ORIGINAL ARTICLE

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Molecular and structural studies of Japanese patients with sialidosis type 1

Received: March 21, 2000 / Accepted: May 10, 2000

Abstract To gain insight into the pathogenesis of sialidosis type 1, we performed molecular investigations of two unrelated Japanese patients. Both of them are compound heterozygotes for base substitutions of ⁶⁴⁹G-to-A and ⁷²⁷G-to-A, which result in amino acid alterations V217M and G243R, respectively. Using homology modeling, the structure of human lysosomal neuraminidase was constructed and the structural changes caused by these missense mutations were deduced. The predicted change due to V217M was smaller than that caused by G243R, the latter resulting in a drastic, widespread alteration. The overexpressed gene products containing these mutations had the same molecular weight as that of the wild type, although the amounts of the products were moderately decreased. A biochemical study demonstrated that the expressed neuraminidase containing a V217M mutation was partly transported to lysosomes and showed residual enzyme activity, although a G243R mutant was retained in the endoplasmic reticulum/Golgi area and had completely lost the enzyme activity. Considering the data, we surmise

that the V217M substitution may be closely associated with the phenotype of sialidosis type 1 with a late onset and moderate clinical course.

Key words Sialidosis · Lysosomal neuraminidase · Homology modeling · Protective protein / cathepsin A

Introduction

Lysosomal neuraminidase (sialidase, EC 3. 2. 1. 18) is an acid glycosidase that catalyzes the removal of the terminal sialic acid residues of glycoconjugates (Verheijen et al. 1982, 1985, 1987; Hiraiwa et al. 1988; van der Horst et al. 1989). Mammalian lysosomal neuraminidase is associated with protective protein/cathepsin A (PPCA; EC 3. 4. 16. 1) and β -galactosidase (EC 3. 2. 1. 23), and essentially requires PPCA for its activation (d'Azzo et al. 1982; van der Spoel et al. 1998). Defects in the *PPCA* gene result in a human metabolic disease, galactosialidosis, associated with secondary deficiencies of lysosomal neuraminidase and β -galactosidase (reviewed by d'Azzo et al. 1995).

The primary defect of the lysosomal neuraminidase causes another autosomal recessive genetic disorder, sialidosis (reviewed by Thomas et al. 1995). The disease is clinically divided into two groups: late-onset sialidosis type 1, characterized by macular cherry-red spots and myoclonus, and early-onset sialidosis type 2, distinguished from type 1 by the presence of dysmorphic features besides neurological symptoms and cherry-red spots. The molecular basis of the disease has remained obscure. Recently, human lysosomal neuraminidase cDNA was isolated (Bonten et al. 1996; Pshezhetsky et al. 1997; Milner et al. 1997), and studies for clarifying the pathogenesis of sialidosis were started. So far, six different mutations in the neuraminidase gene have been reported (Bonten et al. 1996; Pshezhetsky et al. 1997). However, little has been elucidated regarding the enzyme proteins caused by the mutant genes.

In this study, we characterized the molecular defects in lysosomal neuraminidase caused by gene mutations, which

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were newly identified in two unrelated Japanese patients with sialidosis type 1.

Patients and methods

Patients

Patient 1. This patient is a 25-year-old female offspring of nonconsanguineous parents. At the age of 17 years, she developed an ataxic gait, and speech disturbance. There were subsequent episodes of generalized convulsions and visual disturbance. Ophthalmological examination disclosed macular cherry-red spots. Her neurological symptoms rapidly progressed, and she could not stand without support at the age of 18 years, and is bed-ridden at present. Her younger sister exhibited a similar clinical course, although her brother had a milder clinical course (Mitoma et al. 1993). Lysosomal enzyme assay revealed an isolated neuraminidase deficiency, but β -galactosidase activity was normal in these three siblings (Yamamoto et al. 1995).

Patient 2. This patient is a 42-year-old man. He was born to nonconsanguineous parents. He developed normally after birth until the age of 32 years, when involuntary movements began in the upper extremities. Then, he developed gait disturbance and slurred speech. Physical examination at age 41 years revealed dysarthria, action myoclonus, and hyperactive deep tendon reflexes. Lens opacities and macular cherry-red spots were detected. Neither computed tomography nor magnetic resonance imaging of the head revealed any abnormality. Lysosomal enzyme assay disclosed a neuraminidase deficiency. Other enzyme activities, including those of β -galactosidase and cathepsin A, were normal. His brother has shown similar clinical manifestations, but detailed data for the brother, including neuraminidase activity, are not available.

