absence of PPCA, neuraminidase is found only in light organelles and is completely inactive. These data suggest that PPCA activates neuraminidase first by interacting with it in a pre-lysosomal compartment, and then by mediating its transport to dense lysosomes.

Various mechanisms govern intracellular transport of lysosomal proteins, which depends on their primary structure and solubility. Integral membrane proteins have a hydrophobic transmembrane domain and a specific lysosomal targeting motif in their cytoplasmic tails, containing the characteristic G-Y-X-X-hydrophobic sequence (reviewed in Hunziker and Geuze, 1996). If the cytoplasmic tails of lamp-1, lamp-2, limp-1 and acid phosphatase, for example, are introduced into integral plasma membrane proteins, the latter are re-routed to lysosomes. Lysosomal enzymes without a transmembrane domain are tagged in the Golgi with the M6P recognition marker (Hille-Rehfeld, 1995). Such proteins are dependent on their phosphomannosyl residues for intracellular transport, with the exception of the G_{M2} activator protein, cathepsin D, the sphingolipid activator protein (SAP) precursor and aspartylglucosaminidase that apparently can reach the lysosome even in the non-glycosylated state (Rijnboutt *et al.*, 1991b; Tikkanen *et al.*, 1995; Vielhaber *et al.*, 1996; Glombitza *et al.*, 1997). For cathepsin D and the SAP precursor, transient membrane association is thought to play a role in lysosomal transport (Rijnboutt *et al.*, 1991a). Hydropathy analysis of neuraminidase did not demonstrate a prominent hydrophobic domain besides the signal sequence (data not shown). Furthermore, it is unlikely that phosphorylation mediates routing of neuraminidase, since the enzyme is poorly phosphorylated, even in the presence of PPCA. Our data suggest that the oligosaccharide-linked phosphates on neuraminidase are only partially unmasked; in addition, those that are unblocked may not be sufficiently multivalent, a requirement for high-affinity binding to M6P receptors (reviewed in Hille-Rehfeld, 1995). This may explain why the M6P marker on neuraminidase is not functional in receptor-mediated endocytosis of this enzyme. We also demonstrated that neuraminidase is readily secreted from transfected cells. Thus, our results indicate that neuraminidase does not behave as a membrane-associated protein, contrary to what was suggested earlier (Pshezhetsky *et al.*, 1997), and that it is co-transported with PPCA along the endosomal/lysosomal pathway through its association with this protein.

Evolutionarily, sialic acids are relatively young, occurring almost exclusively in vertebrates and higher invertebrates, and, apart from a few exceptions, not in plants or lower invertebrates (Schauer *et al.*, 1995; Reuter and Gabius, 1996, and references therein). Sialidases, on the other hand, occur not only in species that synthesize neuraminic acid, but also in various microorganisms that do not make this monosaccharide. Many of these organisms have contact with sialic acid-synthesizing animals, and are thought to have obtained the neuraminidase gene through horizontal gene transfer (for reviews, see Roggentin *et al.*, 1993; Saito and Yu, 1995). Considering the homology between lysosomal neuraminidase and various bacterial sialidases (Bonten *et al.*, 1996), many of which are secretory enzymes (Corfield, 1992; Schauer *et al.*, 1995), it is possible that all of these proteins derive from a common secreted precursor, and that the lysosomal enzyme has acquired the means to be intracellularly compartmentalized: through the help of another protein, PPCA. The use of targeting signals from a secondary protein is well documented in the case of MHC class II antigens, which rely on the cytoplasmic domain of the associated invariant chain for their endosomal/lysosomal localization (Wolf and Ploegh, 1995). The α-chain of lysosomal β-hexosaminidase may need the β-hexosaminidase β-chain for lysosomal localization (d'Azzo *et al.*, 1984); however, this has not been demonstrated directly.

The molecular nature of the catalytic activation of neuraminidase is not clear. In pre-lysosomal compartments, initial association of the enzyme with PPCA and mild acidification may induce conformational changes that result in a low catalytic capacity. In mature lysosomes, partial processing by proteases may be needed for the acquisition of full enzyme activity, and this processing can only occur after lysosomal proteins have been segregated from the secretory pathway (Hasilik, 1992). The carboxypeptidase activity of PPCA itself does not seem to play a role in the activation of neuraminidase, because a catalytically inert mutant of PPCA retains the capacity to activate neuraminidase and β-galactosidase (Galjart *et al.*, 1991); instead, cathepsin C and an unidentified acidic aminopeptidase have been implicated in this process (D'Agrosa and Callahan, 1988; Hiraiwa *et al.*, 1993). Alternatively, structural rearrangements may occur, as has been described for influenza virus neuraminidase. This enzyme undergoes various maturation steps from an inactive monomer to an active tetramer (Hogue and Nayak, 1992; Saito *et al.*, 1995). Initial dimerization, intermolecular disulfide linking and Ca^{2+} binding are thought to be followed by a conformational change that confers enzymatic activity to the oligomeric forms of the protein (Burmeister *et al.*, 1992; Saito *et al.*, 1995). Since active neuraminidase remains associated with mature PPCA, this interaction apparently is needed to maintain catalytic activity, in agreement with previous purification studies (Verheijen *et al.*, 1985; Yamamoto and Nishimura, 1987; Pshezhetsky and Potier, 1994). The relatively short half-life of lysosomal neuraminidase, on the other hand, may

be important to control neuraminidase activity in the endosomal/lysosomal pathway.

Surprisingly, the β-galactosidase precursor also co-precipitated with the neuraminidase polypeptide. This interaction is intriguing because there is no genetic evidence that it is required for the functioning of either protein. However, the direct interaction between neuraminidase and β-galactosidase may again provide a means of subtle regulation of the two enzyme activities, or it may enhance the stability of the multi-enzyme complex.

We previously have reported that β-galactosidase in transfected COS-1 cells depends on PPCA for intralysosomal stability, since the amount of its mature form is strongly reduced both in the absence of PPCA and when combined with a transport-incompetent PPCA mutant (Morreau *et al.*, 1992). Our subcellular fractionation experiments have now confirmed these results (unpublished observation). In a recent report, Pshezhetsky and Potier (1996) have described the isolation of a 1.27 MDa enzyme complex from human placenta that contains *N*-acetylgalactosamine-6-sulfate sulfatase (GALNS) in addition to β-galactosidase, PPCA and neuraminidase. Significantly, these authors noted a partial deficiency of GALNS in GS fibroblasts. It would be interesting, therefore, to investigate whether PPCA is required for the lysosomal transport or for the intralysosomal stability of GALNS.

Deficiencies of PPCA are the primary cause of GS. The pathology of this disorder, however, derives mainly from a secondary deficiency in neuraminidase and from the subsequent accumulation of undegraded substrates (d'Azzo *et al.*, 1995). Our findings allow us now to link these issues. In GS, either the PPCA gene is not transcribed or PPCA mutants are synthesized that are partially or completely impaired in their lysosomal transport. In both cases, lysosomal localization and subsequent activation of neuraminidase are affected. We can infer from our data that in patients' cells that synthesize transport-incompetent PPCA, neuraminidase is retained to a large extent in an early biosynthetic compartment. On the other hand, the restoration of neuraminidase activity in GS fibroblasts after internalization of exogenous PPCA precursor (Galjart *et al.*, 1988, 1991) implies that the two proteins can interact where the biosynthetic and endocytic pathways merge. In future studies, structural analysis of the PPCA–neuraminidase complex should provide further insight into the role of PPCA in protein transport and in the activation of neuraminidase.

Materials and methods

Cell culture

Normal and sialidosis fibroblasts were obtained from the European Cell Bank, Rotterdam (Dr W.J.Kleijer). Fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotics. COS-1 cells (Gluzman, 1981) were grown in the same medium in 5% serum.

Transfections and metabolic labeling

cDNAs encoding human lysosomal neuraminidase (Bonten *et al.*, 1996), β-galactosidase (Morreau *et al.*, 1989), PPCA (Galjart *et al.*, 1988), PPCA-Phe412Val (Zhou *et al.*, 1991), PPCA-Tyr221Asn, PPCA-Val104-Met and PPCA-Leu208Pro (Zhou *et al.*, 1996), subcloned into the mammalian expression vector pSC-TOP (Fornerod *et al.*, 1995), and β-hexosaminidase β-chain, subcloned into pcDNA1.1 (Invitrogen) (kindly provided by Dr R.Proia), were transfected into COS-1 cells using calcium phosphate precipitation, as described (Chen and Okayama, 1987), or the Superfect reagent, according to the manufacturer's instructions (Qiagen). For steady-state labeling, at 48 h post-transfection cells were metabolically labeled with L-[4,5-^{3}H]leucine (50 μCi/ml culture medium) for 16 h, or at 64 h post-transfection cells were labeled with [^{32}P]orthophosphate (100 μCi/ml culture medium) for 8 h. Pulse–chase labeling was performed as described (Morreau *et al.*, 1992).

Immunoprecipitation, deglycosylation and dephosphorylation

Transfected and metabolically labeled COS-1 cells were used for immunoprecipitation essentially as described (Proia *et al.*, 1984; Zhou *et al.*, 1996). Cell lysates were prepared according to the method of Proia *et al.* (1984). COS-1 cell lysates and medium samples were incubated with antiserum for 16–18 h. Immunoprecipitations were done with rabbit antisera against neuraminidase (anti-Neur) (Bonten *et al.*, 1996) and PPCA (Bonten *et al.*, 1995). Two antisera against β-galactosidase were combined: αn64, raised against the mature form of the enzyme, isolated from human placenta, and anti-85, raised against the β-galactosidase precursor, overexpressed in insect cells. Recovered proteins were resolved on 12% SDS–polyacrylamide gels under denaturing and reducing conditions and visualized by autoradiography (for ^{32}P-labeled samples) or by fluorography of gels impregnated with Amplify (Amersham) (for ^{3}H-labeled samples). Apparent molecular weights were calculated by comparison with marker proteins (Life Technologies). Dephosphorylation of ^{32}P-labeled proteins was carried out as described (Isidoro *et al.*, 1991), using EIA-grade CIP (Boehringer Mannheim). Densitometry scanning of autoradiographs was performed on a BioImage Visage 110 system.

Immunotitration

Fixed *S.aureus* cells, activated as described (Zhou *et al.*, 1996) and extensively washed, were resuspended in phosphate-buffered saline (PBS), and loaded with IgG by incubating the bacteria in an excess volume of either pre-immune rabbit serum or anti-PPCA antiserum for 2 h at 4°C. The bacteria were then washed twice in PBS and twice in extraction buffer (20 mM sodium acetate, pH 5.5, 150 mM NaCl and 0.4% NP-40), and resuspended in the latter buffer to make a 20% (v/v) suspension. Transfected COS-1 cells were lysed in extraction buffer, and insoluble material was removed by centrifugation at 16 000 *g* for 2 min. Immunotitration was performed by mixing 15 μl of the COS-1 cell extract (0.5 mg protein/ml) with increasing volumes of one of the IgG-loaded *S.aureus* suspensions, in a total volume of 25 μl. Following incubation at 4°C for 2 h, the reaction mixtures were spun at 16 000 *g* for 1 min, and supernatants were assayed for neuraminidase and cathepsin A activities.

Subcellular fractionation

Transfected COS-1 cells were washed twice with PBS and once with a HEPES-buffered sucrose solution [HBS: 250 mM sucrose, 10 mM HEPES–NaOH, pH 7.4, 1 mM EDTA (Gieselmann *et al.*, 1983)]. Cells were harvested in HBS by scraping and were homogenized by 30 strokes in a tight-fitting dounce (Kontes). Nuclei and unbroken cells were removed by centrifugation at 600 *g* for 10 min, and the post-nuclear supernatant (400 μl) was loaded onto 8.6 ml of isotonic, HEPES-buffered Percoll [containing 29.74 ml Percoll (Pharmacia) per 100 ml, 210 mM sucrose, 8.4 mM HEPES–NaOH, pH 7.4, and 1 mM EDTA], prepared according to the manufacturer's instructions and to Gieselmann *et al.* (1983) and Vincent and Nadeau (1984). Subcellular organelles were separated in self-generating density gradients, as described (Gieselmann *et al.*, 1983). Six 1.5 ml fractions were collected from the bottom of each gradient, and Percoll was removed by diluting each fraction in 10 vols of sucrose wash buffer [250 mM sucrose, 10 mM HEPES–NaOH, pH 7.4, 1 mg/ml bovine serum albumin (BSA)] and centrifugation for 15 min at 20 000 *g* (Pisoni *et al.*, 1987). The pelleted material was then resuspended in 0.5 ml of sucrose wash buffer and spun for 12 min at 15 600 *g*. The final pellets were resuspended in 50 μl of 50 mM sodium acetate, pH 5.0, 0.25% glycodeoxycholate, 100 mM NaCl and 1 mg/ml BSA, and assayed for neuraminidase and cathepsin A activities (Kleijer *et al.*, 1996). Density was determined using density marker beads (Pharmacia). Equal aliquots from each fraction were subjected to Western blot analysis using anti-Neur and anti-PPCA antisera, and developed with either a colorimetric or a chemiluminescent substrate (Renaissance, Dupont) as described earlier (Bonten *et al.*, 1995).

Immunofluorescence

COS-1 cells were seeded in 6-well dishes at a density of 1×10^5 cells per well. Cells were transfected with increasing amounts of PPCA cDNA (ranging from 0.25 to 1.5 μg), or co-transfected with the cDNAs for both PPCA and neuraminidase, in which case the amount of neuraminidase plasmid was fixed at 0.25 μg while PPCA was added in increasing quantities from 0.25 to 1.5 μg. For indirect immunofluorescence, the cells were trypsinized 48 h post-transfection and aliquots were seeded on Superfrost/Plus microscope glass slides (Fisher). The next day, the slides were processed according to the method of van Dongen *et al.* (1985), using affinity-purified anti-Neur antibodies (Bonten *et al.*, 1996) and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG secondary antibodies (Sigma). A minimum of 70 transfected cells per transfection were examined microscopically and scored for the presence of either a punctated, ER/Golgi or crystal-like staining pattern, as described earlier (Bonten *et al.*, 1996). Transfection efficiencies were determined by counting both the transfected and untransfected cells. Cell lysates were assayed for neuraminidase and cathepsin A activities as described above. Total protein concentrations were quantitated using the BCA kit (Pierce Chemical Co.), following the manufacturer's guidelines.

HRP Internalization

Uptake experiments with HRP were performed as described by Tulp *et al.* (1993). Briefly, confluent COS-1 cells were incubated for either 4 or 10 min with 2 mg/ml HRP (Sigma, Type VI-A) in 10 mM glucose, 10 mM HEPES–NaOH, pH 7.4, in DMEM. After the 4 min pulse, cells were transferred to ice. Alternatively, cells were washed three times with PBS, chased for 3 h in DMEM containing 5% fetal bovine serum and antibiotics, and then transferred to ice. All cells were then fractionated on Percoll gradients as described above. Gradient fractions were assayed for HRP content using *o*-phenylenediamine (Sigma) as a substrate, according to Amigorena *et al.* (1994). Following a 1 h incubation at room temperature, assays were read at 450 nm without terminating the reactions with HCl. A solution of HRP (5 ng/ml) was used as the standard.

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Lysosomal protective protein/cathepsin A: role of the "linker" domain in catalytic activation.

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Lysosomal Protective Protein/Cathepsin A

ROLE OF THE "LINKER" DOMAIN IN CATALYTIC ACTIVATION*

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Lysosomal protective protein/cathepsin A is a serine carboxypeptidase that forms a complex with β-galactosidase and neuraminidase. The enzyme is synthesized as a 54-kDa precursor/zymogen and processed into a catalytically active 32- and 20-kDa two-chain form. We have expressed in baculovirus-infected insect cells the human one-chain precursor as well as the two separate subunits in order to establish the mode of catalytic activation of the zymogen and the assembly and activation of the two subunits. Infected insect cells synthesize large quantities of the exogenous proteins, which are glycosylated and secreted but not processed. Co-expression of the two subunits results in their assembly into a two-chain form of 34- and 20-kDa with negligible enzymatic activity. Limited proteolysis with trypsin of the 54-kDa precursor and the reconstituted 34- and 20-kDa form gives rise to a fully active 32- and 20-kDa product. These results enabled us to map the sites of proteolytic cleavage needed for full activation of the cathepsin A zymogen. They further indicate that the 34- and 20-kDa form is a transient processing intermediate that is converted into a mature and active enzyme by removal of a 2-kDa "linker" peptide from the COOH terminus of the 34-kDa subunit.

