Protective protein/cathepsin A, neuraminidase and β-galactosidase: interacting enzymes involved in lysosomal disorders

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Protective protein/cathepsin A, neuraminidase en β-galactosidase: samenspelende enzymen betrokken bij lysosomale ziekten

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Contents

Scope of this thesis

Lysosomal storage diseases comprise a large group of genetic disorders of metabolism caused by an impaired lysosomal system, which ultimately leads to altered cellular homeostasis and organ dysfunction. Galactosialidosis, sialidosis and G_{M1}-gangliosidosis are three examples of this type of disorder that are often discussed together because they share clinical and biochemical characteristics. They are associated with either single or combined deficiency of three hydrolases, β-galactosidase, neuraminidase and protective protein/cathepsin A (PPCA). These enzymes form a high molecular weight lysosomal complex. To better understand the bases of the lysosomal disorders caused by the malfunctioning or absence of these enzymes, it is crucial to elucidate the molecular interplay of these lysosomal components. The objective of this thesis was to study the composition of the β-galactosidase/neuraminidase/PPCA complex, characterize normal and disease-associated forms of its constituent polypeptides and investigate their mode and site of association. Instrumental to the experimental work was the isolation and characterization of the cDNA coding for the human lysosomal neuraminidase. Through these studies we have now established that the intracellular routing, catalytic activation and stability of the three enzymes are regulated at least in part by their mutual interaction, and we discovered that PPCA may act as a transport protein.

Chapter 1

General introduction

General introduction

1.1 Lysosomes

Lysosomes are physically and biochemically distinct cytoplasmic organelles, delineated by a single phospholipid bilayer membrane (for reviews see Bainton, 1981; de Duve, 1983; Kornfeld and Mellman, 1989; Lloyd, 1996a; Storrie and Desjardins, 1996). These organelles are the major site of intracellular compartmentalized degradation: all but resident lysosomal components are rapidly degraded to small molecules. Lysosomes are acidic (pH 4-6), and contain over 70 different hydrolases, including glycosidases, lipases, nucleases, phosphatases, phospholipases, proteases and sulfatases. Most of these enzymes are soluble, while a few are membrane-associated. Other typical lysosomal membrane proteins are lysosome-associated membrane proteins, lysosomal integrated membrane proteins (Hunziker and Geuze, 1996), and various transmembrane proteins that are responsible for the transport of for small molecules, like amino acids, monosaccharides, ions and nucleosides (Chou *et al.*, 1992; Lloyd, 1996b). Lysosomes have an exceptionally high specific density, which allows their separation from other cytoplasmic organelles and they vary widely in shape and size. On average, a eukaryotic cell may contain as many as several hundred lysosomes.

The lysosomal compartment is a convergence point of various distinct intracellular trafficking pathways, all contributing lysosomal constituents. Solutes, receptor-bound ligands, and plasma membrane components are delivered to lysosomes via vesicle transport along the endocytotic pathway (Mellman, 1996), or across the lysosomal membrane (Lloyd, 1996b). A specific subset of cytosolic proteins is directly imported into lysosomes through a chaperone-mediated import system, which is activated under starvation conditions (Cuervo and Dice, 1998; Terlecky, 1994). Particles, aging cells, and infecting bacteria are brought to lysosomes through phagocytic processes, while intracellular organelles like mitochondria are subjected to lysosomal degradation after autophagy. Finally, newly synthesized proteins and lipids are compartmentalized into the acidic organelle via the biosynthetic pathway (Kornfeld and Mellman, 1989; Hunziker and Geuze, 1996).

Significantly, efflux out of lysosomes is strictly limited to the degradative end products and selected membrane components. Soluble low-molecular weight compounds exit mainly to the cytoplasm through the transmembrane transporters, while lipids and membrane-associated molecules traffic in membrane vesicles, to endosomes or the plasma membrane. Lysosomal membrane proteins have been shown to recycle between lysosomes, the cell surface and endosomes (Riederer

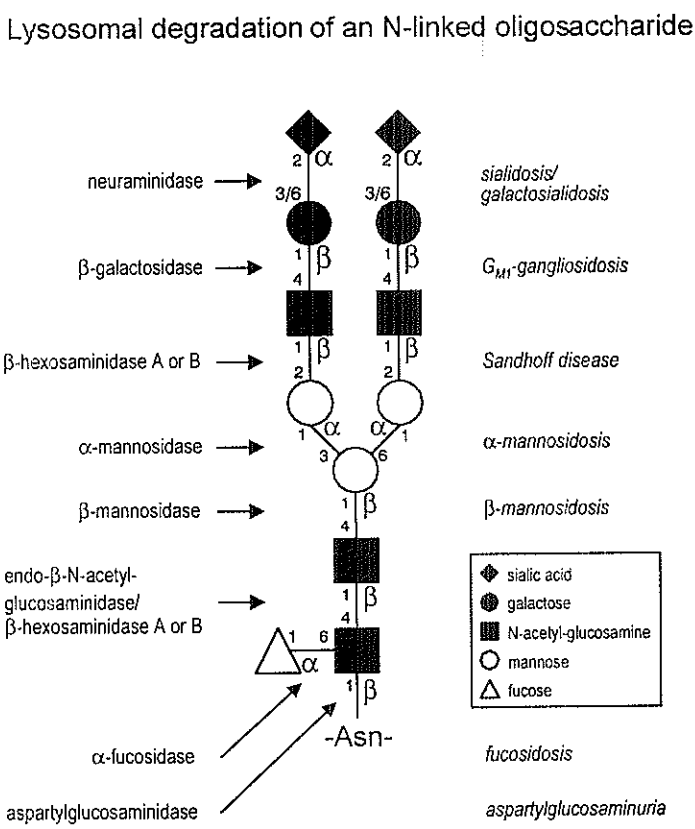

Fig. 1. Scheme of lysosomal degradation of a complex N-linked oligosaccharide of the biantennary type. At left are indicated the lysosomal hydrolases that cleave the specific linkages in the oligosaccharide structure. Deficiencies of these enzymes cause specific *lysosomal storage disorders*, which are indicated at right. Asn: asparagine. Adapted from (Thomas and Beaudet, 1995).

et al., 1994; Traub *et al.*, 1996 and references therein).

The capacity of lysosomes in a specific cell to catabolize different substrates is maintained by the continuous synthesis of the appropriate complement of enzymes. Resident lysosomal proteins are transported to the acidic organelle through a number of distinct trafficking processes (reviews: Kornfeld and Mellman, 1989; Sabatini and Adesnik, 1995). They are synthesized and co-translationally N-glycosylated in the endoplasmic reticulum (ER); in this compartment they also fold into their native tertiary structure, after which they are transported to the Golgi system. In the *cis*-Golgi cisternae the N-linked carbohydrate chains of many soluble lysosomal enzymes acquire a characteristic determinant, when several terminal mannose residues are modified into mannose-6-phosphate groups. After binding to mannose-6-phosphate receptors in the *cis*- or *trans*-Golgi cisternae, or in the *trans*-Golgi network, these enzymes are segregated from the bulk of secretory proteins and transported to endosomes and lysosomes (reviewed in Kornfeld and Mellman, 1989; Hille-Rehfeld, 1995). In certain cell types a mannose-6-phosphate-independent process also operates to correctly guide several soluble enzymes to lysosomes (see discussion section of Chapter 5; for review see Kornfeld and Sly, 1995). Transmembrane proteins are targeted to lysosomes through a small motif in their cytoplasmic tails (Hunziker and Geuze, 1996). Most lysosomal enzymes undergo intralysosomal proteolytic processing, which is, especially for many proteases, a prerequisite to become catalytically active (Hasilik, 1992).

In addition to its essential role in substrate catabolism, the lysosomal compartment has been implicated in other cellular processes; examples are the de-esterification of cholesterol-esters that are endocytosed in low-density lipoprotein particles, the loading of major histocompatibility complex class II molecules with antigenic peptides (Wolf and Ploegh, 1995), and the internalization of *Trypanosoma cruzi* (Tardieux *et al.*, 1992).

1.2 Lysosomal glycoconjugate catabolism

Oligo- and polysaccharides are two of the many types of macromolecules that are intralysosomally degraded. Among these compounds are polysaccharides like glycogen as well as the carbohydrate moieties of glycoconjugates, such as glycoproteins, proteoglycans and glycosphingolipids. N-linked oligosaccharides found on glycoproteins vary widely in structure, from the so-called 'high-mannose' form, having a $GlcNAc_2$-Man_6 composition (Kornfeld and Kornfeld, 1985), to complex forms, as depicted schematically in Fig. 1. Proteoglycans are highly glycosylated glycoproteins, containing up to 95% carbohydrate, which occur in the form of many glycosaminoglycan (GAG) chains: long unbranched polymers of a

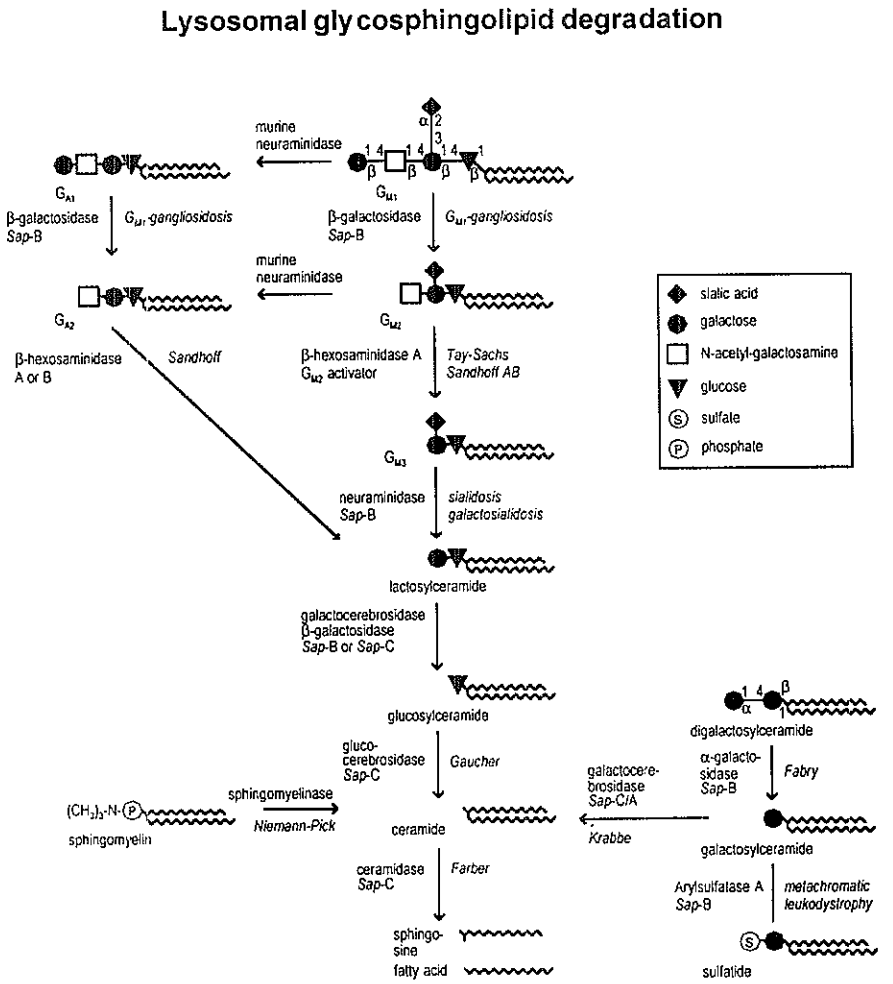

Fig. 2. Scheme of lysosomal glycosphingolipid degradation. The different catabolic steps are represented by arrows. The enzymes that catalyse these reactions are indicated above or to the left of the arrows, as well as the auxiliary proteins involved in some of the conversions; below or to the right of each arrow is indicated the storage disorder which ensues when the corresponding hydrolase is deficient. Adapted and compiled from (Sandhoff and van Echten, 1993; Klein *et al.*, 1994; Sandhoff *et al.*, 1995; Sango *et al.*, 1995; Phaneuf *et al.*, 1996; Sandhoff and Kolter, 1996; Burkhardt *et al.*, 1997; Chatelut *et al.*, 1997; Hahn *et al.*, 1997; Harzer *et al.*, 1997).

disaccharide unit of either a GlcNAc or a GalNAc residue which is often sulfated, and either a glucuronic, iduronic acid, or a galactose residue. GAGs are linked to specific serine residues and contain on average 80 monosaccharide residues. Depending on their composition, various types of GAGs have been described, including dermatan sulfate, heparan sulfate and keratan sulfate (Neufeld and Muenzer, 1995).

Glycosphingolipids (GSLs) are made up of the hydrophobic compound ceramide and an oligosaccharide, examples of which are shown in Fig. 2 (for GSL nomenclature see Svennerholm, 1994). The ceramide moieties of GSLs are embedded in cellular membranes, while their carbohydrate parts extend into the aqueous environment. These compounds are synthesized in the ER and the Golgi system, and are localized in the outer leaflet of the plasma membrane (reviewed in Sandhoff and van Echten, 1993; van Meer, 1998). Interestingly, GSLs are thought to accumulate together with cholesterol in distinct membrane domains, which associate with various proteins, and may function in biosynthetic and endocytotic traffic, and in transmembrane signalling (reviewed in Simons and Ikonen, 1997; van Meer, 1998).

The lysosomal degradation of N-glycans, GSLs and GAGs proceeds in a stepwise fashion, and involves exohydrolases that are both residue- and linkage-specific. In Figs. 1, 2 and 3 examples of degradative pathways of these compounds are schematically depicted, together with the enzymes catalyzing the individual steps. Some enzymes, like β-galactosidase, neuraminidase and β-hexosaminidase are involved in multiple pathways; they not only degrade glycosphingolipids, but also glycosaminoglycans, and/or glycoproteins. Lysosomal neuraminidase catalyzes the removal of terminal sialic acid residues (Section 1.8) that are α-ketosidically linked to oligosaccharides of glycoconjugates; thus, this enzyme often initiates the degradation of oligosaccharide chains. Beta-galactosidase cleaves β-linked galactosyl residues (Section 1.7), whereas β-hexosaminidase cleaves both β-linked N-acetyl-glucosamine and N-acetyl-galactosamine residues.

Glycoproteins and proteoglycans are transported to lysosomes as solutes or are delivered by specific receptors that release their ligands intralysosomally; these compounds are thus readily accessible for lysosomal hydrolases. For the transport of GSLs to lysosomes two models have been put forward (reviewed: Sandhoff and van Echten, 1993; van Echten and Sandhoff, 1993; Sandhoff and Kolter, 1996). From the plasma membrane, GSLs may travel by membrane flow along the endosomal-lysosomal pathway and end up in the lysosomal membrane. Alternatively, they may become localized in intraendosomal vesicles by being included in typical invaginations of the endosomal membrane, possibly facilitated by their preferential partitioning in distinct membrane domains. In this way, GSLs

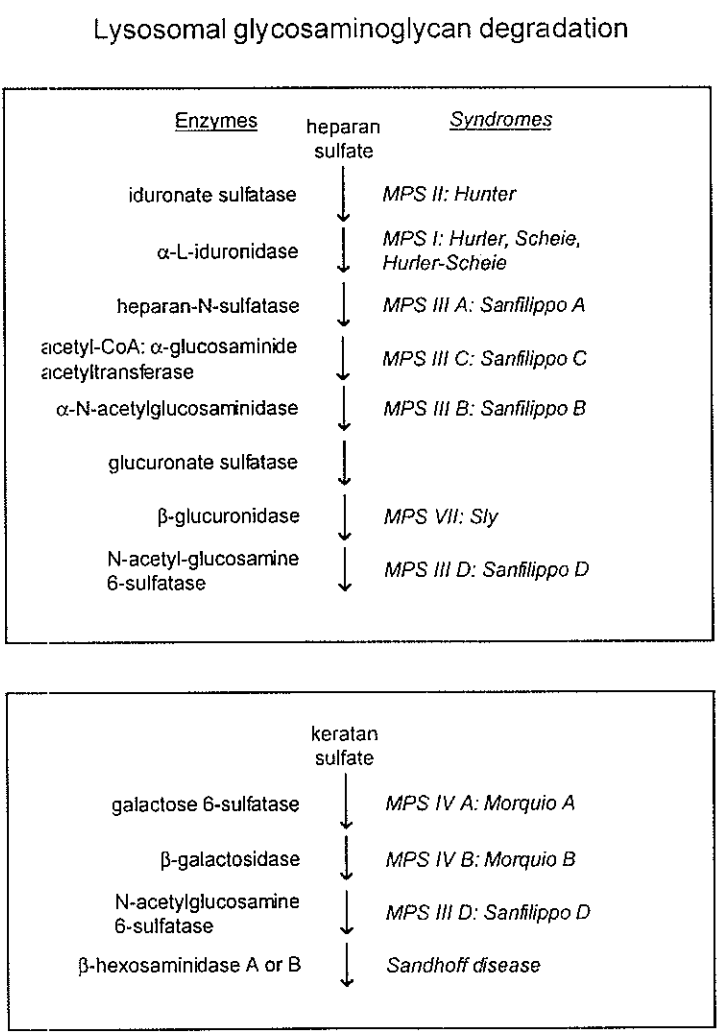

Fig. 3. Schematic representation of the first steps of the lysosomal degradation of the glycosaminoglycans heparan sulfate and keratan sulfate. The enzymes involved in these pathways and the *lysosomal storage disorders*, which are caused by their deficiencies are indicated as in Fig. 2. MPS: mucopolysaccharidosis. Adapted from (Neufeld and Muenzer, 1995).

end up in intralysosomal vesicles and as such may be more accessible to lysosomal enzymes.

The degradation of the carbohydrate moieties of GSLs differs to some extent from those of glycoproteins and proteoglycans (Sandhoff and Kolter 1997). GSLs are efficiently degraded by soluble lysosomal exoglycosidases until four or less oligosaccharide residues remain. For the last steps of their degradation specific auxiliary proteins are needed, five of which have been identified: "Sphingolipid Activator Proteins" (*Saps* or saposins) A, B, C and D, and G_{M2} activator protein (Sandhoff and van Echten, 1993; Klein *et al.*, 1994; Sandhoff *et al.*, 1995; Sandhoff and Kolter, 1996; Burkhardt *et al.*, 1997; Chatelut *et al.*, 1997; Harzer *et al.*, 1997). *Sap*-A, -B, -C and -D are encoded by a single gene and are derived from the primary translation product (prosaposin) by proteolytic processing. Both *Sap*-B and G_{M2} activator facilitate degradation of specific GSLs by solubilizing these compounds: the activator proteins bind to glycolipids that are located in membranes, and temporarily extract them out of the membrane in water-soluble complexes (Sandhoff *et al.*, 1995). *In vitro* these two activators can transfer glycolipids from one vesicle to another. The G_{M2} activator itself also binds to the G_{M2}-cleaving enzyme β-hexosaminidase A (Kytzia and Sandhoff, 1985; Yadao *et al.*, 1997). The mechanisms of action of *Sap*-A, -C and -D are less well defined. *In vitro*, these cofactors activate various GSL-degrading enzymes, which is thought to occur through direct interaction between activator and enzyme (O'Brien and Kishimoto, 1991; Sandhoff *et al.*, 1995).

1.3 Lysosomal storage disorders

A single deficiency of any of the enzymes or auxiliary proteins involved in the abovementioned degradative pathways is sufficient to disrupt the lysosomal catabolic process. This is accompanied by intralysosomal accumulation and urinary excretion of the substrates normally cleaved by the affected enzyme. The excessive intralysosomal storage causes cell and organ dysfunction, which leads to varying clinical manifestations known as lysosomal storage disorders (LSDs). Over 40 of these diseases have been described, and all but two are inherited as autosomal recessive traits (reviewed in Neufeld, 1991; Gieselmann, 1995; Scriver *et al.*, 1995; Sandhoff and Kolter, 1997). As a group, their incidence in the population can be estimated to be about 1:8000 live births (Scriver *et al.*, 1995). The carrier frequency is generally low, but in some forms of LSDs it may be very high in specific ethnic populations (see for example Kronn *et al.*, 1998). Most patients present with pathology and developmental abnormalities of multiple organs; depending on the specific enzyme deficiency, central/peripheral nervous system, reticuloendothelial system, liver, spleen, kidney, skeleton, eyes, skin,

and/or heart and blood vessels may be involved. In Figures 1, 2 and 3 examples are given of disorders that are caused by deficiency of enzymes involved in the catabolism of N-linked glycans, glycosphingolipids or glycosaminoglycans.

For many lysosomal storage disorders clinically heterogeneous forms have been described, differing widely in severity and age of onset (reviewed in Scriver *et al.*, 1995). The spectrum ranges from fatal early infantile to milder adult variants. The latter present with very mild symptoms, progress slowly, and have a near-normal life span. The main factor determining disease severity is the rate of substrate accumulation, which, in turn, is dependent on the balance between the rate of substrate influx into the lysosomal compartment, and the level of residual enzyme activity in this organelle. Importantly, both these parameters may vary between tissues and over time (Conzelmann and Sandhoff, 1991; Leinekugel *et al.*, 1992). In early infantile forms the enzyme deficiency is usually complete, due to a lack of enzyme synthesis, or due to the synthesis of nonfunctional enzymes. In contrast, patients with milder symptoms often have some level of residual activity (measured in leukocytes or cultured fibroblasts), which is exerted by partially functional mutant enzymes. Thus, the level of residual enzyme activity is informative in discriminating severe early infantile forms from later-onset variants. However, this parameter cannot allways predict the relative disease severity of the late-onset patients, as the wide clinical heterogeneity within this group (late-infantile to late-adult) is often associated with only a narrow range of residual enzymatic activity (Conzelmann and Sandhoff, 1991). Alternatively, analysis of the genetic lesions present in LSD patients may give an indication of their clinical outcome. Specific genotypes can, on an empirical basis, be associated with a particular level of disease severity. However, the clinical outcome remains difficult to predict in patients who have previously uncharacterized combinations of alleles.

Conzelmann *et al.* have proposed a simple kinetic model ([1]), which describes a relationship between residual enzyme activity and rate of substrate accumulation (Conzelmann and Sandhoff, 1991; Leinekugel *et al.*, 1992). These authors defined as critical "threshold" activity of a lysosomal enzyme the level of activity that is equal to the rate of influx of its substrate into the lysosome. They estimated that in normal individuals the activity of a lysosomal enzyme is 20-fold higher than its threshold activity. Thus, heterozygotes with half the normal level, and pseudodeficient individuals with only 10% of normal activity, are still above the threshold level, and are not ill. According to the model, only when the activity of a lysosomal enzyme is lower than its threshold activity, the rate of substrate

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([1]) $V_{acc} = V_i(1-a_r/a_t)$

where V_{acc} is the rate of substrate accumulation, V_i the rate of substrate influx, a_r the residual enzyme activity, and a_t the threshold activity.

accumulation is inversely proportional to the residual activity, and small changes in it lead to dramatic changes in the rate of substrate turnover. The authors mention that the obvious limitation of the analysis of substrate turnover is that the pathologically relevant accumulation in patients usually does not take place in skin fibroblasts, but in other, experimentally and diagnostically inaccessible tissues.

While in most LSDs the activity of a single enzyme is reduced, a number of LSDs has been described in which multiple lysosomal enzymes are deficient. Examples are I-cell disease, pseudo-Hurler polydystrophy, and multiple sulfatase deficiency, all of which are caused by defects in specific posttranslational processing events. In the former two conditions, the deficient enzyme normally catalyzes the first step in the attachment of the mannose-6-phosphate marker on N-glycans of lysosomal enzymes (Kornfeld and Sly, 1995). This prevents many of these proteins from reaching the lysosome and leads to a severe reduction of their activities. Normally, lysosomal sulfatases are post-translationally modified by oxidation of a specific cysteine residue (Dierks *et al.*, 1997). Omission of this step leaves these enzymes catalytically inactive, causing multiple sulfatase deficiency (Recksiek *et al.*, 1998).

1.4 G_{M1}-gangliosidosis, sialidosis and galactosialidosis

This thesis focuses on three lysosomal enzymes: protective protein/cathepsin A (PPCA), N-acetyl-α-neuraminidase, and β-D-galactosidase. Genetic deficiencies of these three enzymes are associated with three distinct lysosomal storage disorders, that share clinical and biochemical features. Absence or malfunctioning of PPCA is the cause of galactosialidosis (GS), while neuraminidase deficiency is responsible for sialidosis, and β-galactosidase is affected in G_{M1}-gangliosidosis. These diseases are all autosomal recessive multisystemic disorders with a severe CNS involvement and a broad clinical heterogeneity (Table 1) (reviewed in d'Azzo *et al.*, 1995; Suzuki *et al.*, 1995; Thomas and Beaudet, 1995). Biochemical and molecular biological aspects of PPCA, β-galactosidase, and neuraminidase will be further described in the next sections.