Materials

1,2-Dimyristyloxypropyl-3-dimethyl-hydroxy ethylammonium bromide (DMRIE)-C reagent for transfection was purchased from GIBCO/BRL (Grand Island, NY, USA). We purchased leupeptin from the Peptide Institute (Osaka, Japan); N-benzoyloxycarbonyl-L-phenylalanyl-L-leucine (Z-Phe-Leu) from Sigma (St. Louis, MO, USA); and 1,4-diazabicyclo-[2,2,2]octane (DABCO) from Wako Pure Chemical Industries (Osaka, Japan). Restriction enzymes were purchased from New England Biolabs (Beverly, MA, USA).

Cell culture

Human skin fibroblasts derived from healthy Japanese subjects and patients with sialidosis type 1 were maintained in Ham's F-10 medium supplemented with 10% fetal calf serum (FCS) and antibiotics, as described previously (Sakuraba et al. 1999). For transient expression of the wild-

type and mutant lysosomal neuraminidase cDNAs, a fibroblast cell line derived from a galactosialidosis patient (ASVGS-1) (Shimmoto et al. 1993; Gluzman et al. 1981) or COS-1 cells (kindly supplied by Dr. Y. Nabeshima, National Institute of Neuroscience, NCNP, Tokyo, Japan) were cultured in the same medium.

Gene analysis

Total RNA and genomic DNA were isolated from the cultured fibroblasts, as described previously (Davis et al. 1986). Lysosomal neuraminidase cDNA, including the entire protein coding sequence, was obtained by reverse transcription (RT)-polymerase chain reaction (PCR) (Kawasaki 1990), using an oligo(dT) primer and an RT-PCR kit (Amersham, Buckinghamshire, England), followed by cDNA amplification, using a pair of primers (sense, 5'-AGTCTAGCTGC CAGGGTTCGC-3' and antisense, 5'-AGTCCTGAAGGC AGAATACC-3'). The amplified lysosomal neuraminidase cDNA was subcloned into the pGEM-T vector (Promega, Madison, WI, USA). Nucleotide sequencing was performed with a DyeDeoxy Terminator Cycle Sequencing Kit and an automated sequencer (Applied Biosystems, Foster City, CA, USA). As described under "Results", ⁶⁴⁹G-to-A (V217M) and ⁷²⁷G-to-A (G243R) substitutions were detected in both the patients. These base substitutions are located in exon 4 of the lysosomal neuraminidase gene. To confirm the presence of these mutations, genomic DNA fragments, including exon 4, were amplified by PCR, using sense (5'-TGAGCCCCTAGAGTCTCC-3') and antisense (5'-GGCAGGGAGGGTCAAATG-3') primers. The amplified DNA fragment was analyzed by direct sequencing.

Transient expression of lysosomal neuraminidase cDNA

An expression vector for the wild-type neuraminidase was prepared by inserting the full-length human lysosomal neuraminidase cDNA into the *Not* I site of the pCMV expression vector (MacGregor and Caskey 1989), and designating this as pCMV-sialidase, as previously described (Vinogradova et al. 1998). For expression of the mutant lysosomal neuraminidase cDNA, the DNA fragment obtained from the mutant cDNA subclone, containing either ⁶⁴⁹G-to-A (V217M) or ⁷²⁷G-to-A (G243R), was double-digested with *Bst*EII and *Kpn* I, and substituted for the pCMV-sialidase plasmid. The cloned plasmids were designated pCMV-sialidase[V217M] and pCMV-sialidase[G243R], respectively.

For enzyme assay and immunoblotting, ASVGS-1 and COS-1 cells were seeded onto 60-mm dishes (2×10^5 cells), 1 day before transfection. For immunocytochemistry, COS-1 cells were seeded onto two-well chamber slides (Lab-Tek; Nunc, Naperville, IL, USA) (1×10^5 cells). The transfection was performed using DMRIE-C reagent (GIBCO/BRL) according to the manufacturer's protocol. Briefly, a mixture comprising the plasmid DNA (5 μ g) and DMRIE-C reagent (10 μ l) was added to cells cultured in FCS-free Ham's F-10 medium, followed by incubation for 5 h. Then, the culture

medium was replaced with Ham's F-10 medium containing 10% FCS, and the cells were cultured for 4 days. As for ASVGS-1 cells for enzyme assay and COS-1 cells for immunocytochemical analysis, a pCAGGS vector (Miyazaki et al. 1991) containing human wild-type PPCA cDNA (pCAGGS-PP, 5 μ g) (Shimmoto et al. 1993) was co-transfected with the wild-type or mutant lysosomal neuraminidase cDNA.

