The EMBO Journal Vol.17 No.6 pp.1588–1597, 1998

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

Aarnoud van der Spoel, Erik Bonten and Alessandra d'Azzo[1]

Department of Genetics, St Jude Children's Research Hospital, 332 North Lauderdale, Memphis, TN 38105, USA

[1]Corresponding author
e-mail: alessandra.dazzo@stjude.org

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

Keywords: activation/lysosomes/neuraminidase/protective protein/transport

Introduction

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

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

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

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

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

Results

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

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

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

Neuraminidase is secreted into the extracellular space

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

PPCA controls the intracellular routing of neuraminidase

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

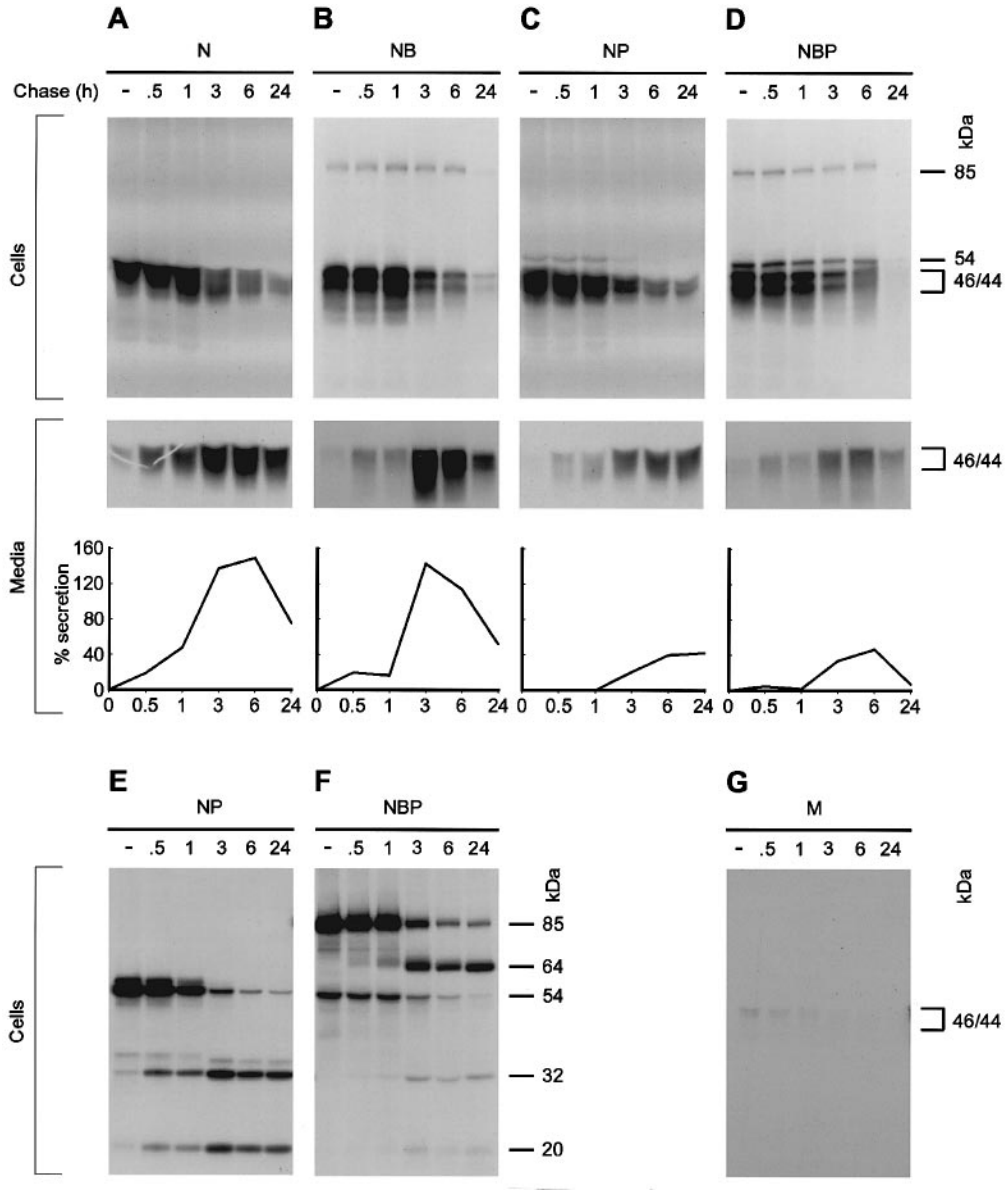