A primary defect of lysosomal protective protein/cathepsin A (PPCA)[1] in humans causes the metabolic storage disorder galactosialidosis (Ref. 1; reviewed in Ref. 2). This disease is characterized by severely reduced activities of the enzymes β-D-galactosidase and *N*-acetyl-α-neuraminidase, secondary to absent or abnormal PPCA. The reason for the additional combined deficiency relates to one of the functions of PPCA protein, which is to associate with and protect the two glycosidases, modulating their activity and stability in lysosomes (1, 3–5). The primary structures of human, mouse, and chicken PPCAs are highly conserved (6–8) and bear homology to yeast and plant serine carboxypeptidases (Ref. 6; for reviews see Refs. 9 and 10). Mammalian PPCAs have cathepsin A activity at lysosomal pH but maintain a deamidase/esterase activity at neutral pH (8, 11). Furthermore, the human enzyme, purified from platelets and lymphocytes, has been shown to function both in *in vitro* and *in vivo* assays on the inactivation of selected neuropeptides, like substance P, oxytocin, and endothelin I (11–13).

PPCA is synthesized as a 54-kDa precursor that is glycosylated on two Asn residues and is enzymatically inactive (1, 6, 8). The precursor dimerizes at neutral pH shortly after synthesis and is likely to be transported as a dimer to the lysosomes (14). Once in the acidic lysosomal environment, the zymogen is cleaved into an enzymatically active 32- and 20-kDa two-chain form. However, the events involved in proteolytic activation have not been established until now. Here we have used baculovirus (BV)-expressed human precursor and two separate subunits to map the sites of cleavage and processing of human PPCA zymogen. We have identified a "linker" domain in the precursor molecule, located at the COOH terminus of the large subunit, which needs to be removed for full catalytic activation of the enzyme.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—AcMNPV transfer plasmids pJR2 and pBC3 are derivatives of plasmid pAc373, which includes the entire polyhedrin gene (15). They both contain a polylinker with multiple cloning sites, inserted directly 3′ of the polyhedrin promoter. In pJR2 the polylinker substitutes a 33-nucleotide deletion spanning the ATG, whereas in pBC3 only the ATG codon is mutated to ACG. Full-length human PPCA cDNA, HPP54 (6), and the two deletion cDNA mutants, HPP32(Δ20) and HPP20(Δ32) (8), were subcloned either in pJR2 or pBC3 as *Eco*RI fragments, using standard procedures (16). The HPP20(Δ32) deletion mutant was tagged with the human PPCA signal sequence, as reported earlier (8). All cDNA fragments were engineered to have short 3′- and 5′-untranslated regions (<10 base pairs).

Generation of Recombinant Baculoviruses—Spodoptera frugiperda insect cells (IPLB-SF21) were cultured in monolayers at 27 °C in TNM-FH medium (17) supplemented with 10% fetal bovine serum and antibiotics (complete medium). Wild-type AcMNPV virus strain E2 (18) and recombinant baculoviruses were propagated on confluent monolayers of Sf21 cells. Recombinant baculoviruses were generated by co-transfecting Sf21 cells with 1 μg of wild-type AcMNPV DNA and 10 μg of plasmid DNA as described (19). They were selected and purified by sequential plaque assays and verified by Southern blot analysis (19). Large quantities of inoculum were produced by infection of insect cells at 25–50% confluence with recombinant virus at a multiplicity of infection of < 1 plaque-forming unit/cell. After 3–6 days at 27 °C, when all cells appeared infected, the medium was harvested and centrifuged for 5 min at 1000 rpm to remove detached cells. The titre of the inoculum was determined by plaque assay analysis.

Metabolic Labeling of Infected Cells—For biosynthetic labeling studies, Sf21 cells were seeded in 6-well plates and grown until 80–90% confluence. Cells were infected with baculoviruses at a multiplicity of infection of 5–10 plaque-forming units/cell and radiolabeled for 4 h with 50 μCi/ml [^{35}S]methionine (Amersham Corp.). Immunoprecipitations were carried out with anti-human PPCA precursor antibodies (anti-54) (8) and formalin-fixed *Staphylococcus aureus* cells (Immunoprecipitin, BRL) as reported earlier (8, 20). Immunoprecipitated proteins were

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[1] The abbreviations used are: PPCA, protective protein/cathepsin A; BV, baculovirus; DFP, diisopropyl fluorophosphate.

resolved on 12.5% SDS-polyacrylamide gels under reducing or nonreducing conditions (21) and visualized by fluorography of gels soaked in Amplify (Amersham Corp.).

Immunoelectron Microscopy—Sf21 cells infected with recombinant baculoviruses were fixed two days postinfection in 0.1 M phosphate buffer, pH 7.3, 1% acrolein, and 0.4% glutaraldehyde. Further embedding in gelatin, preparation for ultracryotomy, and methods for immunoelectron microscopy were as reported earlier (22). Ultrathin sections were probed with antibodies (anti-32) raised against the denatured 32-kDa chain of human PPCA (6).

Development of Anti-peptide Antibodies—A 15-amino acid peptide (NH_2-Cys-Met-Trp-His-Gln-Ala-Leu-Leu-Arg-Ser-Gly-Asp-Lys-Val-Arg-COOH) based on the COOH-terminal sequence of the 34-kDa subunit (amino acids 285–298) (6) was synthesized on a peptide synthesizer (Applied Biosystems) and covalently coupled via the NH_2-terminal Cys residue to the carrier protein *Keyhole Limpet Hemocyanin* using the Imjet-activated immunogen conjugation kit as recommended by the manufacturer (Pierce). Polyclonal antibodies (anti-pep) were raised in rabbits against the conjugated product and tested on immunoblots or immunoprecipitations of BV-produced proteins.

Limited Proteolysis with Trypsin—Infections with baculovirus constructs were performed as described above. Medium samples were concentrated 5–20-fold by ammonium sulfate precipitation and desalted on a Sephadex G50 column (20). Aliquots of 15 μl of medium concentrates were diluted to 200 μl with 10 mM sodium phosphate buffer, pH 6.8, and 1 mg/ml bovine serum albumin. Samples were subjected to limited proteolysis with 1 μg of trypsin (Sigma) for increasing periods of time as described by Galjart *et al.* (8). Reactions were stopped by the addition of 3 μg of bovine pancreas trypsin inhibitor (Sigma). Aliquots of each reaction mixture (10 μl) were assayed for cathepsin A activity using the *N*-blocked dipeptide benzyloxycarbonyl-phenylalanyl-alanine as described by Galjart *et al.* (8). 20-μl aliquots of trypsin-digested proteins were resolved on SDS-polyacrylamide gels and transferred from gels to Immobilon polyvinylidene difluoride membranes (Millipore Corp.) using a semi-dry blotter (W. E. P. Company). Blots were incubated for at least 12 h in blocking buffer (0.01 M Tris-buffered saline, pH 8.0, 0.05% Tween 20, and 3% (w/v) bovine serum albumin) and subsequently probed with anti-54 or anti-pep antibodies followed by alkaline phosphatase conjugate anti-rabbit IgG second antibodies (Sigma). Proteins were stained using the colorimetric substrate for alkaline phosphatase (Sigma). For the DFP-binding assay, 40-μl aliquots were incubated for 1 h at room temperature with 1 μCi of [^{3}H]DFP as described earlier (8). Radiolabeled proteins were immunoprecipitated with anti-54 antibodies and resolved by SDS-polyacrylamide gel electrophoresis and fluorography.

RESULTS

SF21 insect cells were infected with recombinant baculoviruses AcHPP54, AcHPP32, and AcHPP20 separately or co-infected with AcHPP32 and AcHPP20. Metabolic labeling and immunoprecipitation analysis showed that PPCA precursor was synthesized in large quantities and efficiently secreted but was not or poorly processed to the mature two-chain product (Fig. 1, *lanes 1* and *5*). Its estimated molecular mass of ~54 kDa was similar to wild-type precursor from human cultured fibroblasts (1). Trace amounts of low molecular weight polypeptides, visible both intra- and extracellularly, were products of aspecific proteolysis, because pulse-chase experiments showed no time-dependent conversion of the precursor into mature protein (not shown). Single infections with AcHPP32 and AcHPP20 resulted in the production of truncated polypeptides with molecular masses of 34, 20, and 18 kDa, respectively (Fig. 1, *lanes 2* and *3*). Only the former, which was 2 kDa larger than the corresponding wild-type subunit, was secreted to some extent (Fig. 1, *lane 6*). The two chains of 20 and 18 kDa (Fig. 1, *lane 3*) were different glycosylation forms of the small subunit. Under nonreducing conditions the secreted large chain migrated as a doublet, suggesting that within this polypeptide partial and/or different intrachain disulfide bridges may have formed (Fig. 1, *lane 10*). Co-infected cells synthesized a large subunit of 34 kDa and three small subunits of 20, 19.5, and 18 kDa, respectively. The intermediate 19.5-kDa species was unique for co-infected cells (compare *lanes 3* and *4*) and was the

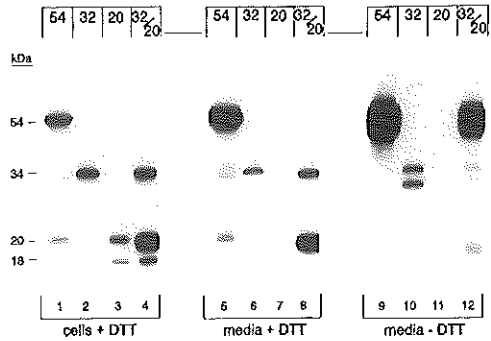

FIG. 1. **Metabolic labeling of PPCA precursor and separate subunits in insect cells.** Sf21 cells were either infected with baculovirus construct AcHpp54 (54), AcHpp32 (32), or AcHpp20 (20), or co-infected with AcHpp32 and AcHpp20 (32/20). 2 days after infection newly synthesized proteins were labeled with [^{35}S]methionine. Labeled proteins were immunoprecipitated from cells and media using anti-54 antibodies. Proteins were separated by SDS-polyacrylamide gel electrophoresis under reducing (*lanes 1–8*) or nonreducing (*lanes 9–12*) conditions and visualized by fluorography. Exposure time was 24 h. Molecular sizes are indicated. *DTT*, dithiothreitol.

only one of the small subunits found in the medium (Fig. 1, *lanes 8* and *12*). Co-expressed polypeptides were immunoprecipitated in larger quantities both intra- and extracellularly, compared with single infections and under nonreducing conditions were resolved as one product of ~54 kDa, similar in size to the wild-type precursor (Fig. 1, *lanes 9* and *12*). These results suggest an early intracellular association of the two separately synthesized polypeptides, which are secreted in an associated state. Although they assemble, the 34-kDa subunit remains 2 kDa bigger (Fig. 1, *lane 8*) and is not further processed to the mature size.

To determine whether the subcellular localization of over-expressed proteins could account for the lack of maturation, singly infected or co-infected cells were analyzed with electron microscopy. Immunostaining of ultrathin sections demonstrated an intracellular distribution that was similar for the different over-expressed proteins. Extensive gold labeling was restricted to structures in the cytoplasm corresponding to swollen cisternae of the endoplasmic reticulum (Fig. 2*A*) and the Golgi complex (Fig. 2*B*). Other subcellular organelles, including multivesicular bodies and fibrous structures, usually observed in insect cells infected with either wild-type or recombinant viruses (23, 24), were totally devoid of gold particles. It appears therefore that none of the BV-expressed proteins reach a lysosome-like compartment, where proteolytic processing should occur.

The amino terminus of the 20-kDa chain, isolated from human placenta and human platelets (6, 11), starts with Met-299, which is preceded in the amino acid sequence of the precursor by a conserved arginine. This residue likely represents the site of initial cleavage. Circumstantial evidence has, however, indicated that complete maturation of the precursor may require more proteolytic steps that could occur at Arg-284, Arg-292, or Lys-296. Here we have monitored the processing and catalytic activation of human PPCA precursor and reconstituted subunits by digesting BV-derived secreted proteins with trypsin. To ascertain the occurrence of sequential processing steps, we have used a polyvalent rabbit antibody (anti-pep) raised against a peptide of 15-amino acids, 14 of which are derived from the COOH terminus of the large subunit. Concentrated medium samples containing either the precursor or the 34- and

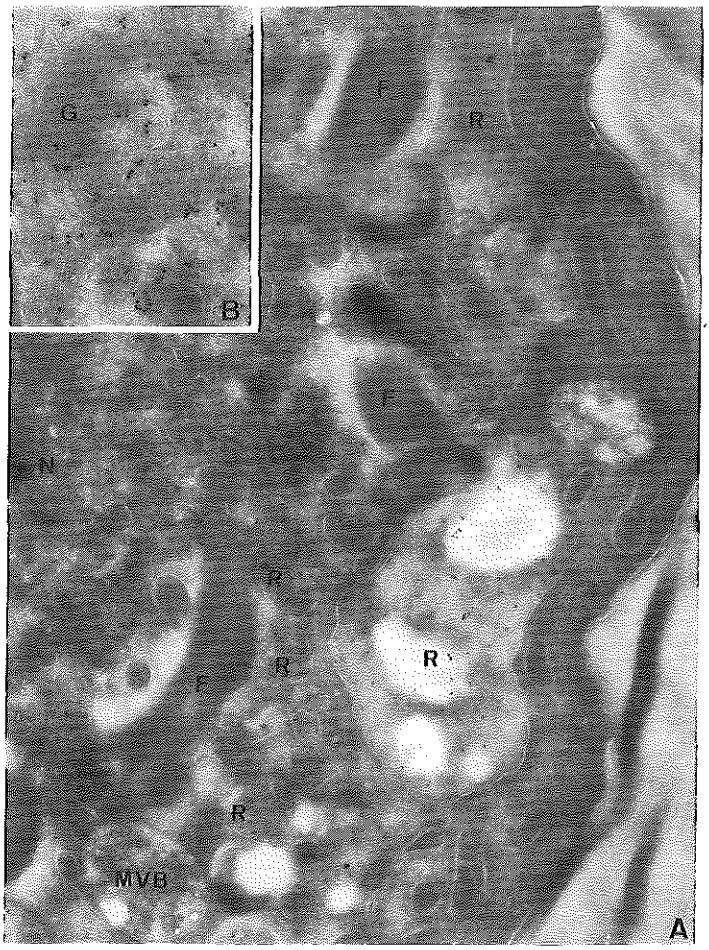

FIG. 2. **Subcellular localization of BV-expressed proteins.** Sf21 cells were infected with AcHPP54, AcHPP32, or AcHPP54 + AcHPP32 and prepared for immunoelectron microscopy 48 h after infection. Cryosections were incubated with anti-32 antibodies followed by goat anti-rabbit IgG gold labeling. *A*, in all three cases the over-expressed proteins were localized in swollen cisternae of the endoplasmic reticulum (*R*), which have the appearance of large vacuoles. *B*, gold particles were also clearly present in the Golgi complex (*G*). *n*, nucleus; F, fibrous structures; *MVB*, multivesicular bodies. The magnifications were: 38,000× for *A* and 79,000× for *B*.

20-kDa associated protein were incubated with a fixed amount of trypsin for increasing periods of time, and each reaction was stopped by the addition of trypsin inhibitor. Aliquots of each sample were tested on Western blots immunostained with anti-54 and anti-pep antibodies and were assayed for cathepsin A activity. As seen in Fig. 3 (*upper left panel*), after 0.5 min of incubation with trypsin, part of the one-chain precursor was cleaved into a two-chain product of 34 and 20 kDa. Between 2 and 5 min, the 34-kDa form was gradually converted into a 32-kDa derivative, and complete maturation was achieved after 10 min. In contrast, the size of the 20-kDa chain did not vary. Prolonged digestion periods (15 and 30 min) led to aspecific degradation and lower yield of both subunits. Using anti-pep antibodies, only the 54- and 34-kDa polypeptides were detected, indicating that the COOH-terminal peptide of the 34-kDa species was lost upon conversion to the 32-kDa form (Fig. 3, *middle left panel*). Step-wise maturation of the precursor into a fully processed product was paralleled by a clear increase in cathepsin A activity, which was maximal after 15 min of digestion (Fig. 3, *lower left panel*). Similar maturation steps were observed for the reconstituted 34- and 20-kDa protein. Upon trypsin cleavage, the large chain was again converted from a 34- to a 32-kDa product, whereas the size of the small subunit did not change (Fig. 3, *upper* and *middle right panels*). However, the amount of both polypeptides, as detected on immunoblots, was significantly less than for wild-type precursor, resulting in an overall reduced cathepsin A activity. This was probably due to both a lower secretion of the reconstituted two-chain protein as well as a reduced stability. However, also in this case a clear increase in enzymatic activity was measured after digestion (Fig. 3, *lower right panel*).

