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NAGLU Mutations Underlying Sanfilippo Syndrome Type B

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Summary

Sanfilippo syndrome type B (mucopolysaccharidosis III B) is a rare autosomal recessive disease caused by deficiency of α-*N*-acetylglucosaminidase, one of the enzymes required for the lysosomal degradation of heparan sulfate. The gene for this enzyme, *NAGLU*, recently was isolated, and several mutations were characterized. We have identified, in amplified exons from nine fibroblast cell lines derived from Sanfilippo syndrome type B patients, 10 additional mutations: Y92H, P115S, Y140C, E153K, R203X, 650insC, 901delAA, P358L, A664V, and L682R. Four of these mutations were found in homozygosity, and only two were seen in more than one cell line. Thus, Sanfilippo syndrome type B shows extensive molecular heterogeneity. Stable transfection of Chinese hamster ovary cells, by cDNA mutagenized to correspond to the *NAGLU* missense mutations, did not yield active enzyme, demonstrating the deleterious nature of the mutations. Nine of the 10 amino acid substitutions identified to date are clustered near the amino or the carboxyl end of α-*N*-acetylglucosaminidase, suggesting a role for these regions in the transport or function of the enzyme.

Introduction

The mucopolysaccharidoses are a family of lysosomal disorders characterized biochemically by failure to degrade one or more glycosaminoglycans, because of a deficiency of the appropriate lysosomal enzyme. In Sanfilippo syndrome (mucopolysaccharidosis III) the degradation of heparan sulfate is specifically affected by deficiency of one of four enzymes needed to remove its modified glucosamine residues: heparan *N*-sulfatase (type A), α-*N*-acetylglucosaminidase (type B), acetylCoA:α-glucosaminide acetyltransferase (type C), or *N*-acetylglucosamine 6-sulfatase (type D) (reviewed in Neufeld and Muenzer 1995). In the absence of any one of these enzymes, heparan sulfate accumulates in tissues and is excreted in urine. The accumulation of heparan sulfate must be particularly toxic to the brain, since deterioration of the CNS is the most prominent manifestation of Sanfilippo syndrome. Affected children show developmental delay, mental retardation, and, eventually, dementia; they may display nearly uncontrollable hyperactivity and other behavioral disturbances for a period that can last many years (Cleary and Wraith 1993). Magnetic-resonance imaging as well as postmortem studies show that the brain is markedly atrophied, particularly in the cortical region (Tamagawa et al. 1985; Murata et al. 1989). Life span is usually until adolescence, but longer survival occurs among the more mildly affected patients.

The human gene encoding α-*N*-acetylglucosaminidase, *NAGLU*, recently was isolated, characterized, and localized to chromosome 17q21 (Friedman et al. 1995; Zhao et al. 1995, 1996*a;* Weber et al. 1996; Zhao et al. 1996*b*). Seven mutations underlying Sanfilippo syndrome type B have been reported: 503del10, R297X, S612G, R626X, R643H, R674H, and Q706X (Aronovich et al. 1996; Zhao et al. 1996*a*). We now report 10 additional mutations, adding to our understanding of the molecular heterogeneity of this disorder. To verify that the missense mutations are disease producing rather than benign, we studied the expression of similarly mutagenized cDNAs.

Material and Methods

Cell Lines

Fibroblast lines from Sanfilippo syndrome type B patients GM 00737, GM 01426, and GM 02931 and the control line GM 04390 were obtained from the Human Genetic Mutant Cell Repository, Coriell Institute for