Fig. 1. Pulse–chase labeling. COS-1 cells were transfected with vector alone [denoted M in (**G**)] or with cDNAs encoding neuraminidase (N), β-galactosidase (B) or PPCA (P), in various combinations (**A**–**F**), labeled with [^{3}H]leucine for 1 h and chased with cold leucine for the time periods indicated.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

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

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

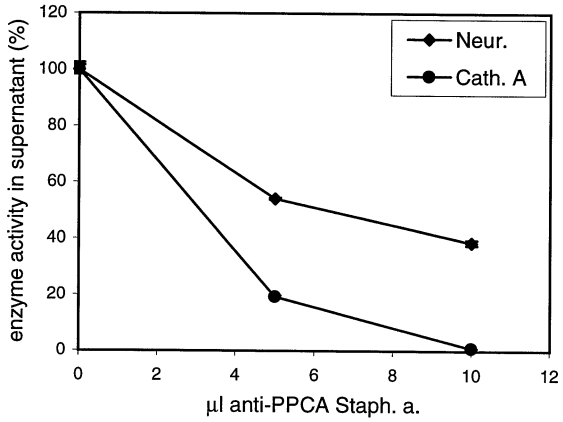

Fig. 2. Precipitation of neuraminidase activity from co-transfected COS-1 cells with anti-PPCA antibodies. Aliquots of lysates of COS-1 cells overexpressing both neuraminidase and PPCA were incubated under acidic conditions with increasing amounts of fixed *S.aureus* cells that had been pre-loaded with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

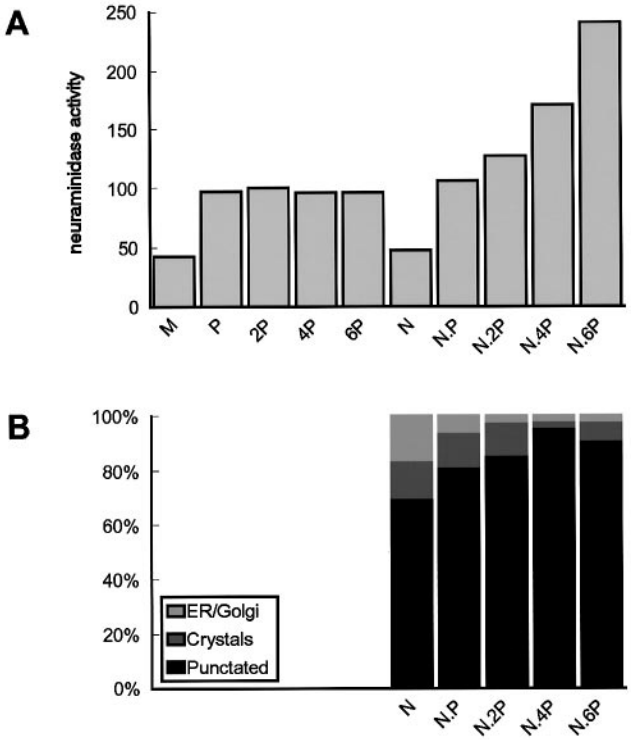

Fig. 3. Titration of neuraminidase. COS-1 cells were transfected with vector alone (M), or with increasing amounts of PPCA (P) cDNA (0.2, 0.4, 0.8 or 1.2 µg per 35 mm dish), alone or in combination with a fixed amount of neuraminidase (N) cDNA (0.2 µg per 35 mm dish). Cells were (**A**) assayed for neuraminidase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