β-galactosidase activity is severely reduced in skin fibroblasts from G_{M1}-gangliosidosis patients, while neuraminidase activity is similarly affected in those of sialidosis patients, and both activities are strongly diminished in GS. Cell-fusion and co-cultivation experiments have shown that the genetic defect underlying the combined enzyme deficiency in GS is different from that causing either of the two single-deficiency disorders. β-galactosidase activity is partially restored in heterokaryons of G_{M1}-gangliosidosis and GS fibroblasts (Galjaard *et al.*, 1975; Hoeksema *et al.*, 1979), and a similar effect is obtained for

Table 1. Overview symptoms and clinical heterogeneity of galactosialidosis, sialidosis, G_{M1}-gangliosidosis, and Morquio B syndrome (adapted with permission from (Rudenko, 1996), with modifications (d'Azzo et al., 1995; Suzuki et al., 1995; Thomas and Beaudet, 1995)).

disorder	*genetic deficiency*	*form*	*onset*	*symptoms*
Galactosialidosis	protective protein/ cathepsin A	early infantile	0 - 3 months	progressive severe phenotype, mental retardation, fetal hydrops, edema, ascites, visceromegaly, skeletal dysplasia, kidney involvement, early death
		late infantile	~ 1 year	hepatosplenomegaly, growth retardation, cardiac involvement, absence of relevant neurological signs
		juvenile/adult	15 years (mean)	myoclonus, ataxia, angiokeratoma, mental retardation, neurologic deterioration, long survival
Sialidosis	neuraminidase	type 1	variable, usually second decade	ocular cherry red spots, generalized myoclonus, gait abnormalities, impaired vision
		type 2 congenital	in utero	fetal hydrops, ascites and
		infantile	0 - 12 months	progressive severe phenotype, dysostosis, coarse facies, visceromegaly, mental retardation, early death
G_{M1}-gangliosidosis	beta-galactosidase	infantile	0 - 6 months	severe neurodegenerative disorder with psychomotor degeneration, bone abnormalities, hepatosplenomegaly, and early death
		late-infantile/juvenile	0.5 - 3 years	same as above, localized skeletal involvement, survival up to 5 years
		adult/chronic	3 - 30 years	gait and speech disturbance, dystonia, intellectually mostly normal; chronic cases: progressive neurological deterioration, protracted course
Morquio B syndrome	beta-galactosidase		5 - 10 years	skeletal dysplasia, corneal clouding, sternal protrusion, no central nervous system involvement

neuraminidase activity after fusing sialidosis and GS cells (Hoogeveen *et al.*, 1980). In the latter studies Hoogeveen *et al.* noticed that neuraminidase activity was also increased in GS fibroblasts after cocultivating these cells with sialidosis fibroblasts. It was suggested that a diffusable "corrective factor" was responsible for this partial restoration, which was later identified as PPCA (see Section 1.6). Subsequent work has established that the β-galactosidase deficiency in G_{M1}-gangliosidosis is due to structural mutations in the β-galactosidase gene (reviewed in Suzuki *et al.*, 1995), and that the combined enzyme deficiency in GS is secondary to genetic defects in the lysosomal protein PPCA (d'Azzo *et al.*, 1982; Galjart *et al.*, 1988, reviewed in d'Azzo *et al.*, 1995). Structural defects in the gene encoding neuraminidase are the cause of sialidosis, as described in Chapter 4.

In sialidosis and galactosialidosis patients, the metabolism of sialoglycoconjugates is similarly impaired. Essentially the same collection of α(2,6)- and α(2,3)- sialylated oligosaccharides has been isolated from urine and fibroblasts of these patients (van Pelt *et al.*, 1988a, 1991), as well as from the placenta of a GS patient (van Pelt *et al.*, 1988b). In addition, increased amounts of the gangliosides G_{M3}, G_{M2}, G_{M1}, and G_{D1a} were detected in peripheral ganglia and spinal cord from GS patients (Miyatake *et al.*, 1979; Yoshino *et al.*, 1990). G_{M3}, G_{D3}, and G_{M4} were also elevated in visceral organs of a 21-year old sialidosis patient (Ulrich *et al.*, 1987). *In vitro* studies further demonstrated that cultured sialidosis and GS fibroblasts accumulated G_{M3}, and to a lesser extent G_{M2}, after loading these cells with G_{M1} (Mancini *et al.*, 1986; Schmid *et al.*, 1992); in addition the lysosomal G_{M3} sialidase activity was reduced in homogenates of sialidosis fibroblasts (Lieser *et al.*, 1989; Schneider-Jakob and Cantz, 1991). Thus, also ganglioside metabolism, especially G_{M3} desialylation, has been found to be impaired in sialidosis and GS.

In G_{M1}-gangliosidosis the degradation of complex N-linked glycans, the glycosphingolipids G_{M1} and G_{A1}, and the glycosaminoglycan keratan sulfate is impaired (Figures. 1, 2 and 3) (Suzuki *et al.*, 1995). Accumulating glycolipids are mainly found in the CNS, while keratan sulfate- or glycoprotein-derived galactosylated oligosaccharides have been identified in patients' liver and urine.

Disruption of the PPCA gene in mice results in a GS-like phenotype. Affected animals present with facial abnormalities, hepatosplenomegaly, progressive and diffuse edema, ataxic movements and tremor; their life span is about 10-12 months (Zhou *et al.*, 1995). Storage of undegraded substrates is evident in their peripheral blood monocytes and lymphocytes, as well as in specific cells of most organs, especially in the kidney. PPCA (-/-) mice secrete excessive amounts of sialylated oligosaccharides in their urine, which is consistent with the severe reduction of neuraminidase activity in all tissues tested.

Surprisingly, β-galactosidase activity is ~20% of normal in fibroblasts, but equal or higher than normal in other organs, indicating that at least in mice the dependence of β-galactosidase on PPCA is significantly less strict than that of neuraminidase. It is unclear if this is also the case in humans, as β-galactosidase and neuraminidase activities in human tissues other than fibroblasts have been measured post mortem only in a few patients (Kleijer *et al.*, 1979). It is noteworthy that both cathepsin A and neuraminidase activity in the knock-out mice can be restored after transplantation with bone marrow from either normal mice, or from transgenic mice that overexpress PPCA in their erythroid cells or in their monocyte/macophage lineage; accordingly, most of the pathological symptoms are prevented by these treatments (Zhou *et al.*, 1995; Hahn *et al.*, 1998).

Beta-galactosidase-deficient mice have been generated, and their pathology is comparable to that of human patients (Hahn *et al.*, 1997). Progressive neuronal accumulation of G_{M1} is seen in the central and peripheral nervous systems; the ganglioside is already detectable in the CNS of three week old mice, and reaches almost five times normal levels at 3.5 months. When older than 4-5 months the animals display neurological abnormalities such as trembling, ataxia, and abnormal gait. Their average lifespan is 7-8 months; the animals become crippled, suffer hindlimb paralysis, and are not able to feed themselves anymore. Some features of the mice are different from those of human patients. The mice have no obvious visceral pathology, other than minor hepatic oligosaccharide storage. Furthermore, the neuronal storage material contains large amounts of the sphingolipid G_{A1}, the desialylated derivative of ganglioside G_{M1}.

1.5 PPCA/neuraminidase/β-galactosidase complex

Several reports describe the copurification of β-galactosidase with PPCA (Lo *et al.*, 1979; d'Azzo *et al.*, 1982; Yamamoto *et al.*, 1982; Hoogeveen *et al.*, 1983; Hubbes *et al.*, 1992). Other authors have shown that also neuraminidase activity is associated with β-galactosidase and PPCA (Verheijen *et al.*, 1982, 1985; Yamamoto and Nishimura, 1987; Hiraiwa *et al.*, 1988). After the identification of PPCA as cathepsin A (see next section), all three enzyme activities were shown to co-purify, using affinity matrices specific for β-galactosidase or PPCA (Potier *et al.*, 1990; Pshezhetsky and Potier, 1994, 1996; Hiraiwa *et al.*, 1996, 1997). Together, these data demonstrate that β-galactosidase, neuraminidase, and PPCA are at least in part physically associated in a multienzyme complex.

The material obtained by affinity chromatography contains various molecular species that are separable by gel filtration (Pshezhetsky and Potier,

1994, 1996; Hiraiwa *et al.*, 1996, 1997). All lysosomal neuraminidase activity present in tissue extracts is found in a high molecular weight form. In contrast, only small fractions (as low as 1-2%) of the total amounts of β-galactosidase and PPCA are present in this complex. A significant portion of β-galactosidase and cathepsin A activities are obtained in a distinct ~680 kDa multimer, which is thought to consist of one β-galactosidase tetramer and four PPCA dimers (Pshezhetsky and Potier, 1993; Pshezhetsky *et al.*, 1995). The remainder of the cathepsin A activity is associated with PPCA dimers, while, depending on the experimental conditions, the remainder of β-galactosidase activity is present in monomeric, or homo-oligomeric forms (Yamamoto *et al.*, 1982; Pshezhetsky and Potier, 1994, 1996; Hiraiwa *et al.*, 1997).

The complex formation of the three enzymes has also been shown in immunotitration experiments: using either anti-PPCA or anti-β-gal antibodies, neuraminidase activity is coprecipitated with cathepsin A and/or β-galactosidase activities (Verheijen *et al.*, 1982, 1985; Pshezhetsky and Potier, 1996; Hiraiwa *et al.*, 1996, 1997). Estimates of the molecular weight of the complex range from ~680 (Potier *et al.*, 1990) to ~1300 kDa (Pshezhetsky and Potier, 1994, 1996), but its stoichiometry is poorly understood even to date.

In two independent studies, N-acetylgalactosamine-6-sulfate sulfatase (GALNS) was also identified as a component of the multienzyme complex containing neuraminidase activity (van der Horst, 1993; Pshezhetsky and Potier, 1996). GALNS is required for the first step of keratan sulfate degradation, prior to the β-galactosidase-catalyzed second step (Fig. 3) (Neufeld and Muenzer, 1995). Remarkably, Pshezhetsky *et al.* have reported that GALNS activity is 6-40% of its normal value in GS fibroblasts, and have found a GS patient, whose urinary secretion of keratan sulfate is comparable to that of patients with a primary GALNS deficiency (Pshezhetsky and Potier, 1996). These and other data suggest that GALNS may also depend on PPCA for its activity (Pshezhetsky and Potier, 1996).

In GS fibroblasts, the half-life of β-galactosidase is about one-tenth of its normal value, due to rapid intralysosomal degradation of this protein (van Diggelen *et al.*, 1981, 1982). Furthermore, the enzyme is only present in mono, di-, and tetrameric forms in these cells (Hoogeveen *et al.*, 1983; Oshima *et al.*, 1994; Pshezhetsky and Potier, 1996). This suggests that the mono/homo-oligomeric forms of β-galactosidase are poorly resistant against intralysosomal proteolysis. This is in agreement with the fact that culturing GS fibroblasts in the presence of protease inhibitors leads to partial restoration of their β-galactosidase activity (Suzuki *et al.*, 1981; d'Azzo *et al.*, 1982; Hoogeveen *et al.*, 1983; Oshima *et al*, 1994; Pshezhetsky and Potier, 1996). This treatment, however, does not affect the aggregation state of the enzyme, which remains mono/homo-oligomeric

(Hoogeveen *et al.*, 1983). In contrast, culturing GS fibroblasts in the presence of exogenous PPCA not only results in the elevation of the β-galactosidase activity (Hoogeveen *et al.*, 1981; d'Azzo *et al.*, 1982; Galjart *et al.*, 1988; Pshezhetsky and Potier, 1996), but also in the generation of the 680 kDa heteromultimeric form of the enzyme (Hoogeveen *et al.*, 1983). Thus, association with the internalized PPCA is sufficient to prevent intralysosomal degradation of β-galactosidase in these cells.

1.6 PPCA

PPCA was identified as a separate biochemical entity by virtue of its physical association with β-galactosidase. An antiserum raised against the purified complex precipitated β-galactosidase and three previously uncharacterized polypeptides of 54-, 32- and 20-kDa from human fibroblasts (d'Azzo *et al.*, 1982). D'Azzo *et al.* observed that, in contrast to normal fibroblasts, the latter molecules were absent from some GS fibroblast strains, and that the medium of these cells lacked the 54 kDa protein (d'Azzo *et al.*, 1982). These authors identified this protein as the "corrective factor" (see Section 1.4) present in conditioned media of normal fibroblasts, which is capable of restoring β-galactosidase and neuraminidase activities in GS cells. In this capacity the protein has a similar effect on β-galactosidase activity as some protease inhibitors (see previous Section); therefore, it was initially named protective protein (d'Azzo *et al.*, 1982).

Cloning of the PPCA cDNA revealed its homology to yeast serine carboxypeptidase Y, the yeast KEX1 gene product and to wheat serine carboxypeptidase (Galjart *et al.*, 1988). The biochemical properties of the protein were suggestive of an earlier characterized serine carboxypeptidase, cathepsin A (Galjart *et al.*, 1991). This enzyme was first described by Fruton and Bergmann, who detected an enzymatic activity in bovine spleen that catalyzed the release of tyrosine from the N-blocked synthetic dipeptide Z-Glu-Tyr at pH 5.5 (Fruton and Bergmann, 1939). The enzyme was purified from various mammalian tissues and was shown to have carboxypeptidase activity directed towards substrates with a hydrophobic amino acid residue at the penultimate position, like Z-Phe-Ala/Val/Phe/Leu (Kawamura *et al.*, 1977, and reviewed in McDonald and Barrett 1986). Cathepsin A activity purified with three polypeptides, estimated at 55, 25 and 20 kDa (Kawamura *et al.*, 1980).

The identity of the protective protein with cathepsin A was further substantiated by the following lines of evidence: (1) isolation of a protein from human platelets that had carboxypeptidase activity and consisted of two disulfide-linked chains with N-terminal amino acid sequences identical to those of the protective protein (Jackman *et al.*, 1990); (2) direct purification of protective

protein on a cathepsin A-specific affinity matrix (Pshezhetsky and Potier, 1994; Matsuzaki *et al.*, 1998); (3) increase in cathepsin A activity after overexpression of the protective protein cDNA in COS-1 cells (Galjart *et al.*, 1991); (4) precipitation of cathepsin A activity with anti-protective protein antibodies (Galjart *et al.*, 1991); (5) lack of cathepsin A activity in GS fibroblasts (Kase *et al.*, 1990; Tranchemontagne *et al.*, 1990; Galjart *et al.*, 1991; Itoh *et al.*, 1995; Kleijer *et al.*, 1996).

The 54 kDa PPCA precursor carries two N-linked oligosaccharide chains, one of which acquires the mannose-6-phosphate recognition marker, and is essential for transport to lysosomes (Morreau *et al.*, 1992). In this compartment the enzyme is processed into the mature, 32/20-kDa form by a two-step process: first endoproteolytic cleavage nicks the protein into a 34/20-kDa intermediate form, followed by the removal of a 2-kDa peptide from the C-terminus of the large 34-kDa chain (Bonten *et al.*, 1995). Elucidation of the crystal structure of the PPCA precursor provided insight into the way the proteolytic processing contributes to the acquisition of catalytic activity (Rudenko *et al.*, 1995). In this model three structural domains are distinguished, the "core" domain containing the active site, the "helical" domain and the "maturation" domain. The latter of these contains the 2 kDa linker segment. In the precursor model, the active site cleft is covered and rendered solvent-inaccessible by a part of the maturation domain, which lies outside the linker peptide. After excision of this 2 kDa fragment, about 35 of the remaining residues are thought to undergo a conformational change to expose the active site (Rudenko *et al.*, 1995). Furthermore, the structure of the core domain is similar to that of a number of other proteins, including the wheat and yeast carboxypeptidases (Rudenko *et al.*, 1995).

PPCA is a multifunctional protein with catalytic activity distinct from its protective function towards β-galactosidase and neuraminidase, (Galjart *et al.*, 1991). The protein has carboxypeptidase activity at acidic pH, and deamidase/esterase activities at neutral pH (Jackman *et al.*, 1990). Interestingly, *in vitro* it can deamidate biologically active peptides as substance P and neurokinin, and act as a carboxypeptidase on endothelin I, bradykinin, and oxytocin-free acid (Jackman *et al.*, 1992). Mature PPCA is released from thrombin-stimulated human platelets and from ionophore-B-stimulated cells of a natural killer cell line, and has been detected in the secretory granules of human IL-2-activated killer cells (Jackman *et al.*, 1990; Hanna *et al.*, 1994). These findings imply that the enzyme may also be involved in extralysosomal processes, like the inactivation of bioactive peptides, or granzyme-mediated cellular cytotoxicity. Thus, although the pathology in GS patients is primarily caused by lysosomal dysfunction, the possibility remains that some of their symptoms are

related to extracellular activities of PPCA.

1.7 β-galactosidase

The amino acid sequences of the human and mouse β-galactosidases (Oshima *et al.*, 1988; Morreau *et al.*, 1989; Nanba and Suzuki, 1990; Yamamoto *et al.*, 1990) are highly homologous. From the primary structure stem point, these mammalian enzymes appear to belong to a family of proteins that occur in very distantly related organisms, including nematodes, bacteria, fungi and plants (Taron *et al.*, 1995; Gutshall *et al.*, 1997; Ito and Sasaki, 1997). The similarities are confined to seven domains: a large 108-residue N-terminal domain, a second, 24-residue N-terminal domain, three smaller central domains and two C-terminal domains, ranging from 14 to 24-residues (Taron *et al.*, 1995).

Biochemical studies have shown that β-galactosidase is synthesized as an 85 kDa precursor, containing 677 amino acid residues, which is processed to a 64 kDa mature form (d'Azzo *et al.*, 1982). Nucleotide sequencing of the β-galactosidase cDNA and partial amino acid sequencing of the mature protein isolated from human placenta have indicated that the 85 kDa precursor is not, or is only minimally processed at its N-terminus (Morreau *et al.*, 1989). This implied that proteolytic cleavage of the precursor molecule normally occurs at a site close to its C-terminus, and that the 64-kDa protein comprises the larger N-terminal part of the precursor. Consequently, the most C-terminal conserved domain of the 85-kDa precursor should be absent in the mature 64 kDa enzyme. It is now clear that the ~20-kDa C-terminal processing product of mammalian β-galactosidase can remain an integral constituent of the mature enzyme and is present in high molecular weight hetero-oligomeric forms of the enzyme (see Chapter 6).

Several mutations in β-galactosidase have been identified in G_{M1}-gangliosidosis and Morquio B syndrome (reviewed in Gieselmann, 1995; Suzuki *et al.*, 1995). These genetic lesions are found throughout the β-galactosidase gene, but cluster in the first three exons, and in the sixth exon (reviewed in Morrone, 1999). Curiously, a number of mutations are located in the very 3' region of the gene (16^{th} exon), all of which (except two) lie in the most C-terminal conserved domain and specifically affect highly conserved residues (Boustany *et al.*, 1993; Hilson *et al.*, 1994; Morrone *et al.*, 1997). The fact that these mutations are causative of G_{M1}-gangliosidosis implies that the C-terminus of lysosomal β-galactosidase might be important for the structure/function of the enzyme. Thus, it was relevant to understand the role of this domain in the expression of lysosomal β-galactosidase activity, both as part of the precursor molecule and as a 20 kDa proteolytic fragment. Our findings will be discussed in Chapter 6 of this thesis.

1.8 Sialidases

Until recently the lysosomal and other mammalian sialidases were poorly characterized at the biochemical and molecular level, in comparison with microbial and viral sialidases. Therefore, the latter enzymes will be briefly introduced, as well as the non-lysosomal mammalian sialidases, as they share some of their features with lysosomal neuraminidase (Chapter 4).

All sialidases target sialic acid residues, which are often located at the termini of the oligosaccharide chains of sialoglycoconjugates, linked to the penultimate monosaccharide residue. These acidic monosaccharides occur in nature in an unusually large number of derivative forms (Varki, 1992; Schauer *et al.*, 1995; Reuter and Gabius, 1996; Schauer, 1997). Three distinct structural roles have been attributed to sialic acid residues in glycoconjugates. They may contribute significantly to the physicochemical properties of these compounds, like the viscosity of mucins (Schauer *et al.*, 1995); alternatively, they are involved in biological recognition processes, as specific ligands (Varki, 1997; Vinson *et al.*, 1997), or as masks of ligands (Schauer, 1985; Schauer *et al.*, 1995). Sialic acid residues present in glycoconjugates have been implicated in a variety of biological processes, including protease or glycosidase resistance, pathogen protection, cell-cell interaction/adhesion, and antigen recognition (reviewed in Corfield *et al.*, 1992; Lasky, 1995; Powell and Varki, 1995; Crocker *et al.*, 1996; Kelm *et al.*, 1996). (In the literature the terms "sialidase" and "neuraminidase" have been used interchangeably, as is the case in this thesis. Strictly speaking, "sialidase" is more appropriate, as most of these enzymes do not cleave off neuraminic acid, but one of its derivatives (Saito and Yu, 1995)).

Sialidases have been found in viruses, bacteria, fungi, protozoa, and mammals (Corfield, 1992; van der Horst, 1993; Schenkman *et al.*, 1994; Saito and Yu, 1995; Taylor, 1996). Together they form a superfamily of hydrolases that share a conserved active site and similar sequence motifs (Crennell *et al.*, 1993; Vimr, 1994; Gaskell *et al.*, 1995). For example, non-viral proteins contain a (F/Y)RIP domain (single letter amino acid code), N-terminally located from two to five copies of an "Asp box" (consensus sequence Ser/Thr-X-Asp-(X)-Gly-X-Thr-Trp/Phe) (Roggentin *et al.*, 1993). Crystallography studies have demonstrated that different sialidases have a common catalytic core of ~40 kDa with a characteristic six-bladed propeller fold (Air and Laver, 1989; Crennell *et al.*, 1993, 1994; Gaskell *et al.*, 1995). In this model, each propeller blade consists of four antiparallel β-strands. The Arg residue in the (F/Y)RIP motif is one of the active site residues, and is located in the center of the molecule, while the Asp boxes are peripherically located. Their function is not exactly known, but these motifs have also been found in unrelated proteins that bind carbohydrates (Rothe *et al.*, 1991);

it is also thought that Asp boxes may have a role in protein secretion (Taylor, 1996).

Some members of the sialidase superfamily contain additional structural domains, such as a stalk region (influenza virus, *Trypanosoma* (Air and Laver, 1989; Schenkman *et al.*, 1994)), lectin-like domains (*Vibrio cholerae, Micromonospora viridifaciens, Macrobdella decora* (Crennell *et al.*, 1994; Gaskell *et al.*, 1995; Luo *et al.*, 1998)), or an immunoglobulin-like domain (*M. viridifaciens* (Gaskell *et al.*, 1995)). Curiously, *V. cholerae* sialidase associates with an endo-β-N-acetylhexosaminidase and a protease to form a mucinase complex, that is able to reduce the viscosity of intestinal mucus (Stewart-Tull and Ollar, 1988).

1.9 Mammalian neuraminidases

In mammalian cells, neuraminidase activity has been detected in lysosomes, the plasma membrane and the cytosol. The enzymes involved differ in their biochemical properties (reviewed by van der Horst, 1993; Saito and Yu, 1995). The lysosomal and plasma membrane neuraminidases have an acidic pH optimum, while cytosolic sialidase is most active at pH 6.0.

The cytosolic enzyme remains active during extensive purification procedures from rat liver and Chinese hamster ovary cells. It has a molecular weight of 43 kDa, is active against glycoproteins, oligosaccharides and gangliosides, and has a preference for α(2,3)-linked sialyl residues (Miyagi and Tsuiki, 1985; Warner *et al.*, 1993). The purified rat protein is monomeric and requires bile acids to cleave sialylated gangliosides *in vitro* (Miyagi and Tsuiki, 1985). cDNA cloning has revealed that the cytosolic sialidases from rat and Chinese hamster are 88% homologous at their amino acid level, and are similar to bacterial sialidases, in that they contain an (F/Y)RIP motif and two Asp-boxes (Miyagi *et al.*, 1993; Ferrari *et al.*, 1994).

Lysosomal neuraminidase has generally been characterized as a very labile, highly freeze/thaw-sensitive enzyme. Various studies have distinguished this enzyme from the plasma membrane neuraminidase, using different detergents and inhibitors. For, example, in human fibroblasts both enzymes can cleave the artificial 4-MU substrate and G_{M3} ganglioside, but Triton X-100 stimulates the plasma membrane sialidase, while sodium glycodeoxycholate enhances the lysosomal enzyme (Lieser *et al.*, 1989; Zeigler *et al.*, 1989; Fingerhut *et al.*, 1992). The detergent effects are only found at specific concentrations. On this basis, assays have been developed that are specific for each of the two enzymes. Thus, the activity stimulated by Triton X-100 is inhibited by exposing whole cells to Cu^{2+}, while the cholate-stimulated activity is only inhibited by the metal ion in cell lysates (Lieser *et al.*, 1989). Furthermore, only

the cholate-stimulated activity appeared to be deficient in sialidosis, galactosialidosis, and I-cell disease fibroblasts (Lieser *et al.*, 1989; Schneider-Jakob and Cantz, 1991).

Recently, the plasma membrane sialidase has been described in biochemical and molecular terms. Kopitz and co-workers have partially purified the sialidase from an octylglucoside extract of the insoluble fraction of human brain grey matter (Kopitz *et al.*, 1997). In presence of this detergent the purified material cleaves G_{M3}, $G_{D1a/b}$ and G_{T1b} (but not G_{M1} or 4MU-Neu5Ac), suggesting that its activity is specific for both $\alpha(2,3)$ and $\alpha(2,8)$ linkages. By photoaffinity labeling, it was identified as a 60 kDa protein, which behaves as a dimer in gel filtration chromatography. Hata *et al.* have succeeded in purifying the membrane-bound enzyme to homogeneity, using bovine brain (Hata *et al.*, 1998). Supplemented with either Triton X-100 or sodium cholate, the 52 kDa enzyme desialylates either gangliosides G_{D3}, G_{D1a}, and G_{T1b}, or G_{M3} and G_{D1b}, respectively. Its apparent mol. wt. is 52 kDa, which is quite close to the mass of the enzyme from human brain. In a preliminary communication, these authors also have reported the molecular cloning of the cDNA of this enzyme (Miyagi *et al.*, 1997).

As stated above, mammalian lysosomal neuraminidase is a particular sialidase since its activity is dependent on the presence of another protein, PPCA, and the active enzyme is isolated in a high molecular weight multienzyme complex. Its activity in tissue and cell extracts is labile, especially in partially purified preparations. This has severely hampered the biochemical and molecular characterization of the protein and gene associated with this activity, and has thus limited our insight into lysosomal sialoglycoconjugate metabolism, sialidosis and galactosialidosis.

