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Molecular Analysis of the Acid Sphingomyelinase Deficiency in a Family with an Intermediate Form of Niemann-Pick Disease

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Summary

A novel point mutation in the lysosomal acid sphingomyelinase gene has been identified in the recently reported Serbian family with a clinically and biochemically atypical intermediate form of Niemann-Pick disease. The mutation was a $T^{1171}\rightarrow G$ transversion resulting in substitution of glycine for normal tryptophan at amino acid residue 391. The coding sequence was otherwise normal. All of the five affected individuals were almost certainly homoallelic, and both of the two obligate heterozygotes studied also carried the same mutation. This mutation is therefore likely to be directly associated with the atypical phenotype of these patients. Expression in COS-1 cells suggested a higher residual activity than that in cultured fibroblasts. A recently developed high-affinity rabbit antihuman sphingomyelinase antibody allowed us to study for the first time the biosynthesis, processing, and targeting of a mutant sphingomyelinase by metabolic labeling of cultured fibroblasts. The mutant enzyme protein was normally synthesized, processed, and routed to the lysosome but was apparently unstable and degraded rapidly once it reached the lysosome. Together with the finding of the relatively high residual activity in COS-1 cells, we interpret our observations to mean that instability and rapid breakdown of the mature mutant enzyme protein, due to the mutation rather than direct inactivation of the catalytic activity, is the primary mechanism for the deficiency of sphingomyelinase activity in these patients. A high prevalence of this mutation in the Serbian population is likely, since the family pedigree indicates that members from four reportedly unrelated families must have contributed the same mutation.

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Introduction

Niemann-Pick disease is an autosomal recessive lysosomal storage disorder caused by a primary deficiency of acid sphingomyelinase (ASM) [E.C.3.1.4.12], resulting in massive tissue accumulation of sphingomyelin (Schuchman and Desnick 1995). Classically, two allelic subtypes with distinct clinical phenotypes have been delineated: a severe neurodegenerative infantile form leading to death by 2–3 years of age (Niemann-Pick disease type A), and a chronic, nonneuronopathic form (Niemann-Pick disease type B) (Crocker 1961). An intermediate, subacute form has also been reported, including rarer patients who satisfy the criteria for Niemann-Pick disease type B but with additional signs of mild neuronal involvement, such as retinal storage, peripheral neuropathy, mild neurological signs, or psychiatric disorders (Spence and Callahan 1989). The molecular or enzymological basis for this broad phenotypic heterogeneity is not yet clear. Cerebral storage of sphingomyelin has been demonstrated in type A but not in type B patients, in spite of essentially similar, very low, residual sphingomyelinase activities measured in brain tissue by the classical in vitro method (Spence and Callahan 1989). A normal Michaelis-Menten constant was found in placental enzyme partially purified from a type A fetus (Rousson et al. 1987). This mutant enzyme protein cross-reacted with a polyclonal antibody against the normal sphingomyelinase (Rousson et al. 1987). Limited studies indicated the presence of immunologically cross-reactive material in crude cell homogenates of both type A and type B patients (Rousson et al. 1986; Callahan and Jobb 1988). On the other hand, cultured fibroblasts from classical type B patients can hydrolyze exogenously added sphingomyelin at significantly higher rates than those from type A patients (Beaudet and Manschrek 1982; Vanier et al. 1985). The availability of the sphingomyelinase cDNA and the gene (Quintern et al. 1987, 1989; Schuchman et al. 1991, 1992) has made it possible to study the precise molecular defects in patients. To date, 12 mutations responsible for Niemann-Pick disease have been reported, among which are 3 with a high prevalence in Ashkenazi Jewish type A and 1 in North African type B patients (Ferlinz et al. 1991; Levran et al. 1991*a*,

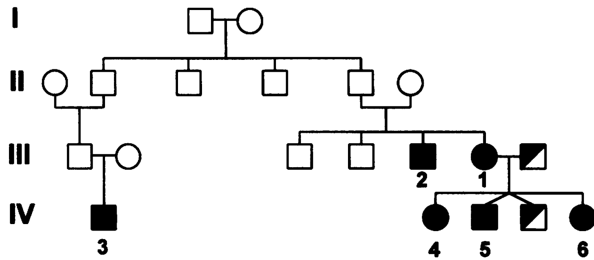

Figure 1 Family pedigree (simplified from Sperl et al. 1994). Filled symbols indicate affected individuals, and half-filled symbols obligate heterozygotes who were tested. Clinical, neurophysiological, and laboratory data of the patients have been reported under the same numbering in Sperl et al. 1994. Patient III/2 was not included in the present investigation.

1991*b*, 1992, 1993*a*, 1993*b*; Takahashi et al. 1992*a*, 1992*b*; Vanier et al. 1993). Several point mutations among which are splice-site mutations associated with a type A or B phenotype and one three-base deletion characteristic of a type B phenotype are thus far known. Mutations can cause deficient steady-state ASM activity not only due to inactivation of the catalytic activity but also due to abnormal processing and stability of the enzyme protein. However, no processing studies of mutant sphingomyelinase have yet been available, due to the lack of suitable anti-ASM antibodies. Anti-ASM antibodies suitable for processing studies have been developed recently and have been used for studies of normal sphingomyelinase (Ferlinz et al. 1994; Hurwitz et al. 1994*b*).