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

Effect of PPCA mutants on neuraminidase compartmentalization

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

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

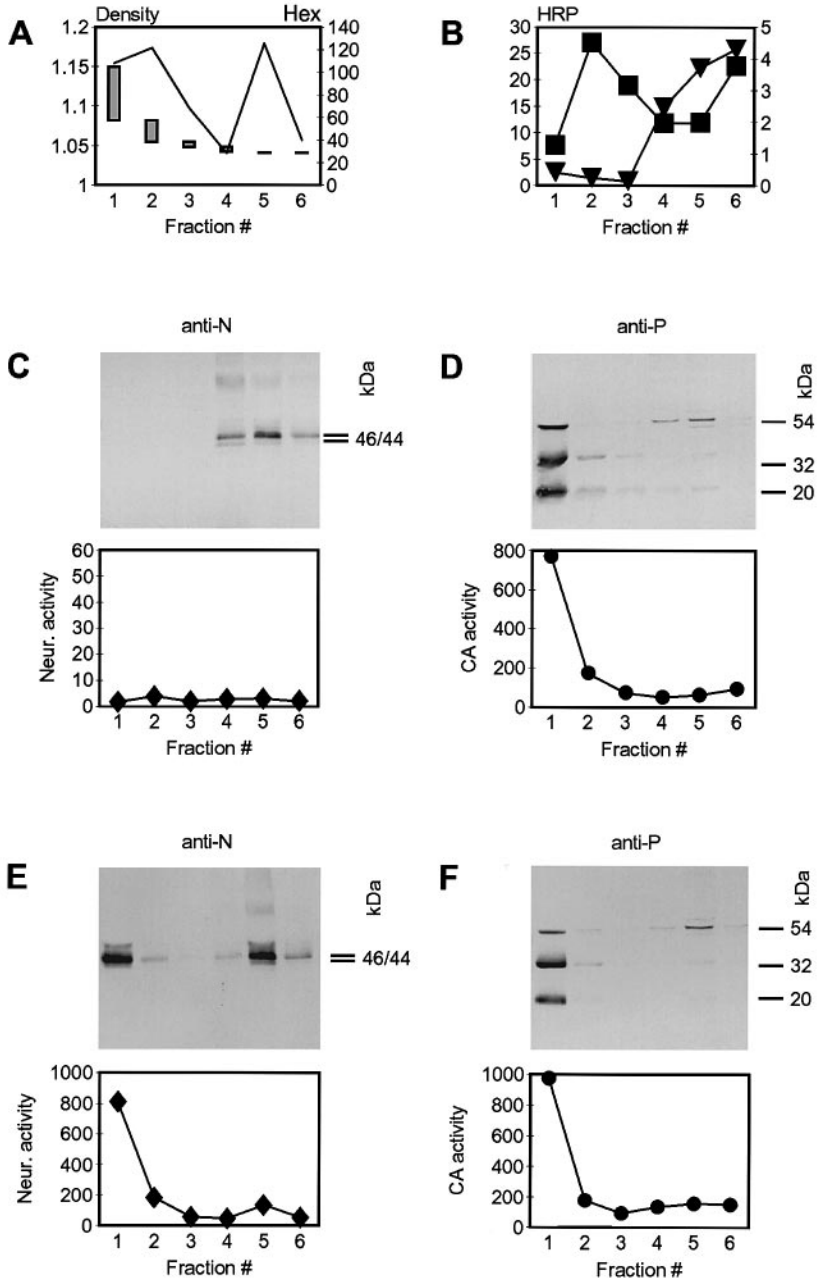

Fig. 4. Subcellular fractionation of COS-1 cells on density gradients. (**A**) Density distribution of Percoll gradients, as determined with density marker beads (Pharmacia), and profile of β-hexosaminidase activity (nmol/h/ml) in COS-1 cells overexpressing β-hexosaminidase β-chain. (**B**) After pulse-labeling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