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Chapter 2

Introduction to the experimental work

Introduction to the experimental work

Research into lysosomal storage disorders (LSDs) is relevant for the future clinical care of patients, since no effective therapy is available for most of these diseases. Also, the study of mutant proteins in patient material is helpful in the understanding of the structure and function of the corresponding normal proteins. In clinical genetics, knowledge of genotype-phenotype correlations may be advantageous in genetic counseling and for the choice of therapeutic approaches (if available), which are most appropriate for specific patients. Understanding genotype-phenotype correlations will involve an explanation why some mutations lead to a complete abrogation of enzyme function, while other mutations only partially impair catalytic activity. More insight into the pathogenesis of LSDs also requires understanding of how intralysosomal accumulation of undegraded compounds affects cells and how this leads to loss of function in tissues and organs. Many hurdles have still to be overcome in the development of therapeutic strategies, either through bone marrow transplantation, enzyme replacement, gene therapy or substrate deprivation.

This thesis aims at increasing the understanding of sialidosis, G_{M1}-gangliosidosis, and galactosialidosis, by focusing on the three enzymes that are deficient in these conditions: neuraminidase, β-galactosidase, and PPCA, respectively, and on the enzyme complex that they form.

As discussed in the Chapter 1, GS provides genetic evidence that in human fibroblasts both neuraminidase and β-galactosidase are dependent on PPCA for their optimal functioning. However, since in GS patients mainly sialylated glycoconjugates accumulate, it is thought that the secondary neuraminidase deficiency is primarily responsable for most of the pathology in this disorder, being comparable to sialidosis in this respect. At the beginning of the experimental work described in this thesis, the sequence of events leading to GS was not fully understood: how different mutations in PPCA correlate with different levels of disease severity, how deficiencies in PPCA lead to decreased activity of neuraminidase, and how different mutations in PPCA result in different degrees of reduction of neuraminidase activity. Furthermore, the molecular basis of sialidosis had not been defined, as neuraminidase had not been sufficiently described in biochemical and molecular terms. Thus, an interesting starting point for this project was the analysis of the genotype-phenotype correlation in GS (Chapter 3), not only because of its clinical importance, but also because this could offer some insight into how PPCA controls neuraminidase activity, since the residual activities of both PPCA and neuraminidase are higher in late-infantile and juvenile/adult GS patients than in early-infantile forms of the disease. To characterize neuraminidase at the biochemical and molecular level two strategies

were followed: on the one hand, screening of databases of randomly cloned cDNAs, on the other hand, purification of the multienzyme complex containing PPCA, β-galactosidase and neuraminidase. The first approach identified the neuraminidase cDNA (Chapter 4), and enabled us to investigate the interaction between PPCA and neuraminidase (Chapter 5). Further, analysis of the multienzyme complex revealed novel information on its composition (Chapter 6).

To identify a genotype-phenotype correlation for GS, mutations in PPCA were analyzed in a group of patients representing the whole spectrum of clinical severity in this disorder (Chapter 3). Following the identification of two point mutations in the PPCA gene that are pathognomonic for the late-infantile phenotype of this disease, the mutant proteins carrying the corresponding amino acid substitutions were overexpressed in COS-1 cells. These experiments showed that the late-infantile PPCA mutants are present in lysosomes at a low but detectable level, while various other mutants found in early-infantile patients were not observed in this compartment. This study indicated that the intralysosomal level of PPCA is a crucial parameter determining the severity of GS, and thus implied that the intralysosomal level of PPCA determines neuraminidase activity.

Benefiting from the systematic sequencing of expressed genes, we identified the neuraminidase cDNA in the dbEST database (Chapter 4). Characterization of this cDNA revealed that the lysosomal enzyme belongs to the sialidase superfamily, and led us to establish the molecular basis of sialidosis. In addition, it opened the way to extensive analyses of neuraminidase. We found that, similar to human fibroblasts, also in the COS-1 cell overexpression system full expression of neuraminidase activity is dependent on the presence of an adequate amount of human PPCA. Using this system, we also observed that the turnover and posttranslational modification of neuraminidase are unaffected by PPCA. However, neuraminidase needs to be localized in the lysosome to be active and the intralysosomal level of neuraminidase positively correlates with that of PPCA (Chapter 5). These findings offer the first explanation of how different mutations in PPCA have different effects on neuraminidase activity.

In the complementary line of enquiry, the multienzyme complex containing neuraminidase, β-galactosidase and PPCA was isolated from mouse liver. Surprisingly, a ~24 kDa fragment of the 85 kDa β-galactosidase precursor was identified as a component of the murine complex (Chapter 6). This fragment is derived from the C-terminus of the β-galactosidase precursor, and is generated when the 85 kDa molecule is proteolytically processed, yielding the 64 kDa N-terminal part. Previously, the C-terminal fragment was thought to be degraded soon after proteolysis of the precursor. However, this polypeptide could also be detected in human fibroblasts. Subsequent overexpression experiments in COS-1 cells demonstrated that the C-terminal domain of the human β-galactosidase

precursor is essential for the biosynthesis of this enzyme.

Chapter 3

Molecular and biochemical analysis of protective protein/cathepsin A mutations:correlation with clinical severity in galactosialidosis

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Molecular and biochemical analysis of protective protein/cathepsin A mutations: correlation with clinical severity in galactosialidosis

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Mutations in the gene encoding lysosomal protective protein/cathepsin A (PPCA) are the cause of the lysosomal disorder galactosialidosis (GS). Depending on age of onset and severity of the symptoms, patients present with either an early infantile (EI), a late infantile (LI), or a juvenile/adult (J/A) form of the disease. To study genotype–phenotype correlation in this disorder, we have analyzed the mutations in the PPCA gene of eight clinically different patients. In two EI and one J/A patient, we have identified four novel point mutations (Val104Met, Leu208Pro, Gly411Ser and Ser23Tyr), that prevent phosphorylation and, hence, lysosomal localization and maturation of the mutant precursors. Two amino acid substitutions (Phe412Val and Tyr221Asn) are shared by five LI patients. These mutations appear to be pathognomonic for this phenotype, and determine the clinical outcome depending on whether they are present together or in combination with other mutations. The latter include a single base deletion and a novel amino acid change (Met378Thr), which generates an additional glycosylation site. Within the LI group, patients carrying the Phe412Val mutation are clinically more severe than those with the Tyr221Asn substitution. This is in agreement with the biochemical behavior of the Asn221-mutant protein, that is, like the Phe412Val protein, phosphorylated, routed to lysosomes and proteolytically processed, but its intralysosomal stability is intermediate between that of wild-type PPCA and Val412-PPCA. Overall, these results may explain the clinical heterogeneity observed in GS patients and may help to correlate mutant allelic combinations with specific clinical phenotypes.

INTRODUCTION

Galactosialidosis (GS), an autosomal recessive lysosomal storage disease found in humans (reviewed in 1), is unlike the majority of these disorders in that it is associated with deficiencies of multiple hydrolases, all of which could contribute to the clinical outcome. Three phenotypic subtypes are distinguished according to age of onset and severity of symptoms: early infantile (EI), late infantile (LI) and juvenile adult (J/A). The clinical features are heterogeneous and include dysmorphism, skeletal dysplasia, visceromegaly, cardiac and renal involvement, progressive neurologic manifestations, ocular abnormalities, angiokeratoma and early death. Of particular interest is the small and well-defined group of LI patients, all of Caucasian origin. They develop symptoms within the first two years of life and their disease progression is slow and mild. Specific features of this subtype include visceromegaly, cardiac abnormalities, growth retardation and, most importantly, absence of relevant neurologic symptoms (2–6).

GS is diagnosed as a combined deficiency of lysosomal β-D-galactosidase and *N*-acetyl-α-neuraminidase (4,7,8). This deficiency causes lysosomal storage of sialylated oligosaccharides and glycopeptides, and, thereby, oligosaccharidiuria (7,9–12). The primary genetic defect, however, lies in the gene encoding a third lysosomal enzyme, the protective protein/cathepsin A (PPCA), an acid carboxypeptidase that normally associates with the two glycosidases to create a fully functional and stable enzyme complex (13–18). PPCA is active at both acidic and neutral pH and functions as cathepsin A/deamidase/esterase on selected neuropeptides, such as substance P, oxytocin and endothelin I (19–21). Because its protective and catalytic activities are distinct, PPCA may act independently of the two

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glycosidases to catabolize specific bioactive peptides (22). It is not known, however, to what extent loss of its serine carboxypeptidase activity contributes to the GS clinical phenotype.

In cultured cells and tissues, the enzyme is synthesized as a 54 kDa precursor/zymogen, which is processed in lysosomes into a mature and active 32/20 kDa two-chain product (13,22,23). Both precursor and mature proteins homodimerize (19,24). PPCA mRNA levels differ among patients with different clinical phenotypes, as does the quality and quantity of immunoprecipitable protein (13,22,25–27). Further, severely reduced or absent cathepsin A/deamidase activity has been detected in fibroblasts and tissues of patients (22,28–31).

A recurrent mutation was identified in the PPCA gene of Japanese patients with the J/A form and mild presentation, but with mental retardation (32–35): a single base substitution at the donor splice site of intron 7 causes aberrant splicing of the mRNA and the omission of exon 7 (SpDEx7). It was postulated that this is a leaky mutation, allowing a small amount of correctly spliced transcript to occur in homozygous patients with adult onset of the disease. In more severe juvenile patients, SpDEx7 occurs in combination with one of three point mutations that result in single amino acid changes: Trp65Arg, Gln49Arg, or Tyr395Cys (35). The latter mutation was also found in two EI severe patients, in combination with substitution Ser60Leu (35). In a few Caucasian patients with the LI condition, two new point mutations have been identified, Phe412Val and Tyr221Asn (24,35,36). Phe412Val impairs dimerization of the mutant PPCA precursor, causing its partial retention in the endoplasmic reticulum, and its rapid degradation following proteolytic processing in lysosomes (24). The second mutation is thought to have a greater effect on the catalytic rather than the protective function of the mutant protein (35). Here, we report a comparative analysis of eight GS patients who collectively represent the full range of severity of the disorder. Although we identified novel mutations in patients of all three clinical subtypes, each of the LI cases has at least one of the previously described point mutations. After investigating post-translational processing, subcellular localization and stability of the mutant proteins, we can arrange the mutations relative to the level of functional PPCA they support. From these studies we can begin to correlate specific combinations of mutant alleles with the severity of the clinical symptoms.

RESULTS

Clinical phenotypes

We have studied eight patients with galactosialidosis: two patients (NG and VE) were affected by the early infantile type of the disease; one patient (LR) showed so far a phenotype intermediate between the early and the late infantile; four patients (RZ, JC, AW and NT) had a late infantile presentation and one patient (KF) was an example of a classical juvenile/adult type. All patients had coarse facies and most of them presented with inguinal and/or umbilical hernias. Table 1 summarizes other clinical features related to the severity and course of the disease, as obtained from case reports. Some recent unpublished information was given to us by the physicians who care for the patients. Within the LI cases, AW and NT are the mildest affected. They are the only patients free

Table 1. Clinical features of patients with galactosialidosis

Initials (Sex) (Reference)	N.G. (male) (68)	V.E. (female) (69)	L.R. (female) (unpublished)	R.Z. (male) (4)	J.C. (female) (2)	A.W. (male) (5)	N.T. (female) (6)	K.F. (male) (8)
Clinical Type Origin	EI Italian	EI German	EI/LI American	LI Italian	LI Canadian	LI Canadian	LI American	J/A Japanese/Dutch
Age of onset	Birth	1 month	Birth	14 months	2 months	1 year	8 months	8 years
Presentation	Fetal hydrops, visceromegaly	Heart and kidney complications	Ascites, edema	Coarse facies, visceromegaly			Visceromegaly	Angiokeratoma
Growth	Abnormal		Normal (early growth disturbance)	Growth disturbance (childhood)	Growth disturbance (after 6 y)	Height <3rd centile (19 y)	Normal	
Skeleton	Osteoporosis	No abnormalities	Joint stiffness	Dysostosis multiplex	Joint stiffness, no dysostosis multiplex	Joint stiffness, dysostosis multiplex	Dysostosis multiplex, normal joints	Dysostosis multiplex
Liver/Spleen	Enlarged	Enlarged	Mildly enlarged	Enlarged	Enlarged only in the first years	Enlarged	Enlarged	No enlargement
Heart	No abnormalities	Heart failure, cardiomyopathy	Thick aortic valves	Thick valves, cardiomyopathy	Thick valves	Mitral and aortic valvular disease	Mitral and aortic valvular disease	
Nervous system	Hypotonia, progess-ve course	Psychomotor retardation, hypotonia	Hypotonia, mild retardation, pyramidal tract signs	Borderline retardation, no deterioration	Mild retardation, no deterioration	No abnormalities, extremity pains	Normal	Ataxia (16 y), myoclonus (22 y), pyramidal tract signs
Eye	Gray disc	No abnormalities	No abnormalities	Cherry-red spots (fading), lens opacity, corneal clouding	No abnormalities	Corneal clouding, no cherry-red spots and lens opacities	No abnormalities	Loss of visual acuity (6 y), corneal clouding, no cherry-red spots
Course	Dead at 2.5 months	Dead at 20 months	Alive at 3.5 years	Alive at 22 years	Alive at 20 years	Alive at 19 years	Alive at 24 years	Dead at 48 years

Note: A blank box stands for not reported.

of central nervous system involvement. Next, JC, RZ and LR are all mildly retarded; however, JC does not show dysostosis multiplex, liver/spleen or eye abnormalities. Although patient LR is still too young to foresee her outcome after 10–15 years, she is the most serious LI patient, presenting severely at birth.

Biochemical characteristics of GS patients

All GS patients included in this study had PPCA mRNA, and in some patients residual cathepsin A activity was measured (Table 2). We first asked whether overt differences could be detected in the biosynthesis, post-translational modifications, and processing of the mutant proteins compared with those of the wild-type PPCA. Immunoprecipitation studies were carried out on radiolabelled cell lysates from the patients' fibroblasts using a monospecific anti-PPCA antibody (anti-54), that recognizes both precursor and mature forms of the enzyme. As seen in Figure 1A, in all GS cells labelled for the 24 h period the level of the various 54 kDa PPCA mutant precursors was higher than that of wild-type precursor, which appeared nearly completely converted to the 32 and 20 kDa mature two-chain protein (Fig. 1A, lanes 1–9). In cells from both the EI (NG and VE) and J/A (KF) cases, the mutant precursors were not proteolytically cleaved (Fig. 1A, lanes 1, 7 and 8), whereas the two mature subunits were clearly detected in fibroblasts from all five LI patients, although the amounts and ratios varied from patient to patient (Fig. 1A, lanes 2–6). In addition, a partially processed intermediate of ~34 kDa was also immunoprecipitated from the latter samples. Among the LI patients, only the sample from LR (Fig. 1A, lane 3) showed an aberrantly sized band of 56 kDa, which was not proteolytically cleaved, since no processed forms of larger molecular weight were detected. The mutation in this precursor protein had apparently introduced a novel glycosylation site, because deglycosylation of the 56 kDa polypeptide resulted in a protein identical in size to the wild-type precursor free of sugars (Fig. 1B). Overall these results point to at least partial retention/accumulation of the mutant precursors in an early biosynthetic compartment. Only the LI patients had residual immunoprecipitable amounts of mature PPCA. However, these findings only partially correlate with the level of residual cathepsin A activity measured in all patients' fibroblasts (Table 2), and do not explain the apparent lack of mature protein in the adult onset patient.

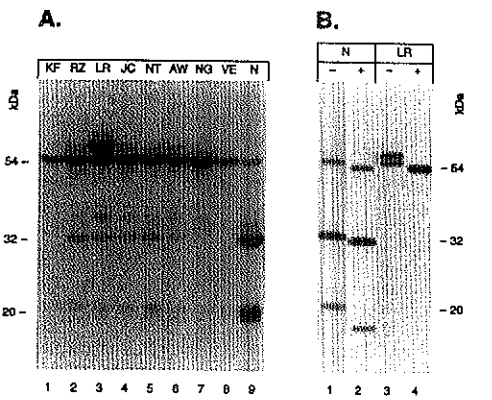

Figure 1. Immunoprecipitation and deglycosylation of PPCA from galactosialidosis and control fibroblasts. (A) Following metabolic labelling of primary cell cultures with [^{3}H]leucine, immunoprecipitation was performed with anti-54 antiserum which recognizes both precursor and mature forms of PPCA. Patients are specified by their initials; N, normal fibroblasts. Molecular sizes of precursor and mature subunits are indicated on the left. Exposure time of the fluorograph was one week. (B) Deglycosylation of immunoprecipitated PPCAs from patient LR and a normal individual (N). Precipitated proteins were incubated without (–) or with (+) N-glycosidase F, as indicated. Exposure times: lanes 1 and 2, 1 month; lanes 3 and 4, 1 week.

Table 2. PPCA mutations in galactosialidosis patients

Clinical phenotype	Patient	Nucleotide mutation(s)	Amino acid change	PPCA mRNA amount	Cathepsin A activity (% of control)
EI	NG	G400→A	Val104Met	normal	ND
		T713→C	Leu208Pro		
EI	VE	G1321→A	Gly 411 Ser	half	ND
		?			
EI/LI	LR	T1324→G	Phe412Val	normal	ND
		T1223→C	Met378Thr		
LI	RZ	T1324→G	Phe412Val	normal	1%
		T1324→G	Phe412Val		
LI	JC	T1324→G	Phe412Val	normal	3%
		T751→A	Tyr221Asn		
LI	AW	T751→A	Tyr221Asn	half	5%
		?			
LI	NT	T751A	Tyr221Asn	half	7%
		ΔC118	+1 frameshift		
J/A	KF	C158A	Ser23Tyr	half	1%
		intron 7: A→G	SpDEx7		

ND, not detectable

Molecular analysis of GS patients

To gain more insight into the molecular lesion(s) in the PPCA gene we analyzed the cDNA derived from the patients' mRNA. Four overlapping fragments, encompassing the entire coding region, were reverse transcribed, amplified by PCR, and directly sequenced. Results were verified through amplification and sequencing of the corresponding genomic regions spanning the cDNA mutations. In addition, the individual exons of the PPCA gene were amplified, using intronic primers, to determine the complete sequence of each exon. A compendium of the mutant alleles from all eight GS patients is shown in Table 2. Two new point mutations were detected in the EI patient (NG), each of which affects one allele and they result in two non-conservative amino acid substitutions, Val104Met and Leu208Pro. The second EI patient (VE) carried yet another single base substitution in one allele, changing Gly411 into Ser. Direct sequencing of the patients' cDNA showed only one base (an aberrant A) at nucleotide 1321, while in genomic DNA both the wild-type G and the mutant A were detected at this position, indicating that the patient was heterozygous for this mutation, and that only the mutant allele was transcribed. In the J/A patient, the only point mutation detected in the cDNA was a C→A transition at position 158, leading to a Ser23Tyr substitution. However, after analysis of genomic DNA, this patient appeared to be a compound heterozygote, having the C158→A mutation in one allele and the SpDEx7 mutation in the other. For the detection of the latter, we used the PCR-based method described by Shimmoto (34), which yields a 142 bp genomic fragment carrying a new *Pst*I restriction site only if amplified from the mutant allele. Indeed, digestion of the amplified fragment from the J/A patient sample gave rise to both the wild-type 142 bp band and a 123 bp digested product, indicating that the patient was heterozygous for the SpDEx7 mutation (not shown). The late onset of the symptoms and mild conditions in this patient must, therefore, be associated with the rare occurrence of a correctly spliced transcript derived from the SpDEx7 allele.

In contrast to the EI and J/A patients, the five LI patients were genetically more homogeneous. Two point mutations, Phe412Val and Tyr221Asn, appeared to define this clinical phenotype (Table 2). We and others have previously identified these two mutations in three of these GS patients (24,35,36). We now find that these mutations can occur in either the homozygous or compound heterozygous state and are diagnostic for the LI phenotype. Three patients had the Tyr221Asn mutation. In patient NT, this amino acid change was encoded by one allele, whereas the second allele carried a deletion of a cytosine at position 118 (C118). The latter caused a frameshift and premature translation termination codon. However, because the deletion was associated with absence of mRNA, no truncated protein was synthesized from this allele, and the expressed mutant PPCA contained the Asn221 change. A similar genotype was found in patient AW, who also carried the Tyr221Asn mutation in one allele. The genomic lesion in the second allele probably caused mRNA instability and was not identified. The Phe412Val mutation was again detected in three of the five LI patients. Patient RZ, described earlier (24), was homozygous for this mutation, whereas JC was compound heterozygous for both Tyr221Asn and Phe412Val mutations. We further used allele-specific oligonucleotide hybridization to screen for the presence of either of these two genetic lesions in a recently diagnosed young patient

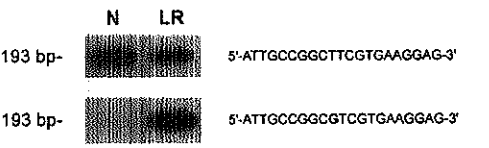

Figure 2. Allele-specific oligonucleotide hybridization. To screen patient LR for the Phe412Val (T1324G) mutation, exon 12 of the PPCA gene was amplified from genomic DNA from the patient and a normal (N) individual using intronic primers. The amplified DNA was hybridized to wild-type (upper panel) and mutant (lower panel) oligonucleotides encompassing the point mutation. The nucleotide sequences of the probes are shown, with the aberrant base indicated in bold. Exposure was for 90 min.

(LR), whose clinical features were suggestive of an LI phenotype. Indeed, we confirmed the presence of the Phe412Val substitution in one of the mutant alleles of this patient. As seen in Figure 2, the wild-type probe gave a heterozygous signal when hybridized to the patient's DNA, while the mutant oligonucleotide only recognized the patient's sample. The mutation in the second allele of this patient, responsible for the over-glycosylation of the precursor protein, was Met378Thr, which generated a new glycosylation site at amino acid Asn376. From these combined data it is apparent that in the LI patients, who have distinctively milder phenotypes and survive longer, the presence of either of these point mutations, Asn221 and Val412, dictates the clinical outcome. Taking into account their most recent clinical evaluation (see Table 1), we can conclude that the Asn221 has a milder effect on the PPCA protein than the Val412 mutation; whereas the combination of both mutations apparently gives rise to an intermediate phenotype. In turn, two copies of the Val412 allele, as seen in patient RZ, seem to confer a less severe condition than only one copy, as in patient LR, who showed clinical manifestations at birth.

Phosphorylation and subcellular localization of mutant PPCAs

Having identified novel point mutations, we wanted to assess their effects on the subcellular distribution of the mutant proteins. It has been demonstrated that phosphorylation of mannose residues on one of the sugar chains of the wild-type PPCA precursor ensures its correct targeting to the lysosome and subsequent processing (24,37). Furthermore, we have shown previously that the Val412 mutant, when overexpressed in COS-1 cells, is phosphorylated in part and delivered to the lysosome. Site-directed mutagenized cDNAs, encoding PPCA proteins that carry one of the amino acid changes described above (i.e., Asn221, Val412, Met104, Pro208, Ser411 or Tyr23), were cloned into a mammalian expression vector and transiently transfected into COS-1 cells. Biosynthetic labelling of these cells with [^{3}H]leucine, followed by immunoprecipitation revealed that Met104-, Pro208-, Ser411- and Tyr23-PPCA precursors were not secreted, whereas Asn221-PPCA was present in the culture medium (results not shown). A similar experiment with ^{32}P-labelled cells showed that only the Asn221 precursor was phosphorylated, secreted and processed, albeit in trace amounts, indicating that it might reach the lysosomes (Fig. 3, lane 3). These results were confirmed by subcellular distribution analysis of the

PPCA mutants with indirect immunofluoresence, using an antibody raised against the denatured 32 kDa subunit (anti-32). Wild-type PPCA exhibited the typical lysosomal punctuated staining (Fig. 4a). In contrast, mutants Tyr23, Met104, Pro208 and Ser411 had an aberrant distribution (Fig. 4b–e); each of these proteins was confined to the endoplasmic reticulum (ER), distributed throughout the cytoplasm, or restricted to the perinuclear area. To ascertain the extent of lysosomal compartmentalization of Asn221-PPCA, its subcellular distribution was monitored by immunoelectronmicroscopy. Ultrathin sections of cells transfected with the Asn221-cDNA construct were probed with the anti-32 antibody, which revealed a clear lysosomal location for this mutant protein (Fig. 4g). However, the number of gold particles in the lysosomes of these cells was ~50% of that in cells expressing wild-type PPCA (Fig. 4f and g; Table 3), although a comparable number of grains was counted in the ER of both sets of cells (not shown). Clearly, the amount of Asn221 protein in lysosomes was higher than that of the Val412 mutant, for which we counted ~6% of the grains seen in cells overexpressing the wild-type protein (24). Interestingly, in cells cotransfected with Asn221 and Val412 cDNAs (Fig. 4h; Table 3), the lysosomal labelling (15%) was close to that of Val412 transfected cells. Using the same procedure, only ER but no lysosomal labelling was detected in cells overexpressing Met104-, Pro208-, Ser411-, and Tyr23-PPCAs (not shown). Taken together these results suggest that the latter mutations lead to drastic conformational changes in the zymogen that prevent its transport to the Golgi complex, where phosphorylation occurs, and, thereby also prevent its correct compartmentalization and processing in lysosomes. On the other hand, a fraction of both Asn221 and Val412 mutant proteins is correctly modified and routed to the lysosomes, although their intralysosomal fate seems to be different.

Table 3. Quantitative assessment of immunolabelled lysosomes in transfected COS-1 cells

Transfected DNA	Counted lysosomes	Gold particles
Hu54 (sense)	31	72 (6)
Hu54 (Tyr221 Asn)	31	38 (4)
Hu54 (Asn221 and Val412)	31	11 (1)
Hu54 (antisense)	31	2 (0.6)

The values represent the average number of gold particles per lysosome. The standard error of the mean (SEM) is indicated in parentheses.

