Mutations in Sialidosis Impair Sialidase Binding to the Lysosomal Multienzyme Complex*

Received for publication, January 17, 2001, and in revised form, February 15, 2001 Published, JBC Papers in Press, February 20, 2001, DOI 10.1074/jbc.M100460200

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Sialidosis is an autosomal recessive disease caused by the genetic deficiency of lysosomal sialidase, which catalyzes the catabolism of sialoglycoconjugates. The disease is associated with progressive impaired vision, macular cherry-red spots, and myoclonus (sialidosis type I) or with skeletal dysplasia, Hurler-like phenotype, dysostosis multiplex, mental retardation, and hepatosplenomegaly (sialidosis type II). We analyzed the effect of the missense mutations G68V, S182G, G227R, F260Y, L270F, A298V, G328S, and L363P, which are identified in the sialidosis type I and sialidosis type II patients, on the activity, stability, and intracellular distribution of sialidase. We found that three mutations, F260Y, L270F, and A298V, which are clustered in the same region on the surface of the sialidase molecule, dramatically reduced the enzyme activity and caused a rapid intralysosomal degradation of the expressed protein. We suggested that this region might be involved in sialidase binding with lysosomal cathepsin A and/or β-galactosidase in the multienzyme lysosomal complex required for the expression of sialidase activity. Transgenic expression of mutants followed by density gradient centrifugation of cellular extracts confirmed this hypothesis and showed that sialidase deficiency in some sialidosis patients results from disruption of the lysosomal multienzyme complex.

Sialidosis (also called mucolipidosis I and cherry-red spot myoclonus syndrome) is an autosomal recessive disease caused by the genetic deficiency of lysosomal sialidase, also called neuraminidase (reviewed in Refs. 1–3). The disease is characterized by tissue accumulation and urinary excretion of sialylated oligosaccharides and glycoproteins (1) and includes two main clinical variants with different ages of onset and degrees of severity. Sialidosis type I or nondysmorphic type is a late-onset mild form, characterized by bilateral macular cherry-red spots, progressive impaired vision, and myoclonus syndrome (4–8). Sialidosis type II or dysmorphic type is the infantile-onset form, which is also associated with skeletal dysplasia, Hurler-like phenotype, dysostosis multiplex, mental retardation, and hepatosplenomegaly (9–12). A severe form of the disease manifests itself prenatally and is associated with ascites and hydrops fetalis (13–15). The age of onset and severity of the clinical manifestations correlate with the amount of residual sialidase activity, suggesting the existence of considerable genetic heterogeneity (1–3).

Although sialidosis was recognized as a deficiency of lysosomal sialidase from the moment of its discovery (16), the molecular mechanism of this disorder was not characterized for the following two decades because the identification and sequencing of sialidase had been hampered by low tissue content and instability of the enzyme. Several works have shown that sialidase is a part of a multienzyme complex containing other lysosomal enzymes such as cathepsin A (protective protein), β-galactosidase, and N-acetylglactosamine-6-sulfate sulfatase (17–19). Because the functional activity of sialidase completely depends on the integrity of its association with cathepsin A, it was hypothesized that cathepsin A supports catalytically active conformation of this enzyme (18). In addition, the complex protects sialidase and β-galactosidase against rapid proteolysis (17, 20, 21) and may also be important for proper sorting and processing of their precursors (22–25). In the autosomal recessive disease galactosialidosis, a primary genetic defect of cathepsin A (17, 21) results in disruption of the complex and causes the combined deficiency of β-galactosidase and sialidase activities. The clinical features and a composition of storage products in galactosialidosis resemble those in sialidosis (8, 9, 26).

Recently the gene coding for sialidase was cloned, and a series of mutations in sialidosis patients was identified (27–31). In particular, we have found two frameshift and eight missense mutations in nine sialidosis patients of multiple ethnic origin (28, 31). To understand the effect of these mutations on sialidase, we modeled the tertiary structure of the enzyme and localized the identified amino acid substitutions (31). Surprisingly, none of mutations directly affected the deduced active site residues or were found in the central core of the sialidase molecule, but all of them involved residues on the surface of the enzyme. Therefore, in most cases it was unlikely that these mutations would introduce electrostatic or steric clashes in the protein core leading to general folding defects of sialidase and its retention in the endoplasmic reticulum/Golgi compartment, as was observed in most of the mutations affecting cathepsin A (32).

In this paper we show that three sialidase mutants that have amino acid substitutions clustered in one region on the surface of the sialidase molecule were correctly processed and sorted but were not associated with the complex and were rapidly degraded in the lysosome. These results permitted us to conclude that the surface region containing these mutations may be involved in the sialidase binding interface with the lysoso-
nal multienzyme complex and that sialidase deficiency in sialidosis patients may be secondary to the disruption of the lysosomal multienzyme complex.