Catalytic activation of precursor and reconstituted products was confirmed by the ability of trypsin-processed proteins to bind the serine protease inhibitor DFP. As shown in Fig. 4 (*lanes 1–6*), only the mature 32-kDa subunit, generated after trypsin digestion of the precursor, was recognized by the radiolabeled inhibitor. The 34-kDa form was apparently unable to bind DFP, although it was present at 0.5–5 min digestion time points (see Fig. 3). The inability to bind the inhibitor was particularly evident in the case of the undigested 34- and

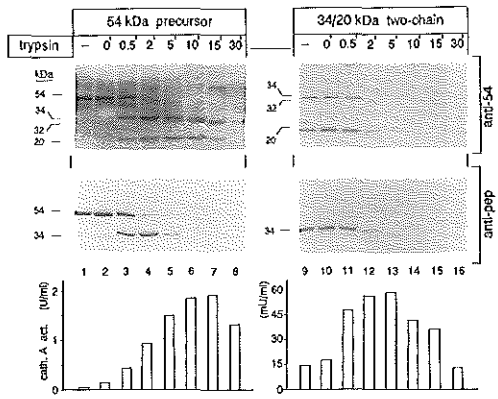

FIG. 3. **Limited proteolysis with trypsin of 54-kDa precursor and 34- and 20-kDa reconstituted two-chain protein.** Aliquots of medium concentrates containing the 54-kDa precursor and 34- and 20-kDa associated protein were incubated at 37 °C with 1 μg of trypsin in the presence of bovine serum albumin (1 mg/ml) for the indicated periods of time. Reactions were stopped with 3 μg of trypsin inhibitor. Samples in *lanes 1* and *9* were untreated. At time 0 (*lanes 2* and *10*), the samples were treated with trypsin inhibitor prior to the addition of trypsin. A portion of each sample was separated by SDS-polyacrylamide gel electrophoresis, followed by electroblotting and immunostaining with anti-54 and anti-pep antibodies. Cathepsin A activity toward the acylated dipeptide benzyloxycarbonyl-phenylalanyl-alanine was measured in each aliquot. One milliunit (*mU*) of activity is defined as the enzyme activity that releases one nanomole of alanine/minute.

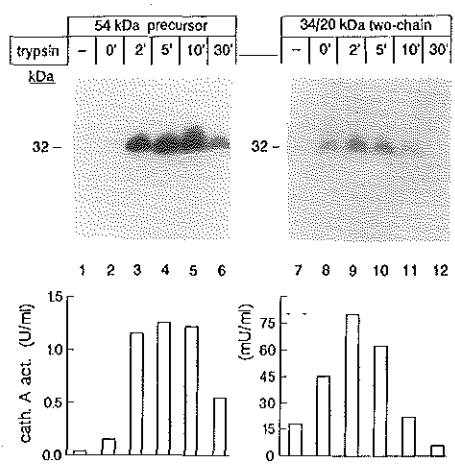

FIG. 4. **Binding of serine carboxypeptidase inhibitor DFP to trypsin digested 54 kDa precursor and reconstituted two-chain protein.** Trypsin digestions of precursor and 34- and 20-kDa two-chain product were performed as described in the legend to Fig. 3. 1 μCi of [^{3}H]DFP was added to 40 μl of trypsin digest, followed by immunoprecipitation with anti-54 antibodies. Immunoprecipitated proteins were separated on SDS-polyacrylamide gels under reducing conditions and visualized by fluorography. Exposure times were 2 weeks for *lanes 1–6* and 3 months for *lanes 7–12*. Molecular sizes are indicated. Cathepsin A activity was measured as described in the legend to Fig. 3.

20-kDa protein (*lane 7*), which showed no radioactive signal, confirming that it has only marginal enzymatic activity. However, this associated form was particularly susceptible to proteolytic cleavage, because immediately after addition of trypsin (time 0, *lane 8*), a substantial conversion to the 32-kDa product took place. For both wild-type precursor and reconstituted protein, the highest levels of cathepsin A activity were measured at digestion time points in which maximal binding was observed (Fig. 4, *lanes 3–5* and *9–10*). As seen in the previous experiment, the overall amount of reconstituted and digested two-chain product was again considerably lower than trypsin-cleaved precursor. All together these data point to the 34- and 20-kDa product as being a processing intermediate, transiently occurring during proteolytic maturation of PPCA precursor. This process is probably mediated by a trypsin-like protease. Removal of a COOH-terminal peptide from the 34-kDa subunit is essential for catalytic activation.

DISCUSSION

PPCA is a lysosomal serine protease with pleiotropic biological properties. It binds in its normal state to the enzymes β-galactosidase and neuraminidase, rendering them stable and active in lysosomes; it also hydrolyzes as carboxypeptidase and/or deamidase/esterase a variety of bioactive peptides, depending upon the pH conditions used in the assay. When deficient or defective in humans, it causes the lysosomal storage disease galactosialidosis. Because its protective and catalytic functions are distinct (8), we and others have postulated a role for PPCA in the local inactivation of selected neuropeptides, with or without the aid of the two glycosidases (8, 11–13). The protein is synthesized in mammalian tissues and cultured cells as a one-chain precursor that is enzymatically inactive. Proteolytic conversion to a disulfide-linked two chain product triggers catalytic activation. To investigate the process of zymogen activation and to assess the capacity of the separately synthesized subunits to assemble into an active enzyme, we have used the baculovirus system (for review see Ref. 25 and references therein) to express human PPCA in insect cells, either as one-chain precursor or as two separate subunits.

BV-encoded proteins are synthesized in large amounts and transported from the endoplasmic reticulum to the Golgi complex but do not seem to be targeted to a lysosome-like organelle. It is conceivable that this transport step either requires a recognition marker on lysosomal proteins specific for insect cells or the process is saturated by the high concentration of newly synthesized proteins. The subcellular distribution of both PPCA precursor and reconstituted subunits could however explain their inadequate intracellular maturation, which may depend on a lysosome-associated protease. Although it is known that the degree of proteolytic processing varies among heterologous proteins expressed with the baculovirus system (25), identical results were obtained by us with mouse PPCA and human β-galactosidase expressed in insect cells[2] and by others with human β-galactosidase, β-glucosidase, and β-hexosaminidase (26–29). Of the three separately synthesized proteins, mainly the wild-type precursor is secreted in a large quantity. Interestingly, however, the co-expressed subunits are able to form interchain disulfide bridges and are secreted only in the associated state. They evidently require conformational changes for their secretion, acquired only when both domains are present. Using baculovirus co-expression vectors, other investigators have demonstrated appropriate formation of functional immunoglobulin heterodimers, which are efficiently secreted, and of disulfide-bridged interleukin 5 homodimers (30, 31).

The focus of the experimental work presented here is the process of enzymatic activation of human PPCA zymogen, which enabled us to map the linker domain between the two chains, whose removal is required for activation. Both wild-

[2] Erik J. Bonten, unpublished data.

type precursor and reconstituted two-chain form can be subjected to partial proteolysis with trypsin, and the cleaved products have maximal cathepsin A activity. Our results suggest that the *in vitro* activation of BV-produced precursor mimics its *in vivo* processing taking place in at least two steps: an endoproteolytic cleavage resulting in a transient intermediate of 34- and 20-kDa, followed by trimming of the last 14 amino acids at the COOH terminus of the large chain. Furthermore, we provide evidence that the 34- and 20-kDa reconstituted product is probably identical to the partially processed intermediate, which is a naturally occurring catalytically inactive form. A similar processing pattern was observed earlier for PPCA precursor over-expressed in COS-1 cells (8, 14). In this case the 34- and 20-kDa intermediate is seen only transiently, indicating that its half-life may be too short and its quantity too low to be detected in normally expressing cultured fibroblasts. There is an interesting analogy between the processing and activation of PPCA and that of other serine carboxypeptidases. This family of proteases comprises single- and two-chain enzymes that are present in different species, ranging from yeast to fungi, plants, and humans (9, 10). Some of the plant peptidases have been purified in an active form composed of two chains that have been sequenced at their NH_2 termini (32). In the case of barley carboxypeptidase I, the two mature subunits of the enzyme originate from a single-chain precursor that in addition contains a stretch of amino acids separating the two chains (33). This closely resembles the processing of PPCA zymogen and provides another verified example in a homologous carboxypeptidase of COOH-terminal trimming after the initial endoproteolytic step. The analogy in maturation events might indicate a similar function for the linker domain in the plant carboxypeptidase and PPCA precursors, namely to keep these forms in an inactive state. In yeast carboxypeptidase Y such an inactivation function resides within its propeptide segment, which must obstruct the access of a substrate to the preformed active site (34). Limited proteolysis with trypsin removes this inactivating segment and exposes the active site. Removal of the linker domain during maturation of the PPCA zymogen may induce major conformational changes on the protein necessary to uncover its active site. Alternatively, this peptide may only mask the active site on an already correctly folded polypeptide. These hypotheses are currently verified on the basis of the three-dimensional structure of the uncleaved precursor molecule.

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Lysosomal neuraminidase: catalytic activation in insect cells is controlled by the protective protein/cathepsin A.

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Lysosomal Neuraminidase

CATALYTIC ACTIVATION IN INSECT CELLS IS CONTROLLED BY THE PROTECTIVE PROTEIN/CATHEPSIN A

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Lysosomal *N*-acetyl-α-neuraminidase is active in complex with the protective protein/cathepsin A (PPCA) and β-galactosidase. The interaction with PPCA is essential for the correct intracellular routing and lysosomal localization of neuraminidase, but the mechanism of its catalytic activation is unclear. To investigate this process, we have used the baculovirus expression system to co-express neuraminidase and PPCA precursors in insect cells, which ensued high enzymatic activity of neuraminidase. Both the 34- and 20-kDa PPCA subunits were required for the activation. We further demonstrated that when expressed alone the neuraminidase precursor remained dimeric (114 kDa) and had low enzymatic activity, but when co-expressed with PPCA and β-galactosidase, multimerized in a complex of ~1350 kDa, together with the other two proteins. The fully active neuraminidase co-precipitated with full-length PPCA and β-galactosidase precursors. However, when co-expressed with the individual PPCA subunits, neuraminidase co-precipitated only with the small 20-kDa polypeptide, which therefore must contain a neuraminidase-binding site. Our finding suggests a model of activation of neuraminidase dependent on its oligomerization at acidic pH that is mediated by interaction with PPCA.

Lysosomal neuraminidase (EC 3.2.1.18) catalyzes the intralysosomal degradation of sialoglycoconjugates by releasing terminal sialic acid from their oligosaccharide side chains. The human enzyme is involved in two genetically distinct lysosomal storage disorders: sialidosis, which is caused by mutations in the lysosomal neuraminidase gene (1,2), and galactosialidosis, which is the combined deficiency of neuraminidase and β-galactosidase, caused by a primary deficiency of the protective protein/cathepsin A (PPCA) (3-5).

In mammalian tissues, neuraminidase is present in a high molecular weight multienzyme complex with PPCA, and β-galactosidase, and copurifies with these enzymes on affinity chromatography columns for either β-galactosidase or PPCA (6-12). Pshezehtsky and coworkers have reported that also N-acetylgalactosamine-6-sulfate sulfatase can be present in the multienzyme complex, although confirmation of this finding or a follow-up has not yet been reported (6). Only a small percentage of PPCA and β-galactosidase activities are consistently measured in the multienzyme complex (1-2% of total), which instead contains all of the neuraminidase activity. Apparently, PPCA and β-galactosidase are active also outside the complex, while neuraminidase is not (6-15). Verheijen et al have shown that human neuraminidase purified from placenta was inactive at neutral pH, but could be reactivated *in vitro* by concentrating the preparation at 37°C and acidic pH, which resulted in a four fold increase in activity for every two fold of concentration (8). On sucrose density gradients the 'stabilized' enzyme coprecipitated with the 32-kDa PPCA subunit and β-galactosidase, suggesting that assembly into a multienzyme complex stabilized/protected the neuraminidase activity. In galactosialidosis fibroblasts, in the absence of PPCA, the 10-15% residual β-galactosidase activity increased upon addition to the culture medium of the protease inhibitor leupeptin, which pointed to a protective role of PPCA against proteolytic degradation of β-galactosidase (6,16,17). In contrast, neuraminidase activity remained undetectable after leupeptin treatment, unless PPCA was added (6,16,17), suggesting a different way for PPCA to influence neuraminidase activity.

We have previously shown that human and mouse lysosomal neuraminidase expressed in COS-1 cells and fibroblasts from patients with sialidosis associate with PPCA shortly after synthesis, and segregate to mature lysosomes, only when accompanied by wild-type PPCA, but not by transport-impaired PPCA mutants (18). Early interaction is needed because neuraminidase is poorly phosphorylated, even in the presence of PPCA, and its mannose-six phosphate-marker (M6P) is not functional in receptor-mediated endocytosis of the enzyme (18). Thus, PPCA functions as an auxiliary transport protein for neuraminidase, and neuraminidase acquires full enzymatic activity in mature lysosomes, only when bound to PPCA. In contrast, others have reported that in galactosialidosis fibroblasts neuraminidase does reach the lysosomes, but is rapidly degraded because of the absence of functional PPCA (19). Thus, the exact mechanism by which PPCA controls both the catalytic activation of neuraminidase, and the maintenance/regulation of its enzymatic activity is unknown.

We have utilized the baculovirus (BV) expression in insect cells to study the mechanism of catalytic activation of PPCA and demonstrated that the one-chain zymogen is cleaved into a two-chain mature enzyme by a trypsin-like protease, removing a linker-peptide between the two subunits (20). The structural basis of this activation mechanism were assessed after the determination of the 3D structure of human PPCA precursor that showed that the active site is preformed in the zymogen, but is blocked by a 'maturation subdomain' (21). Removal of the linker peptide causes conformational changes of the maturation subdomain, exposing the active site (21).

Here we have developed a similar approach to elucidate the mechanism of catalytic activation of neuraminidase in insect cells. When using baculovirus constructs, several factors can influence the level of protein expression in insect cells, including the type of baculovirus vector, the type of insect cells, the multiplicity of infection (M.O.I), the type of culture medium, the time of infection, and the stability of the overexpressed protein (22,23). When high expression levels are achieved the recombinant protein is usually clearly visible on Coomassie stained SDS-gels containing total cell lysates. Taken this into account we expressed neuraminidase in insect cells and studied the *in vivo* and *in vitro* interactions with co-expressed PPCA and β-galactosidase. Our results indicate that neuraminidase is catalytically activated by conformational changes that occur when the enzyme shifts from a 114-kDa dimeric form to an oligomeric complex of ~1350 kDa. This activation occurs exclusively at acidic pH, and is triggered by its interaction with PPCA.

EXPERIMENTAL PROCEDURES

Cell Culture and Generation of Recombinant Baculovirus Constructs – Spodoptera frugiperda insect cells (IPLB-SF21) were cultured in monolayers at 27°C in TNM-FH medium (24), supplemented with 10% fetal bovine serum and antibiotics. Recombinant baculovirus (BV) constructs encoding mouse and human neuraminidase (Moneur and Huneur), human β-galactosidase (βgal) and mouse PPCA (MPP54), were generated through homologous recombination in insect cells, of wild-type BV DNA (AcMNPV) and the respective cDNA's (20,25,26), which were subcloned into the BV-transfer plasmids pJR_2 and pBC_3 (20,27). The generation and characterization of BV-constructs encoding human PPCA (HPP54) and the two separate human PPCA subunits (PP32 and PP20) was described earlier (20).

Development of Antibodies – Polyclonal antibodies were raised in rabbits (Rockland) against BV-expressed 85-kDa human β-galactosidase precursor (anti-βgal), 54-kDa human PPCA precursor (anti-hPPCA), 20-kDa human PPCA subunit (anti-PP20), and 54-kDa mouse PPCA precursor (anti-mPPCA). All secreted recombinant proteins were affinity purified from the insect cell culture medium on Concanavalin sepharose columns (Con A; Pharmacia Biotech), prior to injection in rabbits, with the exception of the 20-kDa PPCA subunit. The latter polypeptide was excised from a preparative SDS-polyacrylamide gel containing PP20 infected cell-lysates. The generation of anti-human neuraminidase antibodies (anti-Neur) was described earlier (2).

Metabolic Labeling of Infected Insect Cells – For biosynthetic labeling studies Sf21 cells were seeded in 6-well dishes and grown until 80-90% confluence, and infected with BV at a multiplicity of infection (M.O.I.) of 5 plaque forming units/cell (pfu/cell). Two days after infection the cells were radiolabeled for 16 h with 50 μCi/ml ^{35}S-methionine. Immunoprecipitations were carried out as described earlier (20,28,29). Immunoprecipitated proteins were resolved on 12.5% SDS-PAGE, and visualized by fluorography of the gels soaked in Amplify (Amersham Corp.).