Stability of the PPCA-YN mutant protein

In order to explain the relative amounts of Asn221 and Val412 mutants in lysosomes, we first performed a pulse-chase experiment using transfected COS-1 cells. Figure 5 shows that the two mutant precursors were synthesized in similar amounts to the wild-type, although the Asn221 appeared to be more stable in the course of the chase. Both mutant precursors were primarily converted into a '34'/20 kDa intermediate. However, the processed forms of the Val412 mutant were clearly more labile than those of the Asn221 mutant, which were, in turn, less stable than the wild-type mature protein. Consistent with the immunoelectronmicroscopy results, the processed forms in Asn221/Val412-cotransfected cells displayed an intermediate

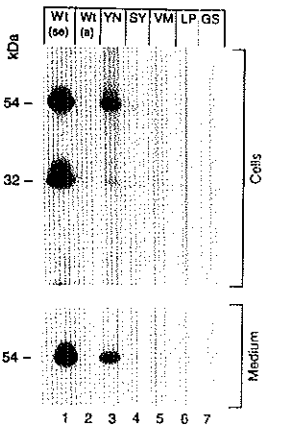

Figure 3. Phosphorylation of mutant PPCAs found in galactosialidosis. COS-1 cells were transfected with various pCD-constructs. WT (sa) and (a) represent PPCA cDNA in the sense and antisense orientation, respectively; YN, SY, VM, LP and GS stand for PPCA cDNAs carrying mutations that encode the single amino acid changes Tyr221Asn (lane 3), Ser23Tyr (lane 4), Val104Met (lane 5), Leu208Pro (lane 6) and Gly411Ser (lane 7), respectively. After metabolic labelling with $^{32}P_i$, COS-1 cells and culture media were harvested and used for immunoprecipitation with anti-54 antibodies. Molecular sizes are indicated on the left. The autoradiograph was exposed for 3 days.

stability, compared with the singly transfected cells. These data suggest that the mutant proteins, alone or in combination, have reduced intralysosomal half-lives when compared with wild-type PPCA. This is most likely due to increased susceptibility of the mutants to proteolytic degradation.

In order to address the latter point, we subjected wild-type and mutant precursors to mild trypsin digest *in vitro* (Fig. 6). We have shown earlier that the normal PPCA precursor can be proteolytically converted into a 32/20 kDa enzyme that has full substrate binding and catalytic activity (38). Equal amounts of radiolabelled wild-type, Asn221 and Val412 precursors, derived from the media of transfected COS-1 cells, were incubated for increasing lengths of time with trypsin. Reactions were terminated by the addition of a trypsin inhibitor, and the samples were tested for cathepsin A activity. Already at 2 min incubation time, wild-type PPCA precursor was converted to the 34/20 kDa intermediate and the 32/20 kDa mature enzyme (Fig. 6, lane 3). Upon prolonged digest, the 34 kDa chain was completely trimmed to a stable protease-resistant 32 kDa molecule (Fig. 6, lanes 3–6). This conversion of the precursor into the two-chain protein was paralleled by a gradual increase in cathepsin A activity (Fig. 6, lanes 2–6, lower panel). In contrast, a 2 min digest of the Val412 precursor produced a major 34 kDa band, and minor amounts of 32 and 20 kDa polypeptides (Fig. 6, lane 15). Prolonged incubation, up to 10 min, caused the rapid disappearance of the 20 kDa chain and the aspecific degradation of the larger proteins (Fig. 6, lanes 16–18). Protease treatment of this mutant precursor produced no cathepsin A activity (not shown). Instead, trypsin digest of the Asn221 precursor readily yielded a mixture of 34 and 32/20 kDa proteins, that, unlike the Val412

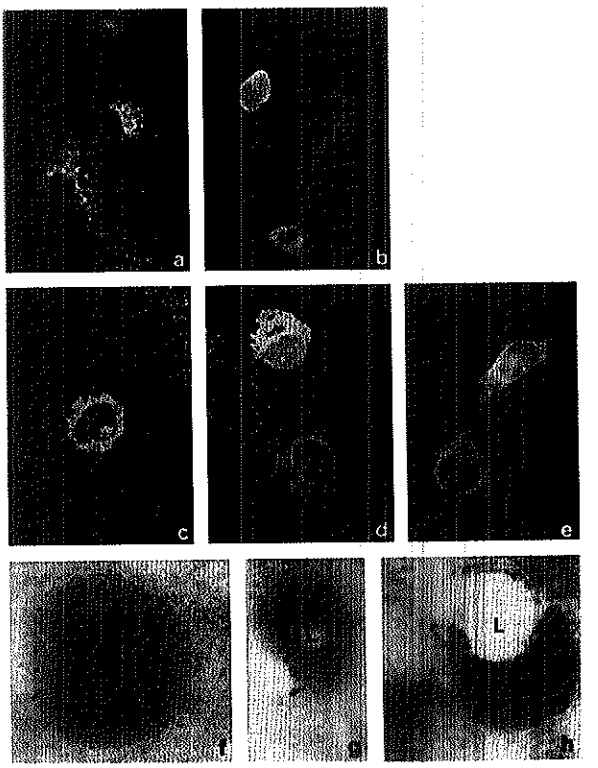

Figure 4. Immunocytochemical localization of normal and mutant PPCAs. COS-1 cells were transfected with pCD-constructs carrying cDNAs of wild-type PPCA (a and f) and mutants Tyr23 (b), Met104 (c), Pro208 (d), Ser411 (e) and Asn221 (g). Alternatively, COS-1 cells were cotransfected to express both Asn221- and Val412-PPCA (h). After transfection, COS-1 cells were either processed for immunofluorescence (a–e) or used for immunoelectronmicroscopy on cryosections (f–h). L, lysosome. Magnification was 1000× (a–e) or 70 000× (f–h). Bar, 0.1 μm.

mutant, were stable up to 10 min (Fig. 6, lanes 9–11). After 5 min, however, the 34 kDa species was not further trimmed to the 32 kDa form, and all three digested polypeptides were slowly degraded at longer incubation points (Fig. 6, lanes 10–12). Asn221-PPCA clearly acquired cathepsin A activity through trypsin digestion (Fig. 6, lane 9–10, lower panel), but lost it following extended protease exposure (Fig. 6, lanes 10–12, lower panel). From these data we may infer that the degree of protease resistance of mature Asn221-PPCA lies between that of wild-type and Val412, which is also consistent with the pulse-chase experiment shown above. Together these results could explain the enhanced stability and higher catalytic activity of the Asn221 mature protein.

Finally, we also compared the intracellular stability of wild-type, Asn221-, and Val412-PPCAs by adding exogenous precursors to the culture medium of GS deficient fibroblasts. It was shown earlier that wild-type precursor is internalized, transported to the lysosome, and properly processed (23). Equal aliquots of radiolabelled mutant precursors, secreted by transfected COS-1 cells, were added to the medium of fibroblasts from an mRNA negative EI patient (23). After 4 days, the cells were lysed and PPCA was immunoprecipitated with anti-54 antibody. As shown in Figure 7, both wild-type enzyme and the Asn221 mutant were resolved in their mature forms, although the latter in significantly reduced amounts (lanes 1 and 2). The mature Val412 protein was instead undetectable (Fig. 7, lane 3). Assuming that the mutant precursors were internalized as efficiently as the wild-type protein, these results further confirm that Asn221-PPCA is less stable than the wild-type enzyme but more stable than the Val412 mutant, and may explain the relatively mild clinical conditions of patients AW and NT.

DISCUSSION

We have investigated the properties of PPCA mutants from a group of GS patients that represents the full scale of disease

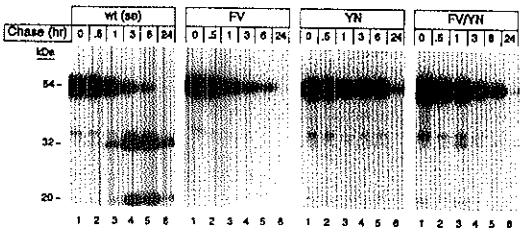

Figure 5. Pulse-chase labelling of normal and mutant PPCAs. COS-1 cells, overexpressing wild-type- [wt (se)], Val412- (FV), Asn221-PPCA (YN) or the combination of the two mutants (FV/YN) were labelled with [^{3}H]leucine for 30 min and chased per the time periods indicated. Radiolabelled proteins were immunoprecipitated with anti-54 antiserum. Molecular sizes are indicated on the left. Exposure was for 5 days.

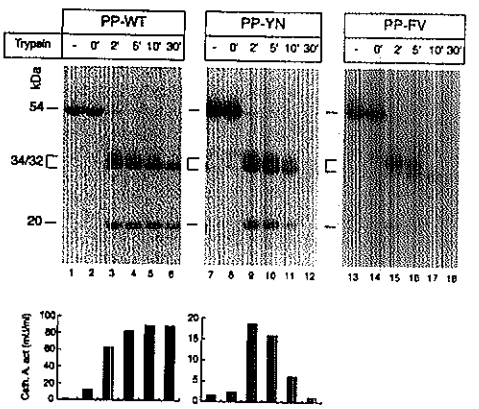

Figure 6. Limited trypsin digestion of normal and mutant PPCA precursors. COS-1 cells were transfected with cDNAs encoding either wild-type (WT), Asn221 (YN) or Val412 PPCA (FV), and were metabolically labelled with [^{3}H]leucine. Culture media containing radioactive PPCA precursors were harvested, concentrated and divided into equal aliquots. Following addition of trypsin, aliquots were incubated at 37°C for the time periods indicated, and reactions were terminated with trypsin inhibitor. Control samples either did not receive any trypsin (lanes 1, 7 and 13) or trypsin inhibitor was added before addition of trypsin (lanes 2, 8 and 14). Samples were analyzed for cathepsin A activity (lower panel) and used for immunoprecipitation using anti-54 antiserum. Immunoprecipitated proteins were separated by SDS-PAGE (upper panel). Molecular sizes are shown on the left. Exposure time of fluorographs was 1 day (lanes 1–6), or 6 days (lanes 7–18).

severity. The results indicate that the main factor determining the clinical course of GS patients is the lysosomal level of mutant PPCA. In the two severely affected EI patients, who died in infancy, we identified three novel point mutations that prevent phosphorylation of the PPCA precursor, and, thereby, its transport to the lysosome. In addition, we found that the J/A patient, who gradually deteriorated after the age of 16 until his death at the age of 48, carried the SpDEx7 mutation combined to a new severe point mutation that again caused retention of the mutant precursor in the ER. Thus, the late onset and long survival of this patient can be attributed to very low levels of lysosomal wild-type PPCA, translated from correctly spliced mRNA derived from the SpDEx7 allele.

All five LI GS patients had at least one allele capable of yielding a PPCA protein that could be phosphorylated and, to some extent, transported to the lysosome. The fact that these patients have a relatively mild phenotype indicates that Val412- and Asn221-PPCAs can associate with β-galactosidase and neuraminidase, protecting them to some extent against immediate degradation/inactivation in the lysosome. Interestingly, however, there is a clear gradient of clinical severity among these patients, from the most severe (patient LR) with only one Val412-expressing allele, to the least severe (patients AW and NT) with only Asn221 protein. The combined occurrence of Val412- and Asn221-PPCA in one patient (JC) apparently results in an intermediate phenotype. We can speculate that in the latter case the simultaneous presence of the two mutant proteins in lysosomes may result in a depletion of the more stable Asn221-PPCA due to a 'dominant negative' effect of the labile Val412 molecules. Since we have evidence that Asn221 is able to dimerize, it might form heterodimers with Val412-PPCA, that are degraded more rapidly than the Asn221 homodimers.

Interestingly, the Met378Thr mutation, present in compound heterozygosity in one of the LI patients, represents the first example among lysosomal proteins of a point mutation that generates a new Asn-linked glycosylation site which is actually utilized. The additional oligosaccharide chain likely affects proper folding and, in turn, compartmentalization of the mutant precursor since no mature protein was found. Several mutations affecting glycosylation sites in lysosomal proteins have been reported, all of which result in the loss of one oligosaccharide side chain (39–43). Elimination of a glycosylation site in arylsulfatase A and α-glucosidase does not seem to affect the correct functioning of these enzymes and, therefore, these mutations are considered polymorphisms (39,40,43). In contrast, in a number of metachromatic leukodystrophy patients with a saposin B deficiency, the absence of the only oligosaccharide chain of this protein is believed to cause its rapid degradation and, in turn, to contribute to the disease (41,42).

Clinical diversity is a hallmark of lysosomal storage disorders. With the isolation of cDNAs and genes of lysosomal proteins, a large number of mutations have been reported. From these compiled studies it has become evident that for some of these disorders a genotype–phenotype correlation can be made. While some mutations completely disable lysosomal enzymes and cause

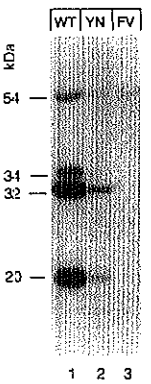

Figure 7. Normal and mutant PPCA precursors after internalization by PPCA deficient fibroblasts. Radiolabelled precursors of wild-type (WT), Asn221- (YN) and Val412-PPCA (FV) were obtained from transfected COS-1 cells cultures as outlined in the legend to Figure 6. Equal c.p.m. of each precursor was added to the culture medium of PPCA mRNA-deficient fibroblasts. After 4 days, internalized radioactive proteins were immunoprecipitated with anti-54 antiserum and proteins were separated by SDS-PAGE. Molecular sizes are shown on the left. The fluorograph was exposed for 49 days.

severe phenotypes, others only partially affect enzyme function and often give rise to milder cases. Thus, small variations in the residual enzyme activity level can significantly alter clinical outcome (for reviews see refs 44–46). For example, similar to our findings, in metachromatic leukodystrophy, which is caused by deficiency of lysosomal arysulphatase A, patients homozygous for a splice site mutation that affects the synthesis of the enzyme have the most severe form of the disease, whereas homozygosity for the Pro426Leu mutation is found in the mild, late onset forms. In the latter case, the mutant arylsulphatase A enzyme, is synthesized in normal amounts, transported to the lysosomes, but has low residual activity and drastically reduced intralysosomal half-life due to digestion by lysosomal cysteine proteinases (47). Analogously, in Niemann–Pick disease, caused by deficiency of sphingomyelinase, various mutations are associated with the A subtype, a severe neurodegenerative disease; whereas deletion of Arg608 and the Gly242Arg substitution apparently select for the B subtype, characterized by late onset and little or no neurological involvement (48,49). In cultured patient fibroblasts, the ΔArg608 allele is able to support a low level of acid sphingomyelinase activity (48), while overexpression in COS-1 cells of Arg242-sphingomyelinase generates 40% of the enzyme activity of the normal protein (49).

The recent determination of the three dimensional structure of the human PPCA precursor (50), will enable us to precisely model the different amino acid substitutions described here at the atomic level and to evaluate their effects on enzyme functioning. Ultimately, the creation of mouse models carrying these specific point mutations in the PPCA gene will hopefully give more insight into the *in vivo* pathologic consequences of these genetic lesions.

MATERIALS AND METHODS

Cell culture

Human skin fibroblast cultures from a normal individual and patients were obtained from the European Cell Bank, Rotterdam (Dr W. J. Kleijer). Fibroblasts were maintained in Dulbecco's modified Eagle's medium/Ham's F10 medium (1:1 vol/vol) supplemented with antibiotics and 10% fetal bovine serum. COS-1 cells (51) were grown in the same medium, supplemented with 5% fetal bovine serum.

Immunoprecipitation and deglycosylation

Human fibroblasts were grown to confluency in 85 mm dishes and labelled with 350 μCi [^{3}H]-4,5-leucine (Amersham) per dish for 24 h. Cell extracts were prepared as described (52), and preabsorbed with normal rabbit serum and affinity purified anti-fibronectin antiserum (Sigma) for 30 min at room temperature. The samples were precleared by incubating them three times for 15 min with 40 μl formalin-fixed *Staphylococcus aureus* suspension (Immunoprecipitin, Gibco-BRL), that had been prepared according to manufacturers' instructions, washed six times in phosphate-buffered saline and resuspended in Tris-buffered saline (10 mM Tris–HCl, pH 7.4, 150 mM NaCl) at 20% (w/v). For specific immunoprecipitation cell lysates were incubated with anti-human PPCA 54 kDa precursor antiserum (anti-54; 22) for 1 h at RT or overnight at 4°C, followed by absorption to 12.5 μl of the *S.aureus* suspension for 30 min. Immunocomplexes were collected by centrifugation, washed and prepared for SDS–polyacrylamide gel electrophoresis (SDS-PAGE) as described (52). Polyacrylamide gels (12% and 12.5%) were used (53,54). After electrophoresis gels were fixed in 40% methanol and 10% glacial acetic acid and treated with Amplify (Amersham) for fluorography of radioactive proteins.

For deglycosylation precipitated proteins were eluted from final washed *S.aureus* pellets by boiling in 70 μl 0.3% SDS in 50 mM sodium phosphate buffer pH 6.8 (PB) for 5 min. Eluates were supplemented with 140 μl 0.75% NP40 and 0.75% β-mercaptoethanol in PB and denatured for 5 min at 95°C. Samples were split into 100 μl aliquots, of which one received 0.2 U recombinant N-glycosidase F (Boehringer Mannheim). Following incubation overnight at 37°C samples were desalted on Sephadex G50 spin columns (equilibrated in 10 mM Tris–HCl, 1 mM EDTA, pH 7.4), lyophilized and processed for SDS-PAGE.

Analysis of amplified cDNA and genomic DNA

For the synthesis and amplification of mutant cDNAs, four sets of oligonucleotide primers were synthesized according to the published cDNA sequence of protective protein (23) on an Applied Biosystems 381A oligonucleotide synthesizer. Total RNA was isolated by the method of Auffray and Rougeon (55). Four overlapping cDNA fragments, encompassing the entire coding region, were synthesized by reverse PCR (56) and amplified on a Perkin-Elmer Cetus thermocycler programmed for 25 cycles. For amplification of genomic DNA, genomic DNA was extracted from cultured fibroblasts and the individual exons of the protective protein gene were amplified by PCR (57). Primers were chosen in the intronic sequences surrounding the exon so that it would be possible to determine the complete sequence of the exons. The following pairs of intronic primers were used to verify the mutations at genomic

level: 5′-TGCTGCACGGAAGCGCTGAG-3′ (sense) and 5′-CTCCCAATCCCGCCCAGAAG-3′ (antisense) for ΔC118 and Ser23Tyr: 5′-TCTCTGAAGTTCTTTCCAGT-3′ (sense) and 5′-CAGTCTGACCTTGCTAACTG-3′ (antisense) for Val104Met: 5′-GGGTATGCTCGCCTCCTCTG-3′ (sense) and 5′-TGTGGTC-CTGTTCTCAGGAT-3′ (antisense) for Leu208Pro and Tyr221Asn: 5′-GGCTTGTTCCACACCCCTCA-3′ (sense) and 5′-CCTGGC-CACTCCCAGGCATA-3′ (antisense) for Met378Thr: 5′-TCTTT-CCTGGTGGGGCAGAT-3′ (sense) and 5′-CCATACAGGGGC-CAGATGGT-3′ (antisense) for Gly411Ser and Phe412Val. For direct DNA sequence analysis, a portion of above PCR amplified cDNA or genomic DNA was subsequently subjected to asymmetric PCR (58), using only one primer, for another 35 cycles. The PCR products were extracted once with phenol/chloroform, followed by filtration through a Centricon-100 membrane (Amicon). The amplified DNAs were sequenced by the dideoxy-chain termination method (59).

Allele specific oligonucleotide (ASO) hybridization

For ASO hybridization, genomic DNA was amplified by PCR using the appropriate intronic primer pairs as described above. The amplified DNA was blotted on Hybond N+ membranes. Hybridization was performed with appropriate ^{32}P-labelled oligonucleotides. After hybridization the membranes were washed in 2× SSC containing 0.1% SDS for 30 min at 3–4°C below the estimated melting temperature.

Two pairs of allele-specific oligonucleotide probes were synthesized, one pair of normal and mutant sequences for Tyr221Asn and one pair of normal and mutant sequences for Phe412Val mutations: Tyr221Asn, 5′-GTGTAACTTCTATGACAACA-3′ (normal) and 5′-GTGTAACTTCAATGACAACA-3′ (mutant); Phe412Val, 5′-ATTGCCGGCTTCGTGAAGGAG-3′ (normal) and 5′-ATTGCCGGCGTCGTGAAGGAG-3′ (mutant).

Site-directed mutagenesis

In vitro mutagenesis of PPCA cDNA was performed as previously described (60) using the whole 1.8 kb PPCA cDNA (23) as a template. After synthesis by PCR of the 5′ 1.5 kb cDNA carrying the complete open reading frame, the presence of the desired mutation was verified by sequencing. The following pairs of mutagenic oligonucleotides were used for site directed mutagenesis: 5′-GTGTAACTTCAATGACAACA-3′ (sense) and 5′-TGTTGTC-ATTGAAGTTACAC-3′ (antisense) for mutagenesis of T751→A; 5′-TCCCCAGCTGGGATGGGCTT-3′ (sense) and 5′-AAGC-CCATCCCAGCTGGGGA-3′ (antisense) for G400→A; 5′-CCA-GTACTACGGCTACCTCA-3′ (sense) and 5′-TGAGGTAGC-CGTAGTACTGG-3′ (antisense) for C158→A; 5′-ATTGC-CAGCTTCGTGAAGGA-3′ (sense) and 5′-TCCTTCTCGAAGC-TGGCAAT-3′ (antisense) for G1321→A; 5′-TCTTCTCCCC-AGACCCACTG-3′ (sense) and 5′-CAGTGGGTCTGGGGA-GAAGA-3′ (antisense) for T713→C. The mutagenized PPCA cDNAs were subcloned into a derivative of the mammalian expression vector pCD-X (23,61).

Transient transfections and immunofluorescence

The pCD-constructs, containing *in vitro* mutagenized cDNAs, were transfected into COS-1 cells using either DEAE-dextran (23,24,62) or the calcium phosphate precipitation method (63,64). To analyze phosphorylation of overexpressed proteins, 48 h after transfection cells were metabolically labelled with [^{32}P]phosphate (100 μCi/ml labelling medium) for 7 h and cell extracts and medium samples were immunoprecipitated with anti-54 antiserum. For indirect immunofluorescence, COS-1 cells were reseeded 48 h post-transfection on Superfrost^{R}/Plus glass slides (Fisher). Next day, cells were processed according to van Dongen *et al.* (65) using an antiserum (anti-32) raised in rabbit against the denatured PPCA 32 kDa chain obtained through overexpression in insect cells (38) and FITC-conjugated anti-rabbit IgG antibodies (Sigma). Pulse-chase experiments were performed as described (37).

Immunoelectronmicroscopy

Transfected COS-1 cells in 100 mm dishes were fixed in 0.1 M phosphate buffer pH 7.3, containing 1% acrolein and 0.4% glutaraldehyde. Further embedding in gelatin, preparation for ultracryotomy and methods for immunoelectronmicroscopy were as previously reported (66). For these experiments we used antibodies (anti-32) raised against the 32 kDa denatured chain of human protective protein, isolated from human placenta (23).

Trypsin digests and uptake studies in human fibroblasts

Cos-1 cells seeded in 85 mm dishes were transfected with pCD-PPCA-WT, -751A (Asn221) and -1324G (Val412). Eighty-four hours post-transfection, cells were metabolically labelled with [^{3}H]-4,5-leucine (50 μCi/ml labelling medium) in the absence or presence of 2.5% dialyzed fetal bovine serum for 16–18 h. Serum-free media containing radiolabelled PPCA precursors were used for limited trypsin digests, as described (24,37). Immunoprecipitation was performed with anti-54 antiserum as above. For uptake experiments serum-containing COS-1 media were concentrated as described (52). Since the secreted PPCA precursors are the major labelled proteins in the medium concentrates, aliquots of these preparations containing equal c.p.m. were added to the media of recipient El GS fibroblasts (23), grown to confluency in 35 mm dishes. After 4 days of uptake, internalized PPCAs were recovered by immunoprecipitation as above.

Enzyme assays

Cathepsin A activity was measured with the synthetic N-blocked dipeptide carbobenzoxy-phenyalanyl-alanine as substrate according to Galjart *et al.* (22) and Kleijer *et al.* (31). Total protein was quantitated with bicinchoninic acid (67) following manufacturers' guidelines (Pierce Chemical Co.).