**EXPERIMENTAL PROCEDURES**

**Expression of Sialidase Mutants in COS-7 Cells—**Site-directed mutagenesis was performed using a Transformer® site-directed mutagenesis kit (CLONTECH), the previously described pCMV-SIAL expression vector, mutagenic primers corresponding to mutant sialidase sequences, and a selection primer used to eliminate a unique ScI I restriction site in the vector according to supplier's protocols. All primers were enzymatically phosphorylated, and for each mutant the corresponding mutagenic primer and the selection primer were annealed to a heat-denatured pCMV-SIAL plasmid. After elongation by T4 DNA polymerase, the modified sialidase was verified by sequencing and transformed into Escherichia coli DH5α. Positive clones were selected after a final ScI I restriction analysis, and the entire sialidase cDNA was sequenced. Up to 80% of transformants contained the desired mutation. DNA fragments of between 300 and 600 base pairs containing the introduced mutations were obtained from the mutant pCMV-SIAL plasmids by double digestion with either BstEII/NaeI, NaeI/KpnI, or KpnI/EcoRV enzymes and subcloned into the parental pCMV-SIAL plasmid. The final constructs were verified by sequencing.

COS-7 cells seeded in 25-mm flasks or 60-mm round dishes were co-transfected with pCMV-SIAL and pCMV-CathA expression vectors (31) using LipofectAMINE Plus reagent (Life Technologies Inc.) in phosphate-buffered saline.

**Centrifugation**—Cells were washed twice with ice-cold PBS, then lysed for 30 min with 0.15 M sodium acetate buffer, pH 5.2, containing 0.5 mg of BSA/ml and 1% (v/v) Triton X-100, washed twice with PBS, and stained with rabbit polyclonal anti-sialidase antibodies and monoclonal antibodies against lysosomal membrane marker LAMP-2 (Washington Biotechnology Inc., Baltimore, MD). Slides were studied on a Zeiss LSM410 inverted confocal microscope (Carl Zeiss Inc., Thornwood, NY).

**Density Gradent Centrifugation of Cell Extracts—**COS-7 cells grown in 7-T5 flasks and harvested 48 h after transfection with wild-type or mutant sialidase were solubilized in 0.2 ml of 0.15 M sodium acetate buffer, pH 5.2, containing 0.5 mg of BSA/ml and 1% (v/v) Triton X-100, washed twice with PBS, and centrifuged at 13,000 × g for 15 min. The supernatants were applied on the top of the density gradient of 30% metrizamide (OptiPrep; Nycomed Amersham) preformed by a 2-h ultracentrifugation at 45,000 rpm in a Beckman SW-55 Ti swinging bucket rotor. After application of the sample, the centrifugation was continued for an additional 17 h at the same speed. Immediately after centrifugation, each tube was divided into 10 fractions using a Beckman tube slicer kit. Each fraction was assayed for activities of sialidase, β-galactosidase, and cathepsin A as well as for the presence of human sialidase and cathepsin A protein by Western blot as previously described (20). The activity of endogenous N-acetyl-β-hexosaminidase in fractions was used as an internal control. The molecular masses of proteins were approximated using the following M₄ standards (Amersham Pharmacia Biotech): thyroglobulin (M₄ = 669,000), catalase (M₄ = 232,000), and BSA (M₄ = 68,000). Thyroglobulin and BSA were covalently labeled with fluorescein isothiocyanate to facilitate their detection in fractions containing proteins from a COS-7 cell.

**Modeling of Sialidase Tertiary Structure—**The modeling was performed using the structures of homologous sialidases from *Micronospora viridifaciens* (Ref. 39; Protein Data Bank file 1eur.pdb), *Salmo nella typhimurium* (Ref. 40; Protein Data Bank file 2sil.pdb), and *Vibrio cholerae* (Ref. 41; Protein Data Bank file 1kit.pdb) as templates. These structures were superimposed with ProSuper King’s Beech Biosoftware Solutions to determine structurally conserved regions. The sequence of human sialidase was manually aligned with the sequences of structurally conserved regions. The modeling was then carried out with Modeler 4 software (Andrej Sali, The Rockefeller University, New York).

**RESULTS**

**Expression and Intracellular Targeting of Sialidase Mutants—**The effect of sialidase mutations on enzyme biogenesis was studied by the transient expression of the mutant cDNA. Mutations were generated by site-directed mutagenesis in the pCMV-SIAL vector previously used for the expression of sialidase (28). Short restriction cassettes containing the mutations were then inserted into the parental pCMV-SIAL vector replacing the corresponding fragments of wild-type sialidase cDNA. The inserts and junction regions of the resulting constructs were verified by sequencing to ensure the correct introduction of mutations. Mutant or wild-type sialidase was co-expressed with human cathepsin A, which is necessary for the expression of sialidase activity. 48 h after transfection, the cell lysates were assayed for sialidase, cathepsin A, and control β-hexosaminidase activities.