Purification of Neuraminidase – Sf21 cells were propagated in 3D bottles (Nunc) until 80-90% confluence and infected at M.O.I. 5 with Moneur. The cells were dislodged two days after infection by tapping the bottles firmly. All steps were carried out at 4°C unless indicated otherwise. The cells were washed in Phosphate buffered Saline (PBS) and pelleted by centrifugation at 1000 rpm. The cells were lysed in water and 1% Nonidet P40 (NP40; Calbiochem) for 30 min. Tris Ethanol Amine (TEA stock solution: 0.2 M, pH 8.75) and water was added to the lysate to obtain final concentrations: 20 mM TEA pH 8.75, 0.1% NP40. The lysate was ultra-centrifugated (Beckman SW28 rotor, 25000 rpm, 1 h), and the supernatant was filtered through a 0.2 μm filter. Neuraminidase was FPLC-purified on a Resource Q column (Pharmacia Biotech.) and eluted with a salt gradient of 0-150 mM NaCl (20 mM TEA pH 8.75). The neuraminidase eluted at ~80 mM NaCl and was concentrated in Centriprep 10 units (Amicon) to 1-3 mg/ml. Neuraminidase was further purified on a Sephacryl S300HR column (Pharmacia Biotech.) in 20 mM TEA pH 8.75/100 mM NaCl. The neuraminidase containing fractions were pooled and concentrated as described above. The column was calibrated with molecular weight markers (Pharmacia Biotech.).

In vitro activation of neuraminidase - Fixed amounts of purified mouse neuraminidase were mixed with increasing concentrations of Con A purified mouse PPCA, human PPCA, human β-galactosidase, or bovine serum albumin (BSA) in 50 mM sodium acetate pH 5.0 or pH 6.8 and 100 mM NaCl, and incubated at room-temperature (RT) for 1 h. Neuraminidase activity was assayed with the artificial 4-methylumbelliferyl substrate (Sigma) according to Galjaard (1980). Aliquots of in vitro activated neuraminidase with and without PPCA were resolved on a Sephacryl S300HR column (50 mM sodium Acetate pH 5.0/100 mM NaCl). Eluted fractions were separated on 12.5% SDS-PAGE, blotted on PVDF membranes (Immobilon, Millipore), and incubated with anti-Neur and anti-PPCA antibodies.

In vivo activation of neuraminidase and gelfiltration - Sf21 cells were infected in 6-well dishes with fixed amounts of Moneur, and increasing amounts of either MPP54, HPP54, PP32, PP20, and PP20 plus PP32 (as described above). Two days after infection the cells were harvested and the neuraminidase activity assayed. The cell-lysates were separated on a Sephacryl S300HR gelfiltration column (50 mM sodium Phosphate pH 7.0/100 mM NaCl) and the eluates were analyzed by SDS-PAGE/western-blotting, and neuraminidase activity assays (as described above).

RESULTS

Baculovirus expression in insect cells of neuraminidase, PPCA, and β-galactosidase, for in vivo catalytic activation of neuraminidase. - In mammalian cells neuraminidase depends on its association with a transport-competent PPCA for lysosomal localization (18). In insect cells, instead, PPCA does not reach the vacuole, but accumulates in the endoplasmic reticulum (ER) and Golgi compartments, and is hardly processed into the mature and catalytically active two-chain form (20). Using BV-infected insect cells to co-express neuraminidase with PPCA and/or β-galactosidase we could now test whether PPCA influences neuraminidase activity in a manner that is independent from its intracellular compartmentalization. For co-expression studies it was crucial to obtain comparable levels of expression of the different components. Previously we over-expressed in Sf21 cells three baculovirus constructs that encode the human PPCA (HPP54), and the two separate PPCA subunits (PP32 and PP20) (20). The expression levels of these proteins were comparable as demonstrated by immuno-precipitation, Western-blotting, and Coomassie staining of SDS-gels (20). We now made baculovirus constructs that encode human neuraminidase (Huneur), mouse neuraminidase (Moneur), mouse PPCA (MPP54), and human β-galactosidase (βgal). To select the best expressing clones, up to 10 baculovirus clones were screened for each protein by infecting the virus in Sf21 cells, and analyzing the recombinant proteins on Coomassie-stained SDS-gels, before and after Con A sepharose purification (Experimental Procedures). Furthermore, the best expressing clones were also tested in metabolically labeled insect cells infected individually with 50 µl of Moneur-, MPP54-, HPP54-, βgal-, HPP32-, and HPP20-recombinant baculoviruses. Two days after infection, cells were labeled for 16 h with ^{35}S-methionine. Radiolabeled proteins were immuno-precipitated with specific antibodies (anti-Neur, anti-PPCA, anti-βgal), and subjected to SDS-PAGE (Fig. 1A). The molecular weights of the expressed and immuno-precipitated proteins are summarized in table 1. The 18-kDa and 75-kDa polypeptides (Fig. 1A, lanes 5 and 8) represented non-glycosylated forms of the small PPCA subunit and the β-galactosidase precursor respectively, since treatment with N-glycosidase F, or inhibition with the N-linked glycosylation inhibitor tunicamycin did not alter their mobility on SDS-PAGE ((20) (data not shown)). These results confirmed that all proteins were expressed at similar levels, with the exception of human neuraminidase (Huneur), which was expressed at considerably lower levels than the other proteins (Fig. 1A, lane 2). For this reason we chose to use the mouse (Moneur) rather than the human neuraminidase construct (Huneur) in co-expression experiments. This approach was further justified by earlier studies demonstrating that interaction of the three enzymes is not species-specific, but each of the human enzymes can be substituted by its murine counterpart in the enzyme-complex (26,30,31).

Table 1. Summary of Immunoprecipitations.

BV-construct	antibody	Mw (kDa)	Fig. 1A
Huneur	anti-Neur	46	lane 2
Moneur	anti-Neur	46	lane 3
PP32	anti-hPPCA	34	lane 4
PP20	anti-hPPCA	20/18	lane 5
HPP54	anti-hPPCA	54	lane 6
MPP54	anti-mPPCA	54	lane 7
β-gal	anti-βgal	85/75	lane 8

Insect cells were co-infected with a constant amount of Moneur (50 µl), and increasing virus concentrations of either MPP54, HPP54, or βgal) (50-300 µl). The cells were harvested two days after infection, and assayed for neuraminidase activity. Both human and mouse PPCA were able to increase the neuraminidase activity in a concentration dependent manner, although mouse PPCA was somewhat more effective in this function (Fig. 1B), whereas β-galactosidase had no effect at all on the neuraminidase activity (Fig. 1B). Aliquots of the different cell-lysates, analyzed on Western-blots probed with anti-Neur antibodies, showed that the amount of the 46-kDa neuraminidase protein was comparable in all samples. Small variations in expression levels and stability of the human and mouse PPCA might account for the differences in neuraminidase activity. Both human and mouse PPCA did not affect the level of expression or the stability of neuraminidase, as shown on Western-blots (Fig. 1B, insert), but their interaction with neuraminidase clearly activated the enzyme.

To determine whether a full-length PPCA was needed for this process or either of the two PPCA subunits (PP20 and PP32) would be sufficient for neuraminidase activation, insect cells were co-infected with a constant amount of Moneur inoculum (50 µl), and increasing concentrations of PP20, PP32, or a combination of the two. Two days after infection, cells were harvested and assayed for neuraminidase activity. The separate subunits did

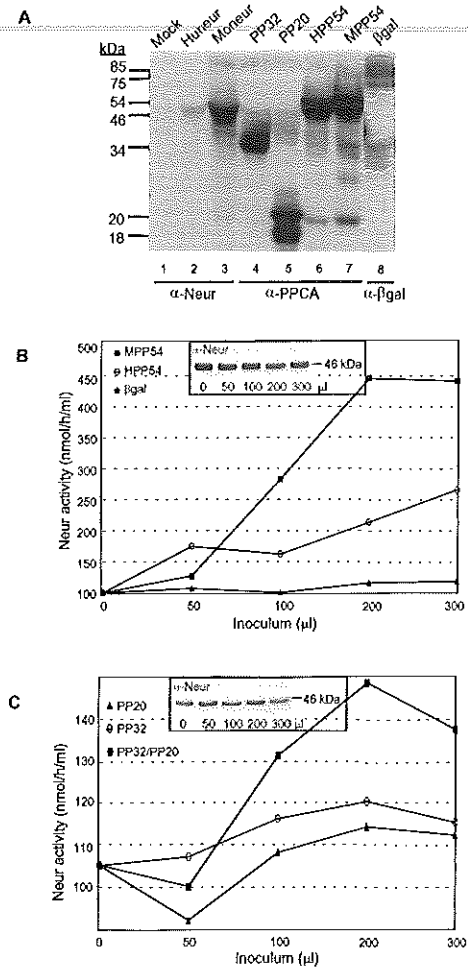

Fig.1. **Baculovirus expression in insect cells of neuraminidase, PPCA, and β-galactosidase, for *in vivo* activation of neuraminidase. (A)** Sf21 cells were single infected with Moneur, Huneur, PP32, PP20, HPP54, MPP54, and βgal. Newly synthesized proteins were radio-labeled with ^{35}S-methionine, two days after infection, followed by immunoprecipitation with either ant-Neur, anti-PPCA, or anti-βgal antibodies. Proteins were separated on SDS-PAGE and visualized by fluorography (eposure time 5 h). **(B)** Sf21 cells were co-infected with constant amount (50 µl) of mouse neuraminidase inoculum (Moneur) and increasing concentrations of either mouse PPCA precursor (MPP54), human PPCA precursor (HPP54), or human βgal precursor (50, 100, 200, 300 µl). Two days after infection the cells were harvested and assayed for neuraminidase activity (nmol/h/ml). The activities are an average of three experiments. Cell-lysates were analyzed for expression of neuraminidase on western-blots, incubated with anti-Neur antibodies (insert). **(C)** Insect cells were co-infected with a constant amount (50 µl) of Moneur, and increasing concentrations of the two human PPCA subunits (PP20 and PP34), or the combination of both (0, 50, 100, 200, 300 µl). Two days after infection the cells were harvested and assayed for neuraminidase activity (nmol/h/ml). The activities are an average of three experiments. The cell-lysates were analyzed for expression of neuraminidase on western-blots incubated with anti-Neur antibodies (insert).

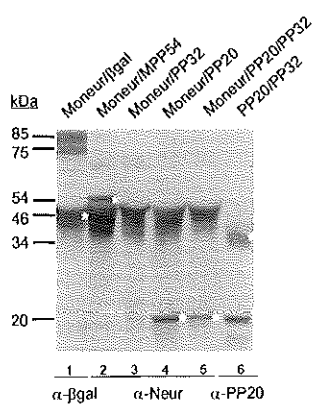

Fig.3. **Metabolic labeling and co-precipitation of BV-expressed neuraminidase, PPCA, β-galactosidase, and the two separate PPCA subunits.** Sf21 cells were co-infected with Moneur and βgal, MPP54, PP32, PP20, and PP32 plus PP20. Newly synthesized proteins were radio-labeled with ^{35}S-methionine, two days after infection, followed by immunoprecipitation with either ant-neur, anti-PPCA, anti-PP32, or anti-βgal antibodies. Proteins were separated on SDS-PAGE and visualized by fluorography (eposure time 5 h). Co-precipitated polypeptides are indicated with a white asterisk (right panel).

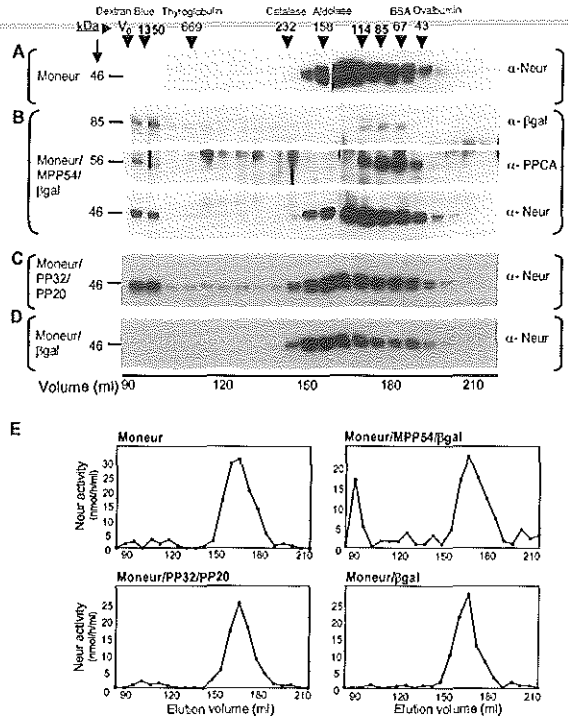

Fig.2. **Gel filtration of BV-expressed neuraminidase.** Insect cells were either **(A)** single infected with Moneur, or co-infected with Moneur and: **(B)** HPP54 and βgal; **(C)** PP32 and PP20; **(D)** βgal. Two days after infection the cells were harvested and the lysates were loaded on a Sephacryl S300HR gelfiltration column (Pharmacia Biotech.). Eluted fractions (90-210 ml) were subjected to SDS-PAGE, blotted on PVDF membranes, and probed with antibodies (indicated at the right). The gel filtration column was pre-calibrated with molecular weight standards (Pharmacia Biotech). Molecular weights of the eluted proteins (native), and molecular weight standards are indicated at top. Molecular weights of the proteins after SDS-PAGE (denatured) are indicated at left. Elution volume is indicated at bottom. **(E)** All gel filtration fractions were assayed for neuraminidase activity (nmol/h/ml).

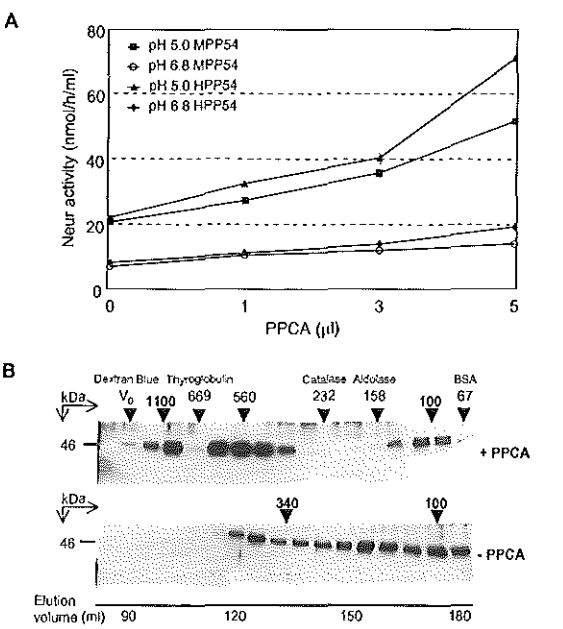

Fig.4. ***In vitro* multimerization and catalytic activation of neuraminidase.** **(A)** Increasing amounts of Con A purified HPP54 and MPP54 were added (0, 1, 3, 5 µl) to purified dimeric mouse neuraminidase and subsequently incubated at room temperature for 4 h, at either pH 5.0 or 6.8. After incubation the samples were assayed for neuraminidase activity (nmol/h/ml). **(B)** Catalytically activated neuraminidase (5 µl HPP54 added to dimeric mouse neuraminidase for optimal activation) and mock activated neuraminidase (BSA added instead of PPCA) were resolved on a Sephacryl S300HR gel filtration column at pH 5.0. Eluted fractions were separated on SDS-polyacrylamide gels, blotted on PVDF membranes, and probed with anti-Neur antibodies. Molecular weights of eluted proteins (native), and molecular weight standards are indicated at top. Molecular weights of proteins after SDS-PAGE (denatured) are indicated at left. Elution volume is indicated at bottom.

not affect the enzyme activity (Fig. 1C). However, a significant increase in activity was observed when both PPCA subunits were co-expressed with neuraminidase (Fig. 1C). The levels of the 46-kDa protein on western-blots were comparable in all samples (fig. 1C, insert). Thus, a full-length PPCA protein, in form of the one-chain precursor or the two-chain mature enzyme, is needed for catalytic activation of neuraminidase.

Complex formation of neuraminidase with PPCA and β-galactosidase. – Our results have shown that PPCA by itself has the ability to activate neuraminidase. We further investigated whether the three-enzyme-complex could assemble also in insect cells, and whether neuraminidase activity would change in the context of this complex. We first analyzed by gel filtration the native molecular weight of mouse neuraminidase alone, or in combination either with the 85-kDa β-galactosidase precursor, the 54-kDa PPCA precursor, or both PPCA subunits. Total cell-lysates from BV-infected insect cells were resolved on a Sephacryl S300HR column, and the eluted fractions were analyzed on western-blots, probed with polyclonal antibodies specific for either of the three proteins (Experimental Procedures). Neuraminidase expressed alone eluted at a molecular weight of ~114 kDa, probably as a dimer (Fig. 2A). When co-expressed with PPCA and β-galactosidase, a small amount of neuraminidase oligomerized and eluted from the column in fractions corresponding to a molecular weight of ~1350 kDa (Fig. 2B, upper panel). These fractions also contained small amounts of PPCA and β-galactosidase, suggesting that the three enzymes were in complex (Fig. 2B, middle and lower panel). However, most of the neuraminidase, PPCA, and β-galactosidase eluted at 114-85 kDa (Fig. 2B, 3 panels), and were not associated. When co-expressed with both PPCA subunits, a small percentage of neuraminidase again eluted at ~1350 kDa (Fig. 2C). However, neither of the two subunits was resolved together with neuraminidase in these fractions, suggesting that they were either only transiently associated with neuraminidase, but just enough to trigger the oligomerization of the enzyme, or they were still in complex with neuraminidase, but their amount was too low to be detected on the western-blot. Co-expression of neuraminidase with only β-galactosidase did not promote oligomerization of the enzyme (Fig. 2D), confirming that β-galactosidase was not directly involved in this process.