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

Neuraminidase is poorly phosphorylated

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

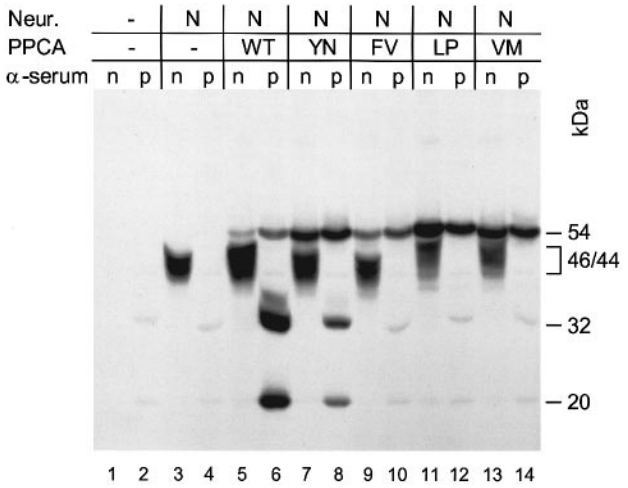

Fig. 5. Co-immunoprecipitation of PPCA mutants with neuraminidase. COS-1 cells, transfected with the neuraminidase cDNA alone, or in combination with a cDNA encoding wild-type PPCA, PPCA-YN, PPCA-FV, PPCA-LP or PPCA-VM, were metabolically labeled with [^{3}H]leucine and used for sequential immunoprecipitations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

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

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

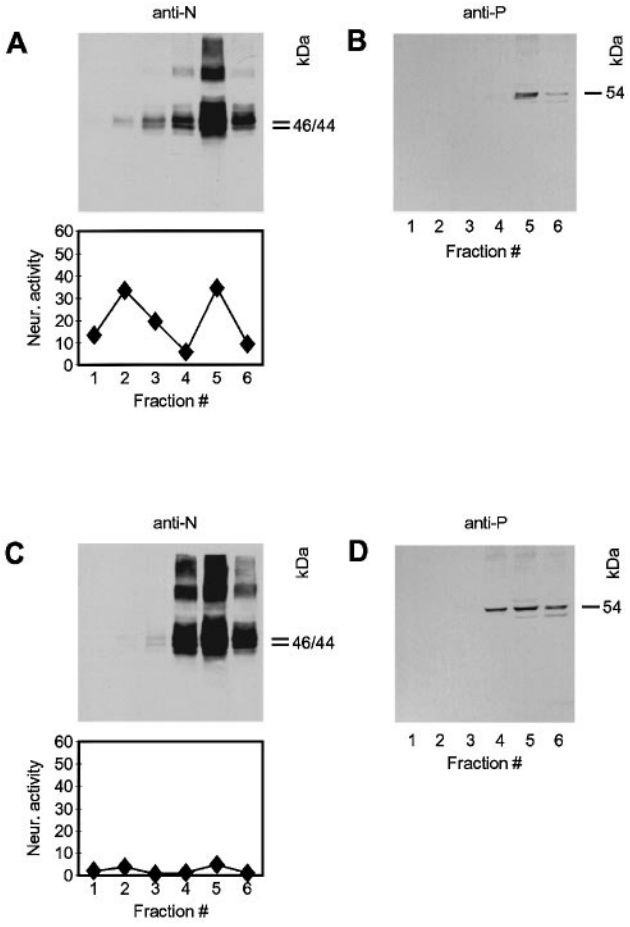

Fig. 6. Density fractionation of COS-1 cells expressing neuraminidase and PPCA mutants. Following co-transfection of COS-1 cells with neuraminidase cDNA and either (**A** and **B**) PPCA-YN or (**C** and **D**) PPCA-LP cDNA, cellular organelles were separated on Percoll gradients. Fractions were used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