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Table 1

Oligonucleotide Primers for PCR Amplification of *NAGLU* Exons

Segment (Exon[s])	PCR Primer	Amplified Product (bp)	Annealing Temperature (°C)
1 (1)	5′-AGACGCCCCAAGGGAGTAT	608	54[a]
	5′-ATTTGGGTGGCAGCGGCTCC		
2 (1)	5′-GGGGCTGCACCGCTACCT	443	55[b]
	5′-TCCGCAGGTTCAAAGAGAAG		
3 (2)	5′-CCCTGCCCATCTGTTAGACT	595	53[b]
	5′-GCACGTTGAAAGCACTTCTA		
3a (1–2)	5′-ACGCCCCCAAGGGAGTATC	1540	55[c]
	5′-GCACGTTGAAAGCACTTCTA		
4 (3)	5′-AGCGCCCAGCACAAAGAAG	374	55
	5′-CCTCATCTCCCAGGATACACG		
5 (4)	5′-GGCCCAGGAAGGGTGGTATTA	309	55[d]
	5′-GGCCCAGAGCTTAAGTTT		
6 (5)	5′-AAACCAGGAGCTGTAGAGAAGT	434	54
	5′-CTGCCTACCCCTACTGACATCT		
7 (6)	5′-GGCCCTCTGTTTCATCACTC	444	55
	5′-AAATCTGGCACTGGGTCCTT		
8 (6)	5′-GCATCAGCCAGAACGAAGTG	426	52
	5′-CCAGCTCCTTGCTCAGGTAG		
9 (6)	5′-CAACCGATCTGATGTGTTTG	386	55
	5′-TTGGCATAGTCCAGGATGTT		
9a (6)	5′-GCTGGCTAGTGACAGCCGCTT	261	54
	5′-CTGGTGCTGTTGGAAAGGGAT		
10 (6)	5′-GCCGAGGCCGATTTCTAC	345	57
	5′-GCGAATCTATCACCAAGAGC		
11 (6)	5′-CGTTCTCAGCAAGCAGAGGTA	373	57
	5′-CAAGCGTGGCAGCAGTGACC		

NOTE.—For each segment, the sense primer is given on the first line, the antisense primer on the second. Unless otherwise indicated, the cycle parameters were as follows: denaturation at 95°C for 1 min and extension at 72°C for 30 s, for 35 cycles.

[a] 10% Dimethyl sulfoxide; 37 cycles.

[b] 37 Cycles.

[c] 40 Cycles.

[d] Extension for 2 s; 37 cycles.

Medical Research. Fibroblast lines IT 41, IT 310, IT 236, IT 421, and IT 424 were from the collection at the University of Naples. Fibroblasts from patient B and family, who are of Ethiopian Jewish origin, were kindly provided by Dr. Gideon Bach. Fibroblasts were cultured as described elsewhere (Paw et al. 1991).

A Chinese hamster ovary (CHO) cell line deficient in dihydrofolate reductase, previously used for the expression of α-L-iduronidase (Kakkis et al. 1994), was cultured in α-minimum essential medium supplemented with ribo- and deoxyribonucleosides and 5% fetal bovine serum, at 37°C and in 5% CO_2.

Identification of Mutations

Conditions for PCR amplification of exons with intron borders and for SSCP analysis (Orita et al. 1989) have been described elsewhere (Zhao et al. 1996*a*). The primers used for PCR amplification are listed in table 1. Those fragments that gave abnormal migration during SSCP analysis were subjected to sequence analysis. However, for five cell lines there were no segments with abnormal SSCP migration, and all of the PCR-amplified segments were sequenced. All sequence determinations were performed on both strands. Manual cycle sequencing using [^{33}P]dCTP was performed by use of either the Amplicycle (Amersham) or the Thermo Sequenase (Perkin-Elmer) sequencing kit. The GC-rich first segment was especially difficult to sequence, and better results were obtained for that region by use of different primers (segment 3a in table 1) and by automated sequence analysis (Aronovich et al. 1996), performed at the University of Minnesota.