Enzyme assay

As mentioned in the "Introduction", a sufficient amount of PPCA is required for the activation of lysosomal neuraminidase, and ASVGS-1 cells co-transfected with PPCA cDNA were used as host cells in this analysis. The transfected ASVGS-1 cells were washed with phosphate-buffered saline (PBS), harvested by scraping, and then suspended in distilled water containing 0.1 mM leupeptin. A homogenate was prepared by pipetting, and then neuraminidase activity was measured immediately, according to a method reported previously (Suzuki et al. 1981). The rest of the homogenate was sonicated and used for other lysosomal enzyme assays and protein determination. Glycosidase activities, including those of neuraminidase, β -galactosidase, and β -hexosaminidase, were assayed fluorometrically, using 4-methylumbelliferyl glycosides as substrates (Suzuki 1987). Cathepsin A activity was measured at pH 5.6, using Z-Phe-Leu as a substrate (Itoh et al. 1991). Protein determination was performed with a dye-binding assay kit (Bio-Rad, Richmond, CA, USA), using bovine serum albumin (BSA) as a standard.

Polyacrylamide gel electrophoresis and immunoblotting

After transfection, COS-1 cells were harvested, sonicated in distilled water containing 0.1 mM leupeptin, and then centrifuged at $10,000 \times g$ for 15 min at 4°C. Aliquots of the resultant supernatant, after reduction with 25 mM mercaptoethanol, were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), according to the method of Laemmli (1970), on a 10% acrylamide gel (Realgel Plate; Biocraft, Tokyo, Japan). Proteins were visualized by immunostaining, using an anti-neuraminidase antibody (Vinogradova et al. 1998) and a Super Signal Ultra Chemiluminescence Kit (Pierce, Rockford, IL, USA). Prestained SDS-PAGE standards (Bio-Rad, Hercules, CA, USA) were used as molecular mass standard proteins: phosphorylase b, 110 kDa; BSA, 84 kDa; ovalbumin, 50 kDa; carbonic anhydrase, 33 kDa; soybean trypsin inhibitor, 24 kDa; and lysozyme, 16 kDa.

Immunocytochemical analysis

The transfected COS-1 cells were fixed with ice-cold 4% paraformaldehyde/PBS (v/v) and then with 80% methanol/PBS (v/v). After nonspecific binding was blocked with 5% goat serum and 1% BSA in PBS, the cells were treated with the anti-neuraminidase antibody (Vinogradova et al. 1998)

overnight at 4°C. Then the cells were washed with PBS, followed by treatment with fluorescein-isothiocyanate (FITC)-labeled F (ab')₂ fragments of goat anti-rabbit IgG (BioSource, Camarillo, CA, USA). After being washed with PBS, the cells were mounted with 55% glycerol/PBS (v/v) containing 3.8% DABCO. The stained cells were examined under a fluorescence microscope (Axiophot; Zeiss, Oberkochen, Germany).

Structural modeling of human wild-type and mutant lysosomal neuraminidases

The crystal structure of neuraminidase from *Salmonella typhimurium* LT2 was determined (Crennell et al. 1993) and deposited in the Protein Data Bank (PDB; Brookhaven National Laboratory, Upton, NY, USA) (Sussman et al. 1998). On the basis of this information (PDB file: 2sil), models of the human wild-type lysosomal neuraminidase and its mutants were built, using molecular modeling software (SYBYL/COMPOSER; TRIPOS, St. Louis, MO, USA), installed on a PowerIndigo2 R8000 workstation (Silicon Graphics, Mountain View, CA, USA). Sequence alignment of the human lysosomal neuraminidase and *Salmonella typhimurium* neuraminidase was performed for definition of the structurally conserved regions of both target and template proteins. A fragment derived from the homologue was used to model the backbone of each structurally conserved region, and the rule-based procedure was used to generate the side chain (Sutcliffe et al. 1987).