The expression results are shown in Table I. All transfected cells had similar cathepsin A activity, suggesting the same transfection efficiency for all cells. Four of the expressed mutants, G68V, G227R, A298V, and L363P, had very low (<10% of normal) sialidase activity. The activity of F260Y and L270F mutants was between 10 and 20% of normal, and that of S182G...
and G328S mutants was between 20 and 40% of normal. Additional experiments showed that F260Y, A298V, and L270F mutants were also significantly less stable than the wild-type sialidase. The half-life of their enzymatic activity in cellular lysates at 37 °C was about 30 min as compared with the 2-h half-life of the wild-type enzyme.

Using immunolabeling, we studied the intracellular distribution of the sialidase mutants expressed in COS-7 cells. To identify the lysosomal late endosomal compartment, the COS-7 cells were treated for 40 min with 75 nM LysoTracker Red DND-99 dye prior to fixation and immunostaining with anti-sialidase antibodies. Alternatively the cells were double-stained with anti-sialidase antibodies and monoclonal antibodies against human LAMP-2. For the wild-type sialidase we have observed the complete co-localization of anti-sialidase immunostaining with lysosomal markers LysoTracker Red (Fig. 1) or LAMP-2 (not shown). The G68V, S182G, F260Y, L270F, A298V, and G328S mutants showed similar localization, suggesting that the mutant protein is able to reach the lysosomes. Although partial co-localization of anti-sialidase and LysoTracker staining was also detectable in the cells transfected with the G227R and L363P mutants, the majority of the anti-sialidase antibodies labeled distinct cellular areas, suggesting that in this case the mutant protein is mostly retained in prelysosomal compartments. This finding is consistent with the results of structural modeling of sialidase mutants that suggested general folding defects and retention in the endoplasmic reticulum/Golgi compartment for both G227R and L363P substitutes (31).

Metabolic Labeling of Sialidase Mutants—The results of sialidase activity assay in COS-7 cells expressing sialidase mutants have shown that some of them, i.e. L270F, A298V, and F260Y mutants, had significantly lower stability in cellular homogenates than the wild-type enzyme. To measure the stability of sialidase mutants in the cell, we performed pulse-chase experiments. The 46–48-kDa polypeptides similar to those previously observed by both immunoprecipitation and Western blotting (20) were precipitated by anti-sialidase antibodies from homogenates of cells transfected with wild-type or mutant sialidase cDNA and pulsed for 40 min (Fig. 2). The intensities of both bands decreased proportionally with the time of chase. By 4 h of chase, normal wild-type sialidase was reduced to 50% of total. In contrast, the degradation rate of F260Y, L270F, and A298V mutants was remarkably increased so that for all these cells 46–48-kDa sialidase bands were already nearly undetectable after 4 h of chase. The same degradation rate was observed with and without leupeptin, a potent inhibitor of lysosomal serine and thiol proteases, suggesting that they are not involved in the degradation of mutant sialidase.

Association of Sialidase Mutants with the Lysosomal Multienzyme Complex—The ability of sialidase mutants to associate with the lysosomal multienzyme complex was studied by the density gradient ultracentrifugation of the cell extracts (Fig. 3). In the extracts of COS-7 cells co-transfected with

### Table 1

| Mutation | Enzymatic activity (%) | Traffic to lysosomes | Short halflife (min) | Complex formation |
|----------|------------------------|----------------------|---------------------|------------------|
| Wild-type | 100                    | +                    | –                   | +                |
| G68V     | ND*                    | +                    | NA*                 | NA               |
| S182G    | 20–40                  | –                    | –                   | –                |
| G227R    | <10                    | –                    | NA                  | –                |
| F260Y    | 10–20                  | +                    | +                   | –                |
| L270F    | 10–20                  | +                    | +                   | –                |
| A298V    | <10                    | +                    | +                   | –                |
| G328S    | 30–40                  | +                    | –                   | +                |
| L363P    | ND                     | –                    | NA                  | NA               |

* ND, not detected. NA, not assessed.