Constructs and Mutagenesis

A 2.5-kb human *NAGLU* cDNA containing the entire coding region and the 3′ UTR was cloned into the expression vector pRC-CMV (Invitrogen) to produce the plasmid pCMV-huNAGLU. The wild-type cDNA was from clone 2a (Zhao et al. 1996*b*); because this clone contained a deletion of a cytosine residue at position 942, it was repaired by substitution of a *Bsp*E1–*Bsp*E1 segment from clone 59 (Zhao et al. 1996*b*), containing

Table 2

Mutations Found in Cell Lines from Patients with Sanfilippo Syndrome Type B

Cell Line	Mutation in Allele 1			Mutation in Allele 2			Method of Analysis
	Codon	Nucleotide	Amino Acid	Codon	Nucleotide	Amino Acid	
GM 00737	CTG→CGG	2045	L682R	TAC→TGC	419	Y140C	PCR, SSCP[a]
GM 01426	GAG→AAG	457	E153K	GAG→AAG	457	E153K	PCR, SSCP[a]
GM 02931	CCG→CTG	1073	P358L	CCG→CTG	1073	P358L	PCR, SSCP[a]
IT 41	CCA→TCA	343	P115S	CCA→TCA	343	P115S	PCR[b]
IT 236	TAC→TGC	419	Y140C	Not identified	…	…	PCR[b]
IT 310	TAC→TGC	419	Y140C	TAC→CAC	274	Y92H	PCR[b]
IT 421	…	901delAA	Frameshift[c]	…	901delAA	Frameshift[c]	PCR[b]
IT 424	…	650insC	Frameshift[d]	CCA→TCA	343	P115S	PCR, SSCP;[a] PCR[b]
B	CGA→TGA	607	R203X	GCG→GTG	1991	A664V	PCR, SSCP[a]

Note.—The mutant nucleotide position in cDNA was calculated from the A of the initiating AUG.

[a] Followed by sequence analysis of segment with abnormal electrophoretic mobility.

[b] Followed by sequence analysis of all fragments.

[c] Termination 55 codons downstream.

[d] Termination 14 codons downstream.

nucleotides 846–1492, for the corresponding segment of clone 2a. The expression construct contained 6 bp of the 5′ UTR and 190 bp of the 3′ UTR. In all but one case, mutations in the *NAGLU* cDNA were generated by two-step PCR (Landt et al. 1990). The first amplification used the *NAGLU* cDNA as a template, a primer containing the desired mutation, and a second oligonucleotide primer (see Appendix, sets 1–4). The product of the first PCR reaction was gel purified and was used as one of the primers for the second PCR reaction. The PCR segment resulting from this second step, which contained the desired mutation, was digested with an appropriate restriction enzyme, was gel purified, and was cloned into the corresponding restriction sites of pCMV-huNAGLU. In one case, Y92H, the mutation was generated by a different method—namely, by reverse-transcriptase PCR using total RNA from cell line IT 310 as a template (see Appendix, set 5). In each case the mutagenized segment and adjoining regions were sequenced to verify that no mutation other than the one desired had been introduced during the procedure.

Transfections

The expression vectors were transfected into CHO cells by electroporation (Bio-Rad Gene Pulser, 240 V, 960 μF). Transfected cells were selected by survival in medium containing 0.75 mg G-418/ml. Approximately 500 positive colonies were pooled for further study. Expression of the transfected cDNA was verified by northern analysis using 10 μg total RNA and a 2.1-kb cDNA probe. For determination of enzyme activity, cells were grown on 100-mm petri plates for 72 h, after being seeded at a density of 2.5×10^6/plate, were transferred to 325 Protein-Free Medium (JRH Biosciences) supplemented with glutamine, and were grown to a dense culture. The cells in each plate were harvested in 1.0 ml of 10 mM Tris-HCl, pH 7.0, containing 0.5% Nonidet P40. The medium was freed of cells by centrifugation. Activity was assayed by the procedure described by Chow and Weissmann (1981). A 25-μl volume of cell suspension or of medium was mixed with 25 μl 0.2 mM 4-methylumbelliferyl-α-*N*-acetylglucosaminide (Calbiochem) in 0.1 M Na acetate buffer, pH 4.3, containing 1 mg bovine serum albumin/ml. After incubation for 1 h at 37°C , the reaction was terminated by addition of 1 ml 0.5 M glycine-NaOH, pH 10.3, for the fluorometric determination of the amount of 4-methylumbelliferone released in the enzyme reaction. Protein concentration was measured by the bicinchoninic acid assay (Smith et al. 1985), by use of a reagent kit from Pierce.