The gel filtration eluates were also assayed for neuraminidase activity (Fig. 2E). The dimeric enzyme had very low activity (Fig. 2E, upper-left panel). The neuraminidase that was eluted at 1350 kDa, together with PPCA and β-galactosidase, was also catalytically active (Fig. 2E, upper-right panel). Given that only a small percentage of neuraminidase was resolved in these high molecular weight fractions, this oligomeric neuraminidase had a substantially higher specific activity (10-20x) than the dimeric form (2B, lower panel). In contrast, the ~1350-kDa oligomeric neuraminidase, resolved on gel filtration after co-expression with the separate PPCA subunits, was catalytically inactive (Fig 2E, lower-left panel), despite the relatively high amounts detected on western-blots (Fig. 2C). Thus, the oligomerization of neuraminidase is not sufficient for its catalytic activation, which apparently requires stable association with PPCA.

Co-precipitation of BV-expressed neuraminidase with PPCA and β-galactosidase. – To demonstrate that neuraminidase was indeed associated with PPCA and β-galactosidase, insect cells were co-infected with the BV-constructs listed in table 1. Two days after infection, cells were metabolically labeled for 16 h with ^{35}S-methionine. The radiolabeled proteins were precipitated with specific antibodies (anti-Neur, anti-PPCA, anti-PP20, anti-βgal), and the immunoprecipitated products were subjected to SDS-PAGE. The 54-kDa PPCA precursor was readily co-immunoprecipitated with neuraminidase using anti-Neur antibodies (Fig. 3, lane 2). Surprisingly, only the 20-kDa and not the 34-kDa subunit of PPCA co-precipitated with neuraminidase (Fig. 3, lane 3 and 4), suggesting that this subunit contains a neuraminidase-interacting domain. It is not clear at this moment whether the large PPCA subunit did not bind at all to neuraminidase, or whether this interaction was too weak to withstand the immunoprecipitation conditions. The 34-kDa subunit did co-precipitate with the 20-kDa polypeptide using antibodies that are specific for the small subunit (Fig. 3, lane 6). Neuraminidase also co-precipitated efficiently with the β-galactosidase precursor using anti-βgal antibodies (Fig. 3, lane 1), indicating that the two proteins did interact strongly, although β-galactosidase did not appear to influence the activity of neuraminidase (Fig. 1 and 2). Mouse neuraminidase associated equally well with the human and mouse forms of PPCA and β-galactosidase (31) (data not shown). As shown in Figure 3 (lane 2), PPCA precursor co-precipitated with neuraminidase only with anti-Neur and not with anti-PPCA antibodies, while neuraminidase co-precipitated with β-galactosidase only with anti-βgal and not with anti-Neur antibodies (Fig. 3, lane 1). This was probably due to the inability of some of the antibodies to recognize the associated proteins, which may no longer expose the epitopes against which the antibodies were raised. Together, the results indicate that neuraminidase, PPCA, and β-galactosidase can physically associate in insect cells, forming a ~1350-kDa enzyme-complex (Fig. 2B).

In vitro catalytic activation of neuraminidase. – The ability to produce in insect cells, and purify large quantities of neuraminidase, PPCA and β-galactosidase (Experimental Procedures), allowed us to test *in vitro* the catalytic activation of neuraminidase. Dimeric mouse neuraminidase (pH 8.75) was mixed with increasing amounts of either mouse or human PPCA, and incubated for 1 h at room temperature at pH 5.0 or 6.8. Afterwards the

samples were assayed for neuraminidase activity. Incubation with both mouse and human PPCA (MPP54 and HPP54) resulted in the increase of neuraminidase activity, but only under acidic conditions (Fig. 4A), while β-galactosidase or BSA had no effect on the activity (data not shown). This demonstrated that PPCA controls the catalytic activation of neuraminidase in a pH dependent manner. To determine whether catalytic activation had any effect on the molecular weight of neuraminidase, we separated neuraminidase, either PPCA-activated or mock-activated, on a Sephacryl S300HR gelfiltration column. The eluates were analyzed on western- blots probed with anti-Neur antibodies (Fig. 4B). PPCA-activated neuraminidase was recovered in two separate pools, as oligomers of ~600 kDa, and as oligomers of ~1100 kDa. In contrast, mock-incubated neuraminidase was mainly recovered in fractions ranging from 100 to 300 kDa (Fig. 4B, lower panel). These results indicate that also *in vitro* PPCA was able to promote the oligomerization of neuraminidase. This process appeared to take place in two steps. Initially, oligomers of ~600 kDa were formed, that probably consisted of 6 dimers, that further multimerized in an ~1100-kDa form. We conclude that the interaction between PPCA and neuraminidase is required for both the oligomerization and catalytic activity of neuraminidase, and the two events cannot be separated.

DISCUSSION

Lysosomal neuraminidase shares significant homology with other bacterial and mammalian members of the sialidase superfamily (2). Most neuraminidases are monomeric, but higher oligomeric states have been reported (Taylor, 1996). The crystal structures of bacterial and viral sialidases revealed that the fold topology of these enzymes is identical, and consists of a six-bladed β-propeller around an axis that passes through the active site (30-33). It is likely that lysosomal neuraminidase shares the same fold. Nevertheless, it is the only member of the sialidase superfamily to require an accessory protein (PPCA) for its transport, catalytic activity and stability in lysosomes

Little is known about the mechanism of complex assembly between neuraminidase, PPCA and β-galactosidase. We have used the baculovirus-expression system to co-express neuraminidase in insect cells, with PPCA and β-galactosidase precursors, and the two separate PPCA subunits (20). BV-encoded PPCA precursor, and the two separate subunits were synthesized in the insect cells in large amounts, and transported from the ER to the Golgi compartment, but were not segregated to the vacuole, which explains their lack of maturation (20). In mammalian cells we could not separate the effect that PPCA had on the transport of neuraminidase to lysosomes (18), from the actual activation event. Since PPCA and neuraminidase likely lack the appropriate signals for vacuolar sorting in insect cells, we were able to test the influence of PPCA on the catalytic activation of neuraminidase, independent from its intracellular transport.

Many lysosomal hydrolases, after segregation to lysosomes, undergo partial proteolytic processing, resulting in the removal of either C-terminal or N-terminal fragments, leading to the mature, active forms of the enzymes (34). Recently we reported the mechanisms of catalytic activation of lysosomal β-galactosidase and PPCA, showing that both are two-subunit enzymes. The 85-kDa β-galactosidase precursor is C-terminally processed by the cleavage of a 24-kDa domain, which remains non-covalently associated to the 64-kDa N-terminal polypeptide (35). The one-chain PPCA zymogen is processed by a trypsin-like protease, removing a ~20 amino acid linker-peptide between the two subunits. The two subunits are linked by disulfide bridges, resulting in a 32/20-kDa two-chain active enzyme (20). The crystal structure of BV-expressed PPCA precursor indicated that the removal of the linker-peptide triggers conformational changes in a subdomain of the PPCA, that in turn expose the catalytic pocket (21). Unlike PPCA and β-galactosidase precursors neuraminidase is catalytically activated by a different mechanism that requires the interaction with PPCA. A pool of neuraminidase, PPCA, and β-galactosidase, when co-expressed in insect cells, assembled into a high molecular weight complex. The exact stoichiometry of this multienzyme complex is unknown, but its molecular weight in insect cells (1350 kDa) is similar to the 1000-1300 kDa molecular weight of the multienzyme complex isolated from human fibroblasts and various other mammalian tissues (6-9,13,36-39). Our *in vitro* studies indicate that the complex probably contains 12 neuraminidase dimers. The number of PPCA and β-galactosidase molecules in the complex remains uncertain. The multimerization of neuraminidase occurs only at acidic pH, which implies that in mammalian cells the high molecular weight complex is assembled in lysosomes.

We have shown earlier that in absence of PPCA, neuraminidase formed crystals when over-expressed in COS-1 cells. However, co-expression of neuraminidase with PPCA prevented crystal formation, and promoted its transport to the lysosomes, and catalytic activation (18,20). Association with PPCA appeared to mediate correct folding and oligomerization of the enzyme, preventing aggregation of partially folded or unfolded molecules. A similar mechanism has been described for the coagulation proteins Von Willebrand Factor (vWF) and Factor VIII (FVIII) (40). Factor VIII needs to associate with vWF for correct folding and segregation into the secretory pathway. In Von Willebrand patients, which are deficient for vWF, FVIII aggregates and is subsequently degraded. The dependence of a molecule on the association with a partner may be an important mechanism to regulate at the protein level its biological activity. Especially for neuraminidase this principle could hold true. Sialic acid residues, being

the terminal sugars on carbohydrate chains, are the first targets for hydrolysis. Thus, it may not always be beneficial to have catalytically active neuraminidase in the lysosomes. An equilibrium between neuraminidase and PPCA could maintain the required level of neuraminidase activity. In turn, this could explain the 'instability' of neuraminidase during enzyme purifications, which is caused either by dissociation from the complex, and/or detachment from PPCA (6-10,12). Given the biological integrity and functionality of BV-expressed neuraminidase, including its association with PPCA and β-galactosidase, this expression system can be of great value not only for biochemical analysis, but also for the crystallization and 3D-structure determination of this enzyme and ultimately of the multienzyme complex.

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

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

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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 ten novel mutations in the neuraminidase gene of 11 sialidosis patients with various degree 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; (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 neuraminidases are found in mammals that 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 catalytically active only 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.

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, including coarse facies and dysostosis multiplex. On the basis of the age of 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 (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 galactosialidosis (GS), which presents with clinical signs strikingly similar to those of sialidosis (reviewed in: (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 less that 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 of 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. However, juvenile /adult GS in contrast, juvenile/adult GS patients have dysmorphic features but milder neurological involvement (reviewed in: (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 that 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 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 11 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, 5, 6, and 7); the type II juvenile phenotype, in one (patient 3); the type II infantile form in three (patients 8, 9, and 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); 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 7, 5 and 6) had slowly progressing disease. The two siblings, 6 and 5, presented with identical symptoms, but only for patient 5 had the disease affected the eyes (Table 1). Despite the relatively mild clinical manifestations, serious central nervous (CNS) disorders, such as ataxia and epilepsy, developed in these patients, whereas patients with 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.

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 (Figure 1, upper panel) (12, 14). To compare the quantity and quality of the different RNA samples, the Northern blot was also hybridized with a PPCA cDNA probe (Figure 1, lower panel). The presence of the 1.9-kb neuraminidase and 2.0-kb PPCA mRNAs was detected in all the patient samples.

Western blots of total homogenates of the cultured fibroblasts were analyzed with affinity purified anti-neur antibodies (12). The size of neuraminidase detected in the patients' fibroblasts (46 kD) was the same as that in wild-type fibroblasts; however, the amount of neuraminidase in the

Table 1. Clinical features of patients with sialidosis

Initials (sex)	1 (female)	2 (male)	3 (female)	4 (female)	5 and 6[12] (female/siblings)	7 (female)	8 (male)	8 (female)[30]	10 (female)[28, 29]	11 (female)
Clinical type	I	I	II juvenile	I	I	I	II infantile	II infantile	II infantile	II congenital/ hydropic
Neur act. (nmol/h/mg)	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 & falling	Coarse facies/ hepatosplenomegaly	Strabismaus, nystagmus	Coarse facies/ hepatosplenomegaly	Hydrops fetalis, joint contractures
Growth	Normal	Normal	Normal	Normal	Normal	Normal		Short stature	Normal	Weight/length under the 3rd percentile
Skeleton	Normal	Normal	Dysostosis multiplex: skull, vertrebal 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	Hepatosplenomegaly		Hepatosplenomegaly	Hepatosplenomegaly
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 abnorm., 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, occular lens opacities, impaired vision	Cherry-red spots (5) and normal (6)	Bilateral cateracts, 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 & 32 years; slowly progressing	Slowly progressing; dead at age 44	Progressing at 4 months	wheel chair at 9 years,dead at 30 years	Progressing at 24 months	exitus at 18 months

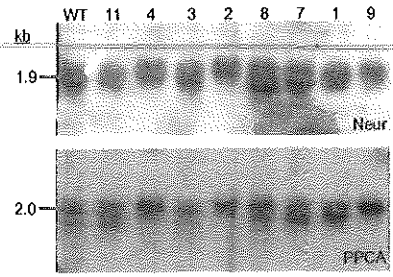
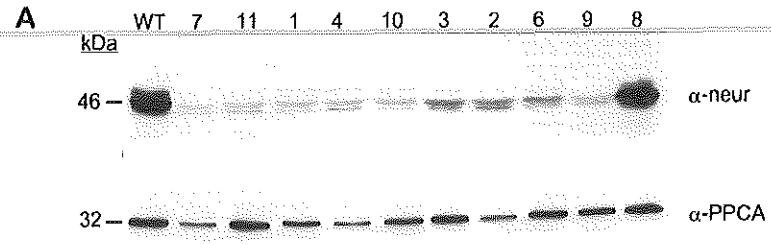


Figure 1. Neuraminidase mRNA expression. Radiolabeled full-length neuraminidase cDNA (upper panel) and PPCA cDNA (lower panel) were used to probe a Northern blot containing RNA (10 µg) isolated from the cultured fibroblasts of a healthy person (WT), four patients with type I sialidosis (patients 1, 2,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

Table 2. Neuraminidase mutations in sialidosis patients

Patient	Clinical phenotype	Nucleotide mutation	Exon	Amino acid change
1	I	C878>T	5	Arg294Ser
		T690>A	4	Leu231His
2	I	C878>T	5	Arg294Ser
		G654>C	4	Gly218Ala
3	II juvenile	G677>A [27]	4	Gly227Arg [27]
		G677>A	4	Gly227Arg
4	I	G677>A	4	Gly227Arg
		G677>A	4	Gly227Arg
5 and 6	I	G159>A	2	Val54Met
		G1127>T [12]	6	Gly378stop [12]
7	I	G980>A [27]	5	Gly328Ser [27]
		dupI1196 ACCACT	6	dupI399HisTyr
8	II infantile	A1107>G	6	Tyr370Cys
		A1107>G	6	Tyr370Cys
9	II infantile	T777>A [14]	4	Phe260Tyr [14]
		T777>A	4	Phe260Tyr
10	II infantile	T777>A	4	Phe260Tyr
		T1086>C [14]	6	Leu363Pro [14]
11	II congenital/ hydropic	C836>T[a]	5	Arg280stop
		C1002>A	5	Pro335Gln

[a] mutation only detected in genomic DNA



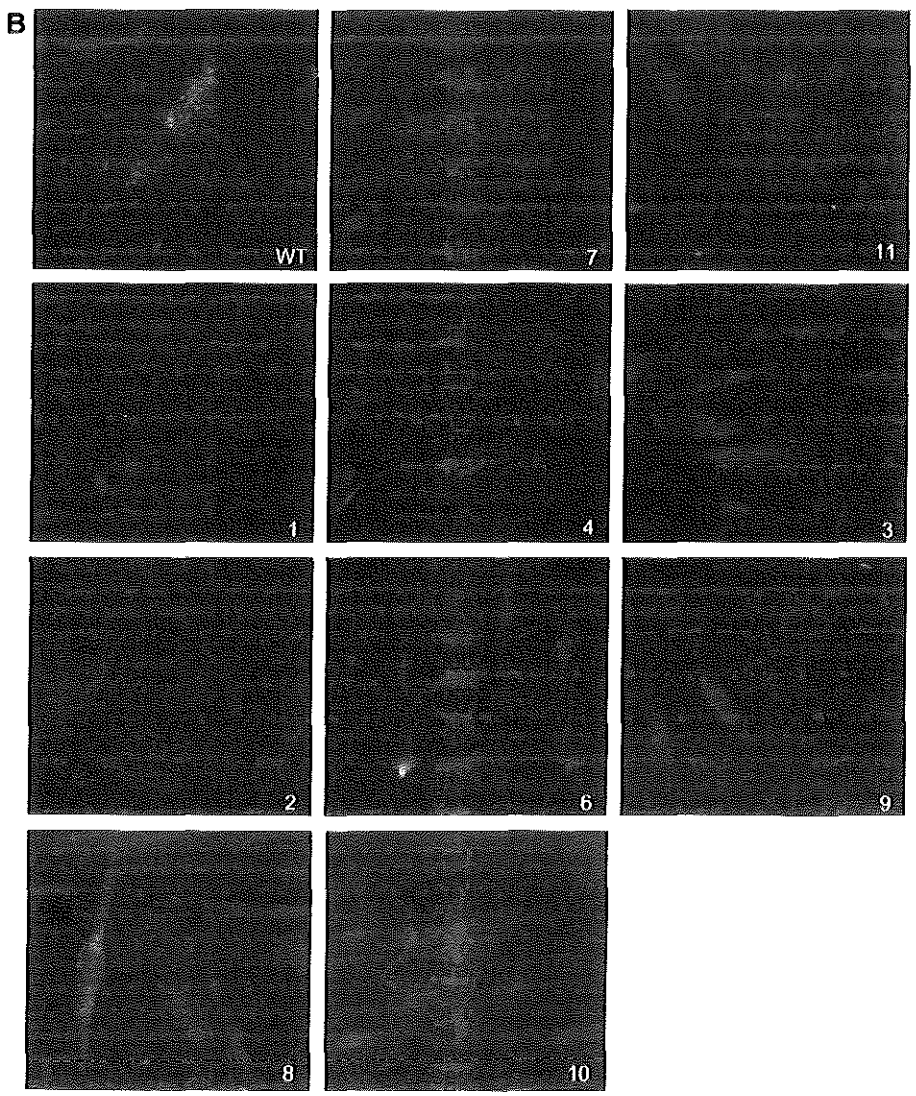


Figure 2. Detection of neuraminidase in fibroblasts of patients. (A) Affinity-purified anti-neur (upper panel) and anti-PPCA antibodies (lower panel) were used to analyze Western blots containing protein (5μg) from fibroblast lysates of a healthy person (WT), five patients with type I sialidosis (patients 1, 2, 4, 6, and 7) and five patients with type II sialidosis (patients 3, 8, 9, 10, and 11) (B) Immunocytochemical localization of neuraminidase in fibroblasts from a healthy person (WT) and from ten patients as indicated. Affinity-purified anti-neur antibodies and FITC-conjugated secondary antibodies were used. The nuclei were stained with DAPI. The magnification is 400x.