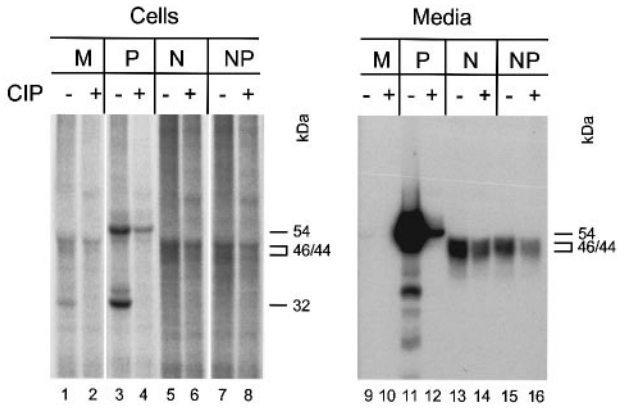

Fig. 7. Phosphorylation of neuraminidase. COS-1 cells were transfected as outlined in the legend to Figure 1, and metabolically labeled with [^{32}P]orthophosphate. Cell lysates and media were used for immunoprecipitation with anti-PPCA antiserum (lanes 3, 4, 11 and 12) or anti-Neur antiserum (all other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

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

Discussion

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

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

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

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

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

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

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

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

Materials and methods

Cell culture

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

Transfections and metabolic labeling

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

Immunoprecipitation, deglycosylation and dephosphorylation

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

Immunotitration

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

Subcellular fractionation

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

Immunofluorescence

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

Acknowledgements

We thank Dr R.Proia (NIH, Bethesda, MD) for providing the β-hexosaminidase β-chain cDNA. We are grateful to Dr Sue Vallance for editing this manuscript and to Charlette Hill for assistance in its preparation. These studies were supported by the American Lebanese Syrian Associated Charities (ALSAC) of St Jude Children's Research Hospital.

References

Amigorena,S., Drake,J., Webster,P. and Mellman,I. (1994) Transient accumulation of new class II MHC molecules in a novel endocytic compartment in B lymphocytes. *Nature*, **369**, 113–120.

Bonten,E.J., Galjart,N.J., Willemsen,R., Usmany,M., Vlak,J.M. and d'Azzo,A. (1995) Lysosomal protective protein/cathepsin A: role of the 'linker' domain in catalytic activation. *J. Biol. Chem.*, **270**, 26441–26445.

Bonten,E., vanderSpoel,A., Fornerod,M., Grosveld,G. and d'Azzo,A. (1996) Characterization of human lysosomal neuraminidase defines the molecular basis of the metabolic storage disorder sialidosis. *Genes Dev.*, **10**, 3156–3169.

Burmeister,W., Ruigrok,R. and Cusack,S. (1992) The 2.2 Å resolution crystal structure of influenza B neuraminidase and its complex with sialic acid. *EMBO J.*, **11**, 49–56.

Chen,C. and Okayama,H. (1987) High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.*, **7**, 2745–2752.

Corfield,T. (1992) Bacterial sialidases—roles in pathogenicity and nutrition. *Glycobiology*, **2**, 509–521.

D'Agrosa,R. and Callahan,J. (1988) *In vitro* activation of neuraminidase in the β-galactosidase–neuraminidase–protective protein complex by cathepsin C. *Biochem. Biophys. Res. Commun.*, **157**, 770–775.

d'Azzo,A., Hoogeveen,A., Reuser,A.J., Robinson,D. and Galjaard,H. (1982) Molecular defect in combined beta-galactosidase and neuraminidase deficiency in man. *Proc. Natl Acad. Sci. USA*, **79**, 4535–4539.

d'Azzo,A., Proia,R.L., Kolodny,E.H., Kaback,M.M. and Neufeld,E.F. (1984) Faulty association of alpha- and beta-subunits in some forms of beta-hexosaminidase A deficiency. *J. Biol. Chem.*, **259**, 11070–11074.

d'Azzo,A., Andria,G., Strisciuglio,P. and Galjaard,H. (1995) Galactosialidosis. In Scriver,C.R., Beaudet,A.L., Sly,W.S. and Valle,D. (eds), *The Metabolic and Molecular Bases of Inherited Disease*. McGraw Hill Inc., New York, pp. 2825–2838.