Results

The mutations identified in this study are presented in table 2. When two mutations were found, they were assumed to be on separate alleles; this was formally verified only in the case of patient B, whose parents' cell lines were shown to each carry only one of the mutations. Several points should be noted: (1) four of the nine cell lines studied were homozygous for a mutation; (2) eight mutations were found in only one cell line; and (3) one mutation (Y140C) was observed in three cell lines, and another (P115S) was observed in two cell lines.

The effect of the missense mutations was investigated in an expression system, by use of CHO cell lines stably transfected with an expression vector containing *NAGLU* cDNA that had been mutagenized to correspond to the missense mutations found in this study, as well as to the two missense mutations (R643H and R674H) identified elsewhere (Zhao et al. 1996*a*) (table 3). Successful transfection of the *NAGLU* expression constructs was verified by northern analysis (data not

Table 3

α-*N*-Acetylglucosaminidase Activity in CHO Cell Lines Transfected with Wild-Type and Mutagenized *NAGLU* cDNA

	α-*N*-Acetylglucosaminidase Activity (nmol/h/mg cell protein)	
Transfection Vector	Intracellular	Secreted[a]
None	2.8	.8
pRCCMV	2.5	1.0
pCMVhuNAGLU (wild type)	61.5	14.9
pCMVhuNAGLU-Y92H	2.7	.6
pCMVhuNAGLU-Y140C	2.5	.8
pCMVhuNAGLU-E153K	2.2	.6
pCMVhuNAGLU-P358L	2.4	.7
pCMVhuNAGLU-A643H	2.5	.7
pCMVhuNAGLU-A664V	3.0	.7
pCMVhuNAGLU-R674H	2.0	.7
pCMVhuNAGLU-L682R	2.8	.8

[a] Over 24 h.

shown). In contrast with CHO cells transfected with the wild-type *NAGLU* cDNA, which had a high level of α-*N*-acetylglucosaminidase activity both inside the cells and in the medium, none of those transfected with cDNA with the respective mutations had activity above the level of CHO cells that had not been transfected or had been transfected with the vector alone. Approximately 2% of the intracellular and approximately 5% of the extracellular enzyme activity of the wild-type construct could have been detected, if present, by the assay used.

Discussion

In figure 1, the 17 mutations identified in Sanfilippo syndrome type B, in this study and in earlier studies (Aronovich et al. 1996; Zhao et al. 1996*a*), are shown at their location on the *NAGLU* gene. They include 10 missense, 4 nonsense, and 3 frameshift mutations. No large deletions or major DNA rearrangements have been found yet. Only three mutations have been observed in more than one cell line: Y140C has been observed three times, P115S twice, and R674H twice. The two patients with the R674H mutation were from the same ethnic group (Arab), but we do not know whether they were related (Zhao et al. 1996*a*). The two patients with P115S come from the same region in southern Italy but are not known to be related; since the P115S homozygote is not from a consanguineous family, the mutation may be common in that region. Two patients with Y140C originated from another area of Italy, but the provenance of the third patient (GM 00737) is not known. Seven mutations were found in homozygosity, with consanguinity known or suspected for three of them. Although the small number of cell lines tested and the dearth of information about cells from the Human Genetic Mutant Cell Repository limits the conclusions that can be drawn about the distribution of *NAGLU* mutations, it appears that Sanfilippo syndrome type B is very heterogeneous at the molecular level (with some mutations common only in specific geographic areas) and that the mutations often may be revealed by consanguineous marriages.

The nonsense codons or the frameshifts that lead to a nonsense codon downstream can be assumed to be disease-producing mutations, since a truncated protein or possibly no protein at all would result from the premature termination. The deleterious nature of nine missense mutations was demonstrated, in this study, by introduction of mutagenized cDNAs into overexpressing cells. Although mRNA was expressed, no functional enzyme was found either in the cells or in the medium.