Loop regions were then constructed by selecting a fragment for modeling loop regions from the general protein database (Jones and Thirup 1986; Claessens et al. 1989). The force field for simulation of proteins (Weiner et al. 1984, 1986) was employed in the minimization steps to optimize the geometry of a molecule. The maximum number of iterations to be performed during the minimization was set as 1000. The minimization procedure was performed to optimize the side chain rotamers, with the backbone and the conserved side chains fixed, and then to optimize the conformation of loops, with the structurally conserved regions fixed. The mutant model was built in the same way as for the wild-type, but based on the primary structure with the amino acid replacement. To determine the influence of the replacement on the model structure, the mutant model was superimposed on the wild-type model by the least-square-mean fitting method (McLachlan 1979). We defined that the structure was influenced by the amino acid replacement when the position of an atom of the mutant differed from that of the wild-type by more than the total root-mean-square distance value.

Results

Gene analysis

The PCR products of the entire cDNA coding sequence for lysosomal neuraminidase were subcloned and sequenced.

Fig. 1A,B. Direct sequencing of genomic DNA. The results show that both patients are heterozygous for **A** the ⁶⁴⁹G-to-A and **B** the ⁷²⁷G-to-A substitutions. The mutation sites are indicated by *arrows*. *P1*, Patient 1; *P2*, patient 2; *N*, a normal subject

Table 1. Transient expression of lysosomal neuraminidase cDNA

Plasmid	Neuraminidase (nmol/h per mg protein)	Cathepsin A (μmol/h per mg protein)	β-Galactosidase (nmol/h per mg protein)	β-Hexosaminidase (μmol/h per mg protein)
None	<1	<0.1	12	2.2
^a pCAGGS-PP	28	5.4	43	1.6
^b pCMV-sialidase and pCAGGS-PP	183	3.7	39	1.5
^c pCMV-sialidase[V217M] and pCAGGS-PP	55	3.1	36	1.2
^d pCMV-sialidase[G243R] and pCAGGS-PP	27	5.0	45	1.6

Lysosomal enzyme activities in a galactosialidosis fibroblast cell line (ASVGS-1 cells) transfected with plasmids, described under “Methods”. Values are the means of two independent measurements

^apCAGGS-PP, a pCAGGS vector including PPCA cDNA

^bpCMV-sialidase and pCAGGS-PP, a pCMV vector including the wild-type lysosomal neuraminidase cDNA and a pCAGGS vector including PPCA cDNA

^cpCMV-sialidase[V217M] and pCAGGS-PP, a pCMV vector including mutant lysosomal neuraminidase cDNA with V217M and a pCAGGS vector including PPCA cDNA

^dpCMV-sialidase[G243R] and pCAGGS-PP, a pCMV vector including mutant neuraminidase cDNA with G243M and a pCAGGS vector including PPCA cDNA

Two types of cDNA clones were detected in both patients, the first having a ⁶⁴⁹G-to-A substitution and the other, a ⁷²⁷G-to-A transition (data not shown). Because both changes were located in exon 4, the genomic DNA fragment including exon 4 was amplified and directly se-

quenced. The results showed that both patients were heterozygous for the ⁶⁴⁹G-to-A and ⁷²⁷G-to-A substitutions (Fig. 1). The results of the cDNA and genomic DNA analyses show that both patients have one allele carrying a G-to-A transition at nucleotide number 649, resulting in the

Fig. 2. Immunoblot analysis of lysosomal neuraminidase expressed in COS-1 cells. COS-1 cells were transfected with the wild-type lysosomal neuraminidase cDNA (*lane 2*), mutant neuraminidase cDNA with a V217M mutation (*lane 3*), and that with a G243R mutation (*lane 4*). Non-transfected cells were used as a control (*lane 1*). The cell extracts were subjected to sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE) under reducing conditions, and then immunoblotted with an anti-neuraminidase antibody, as described under "Methods"

amino acid substitution of methionine for valine (V217M), and the other allele bearing a G-to-A transition at nucleotide number 727, resulting in the substitution of arginine for glycine (G243R).