**Fig. 1. Immunohistochemical localization of sialidase mutants expressed in COS-7 cells.** COS-7 cells were probed with 75 nM lysosomal marker LysoTracker Red DND-99 for 30 min at 37 °C 48 h after transfection with cathepsin A and wild-type (WT) or mutant sialidase cDNAs as indicated and stained with rabbit polyclonal anti-sialidase antibodies and fluorescein isothiocyanate-conjugated secondary antibodies. Slides were studied on a Zeiss LSM410 inverted confocal microscope. Panels show co-localization of anti-sialidase antibodies (green) and LysoTracker marker (red). Magnification × 600.
wild-type sialidase and cathepsin A, all sialidase activity was associated with the peak that sedimented before thyroglobulin ($M_r = 689,000$). This peak, which also contained almost all the cathepsin A and the majority of endogenous $\beta$-galactosidase activities, probably represented the lysosomal multienzyme complex (Fig. 3A). A similar sedimentation profile was observed in the extracts of cells transfected with sialidase G328S or S182G mutants. Although about 3-fold less sialidase activity was detected in the collected fractions as compared with that of the wild-type control, all activity was associated with the high molecular weight fraction. The distribution of sialidase and cathepsin A proteins detected by Western blot (Fig. 3B) followed that of the enzyme activity. In contrast, in the cells transfected with G227R, F260Y, L270F, and A298V mutants, the high molecular weight form of sialidase was not detected. Both sialidase protein and the trace levels of sialidase activity were found in the peak that sedimented together with the low molecular weight marker, BSA ($M_r = 69,000$), suggesting that the mutant enzyme does not associate with the multienzyme complex. Moreover, although a significant amount of sialidase cross-reacting protein was detected in these fractions for WT or mutant sialidase cDNA as indicated on the figure were metabolically labeled with a mixture of [35S]Cys and [35S]Met for 40 min and chased at 37 °C for 0, 1, and 4 h in Eagle's minimal essential medium supplemented with 20% (v/v) fetal calf serum. The sialidase ($SIAL$) was immunoprecipitated from cell lysates with rabbit anti-sialidase antibodies and resolved on SDS-polyacrylamide gel electrophoresis.

### DISCUSSION

Analysis of molecular defects in the sialidase gene in sialidosis patients shows that the spectrum of mutations is very different from that in cathepsin A and $\beta$-galactosidase, which underlies clinically similar disorders galactosialidosis and $\alpha$-glycoprotein deficiency. Most of the sialidosis patients studied so far, 21 of 27, had amino acid substitutions and not frameshift or splicing defects (27, 28, 30, 42). The localization of the missense mutations on the sialidase structural model (Fig. 4) suggested only few of them (shown in blue in Fig. 4) affect active site residues (Y370C) or may interfere with their correct positions (L91R with the active site residue Arg$^{297}$, P80L with Arg$^{297}$, duplication of His$^{399}$ and Tyr$^{400}$ with Glu$^{384}$, P316S with Arg$^{289}$, and P335Q with Arg$^{341}$). In addition, the L363P mutation is situated on a $\beta$-strand adjacent to that containing the active site residue Tyr$^{270}$. The Leu$^{663}$ residue is probably necessary to anchor this $\beta$-strand to the one containing Tyr$^{270}$ so that the L363P mutation can potentially also have an effect on the active site. However, in contrast to cathepsin A mutations in galactosialidosis patients, which mostly affect the enzyme central core and cause unfolding of the protein (32), the majority of sialidase mutations involves residues on the surface of the enzyme and is not supposed to result in significant structural change. Moreover, the distribution of mutations on the sialidase surface is uneven. The region that contains the majority of mutations resulting in complete or almost complete inactivation of the enzyme and causing severe sialidosis type II phenotype is easily detectable (shown in red in Fig. 4). In particular this region contains mutations G227R, F260Y, L270F, and A298V (28, 31), R294S, L231H, and G218A (30), W240R (2), and V217M and G243R (42).

We expressed eight sialidase mutants, four of which contained amino acid substitution in the defined surface patch (G227R, F260Y, L270F, and A298V) and four in the other areas of the sialidase molecule (G68V, S182G, G328S, and L363P) in COS-7 cells and studied trafficking, activity, and stability of the produced protein. We found that in two cases (G227R and...

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2 H. Sakuraba, private communication.
sialidase activity resulted from the disruption of normal protein-protein interactions in the lysosome. Further studies should show if this mechanism is unique for sialidase or extends to other lysosomal enzymes.

Acknowledgments—We thank Dr. Hitoshi Sakuraba, who generously provided unpublished information. We also thank Dr. Mila Ashmarina for critical reading of the manuscript.

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FIG. 4. Schematic diagram of the sialidase model, showing the location of mutations identified in sialidosis patients. Mutations localized in putative sialidase-cathepsin A binding sites are shown in red, mutations in the active site residues or those that may affect the positions of the active site residues are in blue, and mutations that do not cause obvious structural changes are in green.
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J. Biol. Chem. 2001, 276:17286-17290.
doi: 10.1074/jbc.M100460200 originally published online February 20, 2001

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