It is interesting to note that the amino acid substitutions identified here or published earlier are not randomly distributed. Nine of the 10 are clustered in two regions of the protein, 4 between codons 92 and 153 and 5 between codons 612 and 682 (fig. 1) . All the substitutions are of amino acids that are conserved between the human and the mouse cDNAs (the mouse cDNA sequence has been deposited in the GenBank database, accession number MMU85247). Although it is premature to draw conclusions, these regions may be especially important for the folding and transporting out of the endoplasmic reticulum and/or for the acquisition of catalytic activity. The availability of stably transfected cell lines will make it possible to perform studies to discriminate between these possibilities.

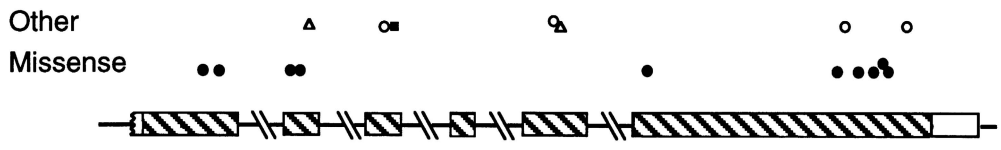

Figure 1 Map of *NAGLU* mutations identified in cell lines of Sanfilippo syndrome type B patients. Boxes denote exons (hatched portions denote the coding regions) and are drawn to scale. Blackened triangles denote deletions (503del110 and 901delAA); the blackened square denotes an insertion (652insC); unblackened circles denote nonsense mutations (R203X, R297X, R626X, and Q706X); and blackened circles denote missense mutations (Y92H, P115S, Y140C, E153K, P358L, S612G, A664V, R674H, and L682R).

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Appendix

Oligonucleotides for Mutagenesis of cDNA

In each of the following sets, the mutant nucleotide is underlined.

Set 1

Antisense oligonucleotides with mutation: P115S, 5′-CGGCACGGCTGACAGTGGCCGCGG; Y140C, 5′-CACACGAAGGAGCAGCTTTGCGTGC; and E153K, 5′-CATCCAGTCTATCTTTCGCTCCCAGCGGG.

Sense primer for first-step PCR: 5′-AAGCCGGGCTTGGACACC.

Antisense primer for second-step PCR: 5′-CTGCAGTCATGGCCTCATAGA.

Restriction sites for cloning: *Pml*I and *Bsu*36I.

Set 2

Antisense oligonucleotide with mutation: P358L, 5′-GCCCCCAGAACTGCAGCTGGTGCTGGAAG.

Sense primer for first-step PCR: 5′-CTGCATTCGCGGGGCATGTT.

Antisense primer for second-step PCR: 5′-TAGCCTCCACGCTGCCCC.

Restriction sites for cloning: *Bsu*36I and *Bpu*1102I.

Set 3

Antisense oligonucleotides with mutation: R643H, 5′-GGTCAGCTGGTAGTGGCTGTTCTGCTC.

Sense primer for first-step PCR: 5′-GCGTGGAGGCTACTGCT.

Antisense primer for second-step PCR: 5′-TTTAATCCCACACTTTGGGTGGT.

Restriction sites: *Pfl*MI and *Pfl*MI.

Set 4

Sense oligonucleotides with mutation: A664V, 5′-CAAGCAGCTGGTGGGGTTGGTGGC; R674H, 5′-CTACTACACCCCTCACTGGCGGCTTTTCC; and L682R, 5′-CTTTTCCTGGAGGCGCGGGTTGACAGTGTGGCC.

Antisense primer for first-step PCR: 5′-TTTAATCCCACACTTTGGGTGGT.

Sense primer for second-step PCR: 5′-GCGTGGAGGCTACTGCT.

Restriction sites: *Pfl*MI and *Pfl*MI.

Set 5

Antisense primer for reverse transcription and PCR: 5′-CTGCAGTCATGGCCTCATAGA.

Sense primer for PCR: 5′-AAGCCGGGCTTGGACACC.

Restriction sites: *Pml*I and *Bsu*36I.

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