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Chapter 4

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-

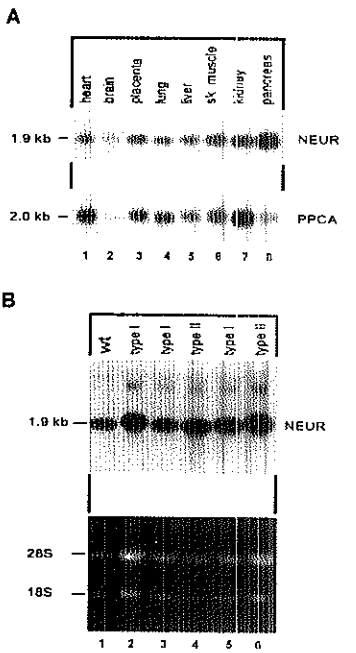

Figure 1. Neuraminidase mRNA expression. (*A*) Sequential hybridization of a multitissue Northern blot with the full-length neuraminidase cDNA (NEUR) and with the protective protein/cathepsin A (PPCA) cDNA. The size of the two transcripts was calculated on the basis of RNA markers. Exposure time for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

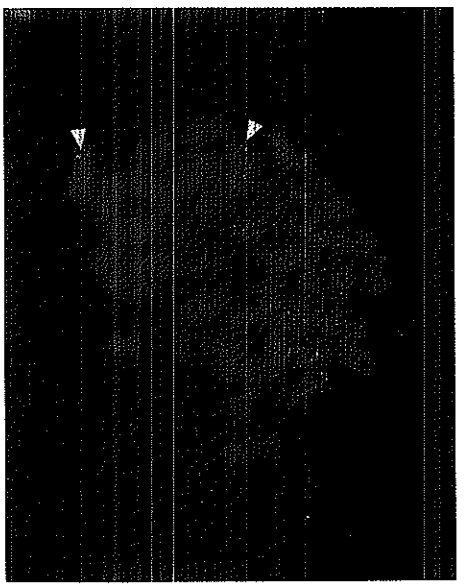

Figure 2. Chromosomal localization of human neuraminidase. Normal metaphase chromosomes were hybridized with a 3.5-kb neuraminidase genomic fragment, labeled with digoxigenin dUTP, and stained with antidigoxigenin antibodies. The white arrowheads indicate the hybridization signals on the chromatids of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

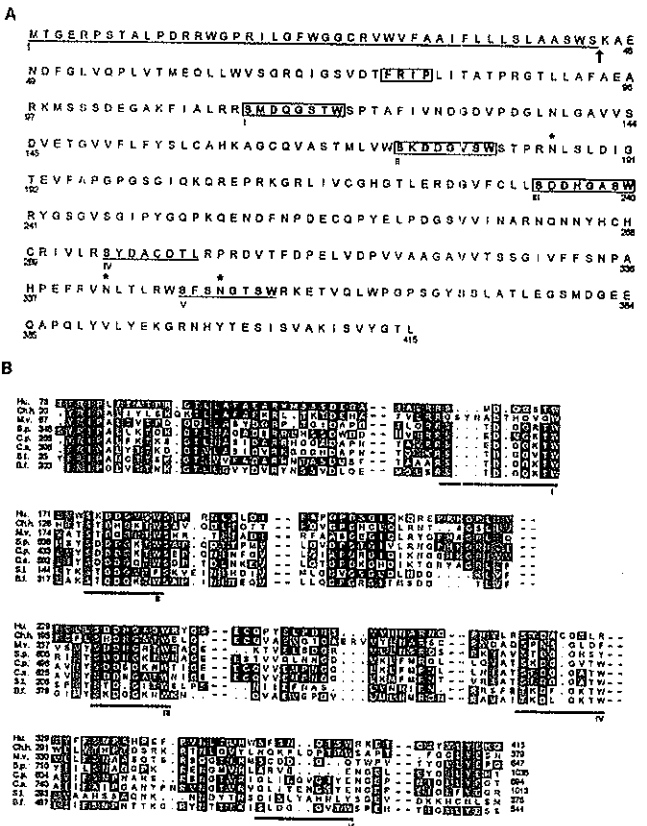

Figure 3. Predicted amino acid sequence of human neuraminidase cDNA and alignment with other sialidases. (*A*) The predicted amino-acid sequence, with residues 1–45 (underlined) representing the signal peptide, and amino acids 46–415 representing the neuraminidase polypeptide. The FRIP domain and three 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-

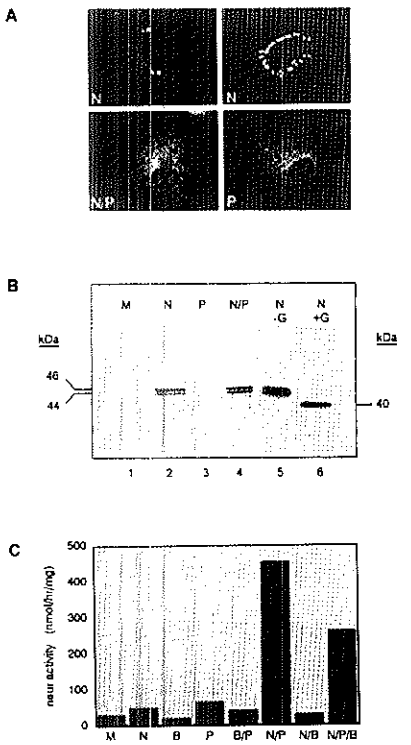

Figure 4. Immunocytochemical localization and neuraminidase activity in transiently transfected COS-1 cells. (*A*) Immunocytochemical localization of neuraminidase in COS-1 cells, transfected with the neur cDNA clone (N), or with both the neur and PPCA cDNAs (N/P), using affinity-purified α-neur antiserum. 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-

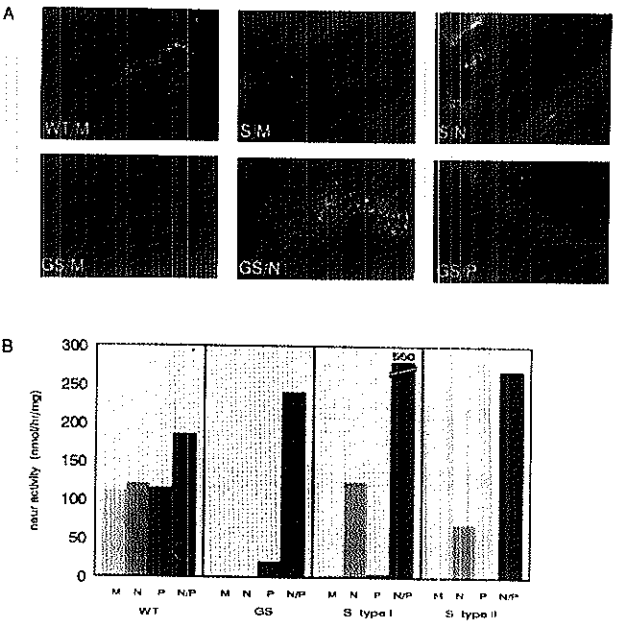

Figure 5. Correction of neuraminidase deficiency in sialidosis fibroblasts. (*A*) Immunocytochemical localization of neuraminidase in fibroblasts of a normal individual (WT), a sialidosis type I patient (S), and a galactosialidosis patient (GS), transfected with vector (M), neur cDNA (N), and PPCA cDNA (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

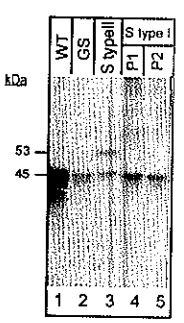

Figure 6. Immunoprecipitation of neuraminidase from sialidosis fibroblasts. Cultured fibroblasts from a normal individual (WT), an E.I galactosialidosis patient (GS), a type II sialidosis patient (S type II), and two siblings with type I sialidosis (S type I, P1, and P2) were labeled metabolically. The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

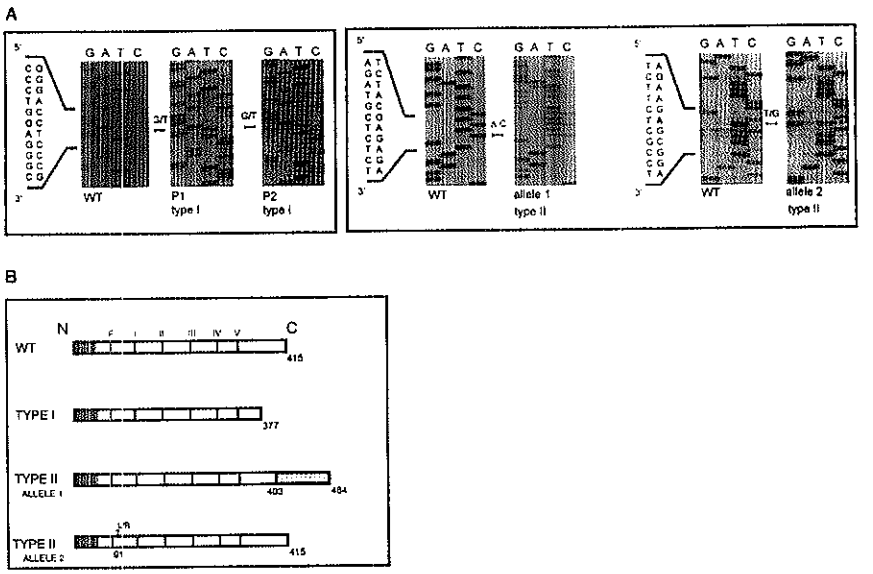

Figure 7. Mutations in the lysosomal neuraminidase gene of type I and type II sialidosis patients. (*A*) Partial nucleotide sequence of the neuraminidase cDNA from sialidosis patients. Total RNA was isolated from the fibroblasts of a normal individual (WT), the two siblings with type I sialidosis (P1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

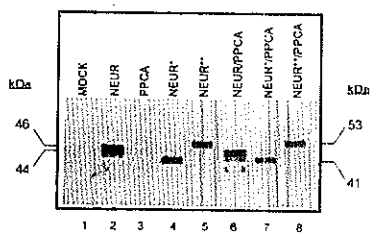

Figure 8. Western blot analysis of mutant neuraminidase from sialidosis patients. Western blot prepared with equal amounts of COS-1 cell lysates, transfected with vector (MOCK), neur cDNA (NEUR), PPCA cDNA (PPCA), or cotransfected with both the neur and PPCA cDNAs (NEUR/PPCA). In addition, two neuraminidase 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; Galiart et al. 1991), which suggests that the carboxypeptidase activity of PPCA is not

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

cDNA construct	Neuraminidase activity (nmol/hr per mg protein)	
	wild-type fibroblasts	sialidosis fibroblasts
mock	58 ± 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 (Froia 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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Chapter 5

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

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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

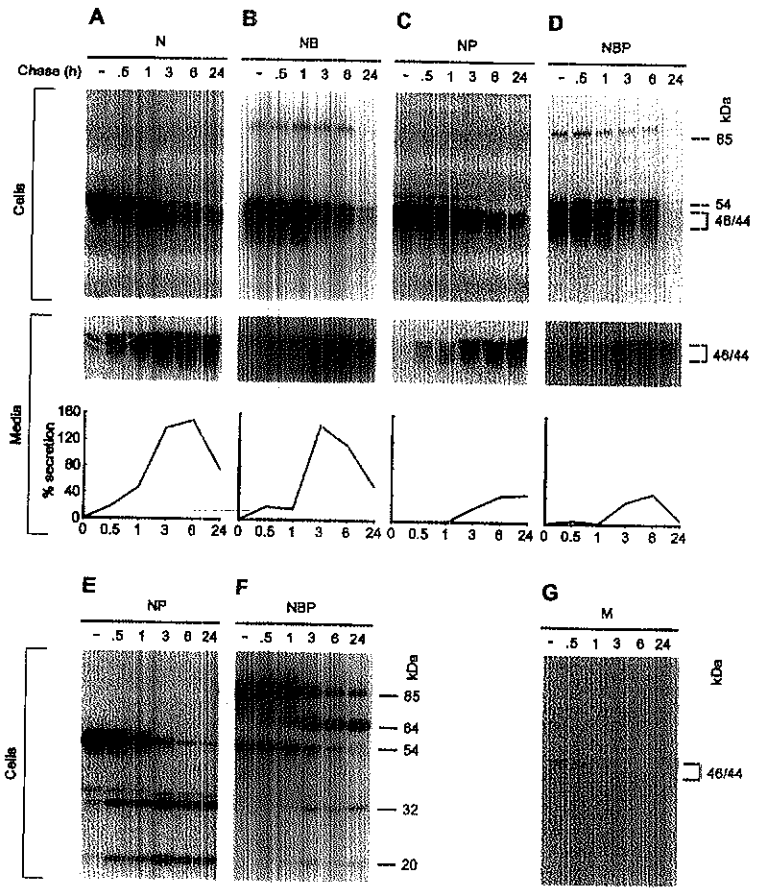

Fig. 1. Pulse-chase labeling. COS-1 cells were transfected with vector alone [denoted M in (G)] or with cDNAs encoding neuraminidase (N), β-galactosidase (B) or PPCA (P), in various combinations (A–F), labeled with [^{3}H]leucine for 1 h and chased with cold leucine for the time periods indicated. Cell 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

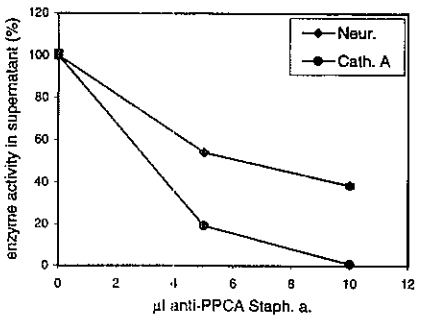

Fig. 2. Precipitation of neuraminidase activity from co-transfected COS-1 cells with anti-PPCA antibodies. Aliquots of lysates of COS-1 cells overexpressing both neuraminidase and PPCA were incubated under acidic conditions with increasing amounts of fixed *S.aureus* cells that had been pre-loaded with 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.

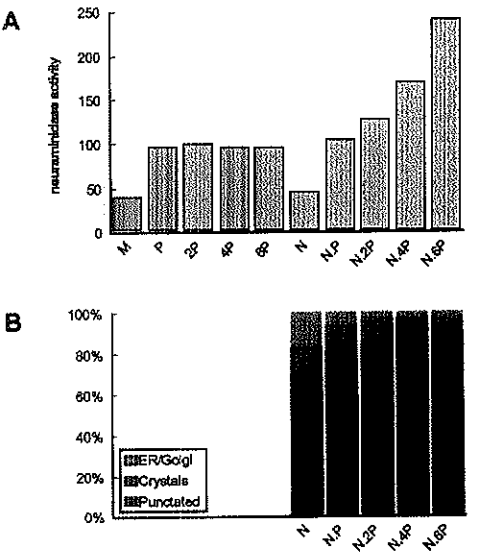

Fig. 3. Titration of neuraminidase. COS-1 cells were transfected with vector alone (M), or with increasing amounts of PPCA (P) cDNA (0.2, 0.4, 0.8 or 1.2 µg per 35 mm dish), alone or in combination with a fixed amount of neuraminidase (N) cDNA (0.2 µg per 35 mm dish). Cells were (A) assayed for neuraminidase 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

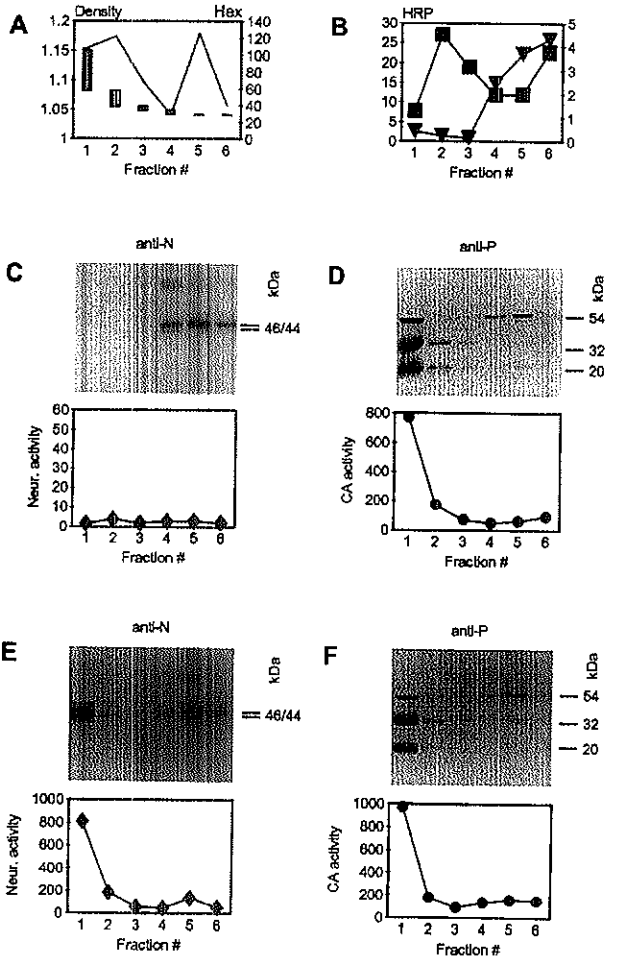

Fig. 4. Subcellular fractionation of COS-1 cells on density gradients. (A) Density distribution of Percoll gradients, as determined with density marker beads (Pharmacia), and profile of β-hexosaminidase activity (nmol/h/ml) in COS-1 cells overexpressing β-hexosaminidase β-chain. (B) After pulse-labeling 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

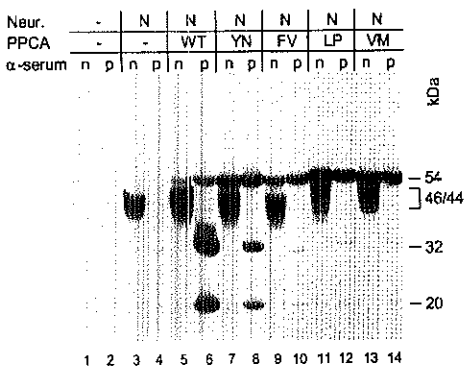

Fig. 5. Co-immunoprecipitation of PPCA mutants with neuraminidase. COS-1 cells, transfected with the neuraminidase cDNA alone, or in combination with a cDNA encoding wild-type PPCA, PPCA-YN, PPCA-FV, PPCA-LP or PPCA-VM, were metabolically labeled with [^{3}H]leucine and used for sequential immunoprecipitations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

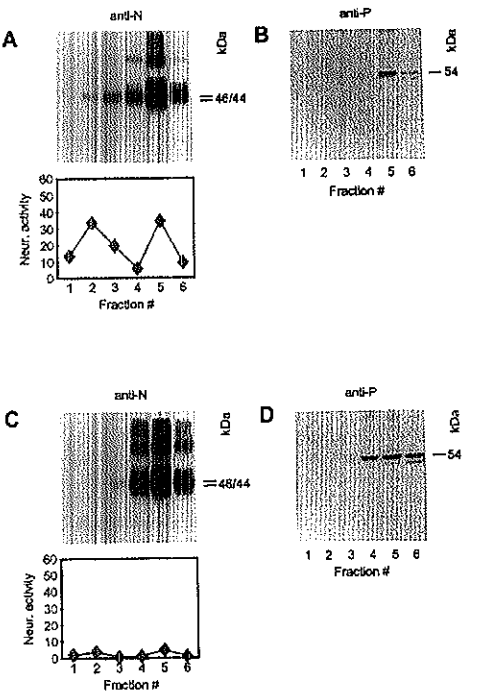

Fig. 6. Density fractionation of COS-1 cells expressing neuraminidase and PPCA mutants. Following co-transfection of COS-1 cells with neuraminidase cDNA and either (A and B) PPCA-YN or (C and D) PPCA-LP cDNA, cellular organelles were separated on Percoll gradients. Fractions were used to assay for neuraminidase 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.

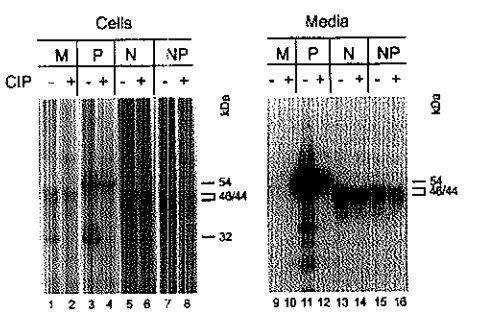

Fig. 7. Phosphorylation of neuraminidase. COS-1 cells were transfected as outlined in the legend to Figure 1, and metabolically labeled with [^{32}P]orthophosphate. Cell lysates and media were used for immunoprecipitation with anti-PPCA antiserum (lanes 3, 4, 11 and 12) or anti-Neur antiserum (all other 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 (Fomerod *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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Chapter 6

Processing of human lysosomal β-D-galactosidase: the C-terminal domain is essential for enzyme function

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Lysosomal β-D-galactosidase, which is deficient in the autosomal recessive disorder G_{M1}-gangliosidosis, is synthesized as an 85 kDa precursor which is C-terminally processed into a 66 kDa intermediate and a 64 kDa mature form. The ~20 kDa proteolytic fragment that is generated was thought to be degraded. We now have evidence that this polypeptide is at least partially maintained and remains boundto the 64 kDa chain. The C-terminal polypeptide was copurified with the high molecular weight β-galactosidase/cathepsin A complex isolated from mouse liver, and was identified by antibody stainingand amino acid sequence analysis. The N-terminal residue of the murine fragment is Ser^{546}, which corresponds to Ser^{544} in the human protein. In addition, this polypeptide was immunoprecipitated from human fibroblasts using anti-β-galactosidase precursor antiserum. Transfection of COS-1 cells with human β-galactosidase cDNA carrying two translation termination signals after codon 543 resulted in the synthesis of the 66 kDa intermediate that was not secreted into the medium, not processed to the 64 kDa form, and did not have enzymatic activity. These deficiencies were partially reversed by co-expression of the truncated protein with the C-terminal fragment starting at Ser^{544}. Our results suggest that the latter domain is required for proper folding, a prerequisite for intracellular transport, enzymatic activation and processing. Taken together, these data providea structural and functionalbasis for the idea of lysosomal β-galactosidase beinga two-subunitmolecule and give new significance to those mutations found in the C-terminal part of the molecule in G_{M1}-gangliosidosis patients.

Human lysosomal β-D-galactosidase (β-gal) is an exoglycosidase that removes β-ketosidically linked galactose residues from the carbohydrate chains of glycoproteins, sphingolipids and keratan sulfate (reviewed in Suzuki *et al.*, 1995). The metabolic storage disorders G_{M1}-gangliosidosis (GM) and Morquio B (MB) are caused by structural deficiencies in the β-gal gene (Suzuki *et al.*, 1995), while in galactosialidosis (GS) reduction of β-galactosidase activity is secondary to deficiencies in the lysosomal carboxypeptidase protective protein/cathepsin A (PPCA) (d'Azzo *et al.*, 1995; Kleijer *et al.*, 1996). Clinically distinct forms of GM have been described, varying in age of onset and severity. In GM patients multiple organs are affected, including central nervous system, skeleton and visceral organs. In contrast, MB patients show no neurologic deterioration or hepatosplenomegaly, but do present with skeletal abnormalities. Oligosaccharides carrying terminal β-linked galactose residues derived from either glycoproteins (in GM) or keratan sulfate (in MB) are the major storage materials in visceral organs, while G_{M1} and, to a lesser extent, G_{A1} gangliosides accumulate in the brains of GM patients (Suzuki *et al.*, 1995). GM pathology and central nervous system involvement is mimicked in β-gal knock-out mice (Hahn *et al.*, 1997).

Depending on the experimental procedure, β-gal can be isolated from mammalian tissues in monomeric, dimeric and tetrameric forms (Norden *et al.*, 1974; Yamamoto *et al.*, 1982), as well as in a high molecular weight complex with PPCA, lysosomal N-acetyl-α-neuraminidase and N-acetyl-galactosamine-6-sulfate sulfatase (Verheijen *et al.*, 1985; Yamamoto and Nishimura, 1987; Pshezhetsky and Potier, 1994, 1996). PPCA protects β-gal against proteolytic degradation, since in GS fibroblasts the half-life of the enzyme is drastically reduced (van Diggelen *et al.*, 1982).

Early immunoprecipitation studies showed that β-gal is synthesized as an N-glycosylated 85 kDa precursor, which is converted into a 66 kDa intermediate and a 64 kDa mature form (d'Azzo *et al.*, 1982; Suzuki *et al.*, 1995). In normal human fibroblasts the intermediate form accumulated when these cells were cultured in the presence of leupeptin, indicating that proteolytic processing could be involved in the conversion of the 66 kDa to the 64 kDa form (d'Azzo *et al.*, 1982). This treatment was sufficient to protect β-gal against degradation in GS fibroblasts, also resulting in the accumulation of the intermediate species (d'Azzo *et al.*, 1982).

A better understanding of β-gal processing was gained by cloning of the β-gal cDNA, which codes for a 76 kDa protein that carries seven potential N-glycosylation sites (Oshima *et al.*, 1988; Morreau *et al.*, 1989; Yamamoto *et al.*, 1990). Comparing the N-terminal sequence of the 64 kDa form and the amino acid sequence, predicted on the basis of the β-gal cDNA, has revealed that only 28

amino acid residues (including the signal sequence) are N-terminally removed during maturation of the precursor molecule. This suggested that the major processing of the β-gal precursor takes place at its C-terminus (Morreau *et al.*, 1989). However, the exact location of the cleavage site at the C-terminus of the precursor molecule has not been determined. Also, little attention has been given to the significance of the C-terminal domain in the biosynthesis of the enzyme, and its fate after maturation of the enzyme. In this report we show that the C-terminal peptide is not degraded after processing of the precursor molecule and that it remains in part associated with the N-terminal fragment. We also demonstrate that the C-terminal domain is required for correct biosynthesis of β-gal.

Experimental procedures

Cell culture – Human skin fibroblasts from a normal individual and patients with early infantile GS (Galjart *et al.*, 1988) or GM are deposited in the European Cell Bank, Rotterdam, The Netherlands (Dr. W. J. Kleijer). Fibroblasts and COS-1 cells (Gluzman, 1981) were maintained in Dulbecco's modified Eagle's medium, supplemented with antibiotics and 10% or 5% fetal bovine serum, respectively.

Enzyme and protein assays - β-gal and cathepsin A activities were measured with 4-methylumbelliferyl-β-D-galactopyranoside and the N-blocked dipeptide carbobenzoxy-phenylalanyl-alanine as substrates, respectively, according to (Galjaard, 1980; Kleijer *et al.*, 1996). Total protein concentrations were quantitated with bicinchoninic acid (Smith *et al.*, 1985) following the manufacturer's guidelines (Pierce Chemical Co.).

Enzyme purification – Mouse livers (600 g wet weight) were extracted from freshly sacrificed C57/Bl6 and Bl6/CBA mice and used for preparation of a lysosomal-mitochondrial extract as described (Scheibe *et al.*, 1985). The extract was then concentrated by ammonium sulfate precipitation (Scheibe *et al.*, 1990) and pelleted proteins were resuspended in 20 mM EDTA, 10 mM tartaric acid, 5 mM β-mercaptoethanol, pH 5.0 (buffer A), and desalted on a BioGel P6DG column (2.5 x 50 cm, equilibrated in buffer A, 1 ml/min). Following concentration in a stirred-cell concentrator (Amicon), proteins were separated on a Sephadex G-200 column (5 x 45 cm, equilibrated in buffer A, 1ml/min); fractions eluting right after the void volume in which both β-gal and cathepsin A activities peaked were pooled and concentrated in Centriprep C-30 concentrators (Amicon). This material was passed through a Sephacryl S-400 column (1.5 x 165 cm, equilibrated in buffer A, 0.4 ml/min); fractions highest in β-gal activity were pooled and loaded on p-aminophenyl β-D-thiogalactopyranoside-agarose (Sigma) column (1 x 8 cm, equilibrated in buffer A containing 100 mm NaCl (buffer B), 0.4 ml/min). This column was washed with buffer B and eluted with buffer B containing 500 mM D-

galactonic acid γ-lactone, and fractions highest in β-gal activitywere pooled. Before SDS-PAGE, D-galactonic acid γ-lactone was removed by dialysis against buffer B.