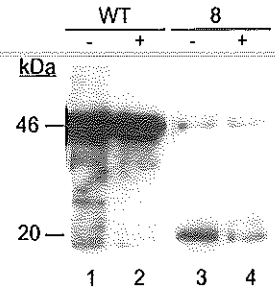


Figure 3. *In vitro* stability of neuraminidase from 8 fibroblasts of patient 8. Fibroblast lysates (5 µg of protein) of a healthy person (WT) and of patient 8 were incubated for 15 min at 37 °C in the absence (-) or presence (+) of protease inhibitors. After incubation, the samples were subjected to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

samples of all patients except one was markedly less than that in wild-type fibroblasts (Figure 2A). Only patient 8 had a normal amount of the 46-kD neuraminidase (Figure 2A; lane 11, upper panel). When the same blot was analyzed with an anti-PPCA antibody that recognizes the 32-kD subunit of the enzyme, similar quantities of fully processed PPCA were detected in wild-type fibroblasts and in fibroblasts of all patients (Figure 2A; lower panel).

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 patients' fibroblasts. The wild-type fibroblasts displayed a punctated staining-pattern that is characteristic of lysosomes (Figure 2B; WT). In contrast, this punctated staining was not detected in any of the patients, with the exception of 8 (Figure 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 (Figure 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 punctated immunostaining in fibroblasts raised the possibility that patient 8 were affected by another lysosomal disorder. We excluded GS since the patients' fibroblasts had normal levels of cathepsin A and β-galactosidase activities, and showed a normal punctated lysosomal localization of PPCA (data not shown). Moreover, we have shown earlier that in 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 the patient's fibroblasts and those of wild-type fibroblasts at 37°C for 15 minutes, in the absence or presence of protease inhibitors to allow 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-kD wild-type neuraminidase was almost unaffected after the incubation at 37°C (Figure 3; lanes 1 and 2), and only minor degradation was observed in the sample without the protease inhibitors (Figure 3, lane 1). In contrast, only a small amount of the 46-kD neuraminidase was present in the samples of patient 8 (Figure 3; lanes 3 and 4). A degradation product of approximately 20 kD was detected in the presence and in the 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 (Figure 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 novel. A compendium of the mutant alleles from all patients is presented in Table 2. Most of the mutant alleles contained point mutations that 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 4 patients from 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.

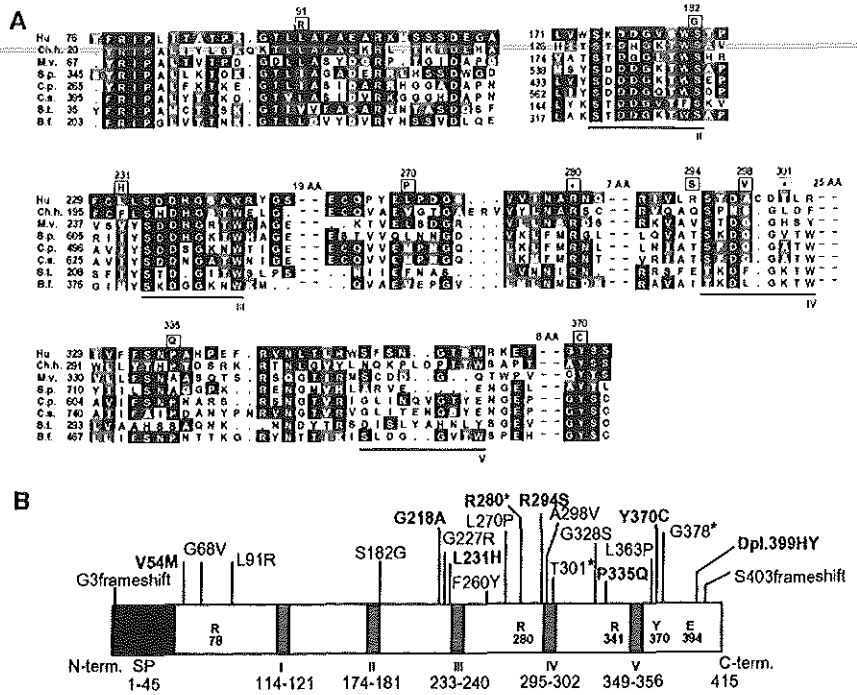


Figure 4. (A) Conserved amino acid residues within the sialidase superfamily that are mutated in sialidosis. Selected regions of human lysosomal neuraminidase (Hu.Lys) are compared with those of other mammalian and bacterial members of the sialidase superfamily: Chinese hamster cytosolic (Ch.h. Cyt.), *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 through V, and amino acid positions are indicated. The five strictly conserved active site residues are indicated in bold print. The 8 novel mutations are indicated in bold print. The signal peptide (SP) is indicated by a black box.

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 (Figure 4A). In particular, Tyr370, which is replaced by a Cys in patient 8, is fully conserved in all sialidases. It is noteworthy that the three-dimensional 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 (Figure 4A: Ser182Gly, Leu231His, Ala298Val, 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 (Figure 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 (Figure 4A and B). 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

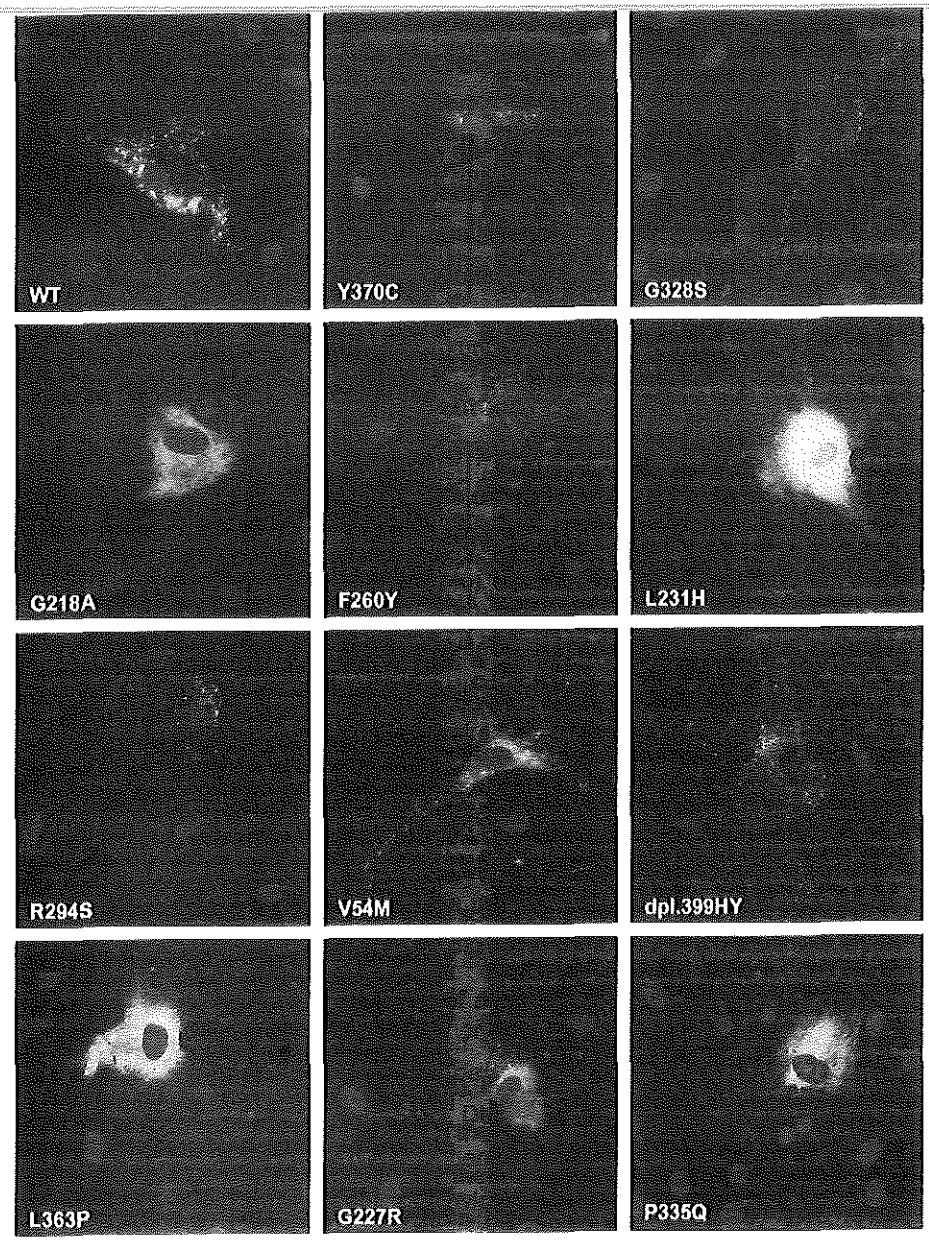


Figure 5. Immunocytochemical localization of neuraminidase overexpressed in sialidosis fibroblasts. Sialidosis fibroblasts were either transfected with the wild-type PPCA and neuraminidase cDNA expression constructs (WT) or with the wild-type PPCA and mutant neuraminidase cDNA expression plasmids as 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 400x.

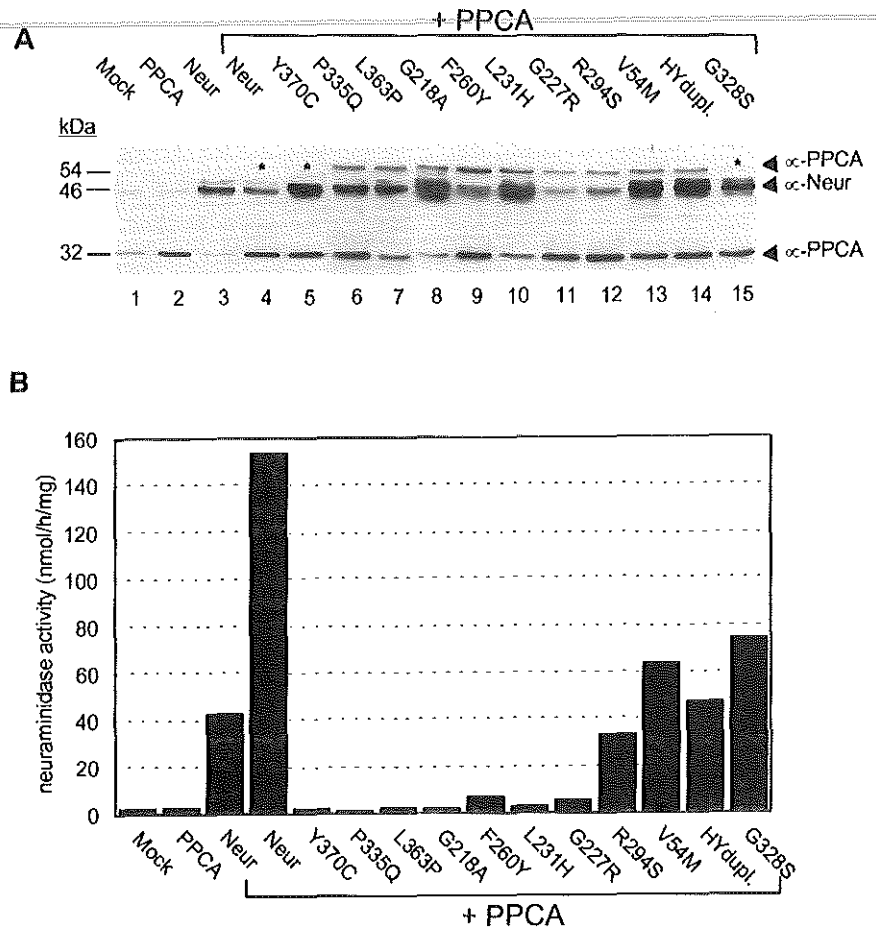


Figure 6. Western blot and catalytic analysis of mutant neuraminidase overexpressed in sialidosis fibroblasts. Sialidosis fibroblasts were transfected with wild-type PPCA and neuraminidase cDNA expression constructs or with wild-type PPCA and mutant neuraminidase cDNA expression plasmids. (A) Western 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-kD 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).

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 less than 1% residual neuraminidase activity and absence of immunofluorescent staining with anti-neur antibodies (12). An expression construct containing the Lac Z gene was used as an internal control for transfection efficiency (Material and Methods). Three days after electroporation, the cells were subjected to immunocytochemical analysis using affinity-purified anti-neur antibodies (12). No punctated staining was observed in four sets of cells expressing mutant proteins (Figure 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 an ER and Golgi staining (not shown), also some punctated staining (Figure 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 (Figure 6A). The blots were subsequently analyzed with anti-PPCA antibodies; all samples contained uniform amounts of the 32-kD PPCA subunit (Figure 6A). However, it was surprising that in all mutants, with the exception of Gly328Ser and Tyr370Cys, we could still detect the 54-kD PPCA precursor (Figure 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 (Figure 6A; lanes 2 and 4). The fact that PPCA precursor was still detected when coexpressed with neuraminidase mutants (Figure 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 (figure 6B). This result was expected for Leu363Pro, Pro335Gln, Gly218Ala, and Leu231His mutants because they did not localize to lysosomes. However, despite the punctated subcellular distribution of Tyr370Cys, Phe260Tyr, and Gly227Arg (Figure 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, dpl.399HisTyr, and Gly328Ser) exhibited substantial residual neuraminidase activity (Figure 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 11 patients with sialidosis (nine unrelated patients and two siblings). The phenotypes of these patients represent the complete spectrum of clinical severity. This number of cases is significant, given that the frequency of diagnosed sialidosis in the population (1 in approximately 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. In the first group, the mutant enzymes are catalytically inactive and do not localize to lysosomes, in the second group, the variants reach the lysosomes but are catalytically inactive, and in the third group 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 one 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 8–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 characterized amino acid substitutions 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 intra-lysosomal 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.

Material and Methods

Cell culture

Human skin fibroblasts from a healthy person and from patients 9 and 7 were obtained from the European Cell Bank, Rotterdam (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. The other fibroblasts strains were isolated in the laboratories of the clinicians who diagnosed the cases (3, 4, 6, and 11). The primary fibroblasts were maintained in DME medium, supplemented with antibiotics and 10% FBS.