Fornerod,M. *et al.* (1995) Relocation of the carboxyterminal part of CAN from the nuclear envelope to the nucleus as a result of leukemia-specific chromosome rearrangements. *Oncogene*, **10**, 1739–1749.

Galjart,N.J., Gillemans,N., Harris,A., van der Horst,G.T.J., Verheijen,F.W., Galjaard,H. and d'Azzo,A. (1988) Expression of cDNA encoding the human 'protective protein' associated with lysosomal beta-galactosidase and neuraminidase: homology to yeast proteases. *Cell*, **54**, 755–764.

Galjart,N.J., Morreau,H., Willemsen,R., Gillemans,N., Bonten,E.J. and d'Azzo,A. (1991) Human lysosomal protective protein has cathepsin A-like activity distinct from its protective function. *J. Biol. Chem.*, **266**, 14754–14762.

Gieselmann,V., Pohlmann,R., Hasilik,A. and vonFigura,K. (1983) Biosynthesis and transport of cathepsin D in cultured human fibroblasts. *J. Cell Biol.*, **97**, 1–5.

Glombitza,G., Becker,E., Kaiser,H. and Sandhoff,K. (1997) Biosynthesis, processing, and intracellular transport of Gm2 activator protein in human epidermal keratinocytes. *J. Biol. Chem.*, **272**, 5199–5207.

Gluzman,Y. (1981) SV40-transformed simian cells support the replication of early SV40 mutants. *Cell*, **23**, 175–182.

Hasilik,A. (1992) The early and late processing of lysosomal enzymes: proteolysis and compartmentation. *Experientia*, **48**, 130–151.

Hille-Rehfeld,A. (1995) Mannose 6-phosphate receptors in sorting and transport of lysosomal enzymes. *Biochim. Biophys. Acta*, **1241**, 177–194.

Hiraiwa,M., Yamauchi,T., Tsuji,S., Nishizawa,M., Miyatake,T., Oyanagi,K., Ikuta,F. and Uda,Y. (1993) Activation of human lysosomal sialidase. *J. Biochem.*, **114**, 901–905.

Hogue,B. and Nayak,D. (1992) Synthesis and processing of the influenza virus neuraminidase A type II transmembrane and glycoprotein. *Virology*, **188**, 510–517.

Hunziker,W. and Geuze,H. (1996) Intracellular trafficking of lysosomal membrane proteins. *BioEssays*, **18**, 379–389.

Isidoro,C., Grassel,S., Baccino,F. and Hasilik,A. (1991) Determination of the phosphorylation, uncovering of mannose 6-phosphate groups and targeting of lysosomal enzymes. *Eur. J. Clin. Chem. Clin. Biochem.*, **29**, 165–171.

Kleijer,W.J., Geilen,G.C., Janse,H.C., van Diggelen,O.P., Zhou,X.-Y., Galjart,N.J., Galjaard,H. and d'Azzo,A. (1996) Cathepsin A deficiency in galactosialidosis: studies of patients and carriers in 16 families. *Pediatr. Res.*, **39**, 1067–1071.

Milner,C., Smith,S., Carrillo,M., Taylor,G., Hollinshead,M. and Campbell,R. (1997) Identification of a sialidase encoded in the human major histocompatibility complex. *J. Biol. Chem.*, **272**, 4549–4558.

Morreau,H., Galjart,N.J., Gillemans,N., Willemsen,R., van der Horst,G.T.J. and d'Azzo,A. (1989) Alternative splicing of beta-galactosidase mRNA generates the classic lysosomal enzyme and a beta-galactosidase-related protein. *J. Biol. Chem.*, **264**, 20655–20663.

Morreau,H., Galjart,N.J., Willemsen,R., Gillemans,N., Zhou,X.Y. and d'Azzo,A. (1992) Human lysosomal protective protein. Glycosylation, intracellular transport, and association with β-galactosidase in the endoplasmic reticulum. *J. Biol. Chem.*, **267**, 17949–17956.