Western blotting and protein sequence analysis – For analytical purposes, western blots were prepared from 12.5% SDS-polyacrylamide gels and probed as described (Bonten *et al.*, 1995). Blots were developed with colorimetric substrates (Bonten *et al.*, 1995). For preparative western blotting, SDS-polyacrylamide gels were prepared with piperazine diacrylamine (BioRad) as crosslinker. Following electrophoresis, proteins were transferred to Problott PVDF membrane (Applied Biosystems) according to manufacturers' instructions. Blots were briefly stained with Coomassie Brilliant Blue and individual bands were excised, which were processed for N-terminal amino acid sequence analysis by automated Edman degradation using an Applied Biosystems 470 sequenator. Initial yield of amino acid derivative was 35 pmol.

Immunoprecipitation and deglycosylation – Metabolic labeling and harvesting of cultured human fibroblasts and COS-1 cells, immunoprecipitation, and deglycosylation of immunoprecipitated proteins were performed as described (Zhou *et al.*, 1996). Rabbit antisera against the β-gal precursor overexpressed in insect cells (anti-BV85), against mature β-gal isolated from human placenta (anti-n64) (Verheijen *et al.*, 1982) and against a bacterially expressed GST-β-gal$^{Ser544-Val677}$ fusion protein (anti-24) were used in immunoprecipitations.

cDNA mutagenesis – The β-gal cDNA (Morreau *et al.*, 1989) was truncated after nucleotide 1679 (corresponding to codon 543) with two stop codons through a PCR-based strategy using sense primer A(5'GTGGATGGGATCCCCCAGGG3', corresponding to β-gal cDNA nucleotides 1389-1407, including the Bam HI site at nucleotide 1397) and antisense primer B (5' CCCCAAGCTTCATCATGAGTTGTGGGCCCAGGCTT3',containing an adapter sequence carrying a Hind III site and two stop codons, and a sequence corresponding to β-gal cDNA nucleotides 1679-1660). Using the full-length β-gal cDNA as template and primers A and B, a fragment corresponding to β-gal nucleotides 1389-1679 was amplified, carrying two translation termination signals and a Hind III site at its 3' end. After Bam HI/Hind III digestion, this fragment was linked to the Bam HI fragment of normal β-gal cDNA ending at nt 1397, to replace all of the β-gal cDNA located 3' of the BamHI site at nt 1397. At its 3' end the PCR product was ligated to a Hind III site in the 3' flanking vector sequence.

To create a cDNA that codes for a β-gal deletion mutant in which the β-gal signal sequence (ending with Tyr^{28}) is linked to Ser^{544}, nucleotide 134 of the β-gal cDNA was linked to nucleotide 1680 by adapting the PCR-based *in vitro* mutagenesis strategy of Higuchi *et al.* (1988). To this end primers were synthesized that span the fusion site: sense primer C (5'CGCAATGCCACCTCCAACTACACGCTCCC3', corresponding to nucleotides

123->134-1680->1696) and antisense primer D (5'CGTGTAGTTGGAGGTGGCATTGCGCAAGC3', corresponding to nucleotides 1691->1680-134->118). Using the β-gal cDNA (cloned into pBluescript) as template in separate PCR reactions, primer D was combined with a sense primer that anneals at the 5' flanking vector sequence, and primer C was combined with an antisense primer that anneals at the 3' flanking vector sequence. Thus, in the first reaction (primer D) a fragment was generated, of which the terminal 24 nucleotides had the same sequence as the first 24 nucleotides of the product of the second reaction (primer C). These two fragments were annealed at their homologous termini and used as template for a third PCR reaction using the primers that anneal at 3' and 5' flanking vector sequences. In this reaction a fragment was amplified that corresponds to the β-gal cDNA lacking nucleotides 135-1679.

The resulting constructs were verified by sequence analysis and subcloned into expession vectors pCD-X (Okayama and Berg, 1982; Galjart *et al.*, 1988) and in pSC-TOP (Rusconi *et al.*, 1990), modified according to Fornerod *et al.* (1995).

Transfections – cDNAs were transfected into subconfluent COS-1 cells using calcium phosphate precipitate, as described by Chen and Okayama (1987), or the Superfect reagent, according to the manufacturer's instructions (Qiagen). Eightyfour hours post-transfection cells were metabolically labeled with L-[4,5-^{3}H]-leucine (50 μCi/ml culture medium) for 16 hours and processed for immunoprecipitation; alternatively, for analysis of enzyme activity cells were harvested by trypsinization 72 hours post-transfection.

Uptake experiments – Early infantile GS fibroblasts (Galjart *et al.*, 1988) were grown to confluency in 35 and 85 mm dishes. PPCA precursors were prepared and added to the culture medium of these cells, as described earlier (Zhou *et al.*, 1996). After four days of uptake, fibroblasts in 35 mm dishes were harvested by trypsinization for analysis of enzyme activities. Cells in 85 mm dishes were cultured for 3 days in the presence of exogenous PPCA precursors; culture was then continued in the presence both of L-[4,5-^{3}H]-leucine (50 μCi/ml) and exogenous PPCA precursors for 18-20 hours before processing cells for immunoprecipitation (Zhou *et al.*, 1996).

Results

Copurification of the β-gal C-terminal fragment with the β-gal 64 kDa chain – During purification of the multienzyme complex containing β-gal, PPCA and neuraminidase, a lysosomal-mitochondrial fraction was prepared from mouse liver and fractionated by gel filtration on a Sephadex G-200 column. All three enzyme activities co-eluted right after the void volume (peak I), while oligomeric forms of β-

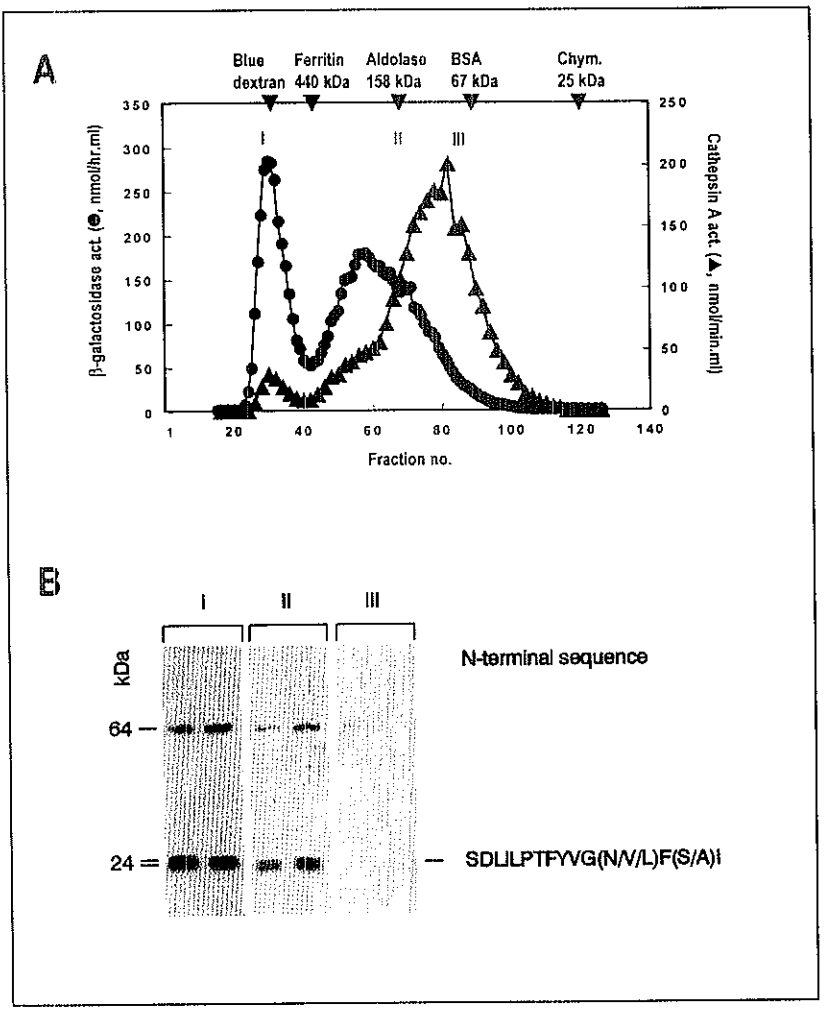

Fig. 1. Gel filtration of lysosomal-mitochondrial extract from mouse liver.
A. Elution profiles of β-galactosidase (●) and cathepsin A (▲) activities. Elution positions of marker proteins are indicated on top (▼). B. Western blots made with fractions from different regions of the elution profile (I, II and III, as indicated in (A)), and probed with anti-β-gal precursor antibodies (anti-BV85). N-terminal sequence of the 24 kDa band is given at right of the blot.

gal and PPCA eluted independently, i.e. PPCA dimers (~104 kDa) and β-gal tetramers (~256 kDa) and dimers (~128 kDa) (Fig. 1A, neuraminidase activity not shown). Surprisingly, in samples displaying β-gal activity not only the 64 kDa form but also two bands with apparent molecular weights of 24 and 22 kDa were clearly

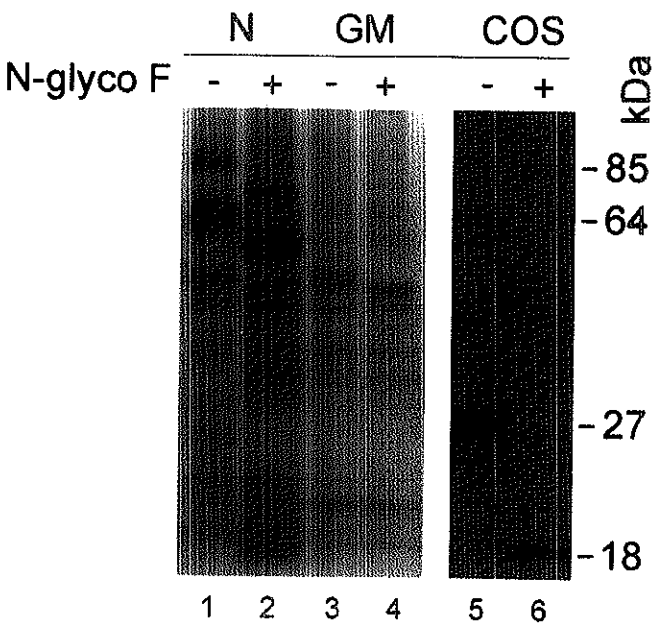

Fig. 2. Immunoprecipitationof the β-galactosidase C-terminal fragment. Cells were metabolically labeled with [^{3}H]-leucine and processed for immunoprecipitation using antibodies that recognize precursor and mature forms of β-galactosidase (anti-BV85 and anti-n64). Precipitated proteins were deglycosylated with N-glyco(sidase) F (+), or mock-incubated (-). Normal (N, lanes 1 and 2) and G_{M1}-gangliosidosis (GM, lanes 3 and 4) fibroblasts were used, as well as COS-1 cells transfected with the β-gal-C cDNA (lanes 5 and 6). From the COS-1 cells also the endogenous mature β-galactosidase polypeptide was immunoprecipitated (64 kDa, lane 5).

recognized by anti-β-gal precursor antibodies (Fig. 1B). Furthermore, these low molecular weight proteins copurified with the high molecular weight β-gal activity (peak I), after further purification of the multienzyme complex on Sephacryl S400 gel filtration and β-gal affinity chromatography columns (data not shown). Subsequent N-terminal amino acid sequence analysis of the 24 kDa protein provided a sequence identical to mouse β-gal residues Ser^{546}-Ile^{560} (Fig. 1B), implying that the 24 kDa band represents a C-terminal fragment of the β-gal precursor molecule.

From this we inferred that the 24 kDa polypeptide is the C-terminal fragment that is generated during processing of the β-gal precursor into the 64 kDa mature

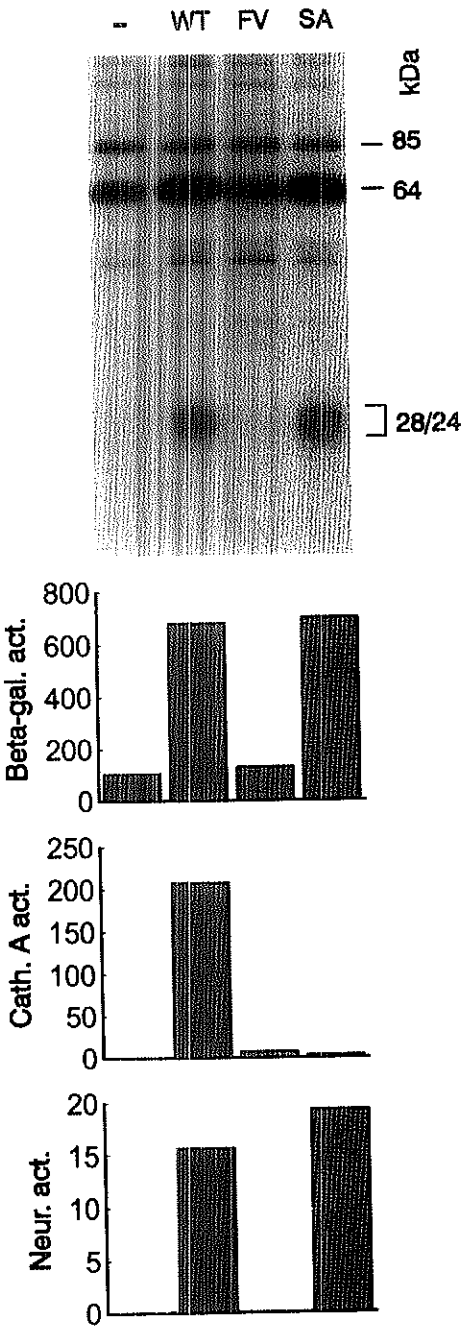

Fig. 3. Uptake experiment using galactosialidosis fibroblasts. PPCA mRNA-negative galactosialidosis fibroblasts were cultured in normal medium (-), or following addition of exogenous PPCA precursors to the medium: either wild-type PPCA (WT), PPCA-Phe412Val (FV), or PPCA-Ser150Ala (SA). After metabolic labeling, cell lysates were extensively precleared and used for immunoprecipitation with anti-BV85 and anti-n64 antisera. Recovered proteins were separated by SDS-PAGE (top panel). Alternatively, cells were harvested with trypsin, washed in PBS, and assayed for β-galactosidase (nmol/hr.mg protein), cathepsin A (nmol/min.mg protein) and neuraminidase (nmol/(hr.mg protein) activities (lower three panels).

form. Thus, the cleavage site in the murine β-gal precursor lies between Ser^{545}-Ser^{546}, corresponding to Ser^{543}-Ser^{544} in the human protein. Endoproteolysis of the human protein at this site would yield two fragments of 66 kDa (Gln^{29}-Ser^{543}) and 19 kDa (Ser^{544}-Val^{677}-COOH), corresponding closely to those experimentally found, adding 2 kDa for each N-linked oligosaccharide. Our data also suggest that the C-terminal fragment is not fully degraded but remains in part associated with the N-terminal chain after processing, both in oligomeric forms and in the multienzyme complex.

Immunoprecipitation of C-terminal fragment from human fibroblasts. - To verify whether the 24 kDa fragment is also retained after normal processing of the human precursor, radiolabeled human fibroblasts were subjected to immunoprecipitation with anti-β-gal antisera that recognize both precursor and mature forms of the protein (anti-BV85 and anti-n64, respectively). Only the 85 kDa β-gal precursor and the 64 kDa mature form were seen in normal fibroblasts (Fig. 2, lane 1). However, since the human C-ter β-gal fragment carries two glycosylation sites, and N-linked oligosaccharides can significantly alter the electrophoretic behavior of proteins, especially of small peptides, the immunoprecipitated material was deglycosylated with N-glycosidase F. This allowed the detection of a ~18 kDa band (Fig. 2, lane 2). Notably, all molecular weight forms of β-gal, including the 18 kDa band, were absent from β-gal mRNA-deficient G_{M1}-gangliosidosis fibroblasts (Fig. 2, lane 4). These results suggest that the C-terminal fragment is retained in the mature form of β-gal in human fibroblasts. The fact that the 64 and 18 kDa bands are not of equal intensity may be due to the 6-fold difference in number of leucine residues between these fragments.

To further ascertain whether the 24 kDa peptide is indeed a component of mature and active β-gal, we used PPCA mRNA-negative GS fibroblasts in the presence of exogenous PPCA precursor. It is well establish ed that endocytosis of PPCA precursor by these cells rescues mature β-gal from rapid degradation and restores its enzymatic activity to nearly normal levels (van Diggelen *et al.*, 1982; Galjart *et al.*, 1988; Galjart *et al.*, 1991). Wild-type PPCA (WT) as well as two PPCA mutants were used in the uptake experiments: PPCA-Phe412Val (FV), originally identified in a mild late-infantile case of GS, is only partially transported to lysosomes, where it is unstable (Zhou *et al.*, 1991; Zhou *et al.*, 1996); PPCA-Ser150Ala (SA) is an engineered active-site mutant, which has no cathepsin A activity, but, like wild-type PPCA, restores β-gal, neuraminidase and cathepsin A activities in PPCA-deficient cells (Galjart *et al.*, 1988; Galjart *et al.*, 1991). GS cells were treated with these unlabeled PPCA precursors for three days. After adding [^{3}H]-leucine to the medium and an additional day of culturing, the cells were used for immunoprecipitation with anti-BV85 and anti-n64 antisera. Fig. 3 shows that,

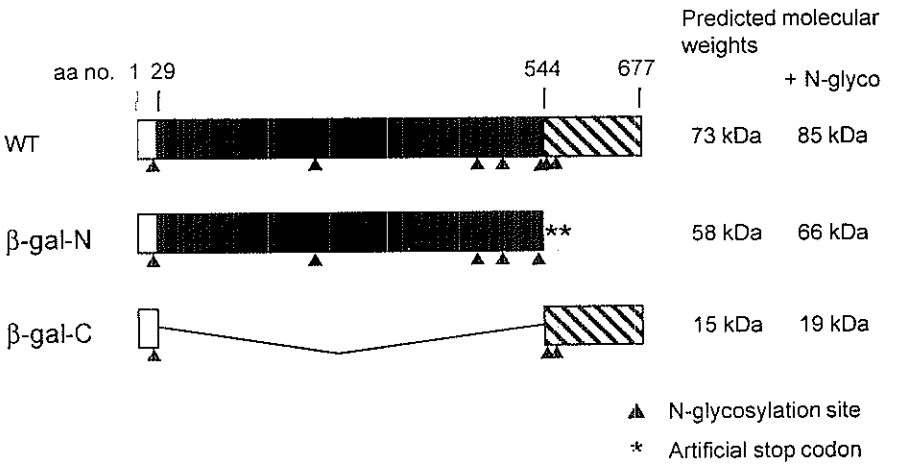

Fig. 4. Scheme of wild-type β-galactosidase and engineered variants. Deletion mutants encoded by cDNA constructs β-gal-N and β-gal-C are compared to wild-type β-galactosidase (WT). Molecular weights of the non-glycosylated forms were calculated on the basis of their predicted amino acid sequences, and for the glycosylated forms 2 kDa was added for each N-glycosylation site.

while the level of the β-gal precursor was comparable in all cells, restoration of β-gal activity was achieved after uptake of PPCA-WT or -SA, but not after uptake of PPCA-FV. The rise in activitywas parralleled by increased amounts of both the 64 and 28/24 kDa forms. Similar results were obtained using an unrelated strain of PPCA mRNA-deficient GS fibroblasts (data not shown). These observations confirm unequivocally that the C-terminal fragment is a part of mature β-gal.

The C-terminal fragment is essential for β-gal biosynthesis – To study the role of the C-terminal domain in the biosynthesis and intracellular routing of β-gal, we constructed cDNAs that separately encode the N-terminal part (Met^{1}-Ser^{543}: β-gal-N), and the C-terminal domain (Ser^{544}-Val^{677}: β-gal-C). To ensure translocation of the latter polypeptide to the endoplasmic reticulum, its cDNA was positioned directly 3' to the β-gal signal sequence (see Experimental Procedures). These polypeptides and their estimated molecular weights are schematically compared with those of wild-type β-gal, before and after N-linked glycosylation in Fig. 4. The mutagenized cDNAs, wild-type β-gal cDNA and PPCA cDNA, were used in single and cotransfections of COS-1 cells. Transfected cells were either assayed for β-

gal activity or metabolically labeled with [^{3}H]-leucine. Radioactive cell lysates were processed for immunoprecipitation using anti-β-gal precursor (anti-BV85) and/or anti-mature β-gal (anti-n64) antisera.

Transfection of COS-1 cells with β-gal-N cDNA gave rise to a polypeptide (Fig. 5A, lane 7), that is comparable in size to the 66 kDa intermediate form, which accumulates in COS-1 cells that overexpress full-length β-gal and are cultured in the presence of leupeptin (Fig. 5A, lanes 4 and 6). Significantly, after coexpression of β-gal-N and β-gal-C the former polypeptide is partially processed to ~64 kDa (Fig. 5A, lane 8), which is also seen in triple-transfected cells that express β-gal-N, β-gal-C and PPCA (Fig. 5A, lane 9). Furthermore, leupeptin also blocks the processing of β-gal-N to 64 kDa (Fig. 5A, lane 10). The 66 kDa form of β-gal-N is precipitated by both anti-β-gal precursor and anti-mature β-gal antisera, but after conversion to the 64 kDa form, it is no longer recognized by the former antiserum (data not shown).

Neither of these antisera precipitated β-gal-N from the culture medium of cells transfected with its cDNA (Fig. 5B, lanes 14 and 15); however, in medium of cells that coexpress β-gal-N and β-gal-C the 66 kDa polypeptide was readily detected (Fig. 5B, lanes 17 and 18), albeit at lower levels than the full-length β-gal precursor (Fig. 5B, lane 12). This demonstrates that the C-terminal domain is required for secretion of β-gal. Significantly, transfection with either β-gal-N or β-gal-C cDNAs did not increase β-gal activity of COS-1 cells, but, when expressed together, these polypeptides generated enzymatic activity slightly above background level (Fig. 6).

A 27 kDa polypeptide was precipitated using anti-β-gal precursor antiserum from cells transfected with β-gal-C cDNA (Fig. 5A, lane 11). Medium samples showed a broad band of 32-28 kDa (Fig. 5B, lane 16), which may be due to differential glycosylation. Secretion of this peptide is markedly reduced when it is co-expressed with β-gal-N (Fig. 5B, lane 18). After deglycosylation, β-gal-C (Fig. 2, lane 6) co-migrated exactly with the deglycosylated 18 kDa band precipitated from normal human fibroblasts (Fig. 2, lane 2).

In COS-1 cells cotransfected with full-lenght β-gal and PPCA cDNAs, the 64 kDa species is stable and readily detectable, and small amounts of the corresponding 20 kDa C-terminal fragment were precipitated from these cells using anti-precursor antiserum (Fig. 5A, lane 5). In contrast, both the 64 and the 20 kDa fragments were rapidly degaded in cells singly transfected with β-gal cDNA (Fig. 5A, lane 3), but were protected against degradation by culturing the cells in presence of leupeptin (Fig. 5A, lane 4). The fact that only a 20 kDa low-molecular weight fragment is precipitated from COS-1 cells overexpressing wild-type β-gal indicates that the C-terminal domain of the precursor molecule synthesized in these cells is differently glycosylated compared to β-gal-C (Fig. 5A,

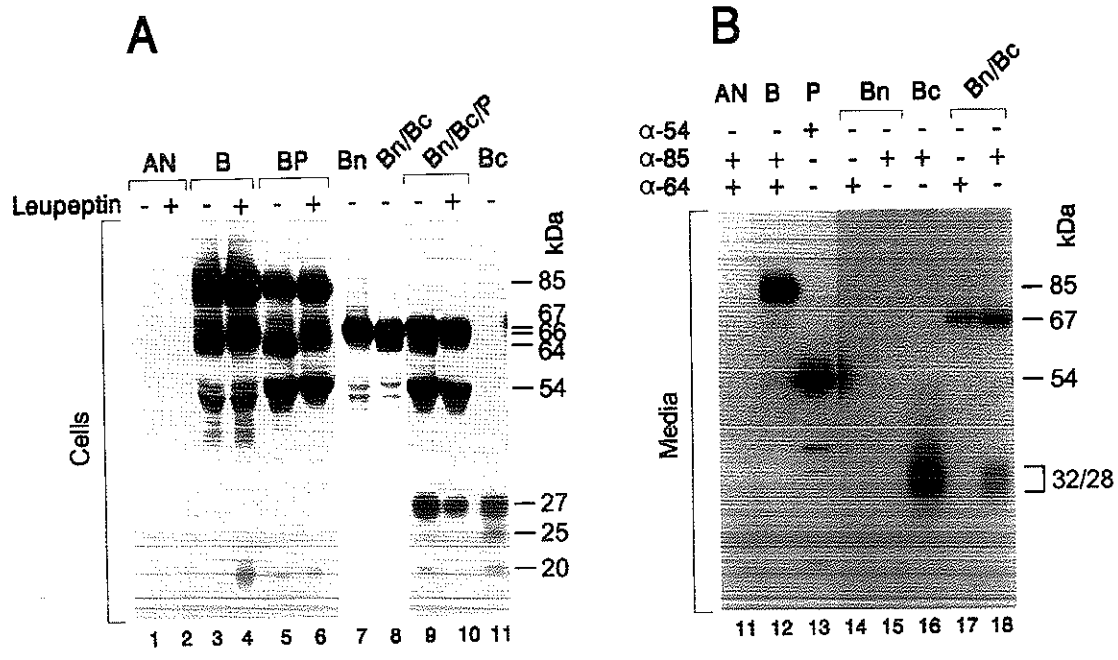

Fig. 5. Transient expression of normal and *in vitro* mutagenized forms of β-galactosidase. COS-1 cells were transfected with the β-galacosidase cDNA either in the sense (B) or antisense (AN) orientation, or with the β-gal-N (Bn), the β-gal-C (Bc), or the PPCA (P) cDNA, or with various combinations of these cDNAs. Cells were cultured in the absence (-) or presence (+) of leupeptin. After metabolic labeling, cell lysates (panel A) and medium samples (panel B) were used for immunoprecipitation with anti-BV85 (α-85) and/or anti-n64 (α-64) and/or anti-PPCA precursor (α-54) antisera.