Transient expression of lysosomal neuraminidase cDNA

To characterize the effect of the amino acid substitutions, V217M and G243R, on lysosomal neuraminidase activity, the wild-type and mutant lysosomal neuraminidase cDNAs were transiently co-expressed with the human PPCA cDNA in a galactosialidosis-derived fibroblast cell line (ASVGS-1 cells). Table 1 summarizes the results. In ASVGS-1 cells, neuraminidase activity, as well as cathepsin A and β -galactosidase activities, were markedly decreased by a genetic defect of PPCA, which is essential for the expression of lysosomal neuraminidase activity. Mock transfection with the PPCA cDNA alone restored the neuraminidase, β -galactosidase, and cathepsin A activities in the cells. Co-transfection of the wild-type lysosomal neuraminidase cDNA and PPCA cDNA caused a further seven-fold increase in neuraminidase activity compared with that in the mock-transfected cells, whereas the activity of β -hexosaminidase, as a control enzyme, did not change significantly. Co-transfection of the mutant lysosomal neuraminidase cDNA containing the ⁷²⁷G-to-A substitution (G243R) and PPCA cDNA did not cause any increase in neuraminidase activity. In contrast, a moderate increase in the enzyme activity was observed when the mutant neuraminidase cDNA containing the ⁶⁴⁹G-to-A substitution

Fig. 3A–D. Immunocytochemical analysis of lysosomal neuraminidase expressed in transformed galactosialidosis cell lines. **A** Mock transfected with protective protein cathepsin A (PPCA) cDNA; **B** co-transfected with the wild-type lysosomal neuraminidase cDNA and PPCA cDNA; **C** co-transfected with the neuraminidase cDNA containing a Y217M mutation and PPCA cDNA; **D** co-transfected with the neuraminidase cDNA containing a G243R mutation and PPCA cDNA. $\times 400$

(V217M) was transfected with PPCA cDNA. In this case, neuraminidase activity was found to be restored to 17% of that in the cells co-transfected with the wild-type lysosomal neuraminidase cDNA and PPCA cDNA.

Immunoblot analysis

As shown in Fig. 2, the wild-type and mutant lysosomal neuraminidase cDNA products expressed in COS-1 cells were analyzed by immunoblotting. We used a polyclonal antibody against a recombinant human lysosomal neuraminidase fragment produced by a bacterial gene expression system that has been reported previously (Vinogradova et al. 1998). For COS-1 cells transfected with the wild-type lysosomal neuraminidase cDNA, a single 48-kDa band was observed (Fig. 2, lane 2). The cells transfected with the mutant V217M and G243R cDNAs produced a gene product of the same size as those transfected with the wild-type cDNA (Fig. 2, lanes 3 and 4), although the intensity of the band was moderately decreased in both these cases. No other immunoreactive bands were observed.

Immunocytochemical analysis

The results of immunocytochemical analysis with the anti-neuraminidase antibody are shown in Fig. 3. Fluorescence microscopy revealed typical lysosomal granular staining in the cells co-transfected with the wild-type lysosomal neuraminidase cDNA and PPCA cDNA (Fig. 3B). In contrast, the expression product of lysosomal neuraminidase cDNA containing a G243R mutation showed a reticular staining pattern (Fig. 3D). On the other hand, both perinuclear reticular and partial punctate cytoplasmic staining were observed in the expression of the mutant with V217M (Fig. 3C).

Structural modeling of wild-type and mutant lysosomal neuraminidases

According to the sequence alignment (Milner et al. 1997), we built a model of the human lysosomal neuraminidase without the N-terminal 47 residues, corresponding to the signal peptide (Milner et al. 1997; Vinogradova et al. 1998). The model was, thus, composed of the 368 amino acids from residue 48 to the terminal residue 415, the amino acid sequence homology with the aligned fragment of *Salmonella typhimurium* LT2 being 39.7%. The modeled lysosomal neuraminidase retains a basic structure composed of six four-stranded antiparallel β -sheets, and resembles a six-bladed propeller with the predicted active site cleft in the middle (Fig. 4). Both V217M and G243R are located in the β -sheet of the third β -sheet-loop unit (Fig. 4). The V217M transversion was deduced to alter the arrangement of 11 amino acid residues around the mutation site (Fig. 5). As to the G243R transversion, it was predicted to cause a drastic structural change with a wide range of up to 38 residues

(Fig. 6). Neither mutation affected the residues involved in the predicted active site (R78, R97, D103, D135, R280, and R347) (Pshezhetsky et al. 1997; Crennell et al. 1993).