Pisoni,R., Flickinger,K., Thoene,J. and Christensen,H. (1987) Characterization of carrier-mediated transport systems for small neutral amino acids in human fibroblast lysosomes. *J. Biol. Chem.*, **5**, 6010–6017.

Proia,R.L., d'Azzo,A. and Neufeld,E.F. (1984) Association of alpha- and beta-subunits during the biosynthesis of beta-hexosaminidase in cultured human fibroblasts. *J. Biol. Chem.*, **259**, 3350–3354.

Pshezhetsky,A.V. and Potier,M. (1994) Direct affinity purification and supramolecular organization of human lysosomal cathepsin A. *Arch. Biochem. Biophys.*, **313**, 64–70.

Pshezhetsky,A. and Potier,M. (1996) Association on *N*-acetylgalactosamine-6-sulfate sulfatase with the multienzyme lysosomal complex of β-galactosidase, cathepsin A, and neuraminidase. *J. Biol. Chem.*, **271**, 28359–28365.

Pshezhetsky,A. *et al.* (1997) Cloning, expression and chromosomal mapping of human lysosomal sialidase and characterization of mutations in sialidosis. *Nature Genet.*, **15**, 316–320.

Reuter,G. and Gabius,H.-J. (1996) Review: sialic acids: structure–analysis–metabolism–occurrence–recognition. *Biol. Chem. Hoppe Seyler*, **377**, 325–342.

Rijnboutt,S., Aerts,H.M., Geuze,H.J., Tager,J.M. and Strous,G.J. (1991a) Mannose 6-phosphate-independent membrane association of cathepsin D, glucocerebrosidase, and sphingolipid-activating protein in HepG2 cells. *J. Biol. Chem.*, **266**, 4862–4868.

Rijnboutt,S., Kal,A., Geuze,H., Aerts,H. and Strous,G. (1991b) Mannose 6-phosphate-independent targeting of cathepsin D to lysosomes in HepG2 cells. *J. Biol. Chem.*, **266**, 23586–23592.

Roggentin,P., Schauer,R., Hoyer,L. and Vimr,E. (1993) MicroReview: the sialidase superfamily and its spread by horizontal gene transfer. *Mol. Microbiol.*, **9**, 915–921.

Sabatini,D. and Adesnik,M. (1995) The biogenesis of membranes and organelles. In Scriver,C.R., Beaudet,A.L., Sly,W.S. and Valle,D. (eds) *The Metabolic and Molecular Bases of Inherited Disease*. McGraw Hill Inc., New York, pp. 459–553.

Saito,M. and Yu,R.K. (1995) Biochemistry and function of sialidases. In Rosenberg,A. (ed.), *Biology of the Sialic Acids*. Plenum Press, New York, pp. 261–313.

Saito,T., Taylor,G. and Webster,R. (1995) Steps in maturation of influenza A virus neuraminidase. *J. Virol.*, **69**, 5011–5017.

Schauer,R., Kelm,S., Reuter,G., Roggentin,P. and Shaw,L. (1995) Biochemistry and role of sialic acids. In Rosenberg,A. (ed.), *Biology of the Sialic Acids*. Plenum Press, New York, pp. 7–67.

Shimmoto,M., Fukuhara,Y., Itoh,K., Oshima,A., Sakuraba,H. and Suzuki,Y. (1993) Protective protein gene mutations in galactosialidosis. *J. Clin. Invest.*, **91**, 2393–2398.

Suzuki,Y., Sakuraba,H. and Oshima,A. (1995) β-Galactosidase deficiency (β-galactosidosis): G_{M1}-gangliosidosis and Morquio B disease. In Scriver,C., Beaudet,A., Sly,W. and Valle,D. (eds), *The Metabolic and Molecular Bases of Inherited Disease.* McGraw Hill, Inc., New York, pp. 2785–2824.