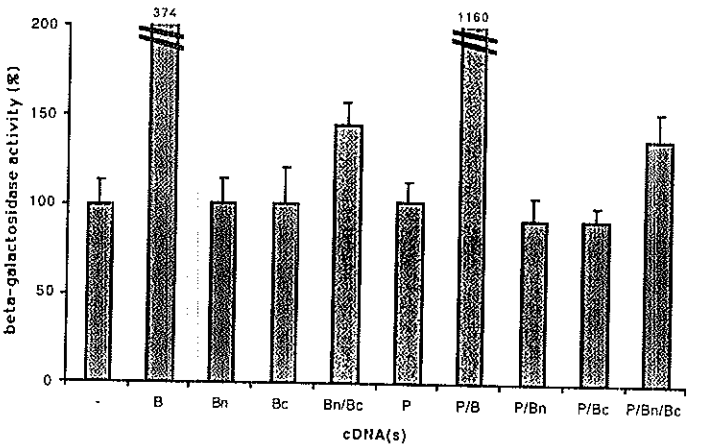

Fig. 6. β-galactosidase activity in transfected COS-1 cells. COS-1 cells were either singly transfected with one of the cDNAs described in the legend to Fig. 5, or cotransfected with various combinations of these cDNAs. Cells were harvested by trypsinization, washed with PBS, and assayed for β-galactosidase activity. Specific activities are expressed as percentage of the endogenous COS-1 cell activity, and are the averages of four to six determinations. Error bars: standard deviation.

lane 11) and the C-terminal fragment precipitated from human fibroblasts (Figs. 2 and 3).

Discussion

The 677aa/85 kDa precursor of human lysosomal β-gal is primarily proteolytically processed at its C-terminus into a 66/64 kDa mature form (d'Azzo *et al.*, 1982). The fate of the ~24 kDa fragment that is generated in this process had not been studied. Interestingly, our purification and immunoprecipitation studies presented here suggest that this polypeptide is retained as part of the mature form of β-gal. At present its specific role is in the catalytic functions of mature β-gal is not clear. We show that the separately expressed N-terminal fragment has no enzymatic activity and is not secreted. The C-terminal domain, co-expressed *in trans*, is able to restore these deficiencies to some extent. Since correct folding is a prerequisite for intracellular transport and catalytic activity (reviewed in Hammond and Helenius, 1995; Thomas and Beaudet, 1995; Brooks, 1997; Gottesman *et al.*, 1997), we infer from our data that the β-gal precursor is dependent on its C-terminal domain for

acquisition of the proper tertiary structure. In addition, the polypeptide may be involved in protein-protein interactions in homooligomeric or heteromultimeric forms of β-gal.

The importance of the β-gal C-terminal domain is illustrated by the finding of amino acid substitutions in this domain in G_{M1}-gangliosidosis patients: R590H, K578R, E632G (Boustany *et al.*, 1993), G640T (Hilson *et al.*, 1994), Y591C, and Y591N (Morrone *et al.*, 1997). However, it is not known how these mutations affect the behavior of the protein, and therefore it will be interesting to analyze the effects of these mutations at the biochemical and structural levels. The importance of the C-terminus of mammalian β-gal is strengthened by the fact that it contains a small domain (residues 577-592) that is conserved in nine β-galactosidases and related proteins from bacterial, fungal, nematode, and plant species (Taron *et al.*, 1995; Gutshall *et al.*, 1997; Ito and Sasaki, 1997). Interestingly, all of the mutations mentioned above except E632G lie in this area. Specifically, Lys^{578} and Arg^{590}, are identical in all organisms except in *Aspergillus niger*, while Tyr^{591}, is maintained in four of these species and is next to the fully conserved Trp^{592}.

The fact that the C-terminal fragment isolated from murine liver starts at Ser^{546} does not exclude the possibility that this peptide may be derived from a larger, primary cleavage product that is trimmed at its N-terminus. However, culturing human fibroblasts in the presence of leupeptin leads to accumulation of the 66 kDa intermediate (d'Azzo *et al.*, 1982). This is in agreement with our findings that the cDNA encoding the large domain of human β-gal (residues 1-543) is translated into a ~66kDa intermediate, which can be further processed into a 64 kDa species. Therefore, it is likely that the human β-gal precursor is initially cleaved at or close to the N-terminal side of Ser^{543}-Ser^{544}, followed by further processing of the large domain at Arg^{530} by a trypsin-like protease, as predicted earlier (Yamamoto *et al.*, 1990). It is noteworthy that the precursor, obtained from overexpressing insect cells, can be readily converted to a 64 kDa form by mild trypsin digestion (Takiyama *et al.*, 1993).

Few examples of C-terminal processing of lysosomal enzymes have been described. Lysosomal α-glucosidase undergoes both N- and C-terminal processing; a ~20 kDa peptide is removed from the C-terminus, but it is not known whether it remains associated with the rest of the mature molecule (reviewed in Hirschhorn, 1995). In contrast, the 18 amino acid residues that are removed from the C-terminus of human lysosomal β-glucuronidase are required for its binding to the microsomal esterase egasyn, by which a fraction of the enzyme precursor remains in the ER (Jackman *et al.*, 1990). In addition, when synthesized without its C-terminus, the enzyme is catalytically impaired, hypophosphorylated and increasingly secreted (Islam *et al.*, 1993). Thus, the C-terminal domain of the β-glucuronidase precursor appears to be essential for its folding process and

compartmentalization.

In contrast to the examples discussed above, many more proteins are known to undergo N-terminal processing (see for reviews Baker *et al.*, 1993; Shinde and Inouye, 1993; Shinde and Inouye, 1994; Eder and Fersht, 1995). The N-terminal proregions of various pro- and eukaryotic proteases, and polypeptide hormones function as intramolecular chaperones; they are known to be required for proper protein folding and (consequentially intracellular transport) by reducing kinetic barriers between un- or partially folded forms and mature molecules. They are also powerfull inhibitors of the proteolytic enzymes, and do not necessarily become superfluous after processing; for example, the extracellular form of cathepsin B remains in a noncovalent complex with its propeptide and this conformation accounts for its stability in this environment (Mach *et al.*, 1994; Cygler *et al.*, 1996). Our findings and those of others now begin to underscore the importance of C-terminal processing.

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Chapter 7

Discussion

Discussion

Structure and transport

The work presented in this thesis suggests that PPCA, neuraminidase and β-galactosidase may be affected in their intracellular localization in lysosomal disease. Using the COS-1 cell overexpression system, we have made the following observations. Whereas a fraction of newly synthesized wild-type PPCA and β-galactosidase precursors is secreted to the extracellular space, the early infantile PPCA mutants as well as a C-terminally truncated form of β-galactosidase are not detectable in the culture medium. For this class of PPCA mutants we have shown that they are neither present in lysosomes, but accumulate in the ER. Furthermore, our immunofluorescence studies have demonstrated that the two late infantile PPCA mutants as well as neuraminidase (in the absence of human PPCA) also accumulate in the ER, but they can, in addition, be immunoprecipitated from the culture medium. Thus the intracellular retention of these polypeptides is not complete. Furthermore, small amounts of the late infantile PPCA mutants can be detected in lysosomes; neuraminidase, however, is not detected in lysosomes when expressed in the absence of PPCA, but when accompanied by one of the late infantile PPCA mutants a small amount of neuraminidase is found in these organelles. Finally, coexpression of the latter protein with wild-type PPCA decreases the level of its ER localization, and significantly augments its lysosomal presence. The (aberrant) trafficking of each of these proteins will be discussed in the following paragraphs.

The observed difference in intracellular transport between the two classes of PPCA mutants may be explained on the basis of structural differences between the various mutants, including those previously identified by Shimmoto *et al.* (1993). The impact of the disease-related amino acid substitutions on the structure of the PPCA molecule has been studied through modeling these alterations in the three-dimensional model of the PPCA precursor (Rudenko *et al.*, 1998). Interestingly, also at the atomic level the early infantile mutations differ from those of late-infantile patients. The nine amino acid residues, that are affected in early infantile PPCA mutants, are located in the protein interior, and their disease-related substitutions are incompatible with proper folding of the PPCA precursor. In contrast, the Phe412Val and Tyr221Asn mutations only locally perturb the structure of PPCA. The former substitution is located on the PPCA dimer interface and introduces a hydrophobic cavity, which destabilizes the association of one monomer with the other; this is in line with the largely monomeric nature of this mutant, which is much less stable than the wild-type dimeric form (Chapter 3 and Zhou *et al.*, 1991). Furthermore, in the PPCA

precursor model (Chapter 1, Section 1.6), Tyr221 interacts with residues that are located immediately C-terminally from the enzyme-inactivating segment that occupies the active site. The Tyr221Asn substitution is expected to introduce small rearrangements in this segment, which may be responsible for the reduced stability of this mutant following proteolytic processing in the lysosome.

Like the PPCA mutants found in GS patients, various mutant proteins identified in other genetic disorders also accumulate in the ER. In various lysosomal storage disorders, mutants of lysosomal enzymes are neither phosphorylated, nor secreted, proteolytically processed, or transported to the lysosome (Neufeld, 1991; Amara *et al.*, 1992; Brooks, 1997). Additional examples of ER-retention are various mutants of the low-density lipoprotein receptor found in class 2 familial hypercholesterolemia, the Z mutant of α_1-antitrypsin associated with hereditary emphysema, and the most common mutant of the cystic fibrosis transmembrane conductance regulator (CFTR), ΔF508 (reviewed in Amara *et al.*, 1992; Brooks, 1997).

As described above for PPCA, the mutations found in other disease-associated ER-accumulating proteins are often incompatible with their proper folding (reviewed in Thomas *et al.*, 1995; Carrell and Gooptu, 1998). For example, *in vitro* thermodynamic studies have shown that a 67 amino acid fragment of CFTR encompassing ΔF508 cannot go through the folding process, as the mutation dramatically reduces the stability of a folding intermediate. Similarly, *in vitro* refolding experiments have revealed a large difference between a peptide fragment of wild-type fibrillin, and that of a fibrillin mutant involved in Marfan syndrome. It is thought that the misfolded polypeptides expressed in inherited diseases are retained by the quality control processes that operate on all proteins synthesized on ER membranes (reviewed in Hammond and Helenius, 1995; Brooks, 1997). Protein folding in this compartment involves transient interactions with resident chaperones as BiP, calnexin, calreticulin, GRP94 and HSP47. Misfolded proteins may expose hydrophobic surface elements, free sulfhydryl groups, or partially glucose-trimmed N-linked oligosaccharides, or may aggregate; through these and other yet unidentified characteristics the molecular misfits remain bound to ER chaperones without being released. Following their ER accumulation, misfolded proteins are often subject to proteolytic degradation. Although not yet fully inderstood, the available data suggest that, after prolonged association with ER chaperones, these proteins are translocated across the ER membrane to the cytoplasm, and hydrolyzed in proteasomes, which are localized on the cytoplasmic face of the ER membrane (reviewed in Brodsky and McCracken, 1997; Kopito, 1997; Suzuki *et al.*, 1998). In eukaryotic cells, the presence of unfolded proteins in the ER may also activate an intracellular signaling pathway, the so-called unfolded protein response, through which the

transcription of genes encoding ER resident proteins is enhanced (reviewed in Chapman *et al.*, 1998).

One example of ER quality control involved in human disease is a myeloperoxidase (MPO) mutant associated with genetic MPO deficiency; this mutant carries an amino acid substitution, does not undergo the proteolytic processing typical for MPO, is not secreted, but binds at length to calreticulin and calnexin, and is degraded in a proteasome-dependent fashion (DeLeo *et al.*, 1998). Another particular example of this phenomenon is a mutant of yeast carboxypeptidase Y, which carries an Arg residue instead of the Gly just downstream of the active site Ser in the conserved GESYAG sequence (single letter amino acid code). This mutant is rapidly degraded by trypsin *in vitro*, indicating that it is structurally different from the wild-type protein (Finger *et al.*, 1993). The aberrant protein is sequestered in the ER, and the chaperone Kar2p (yeast homologue of BiP) is involved in its translocation to the cytoplasm, where it is degraded in a proteasome-dependent manner (Hiller *et al.*, 1996; Plemper *et al.*, 1997). Considering the homology between carboxypeptidase Y and PPCA and the apparent conservation of ER quality control, one can infer, that the severely misfolded PPCA mutants from early-infantile GS patients are efficiently retained by ER chaperones, and have a similar mode of degradation as the CPY mutant. In turn, it is likely that a fraction of the newly synthesized late infantile PPCA mutants, having only minor structural aberrations, passes the ER quality control, becomes phosphorylated and is transported to lysosomes. Nevertheless, as suggested by the *in vitro* limited trypsin digestion experiments (Chapter 3), the modest level of misfolding of these mutants likely inceases their susceptibility for degradation by lysosomal proteases, resulting in their low steady-state levels in the lysosome.

Our studies on the processing of β-galactosidase (Chapter 6) suggest that also a group of β-galactosidase mutants found in G_{M1}-gangliosidosis patients is subject to retention in the ER. During the maturation of the wild-type 85 kDa β-galactosidase precursor, this protein is proteolytically cleaved into an intermediate 66 kDa N-terminal fragment and a small polypeptide derived from the original C-terminus; the larger fragment is further trimmed to a final 64 kDa form. As mentioned above, we discovered that the C-terminal fragment remains at least in part bound to the 64 kDa chain, and is present in the multienzyme complex containing PPCA and neuramindase. To evaluate the function of the C-terminal domain of the β-galactosidase precursor, we overexpressed a truncated form of the precursor (β-gal-N), that lacks this domain, and theoretically corresponds to the 66 kDa intermediate form of β-galactosidase. However, we observed that when β-gal-N is overexpressed in COS-1 cells, it is neither processed further, nor secreted, nor enzymatically active. In contrast, when co-expressed *in trans* with

the β-galactosidase C-terminal domain (β-gal-C), it is clearly secreted, and to some extent processed. On the basis of these data we can speculate that β-gal-N by itself does not fold properly, and is retained in the ER. In turn, one can assume that, when this polypeptide is synthesized in the presence of β-gal-C, its folding is such that it meets the conformational quality criteria of the ER, and is transported further, mostly to the extracellular space, or to lysosomes. Thus, the C-terminal domain of the β-galactosidase precursor seems essential for the folding of the N-terminal part of this polypeptide. Interestingly, it is in this part of the molecule that mutations have been identified in a small number of G_{M1}-gangliosidosis patients (Boustany *et al.*, 1993; Morrone *et al.*, 1997). Therefore, these mutations most likely also impair the folding of the N-terminal domain of β-galactosidase, resulting in prolonged assocation to ER-chaperones, and degradation.

PPCA-neuraminidase

We have shown in Chapter 4 that the dependence of neuraminidase on PPCA is not only found in human or murine cells, but also in transfected COS-1 cells that overexpress these proteins. We further report that in these cells PPCA controls the subcellular localization of neuraminidase (Chapter 5). In the absence of PPCA or co-expressed with one of the PPCA mutants previously identified in early infantile GS patients, neuraminidase is enzymatically inactive and not detectable in mature dense lysosomes. When co-expressed with one of the late infantile PPCA mutants, small amounts of the glycosidase are found in these organelles, while in the presence of wild-type PPCA neuraminidase is readily detectable and active in lysosomes. In neuraminidase/PPCA-cotransfected COS-1 cells, neuraminidase is activated in at least a two-step process, as the enzyme is partially active in a prelysosomal compartment, and fully activated when present in mature lysosomes. Our findings are in line with the observation that murine PPCA (MoPPCA) is less effective in activating human neuraminidase than the human enzyme (HuPPCA): in COS-1 cells that coexpress neuraminidase and MoPPCA, neuraminidase activity is lower than in the company of HuPPCA (Rottier, personal communication). This can be explained by the fact that in these cells the intralysosomal level of MoPPCA is lower than that of HuPPCA; the fraction of the murine protein that occurs in the mature 32/20 kDa form is considerably smaller than that of the human homolog (Rottier *et al.*, 1998).

Obviously, the structural basis for activation of neuraminidase remains to be elucidated. In each of the activation steps, catalytic capacity may be acquired/enhanced through changes in the protein's tertiary or quaternary structure (homo- and heterooligomerization), and/or through subtle posttranslational modifications. The interaction with PPCA is apparently essential

for these changes to take place; they may be effected by PPCA itself, by a change of microenvironment, or by PPCA-dependent interactions with other cellular components.

Our studies and those of others indicate that some of the properties of neuraminidase, related to its interaction with PPCA, are different from those of β-galactosidase. For example, in GS fibroblasts, β-galactosidase activity, but not neuraminidase activity, is partially restored by culturing these cells in the presence of leupeptin (d'Azzo *et al.*, 1982; Pshezhetsky and Potier, 1996). Also, in PPCA-deficient mice, neuraminidase activity is severely reduced in all tissues tested, whereas β-galactosidase activity is about 20% of normal in fibroblasts, but equal to or higher than control values in various other organs (Zhou *et al.*, 1995). Furthermore, pulse-chase experiments have suggested that, in transfected COS-1 cells, human β-galactosidase reaches the lysosomal compartment independently of human PPCA, as in these cells the glycosidase in processed to its mature 64 kDa form with the same kinetics in the absence or presence of PPCA (Van der Spoel, unpublished data). Earlier studies have shown that in GS fibroblasts the processing of β-galactosidase is less efficient than in normal fibroblasts, as in the former cells the 85 kDa precursor has a longer half-life than in the controls (d'Azzo *et al.*, 1982). Nevertheless, in GS cells a significant fraction of newly synthesized β-galactosidase molecules is processed to its mature 64 kDa form within the same lenght of time as in normal fibroblasts (d'Azzo *et al.*, 1982), indicating that in the PPCA-deficient fibroblasts β-galactosidase is in part transported to lysosomes with comparable kinetics as in normal cells. Importantly, the half life of the mature forms of β-galactosidase is significantly extended by the presence of human PPCA; the 64 kDa form is rapidly degraded both in GS fibroblasts and in COS-1 cells that overexpress only β-galactosidase, whereas the level of the glycosidase is constant for a long period of time when it is accompanied by PPCA (d'Azzo *et al.*, 1982 and Van der Spoel, unpublished data). These pulse-chase studies and earlier investigations into the turnover of β-galactosidase in GS fibroblasts (van Diggelen *et al.*, 1981; van Diggelen *et al.*, 1982) suggest that in transfected COS-1 cells as well as in human fibroblasts β-galactosidase is protected against lysosomal degradation by PPCA. In contrast, our pulse-chase studies do not suggest that neuraminidase is subject to enhanced degradation in the absence of PPCA (Chapter 5). Thus, in transfected COS-1 cells the essential interaction of PPCA with neuraminidase appears to take place in a pre-lysosomal compartment.

This conclusion does not exclude the possibility that, following entry of PPCA, neuraminidase and β-galactosidase into the lysosome, PPCA has an additional role in protecting also neuraminidase against lysosomal proteases. Vinogradova *et al* have suggested that this may be the case in human fibroblasts

(Vinogradova *et al.*, 1998). These authors counted the number of gold grains per μm^2 in the lysosomes of fibroblasts that overexpress neuraminidase, using anti-neuraminidase antibodies in immunoelectronmicroscopy. They noted a five-fold difference between normal cells and those from a GS patient. Furthermore, in pulse-chase experiments they determined that in these GS cells the half-life of overexpressed neuraminidase is one-fifth of that in normal fibroblasts. However, since the electronmicroscopy data of these authors may be subject to criticism, further studies are indicated to unequivocally establish the effect of PPCA on neuraminidase in human fibroblasts. For example, this issue can be addressed using GS fibroblasts that overexpress either only neuraminidase or both neuraminidase and PPCA. In addition, both turnover and subcellular localization of neuraminidase in GS cells should also be studied following restoration of neuraminidase activity by endocytosis of the PPCA precursor, as this may offer additional insights in the activation process of the glycosidase.

Further clues to the PPCA-neuraminidase interaction may come from other studies of enzymatic activation by protein hetero-oligomerization. An example is the β-hexosaminidase α-chain. The β-hexosaminidase α-α dimer has only minimal enzymatic activity, whereas after forming a heterodimer with the β-hexosaminidase-β chain this polypeptide is fully active (Gravel *et al.*, 1995). Another example is the association of glucosylcerebrosidase with *Sap*-C, which significantly alters the kinetic properties of the enzyme (Sandhoff *et al.*, 1995). This interaction is essential for the functioning of the enzyme, as *Sap*-C deficiency results in a Gaucher-like LSD. Recently it was reported that, in the presence of negatively charged phospholipids and unsaturated fatty acids, *Sap*-C changes the intrinsic fluorescence spectrum of glucosylcerebrosidase (Qi and Grabowski, 1998). This indicates that the enzyme undergoes a conformational modification upon association with the activator molecule. Further analysis of the cell biological and structural aspects of the activation processes of these two lysosomal enzymes will be interesting. A distinct third example is the activation of cyclin-dependent kinase 2 by its binding to cyclinA; X-ray crystallography has revealed that this association induces large conformational changes in certain domains of the kinase molecule, that bring together active site residues and render the catalytic cleft accessible for substrates (Jeffrey *et al.*, 1995).

In a completely different approach, Ricard *et al.* (1994) have developed a theory, based on statistical thermodynamics, for the changes in kinetic parameters occurring upon the integration of an enzyme, which itself is a homooligomer, into a heteromultimeric protein complex. Their formalism suggests three possibilities: either the V_{max} and K_m values change, the enzyme acquires substrate cooperativity, or the already existing cooperativity is modified. Examples with which the authors illustrate their theory are ribulose bisphosphate

carboxylase-oxygenase from spinach chloroplasts and phosphoribulokinase from *Chlamydomonas* chloroplasts. The V_{max}/K_m ratio of the former enzyme is ten times higher when it is associated with a 5-enzyme multienzyme complex than when it is a free homooligomer. Phosphoribulokinase is almost inactive by itself, and requires association with glyceraldehyde-3-phosphate dehydrogenase to become activated (Lebreton *et al.*, 1997).

Future directions

The identification of the human neuraminidase cDNA by the groups of d'Azzo, Campbell and Potier has removed an essential hurdle for further study of this lysosomal enzyme, and has brought our knowledge of this enzyme to a level comparable to that of the proteins with which it can form a multienzyme complex (Chapter 4 and Milner *et al.*, 1997; Pshezhetsky *et al.*, 1997). Having at hand the cDNAs encoding PPCA, neuraminidase and β-galactosidase, and several molecular and biochemical reagents specific for these proteins and the nucleic acids that encode them, the stage is now set for several lines of investigation. For example, interactions between normal and disease-associated forms of these proteins can be studied in inducible overexpression systems in cell lines and human fibroblasts. Also, it is highly interesting is to perform a more extensive mutational analysis of sialidosis patients and search for a genotype-phenotype relation for this disorder, as Type 1 patients are much less severely affected than Type 2 patients. In addition, projects can be started to study mammalian lysosomal neuraminidase from the atomic level (crystal stucture) to the organismal level (knock-out mice), as murine neuraminidase also has been characterized (Carrillo *et al.*, 1997; Igdoura *et al.*, 1998; Rottier *et al.*, 1998). These and other studies promise very interesting results, and will increase our understanding of PPCA, neuraminidase and β-galactosidase, of lysosomal sialoglycoconjugate catabolism, and of GS, sialidosis and G_{M1}-gangliosidosis.

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Chapter 8

Summary

Summary

The lysosomal catabolism of macromolecules is an essential metabolic process, as its disruption can lead to catastrophic childhood diseases known as lysosomal storage disorders (LSDs). The carbohydrate moieties of glycoproteins, glycosphingolipids and proteoglycans are degraded in lysosomes in a stepwise manner by hydrolases that are residue- and linkage-specific; consequently, a deficiency of only one of them is sufficient to disrupt the metabolic flow and cause the accumulation of undegraded substrates. However, only when the activity of the affected enzyme is below about 10% of normal, substrate storage is such that it leads to disease. At this level of enzyme activity, small differences herein are correlated with dramatic variations in clinical severity and age of onset of symptoms, ranging from early infantile cases with early death to very mildly affected late-adult patients. Thus, like many other metabolic disorders, LSDs are clinically heterogeneous. They are genetic conditions with recessive inheritance, of which more than 30 have been described. Multiple organ systems are often involved in LSD, including central nervous system, skeleton, and visceral organs. At present, few effective therapies for LSDs are available.

Sialidosis and GM1-gangliosidosis are LSDs that are due to structural defects in N-acetyl-α-neuraminidase and β–galactosidase, respectively. The activities of both of these enzymes are also severely reduced in skin fibroblasts of patients with a distinct LSD, galactosialidosis (GS). This disease is caused by genetic deficiencies in protective protein/cathepsin A (PPCA), a lysosomal serine carboxypeptidase. The pathology in GS is mainly caused by the secondary neuraminidase deficiency associated with this disorder; accordingly, disease severity in GS correlates with residual neuraminidase activity, which, in turn, is proportional to that of PPCA. Several mutations have been identified in the mRNAs encoding PPCA and β–galactosidase of GS and GM1-gangliosidosis patients, respectively. Also, mouse models have been generated that closely mimic these two human diseases. Furthermore, using affinity matrices for either β–galactosidase or PPCA, an enzyme complex can be isolated that contains the latter two proteins and the lysosomal neuraminidase activity present in tissue extracts. This thesis focuses on this enzyme complex, its components and their roles in human disease.