Discussion

Few reports on human lysosomal neuraminidase have been published, because the enzyme has low stability during purification and requires PPCA for the expression of its activity (Verheijen et al. 1982, 1985, 1987; Hiraiwa et al. 1988; van der Horst et al. 1989). Recent cDNA cloning for human lysosomal neuraminidase has revealed that it encodes a 45-kDa protein with three potential N-linked glycosylation sites (Bonten et al. 1996; Pshezhetsky et al. 1997; Milner et al. 1997). This information has facilitated the identification of gene mutations causing sialidosis. So far, only one nonsense mutation, E377X, has been found in a patient with sialidosis type 1 (Bonten et al. 1996). In sialidosis type 2, three missense mutations, L91R (Bonten et al. 1996), F260Y (Pshezhetsky et al. 1997), and L363P (Pshezhetsky et al. 1997); a four-base duplication causing a frameshift (Pshezhetsky et al. 1997); and a single-base deletion, resulting in a frameshift and extending the protein by 69 amino acid residues (Bonten et al. 1996), have been reported.

In this study, we identified two novel gene mutations in two Japanese patients with sialidosis type 1. Although these patients are unrelated according to our investigations, they are both compound heterozygotes for the ⁶⁴⁹G-to-A and ⁷²⁷G-to-A alterations. The base substitutions are found at the CpG site, which is known as a mutation hot spot (Cooper and Youssoufian 1988).

Using the information regarding the tertiary structure of bacterial neuraminidase, we modeled the structure of human lysosomal neuraminidase, and deduced the structural changes caused by the identified mutations. Both the V217M and G243R mutations were distant from the deduced active residues. The simulation suggests that the G243R replacement can cause a drastic widespread change in the protein structure. The biochemical study showed that the G243R mutant expressed low amounts of a protein with the same molecular size as in that in the wild type, but it could not be transported to the lysosomal compartment. The enzyme activity of the expressed product with the G243R mutation was completely abolished. The structural change is likely to cause a folding defect, which results in a transport defect and intracellular degradation and/or a defect of activation. On the other hand, the deduced structural change caused by V217M is smaller than that caused by G243R. The V217M mutant was partly transported to lysosomes, and showed residual enzyme activity.

Both patients described here are compound heterozygotes for V217M and G243M. Considering the results of this study, we surmise that the V217M mutation causes a mild change in the protein structure compatible with the presence of residual enzyme activity. Therefore, this mutation may be closely related to the phenotype with a late onset and moderate clinical course.

Fig. 4. Structural model of lysosomal neuraminidase and the locations of the mutations identified in two Japanese sialidosis patients. The backbone is colored according to secondary structures; α -helix (*red*), β -strand (*blue*), and coil (*yellow*). The sequence motifs conserved in the sialidase family, five Asp-boxes (Ser/Thr-X-Asp-X-Gly-X-X-Trp/Phe), a Phe-Arg-Ile-Pro sequence motif, and a C-terminal, Tyr-X-X-hydrophobic residue motif are colored *cyan*, *orange*, and *white*, respectively. The side chains of the predicted active residues are colored *magenta*. The residues of V217 and G243, where the amino acid residue replacements will occur in the patients, are colored *green*

Fig. 5. Conformational change in lysosomal neuraminidase caused by V217M mutation. The amino acid residues influenced by the change from V217 (*green*) to M217 (*red*) are shown; those colored *blue* are the wild-type residues and those colored *yellow* are the mutant residues. The active site residues are colored *magenta*

Fig. 6. Conformational change in lysosomal neuraminidase caused by G243R mutation. The amino acid residues influenced by the change from G243 (green) to R243 (red) are shown; those colored blue are the wild-type residues and those colored yellow are the mutant residues. The active residues are colored magenta

In conclusion, we identified two novel gene mutations in Japanese patients with sialidosis type 1, and characterized the structural and functional defects caused by these missense mutations. Molecular and structural studies will facilitate clarification of the complicated pathophysiology of sialidosis.

Acknowledgments We thank Dr. Michel Potier for helpful advice.

This work was partly supported by grants from the Tokyo Metropolitan Government, the Ministry of Education, Science, Sports and Culture of Japan, and the Ministry of Health and Welfare of Japan.

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