Thomas,G.H. and Beaudet,A.L. (1995) Disorders of glycoprotein degradation and structure: α-mannosidosis, β-mannosidosis, fucosidosis, sialidosis, aspartylglucosaminuria and carbohydrate-deficient glycoprotein syndrome. In Scriver,C.R., Beaudet,A.L., Sly,W.S. and Valle,D. (eds), *The Metabolic and Molecular Bases of Inherited Disease*. McGraw Hill, Inc., New York, pp. 2529–2561.

TikkanenR., Enoman,N., Riikonen,A., Ikonen,E. and Peltonen,L. (1995) Intracellular sorting of aspartyglucosaminidase: the role of N-linked oligosaccharides and evidence of Man-6-P-independent lysosomal targeting. *DNA Cell Biol.*, **14**, 305–312.

Tulp,A., Verwoerd,D. and Pieters,J. (1993) Application of an improved density gradient electrophoresis apparatus to the separation of proteins, cells and subcellular organelles. *Electrophoresis*, **14**, 1295–1301.

van Diggelen,O.P., Hoogeveen,A.T., Smith,P.J., Reuser,A.J. and Galjaard,H. (1982) Enhanced proteolytic degradation of normal beta-galactosidase in the lysosomal storage disease with combined beta-galactosidase and neuraminidase deficiency. *Biochim. Biophys. Acta*, **703**, 69–76.

van Dongen,J.M., Willemsen,R., Ginns,E.I., Sips,H.J., Tager,J.M., Barranger,J.A. and Reuser,A.J. (1985) The subcellular localization of soluble and membrane-bound lysosomal enzymes in I-cell fibroblasts: a comparative immunocytochemical study. *Eur. J. Cell Biol.*, **39**, 179–189.

Verheijen,F.W., Palmeri,S., Hoogeveen,A.T. and Galjaard,H. (1985) Human placental neuraminidase. Activation, stabilization and association with beta-galactosidase and its protective protein. *Eur. J. Biochem.*, **149**, 315–321.

Vielhaber,G., Hurwitz,R. and Sandhoff,K. (1996) Biosynthesis, processing, and targeting of sphingolipid activator protein (SAP) precursor in cultured human fibroblasts. *J. Biol. Chem.*, **271**, 32438–32446.

Vincent,R. and Nadeau,D. (1984) Adjustment of the osmolality of Percoll for the isopycnic separation of cells and cell organelles. *Anal. Biochem.*, **141**, 322–328.

von Figura,K. and Hasilik,A. (1986) Lysosomal enzymes and their receptors. *Annu. Rev. Biochem.*, **55**, 167–193.

Wolf,P. and Ploegh,H. (1995) How MHC class II molecules acquire peptide cargo: biosynthesis and trafficking through the endocytic pathway. *Annu. Rev. Cell Dev. Biol.*, **11**, 267–306.

Yamamoto,Y. and Nishimura,K. (1987) Copurification and separation of beta-galactosidase and sialidase from porcine testis. *Int. J. Biochem.*, **19**, 435–442.

Zhou,X.Y., Galjart,N.J., Willemsen,R., Gillemans,N., Galjaard,H. and d'Azzo,A. (1991) A mutation in a mild form of galactosialidosis impairs dimerization of the protective protein and renders it unstable. *EMBO J.*, **10**, 4041–4048.

Zhou,X.Y. *et al*. (1995) Mouse model for the lysosomal disorder galactosialidosis and correction of the phenotype with over-expressing erythroid precursor cells. *Genes Dev.*, **9**, 2623–2634.

Zhou,X.-Y., van der Spoel,A., Rottier,R., Hale,G., Willemsen,R., Berry,G.T., Strisciuglio,P., Andria,G. and d'Azzo,A. (1996) Molecular and biochemical analysis of protective protein/cathepsin A mutations: correlation with clinical severity in galactosialidosis. *Hum. Mol. Genet.*, **12**, 1977–1987.

Received September 23, 1997; revised and accepted January 20, 1998