Northern blot analysis

Total RNA and polyA$^+$ RNA were isolated from the cultured fibroblasts by using Oligotex and RNAeasy mRNA purification kits (Qiagen). RNA was separated in a 1% agarose gel containing 0.66M formaldehyde. After electrophoresis, the RNA was blotted onto a Zeta-Probe-GT membrane (Bio Rad) and hybridized in ExpressHyb (Clontech) at 68°C with the full-length human neuraminidase cDNA (12) or human PPCA cDNA (40). The blots were washed according to manufacturers' 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). 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 (5μg) were incubated at 37°C in PBS in the presence or absence of a protease inhibitor cocktail ('Complete protease inhibitors', Boehringer Mannheim). The samples were subjected to SDS-PAGE and to Western blotting. Western blots were incubated for 16h with affinity-purified anti-neuraminidase (anti-neur) antibodies (12) and with an affinity-purified anti-PPCA antibody that is specific for the 32-kD 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) and the bound antibodies were detected by using chemiluminescent substrate (Renaissance, DuPont NEN).

For immunocytochemical analysis, fibroblasts were seeded onto Superfrost/Plus glass slides (Fisher) and processed as described by Cullen (42) using anti-neur antibodies (16h at room-

temperature) and FITC-conjugated secondary antibodies (Sigma). The nuclei were stained with 4',6-Diamidino-2-phenylindole, dihydrochloride (DAPI; Sigma-Aldrich), which was added to Vectashield mounting medium (Vector labs).

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 Chemical Co).

PCR analysis

The amplification of mutant neuraminidase cDNAs was performed with PCR primers as previously described (12). Four overlapping cDNA fragments (approximately 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 (*BsmI*, *PstI* and *SmaI*). 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 4-MU 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%.

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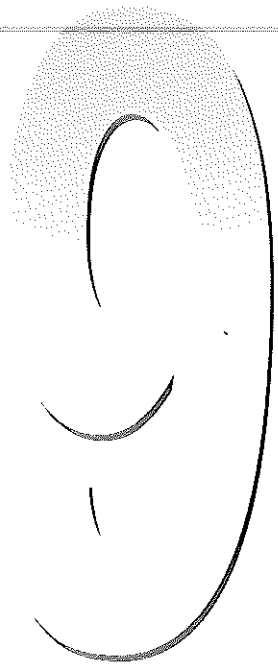
Discussion



Discussion

The results presented in chapters 3-8 demonstrate that human lysosomal neuraminidase is encoded by a gene that is localized on chromosome 6p21 within the HLA locus (Chapter 3). Mouse lysosomal neuraminidase was ~90% identical to the human enzyme and its gene, *neu-1*, was mapped to the H-2 locus on chromosome 17, which is syntenic to the human HLA locus (Chapter 4). Both human and mouse proteins were synthesized as ~46-kDa Asn-glycosylated, catalytically inactive polypeptides that needed to associate with the PPCA to be transported to the lysosomal compartment (Chapters 3-5).

Structural lesions in the neuraminidase gene were responsible for both the lysosomal storage disorder sialidosis and the neuraminidase deficiency in the SM/J mouse strain (Chapters 3, 4 and 8). We established reliable phenotype-genotype correlations in a large number of sialidosis patients (Chapter 8). In addition, we identified an active site mutation in the neuraminidase of a severe type II sialidosis patient. The catalytically inactive mutant protein was present in the patients' fibroblasts in normal amounts and correctly localized in lysosomes (Chapter 8). In contrast, 'mild' type I sialidosis patients apparently had small quantities of catalytically active mutant neuraminidase in their lysosomes (Chapter 8). Thus, the amount of residual catalytically active neuraminidase in the lysosomes is the major factor that determines the severity of the disease. The impact that a mutation has on the folding of the protein will ultimately determine whether it will escape the 'ER quality control', which retains improperly or parially folded proteins (reviewed in: (1)). These polypeptides are usually targeted for degradation by either the proteasome or aggresome pathways (2). By preventing the release of misfolded proteins, the cell diminishes the risks posed by defective protein products. However, mutant proteins that do manage to exit the ER may still be able to reach their final destination and excert partial function.

Not only mutant proteins are subjected to the 'quality control' in the ER. Bip, an ER 'quality control' chaperone, associates with Immunoglobulin heavy-chain molecules and prevents their secretion in the absence of appropriate light-chain molecules (3). This mechanism is reminiscent of the interaction between PPCA and neuraminidase in the ER. Neuraminidase was retained in the ER in the absence of PPCA and formed aggregates (crystals) in over-expressing COS-1 cells (front cover, bottom-right). In turn, this could trigger the activation of the aggresome pathway for the degradation of the misfolded protein (2). The ER-retention of neuraminidase in the absence of PPCA was not complete, since it was secreted in part and also reached the endosomal compartment (Chapter 5).

The bleeding disorder von Willebrand's disease resembles biochemically the combined PPCA/neuraminidase deficiency in galactosialidosis. In plasma, factor VIII (FVIII) was associated and stabilized by non-covalent interactions with Von Willebrand Factor (vWF), which also regulated FVIII activity. Von Willebrand's disease patients' have a combined deficiency of vWF and FVIII, which resulted from a primary defect in the vWF gene. In the absence of vWF, FVIII was retained in the ER and degraded (Kaufman, ASHG annual meeting 1999; (1)). Thus, 'quality control' for protein-partners may be a common mechanism to prevent proteins, which are dependent on a partner for their function, to leave the ER.

Evolution of neuraminidases

The absence of a functional mannose-6-phosphate recognition marker on lysosomal neuraminidase could mean that this enzyme was originally not a lysosomal protein or that it has lost its ability to independently reach the lysosomes. We can speculate that the neuraminidase, like most bacterial sialidases, was originally a secretory protein. Indeed, when the enzyme was over-expressed in COS-1 cells, in the absence of PPCA more neuraminidase was secreted. However, unlike most other secreted lysosomal hydrolases, the secreted neuraminidase could not be re-internalized by the cells. The presence of lysosomal neuraminidase in a multi-enzyme complex and its dependence on PPCA has been conserved in vertebrate species (mammalian and avian) (4). However, neuraminidases appear to be absent in plants and yeast, while the PPCA has two homologs in yeast, the KEX-1 gene product and yeast carboxypeptidase Y (CPY) (5). The analysis of DNA sequences that have been deposited in the DNA-databases reveals that *C. elegans* and isome insect species do have PPCA homologs (E Bonten, unpublished database searches). However, neuraminidases were not found in these species. Thus, it is possible that higher eucaryots have acquired lysosomal neuraminidase, whereas PPCA has evolved from a carboxypeptidase to become a transporter/chaperone/activator of lysosomal neuraminidase and β-galactosidase.

The widespread distribution of neuraminidases in higher organisms, as well as in diverse micro-organisms, but the absence in plants, insects, and yeast, has led to speculation about the existence of a possible common neuraminidase ancestor (6-9). Initially a short sequence of 8 amino acids, 5 of which highly conserved (Ser/Thr-X-Asp-X-Gly-X-Thr-Trp/Phe), was found repeated at 4 positions in several bacterial neuraminidases (6). This domain was called 'Asp-box', since the aspartic acid residue appeared to be the most conserved amino acid (7). Asp-box motifs were eventually also found in neuraminidases and trans-sialidases from non-bacterial species, including *Trypanosoma* and Macrobdella decora (leech), as well as in mammalian species (7, 8, 10)(this thesis). It is

now evident that Asp-box motifs, in combination with a short N-terminal domain (F/Y)RIP, are present in all neuraminidases, with the exception of most of the viral enzymes (7, 8). The arginine in the (F/Y)RIP domain is one of the five strictly conserved catalytic site residues (7, 11-15). Neuraminidases have 2 to 5 Asp-boxes and the distance between the (F/Y)RIP domain and the first Asp-box (35-41 residues) and the spacing between the other Asp boxes are highly conserved (Figure 4).

Some neuraminidases, belonging to the group of high molecular weight enzymes, have additional domains positioned either at the N-terminus (*B. fragilis*), the C-terminus (*M. viridivaciens*) or in the middle of the protein (*V. Cholerae*). These domains, which lack Asp-box motifs (Figure 4), are not essential for the catalytic activity and may reflect additional function(s) of these neuraminidases. Besides the Asp-boxes and the (F/Y)RIP domain, nine other amino acids are strictly conserved among the neuraminidases (Figure 4), six of which are also conserved in the viral enzymes (7).

The function of the Asp-boxes has been much debated in the literature, since the absence of these motifs in most viral neuraminidases indicates that they are not involved in the catalytic mechanism of the enzyme. However, site specific mutations within the Asp-boxes of *Clostridium perfringens* neuraminidase showed changes in K_m, V_{max} and K_I values, which suggest that these residues could be involved in substrate binding (11). Interestingly, Asp-boxes were not exclusively found in neuraminidases, but were also identified in proteins with other functions, including *Xanthus* hemagglutinin (4 Asp-boxes), *S. cerevisiae* invertase (3 Asp-boxes), *B. subtilis* levenase (2 Asp-boxes), *E. coli* penicillin G acylase (1 Asp-box) and spinach oxidoreductase (2 Asp-boxes) (9). The occurrence of these sequences in proteins that are probably unrelated to neuraminidases suggests a more common function for these motifs. The only property that these proteins share is the ability to bind carbohydrates (9). This supports the hypothesis that Asp-boxes are involved in the recognition and binding of carbohydrates/substrates, rather then in the catalytic mechanism. The broad range of sialic acid containing substrates and the diverse substrate specificity of neuraminidases, could explain the differences in the number of Asp-boxes and the differences in their amino acid composition (Figures 4 and 5).

This brings us to the interesting topic of the evolution of neuraminidases. Did the neuraminidases evolve from a common ancestor, and if so, was this common ancestor a protein equiped with Asp-box motifs? Did all Asp-box containing proteins evolve from a common ancestor, or did the neuraminidases and other proteins independently acquire Asp-box motifs? The existence of these motifs in plant and yeast proteins, which do not synthesize sialic acids and do not have neuraminidases, implies that Asp-box motifs are evolutionary older than neuraminidases. However, several lines of evidence suggest that

the neuraminidases did evolve from a common Asp-box containing neuraminidase ancestor and that the neuraminidase superfamily has been spread by horizontal gene transfer. It has been speculated that bacteria, as well as other microorganisms, have acquired the neuraminidase gene from their hosts and that the neuraminidases have originated within higher organisms (7).

Could a mammalian neuraminidase be the common ancestor to microbial neuraminidases? We found that lysosomal neuraminidase is more homologous to bacterial neuraminidases than to the mammalian plasma membrane and cytosolic enzymes (this thesis). In contrast, the cytosolic and plasma membrane neuraminidases are homologous to each other (~40%), more than to lysosomal and bacterial neuraminidases (~19%). Several amino acids in the potential trans-membrane domain of the plasma membrane enzyme are conserved within the cytosolic enzyme. Moreover, both enzymes lack an N-terminal signal sequence (10, 16, 17). Thus, the plasma membrane neuraminidase is in essence a cytosolic protein that may have acquired the capacity to become associated with the plasma membrane.

Overall, these data suggest that the genes encoding cytosolic and plasma membrane neuraminidase are a result of a 'recent' gene-duplication event and that lysosomal neuraminidase is a more distant cousin of these two enzymes. In conclusion, the common neuraminidase ancestor probably was a eucaryotic enzyme equiped with Asp-boxes. The exchange of genomic material between microbial species and their hosts is an essential element that aids their evolution and adaptation to changing environments. Thus, it is likely that several independent events have been responsible for the transfer of eucaryotic neuraminidase genes to microbial species.

Tertiary structure of neuraminidase

The first published crystal structure of a neuraminidase, that of the influenza virus enzyme, revealed a tetrameric association of identical monomers, whose fold was described as a superbarrel or β-propeller (18). Each monomer consists of six four-stranded antiparallel β-sheets, which are arranged as the blades of a propeller around a pseudo six-fold axis (Figure 6A). Structural analyses of the neuraminidases from influenza A virus N2, N6, N8, and N9 subtypes, as well as from two influenza B viruses, have revealed a conserved structure, despite overall low sequence identities (40%). The active site is highly conserved and consists of a rigid catalytic center (19). Several 3D-structures of bacterial neuraminidases have also been solved. The neuraminidases from *M. viridifaciens* (Figure

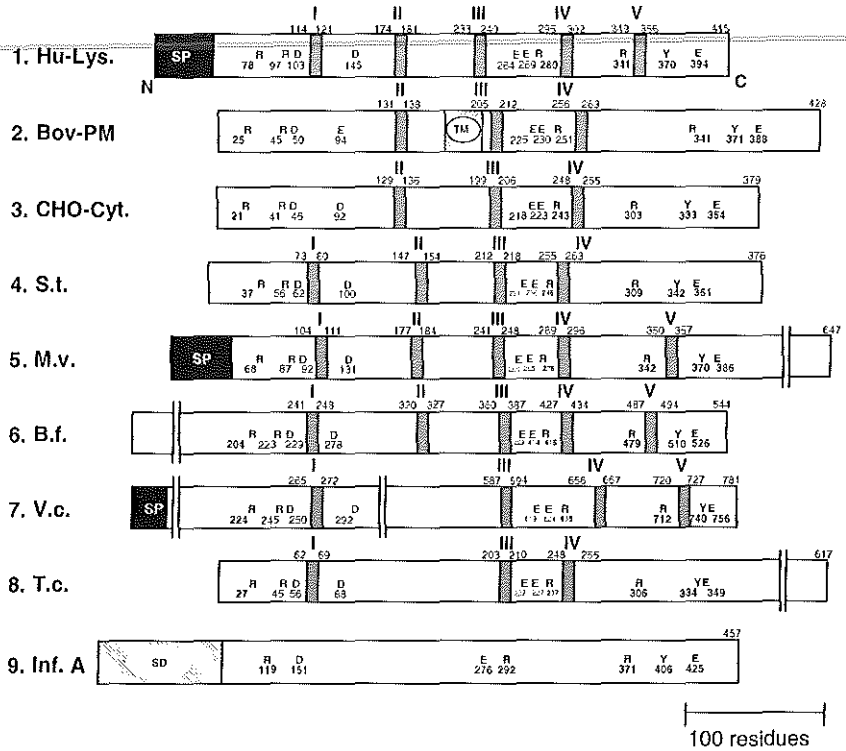


Figure 4. Comparison of the primary structures of neuraminidases: (1) Human lysosomal, (2) Bovine plasma-membrane, (3) Chinese hamster cytosolic, (4) *Salmonella typhimurium LT2*, (5) *Micromonospora viridifaciens*, (6) *Bacteroides fragilis*, (7) *Vibrio cholerae*, (8) *Trypanosoma cruzi* TCNA, (9) Avian influenza A tern N9. Conserved Asp-box motifs are numbered I to V, indicated by their amino acid position, and a gray box. Conserved residues and their positions are indicated in the protein-bars. The five active site residues that are conserved in all neuraminidases are printed in bold. Amino-terminal signal-peptides (SP); trans-membrane domain (TM); stalk-domain (SD).

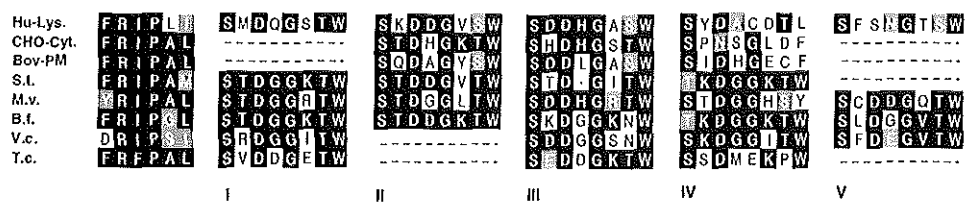


Figure 5. Alignment of the FRIP domains and Asp-box motifs I to V of selected neuraminidases: Human lysosomal (Hu-Lys.), Chinese hamster cytosolic (CHO-Cyt.), Bovine plasma-membrane (Bov-PM), *Salmonella typhimurium LT2* (S.t.), *Micromonospora viridifaciens* (M.v.), *Bacteroides fragilis* (B.f.), *Vibrio cholerae* (V.c), *Trypanosoma cruzi* (T.c).

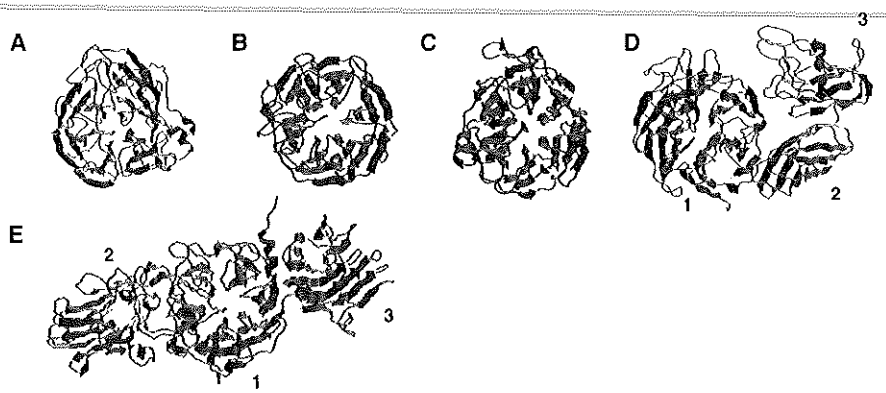


Figure 6. Crystal 3-D structures of neuraminidases: Avian influenza A virus Tern N9 (A), *Micromonospora viridifaciens* small form (B), *Salmonella typhimurium LT2* (C), *Micromonospora viridifaciens* large form (D), *Vibrio Cholerea* (E). Catalytic domains of D. and E. are indicated by number 1, and non-catalytic domains by numbers 2 and 3. Images are created with the program RasWin molecular graphics (RasMol) Version 2.6, copyright © 1993-1995 R. Savle.