We were interested in genotype-phenotype correlations in GS, as this could reveal how distinct mutations in PPCA can be responsable for different levels of disease severity. Further understanding of GS and sialidosis called for a detailed biochemical and molecular characterization of neuraminidase, which was achieved after identification of its cDNA in the dbEST database. This also allowed us to investigate the interaction of PPCA and neuraminidase and address the

question how distinct mutations in PPCA lead to different levels of reduction of neuraminidase activity. In a complementary approach we isolated the multienzyme complex containing these two proteins as well as β–galactosidase. First analysis of the purified material identified a new component of the enzyme complex.

The analysis of the genotype-phenotype correlation in GS is described in Chapter 3. Mutations in PPCA were identified in nine patients, who together represent the entire clinical spectrum of GS. Four new point mutations were found in early-infantile (EI) patients (Val104Met, Leu208Pro, Gly411Ser and Ser23Tyr). In addition, it was noted that two mutations (Phe412Val and Tyr221Asn) are shared by the five late-infantile (LI) patients, who are either homozygous for one of these genetic lesions, or carry both of them, or are compound heterozygous with a distinct mutation not present in other patients. Thus, it appears that one allele encoding either Phe412Val or Tyr221Asn PPCA is sufficient to establish the relatively mild LI phenotype. Further, overexpression experiments in COS-1 cells showed that the EI PPCA mutants are neither phosphorylated, nor secreted or transported to the lysosome, but accumulate in the ER. In contrast, the two LI-specific forms of PPCA are phosphorylated, secreted, and in part become localized in lysosomes. Limited protease assays suggested that these mutants are much more susceptible for intralysosomal proteolysis than wild-type PPCA, explaining their low levels in the lysosome. Taken together, these data show that the clinical severity in GS correlates with the intralysosomal level of PPCA.

Work of others has suggested that also on the atomic level the PPCA mutants associated with EI forms of GS are different from those found in LI patients. Several mutations identified by us and others in EI GS patients were modeled into the three-dimensional structure of PPCA; all of these amino acid substitutions are incompatible with the packing of the molecule, precluding its normal folding. In contrast, the Phe412Val or Tyr221Asn mutations are thought to cause only minor structural alterations. These studies explain the difference in subcellular localization between the EI PPCA mutants and those found in LI patients: the former can be assumed to be withheld in the ER by binding to chaperones involved in "quality control" processes characteristic for this compartment, whereas the latter mutants are transported further to the Golgi system and acquire the mannose-6-phosphate marker.

Chapter 4 describes the molecular cloning of human lysosomal neuraminidase. Sequence analysis of its cDNA revealed that this polypeptide is homologous to bacterial and mammalian sialidases; it contains the F(Y)RIP motif (single letter amino acid code) and so-called "Asp-boxes" that are found in many members of the sialidase superfamily. Electroporation of sialidosis fibroblasts

with the neuraminidase cDNA is sufficient to restore the enzymatic activity in these cells; in contrast, neuraminidase activity in both GS fibroblasts and COS-1 cells is only increased after cotransfection with neuraminidase and PPCA cDNAs. In the latter cells neuraminidase can be detected as a glycoprotein of 46-44 kDa with a polypeptide backbone of 40 kDa, which is in agreement with the presence of three N-glycosylation sites in its amino acid sequence. Significantly, in the neuraminidase mRNAs of a number of sialidosis patients mutations were identified that abolish the catalytic capacity of the protein.

These findings have established the molecular basis of sialidosis. A more extensive mutational analysis of these patients may reveal a genotype-phenotype correlation, and explain the dramatic differences in clinical severity between Type I (late onset, mild) and Type II (early infantile, very serious) sialidosis. Further, generation of a mouse model for this disorder will offer more insight into its pathology.

Chapter 5 describes our investigation into the effects of PPCA and some of the GS-associated PPCA mutants on the behavior of neuraminidase. These studies were performed using the COS-1 cell overexpression system. We found that PPCA does not change the turnover of neuraminidase, nor induces any major structural modifications of this polypeptide, nor affects its phosphorylation. However, subcellular fractionation experiments showed that, only in the presence of transport-competent forms of PPCA, neuraminidase is detectable in mature dense lysosomes. When expressed by itself or coexpressed with an EI PPCA mutant, the polypeptide is not found in lysosomes; furthermore, in the company of wild-type PPCA, its intralysosomal level is dramatically higher than when combined with Tyr221Asn PPCA. These results indicate that the intralysosomal level of neuraminidase is proportional to that of PPCA. Since our pulse-chase experiments do not suggest that neuraminidase is subject to rapid degradation when it is expressed in the absence of PPCA, the data in this chapter imply that, in transfected COS-1 cells, PPCA controls the lysosomal level of neuraminidase by facilitating its intracellular transport.

The subcellular fractionation experiments described in Chapter 5 also revealed that in transfected COS-1 cells full enzymatic activation of neuraminidase is contingent on its lysosomal localization. In the presence of wild-type PPCA, equal amounts of neuraminidase are found in light organelles and in dense lysosomes, but the enzymatic activity of the lysosomal pool is significantly higher than that of the prelysosomal pool. The structural basis of the enzymatic activation of neuraminidase remains to be elucidated. Obtaining the three-dimensional structure of lysosomal neuraminidase may be required to address this issue, and may also reveal to what extent this enzyme differs from other sialidases.

Finally, through purification of the enzyme complex containing PPCA, neuraminidase and β–galactosidase from mouse liver a novel component of this macromolecular assembly was identified (Chapter 6). This is the ~20 kDa C-terminal fragment, which, together with the N-terminal 64 kDa polypeptide, is generated upon proteolytic processing of the 85 kDa β–galactosidase precursor. Previously, this C-terminal fragment was assumed to be degraded; however, we could detect it also in human fibroblasts. To study its significance for the biosynthesis of β–galactosidase, a C-terminally truncated form of the precursor, lacking the ~20 kDa C-terminal part, was overexpressed in COS-1 cells. Although the truncated protein is well expressed, it does not generate enzymatic activity, nor is it secreted into the culture medium. Co-expression of the latter polypeptide with the C-terminal part *in trans* resulted in the secretion of the C-terminally truncated precursor.

The fact that the β–galactosidase precursor requires its C-terminal part in order to be secreted, suggests that this portion may be required for the proper folding of the precursor molecule. We can speculate that the β–galactosidase precursor without its C-terminal part cannot acquire its correct conformation, and is therefore withheld in the ER; in turn, in the presence of the C-terminal part expressed *in trans*, the N-terminal portion seems capable of adopting a more native conformation, passes the conformational quality control of the ER, and can be secreted. The importance of the C-terminal part of β–galactosidase is also underscored by the fact that a number of GM1-gangliosidosis patients carry mutations in this segment. Furthermore, seven domains have been identified which are highly conserved among β–galactosidases from various evolutionary distant species, including mammals, bacteria, a fungus and plants; one of these domains is located in the C-terminal part of the human β–galactosidase precursor. Significantly, the disease-associated mutations in the C-terminal part of human β–galactosidase are substitutions of amino acid residues, which, with two exceptions, are located in the conserved domain.

Taken together, in this thesis significant new findings are described, including the formulation of a genotype-phenotype correlation for galactosialidosis, the molecular cloning of human lysosomal neuraminidase, and an analysis of its interaction with PPCA. These data provide an excellent basis to further investigate the relationships between PPCA, neuraminidase and β–galactosidase, and their roles in genetic diseases in man.

Samenvatting

De lysosomale afbraak van makromolekulen is een essentieel stofwisselingsproces, gezien een verstoring hiervan kan leiden tot de zeer ernstige kinderziekten, die bekend staan als lysosomale stapelingsziekten (LSZn). De koolhydraat-ketens van glycoproteinen, glycosphingolipiden en proteoglycanen worden stapsgewijs afgebroken in lysosomen door hydrolasen die monosaccharide-en binding-specifiek zijn; een deficientie van slechts één van deze enzymen is dus voldoende om de doorstroming van metabolieten te ontregelen, en een opstapeling van niet-afgebroken substraten te veroorzaken. Alleen als de aktiviteit van het betreffende enzym lager is dan ongeveer 10% van de normale waarde, dan is de substraat stapeling zodanig dat het ziekte veroorzaakt. Op dit nivo van enzymaktiviteit zijn kleine verschillen hierin gecorreleerd met aanzienlijke variaties in ernst van de ziekte en leeftijd van presentatie van de symptomen, varierend van jong-overlijdende "early infantile" gevallen tot zeer milde "late-adult" patienten. Dus, net als bij vele andere stofwisselingsziekten is er sprake van klinische heterogeniteit bij LSZn. Dit zijn genetische aandoeningen met recessieve overerving, waarvan meer dan 30 vormen zijn beschreven. Vele orgaansystemen zijn vaak betrokken in LSZn, waaronder het centrale zenuwstelsel, skelet en viscerale organen. Op dit moment zijn weinig effektieve therapieen voor LSZn beschikbaar.

Sialidosis en GM1-gangliosidosis zijn LZSn die veroorzaakt worden door strukturele gebreken in respektievelijk N-acetyl-α-neuraminidase en β–galactosidase. Beide enzymaktiviteiten zijn sterk verlaagd in huid fibroblasten van patienten met een andere LSZ, galactosialidosis (GS). Deze aandoening wordt veroorzaakt door genetische defekten in het protective protein/cathepsin A (PPCA), een lysosomaal serine carboxypeptidase. De pathologie in GS wordt voornamelijk veroorzaakt door de secundaire neuraminidase deficientie, behorende bij deze ziekte; in overeenstemming hiermee is het feit dat de ernst van GS samenhangt met de residuele neuraminidase aktiviteit, die op zijn beurt weer evenredig is met de carboxypeptidase aktiviteit van PPCA. Meerdere mutaties zijn geidentificeerd in de mRNAs voor PPCA en β–galactosidase in repectievelijk GS en GM1-gangliosidosis patienten. Ook zijn muize modellen gemaakt die in grote mate lijken op deze twee menselijke ziekten. Gebruikmakend van affiniteitschromatografie specifiek voor β–galactosidase of voor PPCA kan een enzymkomplex geisoleerd worden dat deze twee enzymen bevat, plus de neuraminidase aktiviteit aanwezig in orgaan-extracten. In dit proefschrift is de aandacht gericht op dit enzymkomplex, de komponenten daarvan en hun rol in ziekten van de mens.

Wij waren geinteresseerd in genotype-phenotype relaties in GS, omdat dit

zou kunnen onthullen hoe verschillende mutaties in PPCA verantwoordelijk zijn voor de verschillende gradaties in ernst van deze ziekte. Voor een beter begrip van GS en sialidosis was het nodig om neuraminidase biochemisch en molekulair-biologisch te karakteriseren, hetgeen bereikt werd na identificatie van het corresponderende cDNA in de dbEST database. Dit stelde ons ook in staat om de interactie tussen PPCA en neuraminidase te onderzoeken en om de vraag te stellen hoe verschillende mutaties in PPCA samenhangen met diverse verlaagde waarden van neuraminidase aktiviteit. In een aanvullende studie isoleerden wij het multienzymcomplex dat deze twee eiwitten bevat plus β-galactosidase. In het verkregen materiaal identificeerden wij een nieuwe component van het enzymcomplex.

De analyse van de genotype-phenotype relatie in GS is beschreven in Hoofdstuk 3. Mutaties werden geidentificeerd in negen patienten, die samen het hele klinische spectrum van GS representeren. Vier nieuwe puntmutaties zijn gevonden in early infantile (EI) patienten (Val104Met, Leu208Pro, Gly411Ser en Ser23Tyr). Bovendien werd opgemerkt dat minstens een van de Phe412Val en Tyr221Asn mutaties bij alle vijf late-infantile (LI) patienten voorkomen, die ofwel homozygoot zijn voor een van deze genetische gebreken, of beide dragen, of heterozygoot zijn in kombinatie met een andere mutatie die niet aanwezig is in de andere patienten. Dus, het blijkt dat een allel, dat ofwel Phe412Val of Tyr221Asn kodeert, voldoende is voor het relatief milde LI fenotype. Overexpressie-experimenten in COS-1 cellen toonden aan dat de EI PPCA mutanten niet gefosforyleerd, uitgescheiden, of naar het lysosoom getransporteerd worden, maar dat zij zich ophopen in het ER. De twee LI-specifieke vormen van PPCA worden gefosforyleerd, uitgescheiden, en ten dele getransporteerd naar lysosomen. Gelimiteerde protease assays duidden erop dat deze mutanten veel gevoeliger zijn voor intralysosomale proteolyse dan wild-type PPCA, hetgeen hun kleine intralysosomale hoeveelheden verklaart. Deze resultaten tonen aan dat de klinische ernst van GS samenhangt met het intralysosomale nivo van PPCA.

Werk van andere auteurs heeft erop gewezen dat ook op atomaire schaal de PPCA mutanten, die geassocieerd zijn met EI GS, verschillen van die van LI patienten. Verschillende mutaties, door ons en anderen geidentifieerd in EI GS patienten, werden gemodelleerd in de drie-dimensionele struktuur van PPCA; al deze aminozuur substituties zijn onverenigbaar met de pakking van het molekuul en verhinderen de normale vouwing. De Phe412Val en Tyr221Asn mutaties lijken slechts beperkte strukturele veranderingen te veroorzaken. Deze studie verklaart het verschil in subcellulaire lokalisatie tussen de EI PPCA mutanten en die van LI patienten: het idee is dat de eersten worden vastgehouden in het ER door te binden aan chaperone-eiwitten die betrokken zijn bij de "kwaliteits kontrole" processen en kenmerkend voor dit kompartiment, terwijl de LI PPCA mutanten

verder vervoerd worden naar het Golgi apparaat en de mannose-6-fosfaat determinant verkrijgen.

Hoofdstuk 4 beschrijft de molekulaire klonering van humaan lysosomaal neuraminidase. Sequentie analyse van het cDNA toonde aan dat dit eiwit homoloog is aan bacteriele sialidasen en die van zoogdieren; het bevat het (F/Y)RIP motief (eenlettercode aminozuren) en zogenaamde "Asp-boxen" die aanwezig zijn in vele leden van de sialidase superfamilie. Electroporatie van sialidosis fibroblasten met het neuraminidase cDNA is voldoende om de enzymactiviteit in deze cellen te herstellen; neuraminidase aktiviteit in GS fibroblasten en COS-1 cellen wordt slechts verhoogd na cotransfectie met de cDNAs voor neuraminidase en PPCA. In COS-1 cellen kan neuraminidase worden aangetoond als een glycoproteine van 46-44 kDa met een eiwitketen van 40 kDa, hetgeen overeenstemt met de aanwezigheid van drie N-glycosylerings plaatsen in de aminozuur volgorde. In het neuraminidase mRNA van een aantal sialidosis patienten werden mutaties geidentificeerd die het eiwit zijn katalytische aktiviteit ontnemen.

Deze resultaten hebben de molekulaire basis van sialidosis vastgesteld. Een meer uitgebreide analyse van mutaties in deze patienten zou een genotype-fenotype relatie kunnen aantonen, en de dramatische verschillen in ernst tussen Type I (late aanvang, mild) en Type II (early infantile, zeer ernstig) sialidosis verklaren. Het genereren van een muize-model voor deze aandoening zal meer inzicht verschaffen in de pathologie ervan.

Hoofdstuk 5 beschrijft ons onderzoek naar de effekten van PPCA en enkele van de GS-geassocieerde PPCA mutanten op het gedrag van neuraminidase. Deze studies zijn gedaan in het COS-1 cell overexpressie systeem. Wij vonden dat PPCA noch de omzetsnelheid van neuraminidase verandert, noch de struktuur van dit eiwit in grote mate wijzigt, en evenmin de fosforylering ervan beinvloedt. Subcellulaire fraktionering experimenten toonden aan dat slechts in de aanwezigheid van transport-kompetente vormen van PPCA neuraminidase kan worden aangetoond in mature zware lysosomen. Als het op zichzelf tot expressie wordt gebracht, of in de aanwezigheid van een EI PPCA mutant, dan wordt het eiwit niet aangetroffen in lysosomen; voorts is, gekombineerd met wild-type PPCA, het intralysosomale nivo van neuraminidase veel hoger dan met Tyr221Asn PPCA. Deze resultaten laten zien dat het intralysosomale nivo van neuraminidase evenredig is met dat van PPCA. Aangezien onze pulse-chase experimenten niet aantonen dat neuraminidase versneld wordt afgebroken in de afwezigheid van PPCA, duiden de resultaten in dit hoofdstuk erop dat, in getransfekteerde COS-1 cellen, PPCA het lysosomale nivo van neuraminidase bepaalt door het intracellulaire transport van dit enzym te bevorderen.

De subcellulaire fraktionering experimenten beschreven in Hoofdstuk 5 tonen ook aan dat in getransfekteerde COS-1 cellen neuraminidase voor volledige enzymatische aktivatie afhankelijk is van de lysosomale lokalisatie. In de aanwezigheid van wild-type PPCA werden gelijke hoeveelheden neuraminidase gevonden in lichte organellen en in zware lysosomen, de enzymatische aktiviteit van de lysosomale pool was echter beduidend hoger dan die van de prelysosomale pool. De strukturele basis van de enzymatische aktivering van neuraminidase moet nog opgehelderd worden. Het verkrijgen van de drie-dimensionele struktuur van dit enzym is wellicht hiervoor noodzakelijk, en zou ook kunnen aantonen in welk opzicht het verschilt van andere sialidasen.

Door middel van de opzuivering van het PPCA/neuraminidase/β-galactosidase complex uit muizelevers werd een nieuwe komponent van dit macromolekulaire complex geidentificeerd (Hoofdstuk 6). Dit is het ~20 kDa C-terminale fragment, dat, samen met het N-terminale 64 kDa polypeptide, wordt gegenereerd bij de proteolytische processing van de 85 kDa β-galactosidase prekursor. Voorheen werd aangenomen dat dit C-terminale fragment werd afgebroken; wij konden het, in tegendeel hiermee, ook aantonen in humane fibroblasten. Om de betekenis ervan te onderzoeken voor de biosynthese van β-galactosidase werd een C-terminaal afgekorte vorm van de prekursor, dat het 20 kDa C-terminale gedeelte mist, tot expressie gebracht in COS-1 cellen. Hoewel het afgekorte eiwit goed geexpresseerd werd, genereerde het geen enzymatische aktiviteit, noch werd het uitgescheiden in het kweek medium. Co-expressie van dit eiwit met het C-terminale fragment *in trans* resulteerde in de uitscheiding van de C-terminaal afgekorte prekursor.

Het feit dat de β-galactosidase prekursor het C-terminale domein nodig heeft om uitgescheiden te worden duidt erop dat dit gedeelte wellicht betrokken is bij de vouwing van het prekursor molekuul. We kunnen speculeren dat de β-galactosidase prekursor zonder het C-terminale domein niet zijn juiste conformatie kan aannemen, en dat het daarom wordt vastgehouden in het ER; in de aanwezigheid van het *in trans* ge-expresseerde C-terminale deel lijkt het N-terminale deel een meer normale konformatie te kunnen aannemen, de kwaliteits controle van het ER te kunnen passeren en worden uitgescheiden. Het belang van het C-terminale domein van β-galactosidase wordt ook onderstreept door het feit dat een aantal GM1-gangliosidosis patienten mutaties in dit segment dragen. Bovendien zijn zeven domeinen geidentificeerd die sterk gekonserveerd zijn bij een aantal β-galactosidasen van diverse, evolutionair niet-verwante soorten, waaronder zoogdieren, bakterien, een schimmel en planten; een van deze domeinen bevindt zich in het in het C-terminale deel van humaan β-galactosidase. De ziekte-gerelateerde mutaties in dit segment zijn aminozuursubstituties, die zich, op twee uitzonderingen na, bevinden in het gekonserveerde domein.

In dit proefschrift worden belangrijke nieuwe resultaten beschreven, waaronder het opstellen van een genotype-fenotype relatie voor galactosialidosis, de molekulaire klonering van humaan lysosomaal neuraminidase, en een analyse van de interaktie van dit enzym met PPCA. Deze gegevens bieden een uitstekende basis voor verder onderzoek naar de relaties tussen PPCA, neuraminidase en β-galactosidase, en naar hun betekenis voor erfelijke ziekten van de mens.

Abbreviations

C-terminal	carboxy-terminal
CFTR	cystic fibrosis transmembrane conductance regulator
CNS	central nervous system
CPY	carboxypeptidase Y
dbEST	database expressed sequence tags
EI	early-infantile
ER	endoplasmatic reticulum
GAG	glycosaminoglycan
GalNAc	N-acetyl-galactosamine
GALNS	N-acetyl-galactosamine-6-sulfate sulfatase
GlcNAc	N-acetyl-glucosamine
GS	galactosialidosis
GSL	glycosphingolipid
HuPPCA	human PPCA
kDa	kilodalton
LI	late-infantile
LSD	lysosomal storage disease
LSZ	lysosomale stapelingsziekte
Man	mannose
MoPPCA	murine PPCA
MPO	myeloperoxidase
N-glycan	asparagine-linked glycan
N-linked	asparagine-linked
N-terminal	amino-terminal
PPCA	protective protein/cathepsin A
Sap	sphingolipid activator protein

Thank you. Thank you very much.

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I realize that, by now, when I say "neuraminidase" loud enough, it really sounds atrocious. I can only say that I hope to have a zip-a-dee-do-dah summer with you and our little wonder.

"Be well,
do good work,
and keep in touch!"

(G. Keillor)

Curriculum vitae

The author was born on May 26, 1963, in Noordwijk, Noord-Holland, the Netherlands. He spent his childhood in the North-Sea dunes and on the white beaches of Aruba, Netherlands Antilles, in the Caribbean. Following his return to the Netherlands in 1975, he attended the Elshofcollege in Nijmegen and obtained the Gymnasium-β diploma in 1981. That same year he began the study of Biology at the Catholic University of Nijmegen, and continued from 1984 with the interdisciplinary Medical Biology module from the University of Utrecht. His minor research internships were with Dr W. Spaan at the Laboratory of Virology of the Veterinary Faculty at the same university, and with Dr C. Heijnen at the Immunology Laboratory, Wilhelmina Children's Hospital, Utrecht. His major research internship was with Dr H. Ploegh at the Department of Cellular Biochemistry, Netherlands Cancer Institute, Amsterdam. After graduation in January 1989, he fulfilled, for 18 months and 20 days, the alternative national service with Prof. Dr D. van den Eijnden at the department of Medical Chemistry, Free University, Amsterdam, by studying a murine Golgi galactosyltransferase. In 1991 he started as graduate student (Onderzoeker in Opleiding) with Dr A. d'Azzo and Prof. Dr H. Galjaard in the department of Cell Biology and Genetics, Erasmus University, Rotterdam, focusing on mammalian lysosomal glycosidases. In 1993, he continued with this project when he moved with Dr d'Azzo to the newly started Department of Genetics of St. Jude Children's Research Hospital, Memphis, USA. In 1997 he was appointed Laboratory Research Specialist in this department. From March 1998 the author works as Research Associate with Drs F. Platt and T. Butters at the Glycobiology Institute, Department of Biochemisty, University of Oxford, United Kingdom.

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Propositions belonging to the thesis:

Protective protein/cathepsin A, neuraminidase and β-galactosidase: interacting enzymes involved in lysosomal disorders

1 Human lysosomal neuraminidase belongs to the superfamily of sialidases.
this thesis

2 Human lysosomal neuraminidase does not have the characteristics of an integral membrane protein.
this thesis

3 The suggestion of Miyagi *et al.* that bovine plasma membrane sialidase is integrated in the phospholipid bilayer by a hydrophobic 21-residue transmembrane domain centrally located in the molecule is very difficult to envisage, when one assumes that, like various other sialidases, this protein also has a six-bladed propeller fold.
Miyagi et al. (1999) J Biol Chem ***274****: 5004-5011*

4 Considering that bovine plasma membrane-associated sialidase is in part found in lysosomes, and that it has a very acidic pH optimum, it seems likely that this enzyme is responsible for the intralysosomal cleavage of α2→8 sialyl linkages present in complex gangliosides.
Miyagi et al. (1999) J Biol Chem ***274****: 5004-5011*

5 The notion of substrate channeling by the beta-galactosidase/neuraminidase/ galactosamine-6-sulfate sulfatase/PPCA-complex remains speculative until any of the known structural features associated with this phenomenon (the presence of a physical tunnel, covalent substrate binding, or electrostatic substrate guidance), or a novel mechanism of substrate confinement, is described for this macromolecular assembly.
Hammes (1981) Biochem Soc Symp ***46****: 73-90; Mattevi et al. (1992) Science* ***255****: 1544-1550; Stroud (1994) Nature Struct Biol* ***1****: 131-134; Pan et al. (1997) TIBS* ***22****: 22-27; Holden et al. (1998) Curr Opin Struct Biol* ***8****: 679-685*

6 Cellular life cannot be understood from DNA.
RJ Williams (1998) Novartis Found Symp ***213****: 15-24*

7 A more accurate metaphor (for the phrase "the survival of the fittest") would be the survival of the **fitting**, the fitting being what fits into a niche in an ecosystem.
K.E. Boulding, in: Ecodynamics: A new theory of societal evolution

8 The multiregional hypothesis of Wolpoff and others about the descent of *Homo sapiens* can be compared with, and is as likely as, the hypothesis that the English language has independently arisen on three continents, and is a renewed attempt to make an exception for humans in evolutionary theory.

9 The suggestion of During *et al.* that oral gene therapy is a suitable approach for intestinal lactase deficiency is at odds with the effectiveness and relative ease of dietary lactose restriction, and merely serves the interests of the dairy industry.
During et al. (1998) Nature Medicine ***4****: 1131-1135*

10 Chicken is chicken, but the wing is the thing.
Memphian mural poetry