6B and D), *S. typhimurium* (Figure 6C), and *V. cholerae* (Figure 6E) showed a high degree of structural similarity to the viral neuraminidase structures, with conserved six-bladed β-propeller (12-15). However, the neuraminidases from *M. viridifaciens* and *V. cholerae* have, besides the catalytic domain, two additional separately folded domains (Figure 6D and E). These domains either have lectin-like or immunoglobulin-like folding characteristics. Their functions are yet unclear, but they are not essential for the catalytic activity. In common with the viral neuraminidases, the active site of the bacterial enzyme also consists of an arginine triad, a hydrophobic pocket and a key-tyrosine and glutamic acid (12). The tyrosine at amino acid position 370 in human lysosomal neuraminidase corresponds to the key-tyrosine in the viral and bacterial neuraminidases (Chapter 8). The substitution of this residue for a cysteine, as found in the neuraminidase from a severe type II sialidosis patient, leads to the complete loss of catalytic function. The Asp-boxes are located at equivalent positions in the fold of the protein, far from the active site, with the aspartic acid residues exposed to the solvent. Their peripheral position confirms that they are not involved in the catalytic mechanism, but they may determine the fold and the integrity of the propeller (12). It is noteworthy that several mutations map near or at the Asp-box motif's of lysosomal neuraminidase in sialidosis patients (Chapter 8). The impact of these mutations on the structure of the enzyme is yet unknown. However, they most likely do not directly affect the catalytic machinery, but instead may cause misfolding or interfere with substrate recognition.

The ability to produce large quantities of lysosomal neuraminidase in insect cells (Chapter 7) could potentially be used to crystallize the enzyme and determine its tertiary structure. It will be interesting to compare its structure with those of the bacterial and viral neuraminidases. These studies may lead to the identification of structural elements that are unique for lysosomal neuraminidase, and that potentially are involved in the interaction with PPCA and β-galactosidase.

The work presented in this thesis has contributed to a better understanding of the properties of lysosomal neuraminidase, including its interactions with PPCA and β-galactosidase for the transport to lysosomes and its mechanism of catalytic activation. We also gained insight into the role that neuraminidase mutants play in the lysosomal storage disorder sialidosis. The generation of murine models for both type I and type II sialidosis will be essential to obtain a further understanding of the clinical impact of a lysosomal neuraminidase deficiency.

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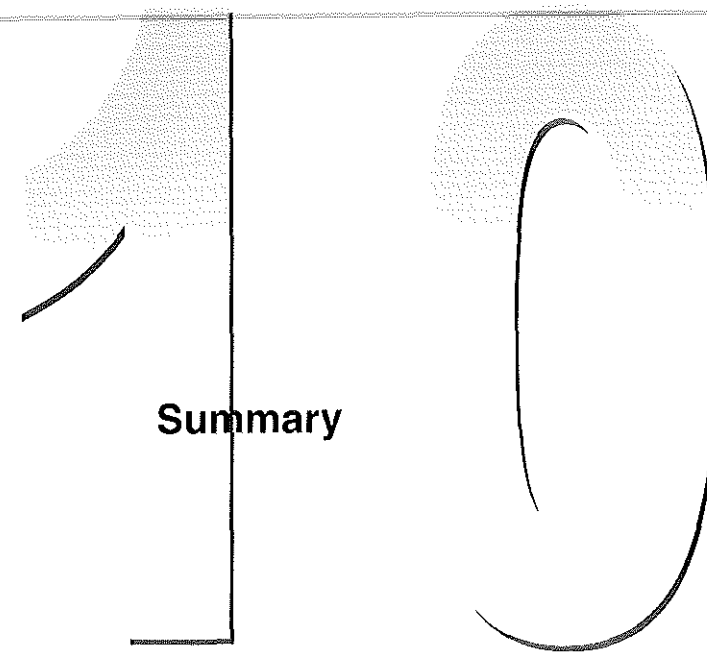

Summary

Summary

Neuraminidase is the first hydrolase that is needed for the step-wise degradation of oligosaccharides, on which its substrate, sialic acid, is terminally positioned. The lysosomal storage disorder (LSD) sialidosis is caused by lesions in the neuraminidase gene. Patients that are affected by this disease present, depending on the severity, with multi-systemic manifestations, which may include retinal cherry-red spots (loss of vision), deafness, coarse facies, skeletal abnormalities, organomegaly, myoclonus, seizures and mental retardation. Many of these symptoms are also commonly seen in other lysosomal storage disorders. A primary genetic deficiency of lysosomal protective protein/cathepsin A (PPCA) results in a secondary deficiency of neuraminidase and β-galactosidase, which in turn leads to galactosialidosis (GS). GS patients share many clinical features with sialidosis patients, likely as a result of the secondary neuraminidase deficiency.

A cDNA encoding for a neuraminidase was identified in the dBEST database because of its homology to other bacterial and mammalian sialidases (Chapter 3). Electroporation of this cDNA into sialidosis fibroblasts restores the neuraminidase activity and gives a punctated lysosomal-like intracellular distribution. In contrast, the expression of this cDNA in GS fibroblasts does not restore neuraminidase activity. The co-expression of PPCA with neuraminidase is needed to generate catalytic activity in these cells. These experiments show that the dBEST cDNA clone is encoding lysosomal neuraminidase. Furthermore, we identified three mutations in the neuraminidase cDNA of sialidosis patients. Overexpression of these mutations result in a complete abolishment of both the catalytic activity and lysosomal localization of the mutant enzymes (Chapter 3).

In Chapter 4 we present the cloning of the cDNA and gene encoding murine lysosomal neuraminidase, and the identification of a point mutation (Leu209Ile) in the neuraminidase gene of the mouse strain SM/J, that causes a partial neuraminidase deficiency in these mice. We show that the mutation interferes with the efficiency of the enzyme to cleave natural substrates, but it has no effect on the substrate specificity.

In Chapter 5 we describe in detail the interaction between neuraminidase and PPCA, co-expression them in COS-1 cells. We show that association of neuraminidase with PPCA in an early biosynthetic compartment is essential for the routing of neuraminidase to lysosomes, and that the enzyme lacks a functional mannose-6-phosphate recognition marker that is required for routing to the lysosomes. When neuraminidase is expressed in COS-1 cells in the absence of PPCA, it accumulates in the ER, while co-expression with PPCA restores the lysosomal localization. In contrast, when neuraminidase is co-expressed

with transport-incompetent PPCA mutants, it is unable to reach the lysosomal compartment.

In chapters 6 and 7 we utilize the baculovirus expression system to express the PPCA precursor, the two separate PPCA subunits, the β-galactosidase precursor, and lysosomal neuraminidase. The PPCA precursor is catalytically activated *in vitro* by the proteolytic removal of an internal linker-peptide. The activated mature PPCA is a two-chain molecule that is non-covalently associated through disulfide bridges. When co-expressed, PPCA, neuraminidase and β-galactosidase are present as a multienzyme complex of >1000 kDa. PPCA associates with neuraminidase through the C-terminal 20-kDa PPCA subunit, promoting oligomerization and catalytic activation of neuraminidase, but only at acidic pH. Neuraminidase is also associated with β-galactosidase but, in contrast, this association has no effect on either the oligomeric state or catalytic activity of neuraminidase.

Finally, in Chapter 8 we identified several novel mutations in the neuraminidase cDNA and gene of eleven sialidosis patients, belonging to different clinical subtypes. The properties of the mutant proteins were analyzed by over-expression in deficient sialidosis fibroblasts. We studied the impact of the mutations on the subcellular localization and catalytic activity of neuraminidase. We are able to divide the mutant enzymes into three groups. The first group includes mutant proteins that lack residual activity and show no lysosomal localization. Mutants in the second group show lysosomal localization but no residual catalytic activity, while proteins in the third group show both lysosomal localization and significant residual catalytic activity. One of the identified mutations involves one of the five strictly conserved active site residues (Tyr370Cys). The patient that was homozygous for this mutation had a normal amount of correctly localized neuraminidase that however was completely inactive. As a result this individual is a severe type II sialidosis patient. We can establish reliable phenotype-genotype correlations for all sialidosis patients. Mutations belonging to the first group are present in severe type II sialidosis patients. In contrast, mutations from the third group are sufficient to give a mild type I phenotype. Thus, the phenotype correlates with the amount of residual neuraminidase activity and presence or absence of lysosomal localization.

The studies presented in this thesis have provided new understanding about the characteristics of lysosomal neuraminidase, including its interactions with PPCA and β-galactosidase, and its involvement in the lysosomal storage disorders sialidosis and galactosialidosis. The genotype-phenotype correlation in sialidosis patients may provide a better understanding about the clinical manifestations of these patients. The increased

knowledge about neuraminidase and its impairment in sialidosis patients may become valuable for the future development of therapeutic strategies for sialidosis.

Neuraminidase is het eerste enzym dat benodigd is voor de stapsgewijze afbraak van koolhydraatketens, waarop het substraat, siaalzuur, een terminale positie heeft. De lysosomale stapelingsziekte (LSZ) sialidosis wordt veroorzaakt door mutaties in het lysosomaal neuraminidase gen. Afhankelijk van de ernst van de ziekte lijden de patienten aan meervoudige gebreken waarbij ondere andere de ogen, het gehoor, het skelet, het centrale zenuwstelsel en de viscerale organen betrokken zijn. De LSZ galactosialidosis (GS) wordt veroorzaakt door een defect van het lysosomale protective protein/cathepsin A (PPCA). Als PPCA niet goed functioneert kunnen neuraminidase en β-galactosidase het lysosoom niet bereiken, omdat zij afhankelijk zijn van PPCA voor hun transport naar het lysosoom. Dit heeft tot gevolg dat het GS ziektebeeld veel lijkt op dat van sialidosis patiënten.

Op grond van de homologie met andere bacteriële neuraminidases werd het cDNA dat codeert voor het lysosomale neuraminidase geidentificeerd in de dBEST database (Hoofdstuk 3). De electroporatie van dit cDNA in de gekweekte fibroblasten van sialidosis patiënten herstelt de neuraminidase activiteit en vertoont een typische lysosomale lokalisatie van het eiwit. Dit is in tegenstelling tot de electroporatie in GS fibroblasten waarbij de activiteit van dit enzym niet hersteld wordt. Alleen wanneer ook PPCA wordt geëxpresseerd in deze cellen kan de neuraminidase activiteit hersteld worden. Deze experimenten tonen aan dat het geidentificeerde cDNA codeert voor het lysosomale neuraminidase. Vervolgens vonden we drie mutaties in de neuraminidase cDNAs van sialidosis patienten. Overexpressie van deze neuraminidase mutanten in zowel COS-1 en sialidosis fibroblasten veroorzaakt een volledig gebrek aan enzym activiteit en lysosomale lokalisatie (Hoofdstuk 3).

In Hoofdstuk 4 beschrijven we de klonering van het cDNA dat codeert voor het lysosomale neuraminidase van de muis en de identificatie van een mutatie (Leu209Ile) in het betreffende gen in de muizenstam SM/J. Deze aminozuur verandering veroorzaakt een verlaging van de enzymactiviteit. We tonen aan dat de mutatie de verwerking van natuurlijke siaalzuur substraten negatief beinvloedt maar dat het geen invloed heeft op de specificiteit van dit proces.

In Hoofdstuk 5 beschrijven we de interactie tussen neuraminidase and PPCA waarbij we gebruik maken van expressie in COS-1 cellen. We tonen aan dat deze interactie plaats vindt direct na de eiwitsynthese en dat dit benodigd is voor het transport van neuraminidase naar het lysosoom. De koolhydraatketens van neuraminidase hebben geen functionele mannose-6-fosfaat determinant dat benodigd is voor het transport naar het lysosoom,

waardoor overexpressie van neuraminidase in COS-1 cellen leidt tot accumulatie van het eiwit in het endoplasmatische reticulum (ER). De co-expressie met PPCA herstelt de lysosomale localisatie van neuraminidase. Echter, de co-expressie van neuraminidase met PPCA mutanten, die zelf niet tot correct transport in staat zijn, heeft geen effect op het lysosomale transport van dit enzym.

In de hoofdstukken 6 en 7 expresseren we neuraminidase, β-galactosidase en PPCA in insekten cellen middels baculovirus constructen. De PPCA precursor wordt *in vitro* geactiveerd door de enzymatische verwijdering van een intern peptide. Het enzymatisch actief PPCA bestaat uit twee eiwit ketens die niet-covalent verbonden zijn door disulfide bruggen. Door de gelijktijdige expressie van PPCA, neuraminidase en β-galactosidase wordt een multienzym complex gevormd (>1000 kDa). De PPCA precursor bindt neuraminidase via het C-terminale 20-kDa PPCA domein en bevordert de oligomerisatie en activatie van neuraminidase. Neuraminidase kan ook binden aan β-galactosidase maar dit heeft geen effect op de enzym activiteit en de oligomerische staat.

Tenslotte, in Hoofdstuk 8 beschrijven we de identificatie van nieuwe mutaties in neuraminidase van elf patiënten met de verscheiden klinische vormen van sialidosis. De biochemische eigenschappen van deze gemuteerde enzymen worden bepaald door de overexpressie in sialidosis fibroblasten waarbij vooral gekeken wordt naar het effect op de subcellulaire localisatie en de enzym activiteit. Op grond hiervan kunnen de mutaties ingedeelt worden in drie verschillende groepen. De eerste groep bevat eiwitten zonder activiteit en een totaal gebrek aan lysosomale localisatie. De tweede groep bestaat uit enzymen die wel in het lysosoom belanden maar geen activiteit hebben, terwijl de derde groep mild is met eiwitten die lysosomaal zijn en enigszins actief. Eén mutatie betrof één van de vijf geconserveerde aminozuren die het actieve centrum van neuraminidase vormen (Tyr370Cys). De betreffende patiënt is homozygoot voor deze mutatie en heeft een normale hoeveelheid enzym dat aanwezig is in het lysosoom, alhoewel zonder enzym activiteit. Als gevolg van deze mutatie heeft deze patiënt de ernstige vorm van het type II sialidosis. We kunnen voor alle patiënten betrouwbare genotype-phenotype correlaties aantonen. Mutaties behorende tot de eerste groep worden aangetroffen in type II sialidosis patiënten, terwijl mutaties uit de derde groep aanleiding geven tot het veel mildere type I ziektebeeld. Het ziektebeeld wordt bepaald door de aanwezigheid van een zeer geringe hoeveelheid neuraminidase activiteit in de lysosomen van de patiënten. Echter, het is bekend dat ook andere genetische en niet-genetische factoren de ziekte kunnen beinvloeden.

De in dit proefschrift beschreven studies geven een nieuw inzicht in de eigenschappen van lysosomaal neuraminidase, inclusief de interacties met PPCA en β-galactosidase en de betrokkenheid in de LSZn sialidosis en GS. De genotype-phenotype correlaties van sialidosis patienten zal een beter begrip geven van het ziektebeeld van deze patiënten. De verworven kennis over neuraminidase en de betrokkenheid in sialidosis kan tevens van groot belang zijn in de toekomstige ontwikkeling van therapieën voor deze patiënten.

Abbreviations

AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
Bip	immunoglobulin binding protein
(c)DNA	(complementary) deoxyribonucleic acid
CMP	cytidine 5'-monophosphate
CPW	wheat carboxypeptidase W
CPY	yeast carboxypetidase Y
C-terminal	carboxy-terminal
dBEST	database expressed sequence tags
ER	endoplasmic reticulum
FVIII	factor FVIII
GalNAc	N-acetyl-galactosamine
GALNS	N-acetyl-galactosamine-6-sulfate sulfatase
GlcNAc	N-acetyl-glucosamine
GS	galactosialidosis
kb	kilo base
kDa	kilo Dalton
LSD	lysosomal storage disease
Man	mannose
ManNAc	N-acetylmannosamine
MLIV	mucolipidosis IV
(m)RNA	(messenger) ribonucleic acid
MU	methylumbelliferyl
Neu5Ac	N-acetylneuraminic acid
N-linked	asparagine-linked
N-terminal	amino-terminal
PPCA	protective protein/cathepsin A
vWF	Von Willebrand factor

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Curriculum vitae

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