We recently described a Serbian family with six members affected by an atypical intermediate form of Niemann-Pick disease with a visceral course and an additional macular halo syndrome (Sperl et al. 1994). We report here identification of a novel point mutation in the ASM gene responsible for the disease in this family. All affected members examined were almost certainly homoallelic for the mutation. Synthesis and processing of this mutant enzyme protein were also studied in cultured fibroblasts.

Material and Methods

Subjects and Biological Material

The family pedigree (fig. 1) includes six patients with an intermediate form of Niemann-Pick disease, all members of the same clan with its roots in a small village of Serbia. They showed a visceral course of the disease with additional retinal changes (macular halos) indicative of neuronal storage and a very low degradation rate of sphingomyelin in skin fibroblasts. Their detailed clinical and enzymatic phenotype has been published recently (Sperl et al. 1994). The present study included seven subjects: one affected woman (patient III/1), her four children, three of them affected (patients IV/4, IV/5, and IV/6), her supposedly unrelated husband, and an affected second degree cousin (patient IV/3). Blood samples from patient IV/3 were provided by Dr. W. Sperl, Innsbruck. Skin fibroblast cultures from all other subjects were previously supplied by Dr. U. Wiesmann, Bern, and Dr. G. Utermann, Innsbruck.

Material

DNA-modifying enzymes and N-[1-(2-3-Dioleoyloxy)propyl]-N,N,N-trimethyl-ammonium methylsulfate (DOTAP) lipofection reagent were obtained from Boehringer, cell culture media and reverse transcriptase from Gibco-BRL, and *Taq*-DNA polymerase from Cetus–Perkin Elmer. Radioisotopes were purchased from Amersham. A Sequenase version 2.0 kit was obtained from USB, and coated magnetic beads for DNA single-strand and mRNA isolation were obtained from Dynal. All other standard chemicals were of the highest quality available. The preparation and characterization of the anti-ASM antibody have been reported elsewhere (Hurwitz et al. 1994*b*).

RNA Extraction, cDNA Synthesis, and PCR Amplification

Total RNA and genomic DNA from fibroblasts and blood was prepared according to standard protocols (Ausubel et al. 1987; Sambrook et al. 1989). mRNA was isolated by incubation of total RNA with oligo-dT-coated magnetic beads and subsequent separation of solid-phase-bound mRNA by magnetic attraction (Dynal). Reverse transcription (200 U MuML V-RNase H^- reverse transcriptase, 43°C, 60 min) of 2 μg mRNA to partial cDNAs was performed using 20 pmol of an internal sequence-specific primer. Northern analysis of 1 μg of poly A^+RNA was done with a full-length sphingomyelinase cDNA as the probe.

PCR amplification of the two portions of cDNAs was done with a primer set consisting of a 5′-biotinylated and a normal oligonucleotide, each (A1bio/A2 and B1/B2bio, in table 1). This yielded two overlapping PCR products spanning the total protein-coding sequence as well as most of the 3′-UTR. Amplification of 1 μg of cellular DNA was carried out using an intronic 3′-sense primer and a 5′-biotinylated antisense primer (I/B2bio in table 1), with 30 cycles of heat denaturation at 95°C for 30 s, annealing at 54°C for 2 min, and extension at 72°C for 2.5 min. The amplified PCR products were evaluated by agarose gel electrophoresis and were subsequently purified by extraction of excised bands using silicon-beads (Qiagen).

DNA Sequence Analysis

Purified biotinylated PCR DNA was bound to Dynabeads M280-Streptavidin and the nonbiotinylated

Table 1

Oligonucleotide Sequences

Oligonucleotide	Coordinates[a]	Sequence
A1bio	−29/−11	5′-Biotin-CGAACCAGCCCCGTGTAGG-3′
A2[b]	1083/1063	5′-GAGGGTGCGCAGGGCTTCGGC-3′
B1	761/778	5′-TGAGGACCCTGGAGAGCC-3′
B2[b]	1984/1967	5′-GTGGATTCACCGGATGATCTTGCCT-3′
B2bio[b]	1979/1967	5′-Biotin-TTCACCGGATGATCTTGCCT-3′
S	1070/1087	5′-CCTGCGCACCCTCAGAAT-3′
X[b]	1183/1159	5′-TGATCAAGAGCCCGAAGTTCTCACG-3′
Y	1159/1183	5′-CGTGAGAACTTCGGGCTCTTGATCA-3′
I	intron 2	5′-ataagcttggaacaagtgttgacctc-3′

[a] Counted from the adenosine residue of the first putative initiation codon. Sequences given in upper-case characters are exon sequences, and those in lower case are intron sequences. The underlined areas were altered from the natural sequences in order to generate new restriction sites.

[b] Oligonucleotides corresponding to the antisense strand of DNA.

strand released essentially according to the manufacturer's instruction (Dynal). Solid phase-bound single-stranded DNA, as well as soluble DNA, were neutralized and sequenced by the chain-termination method (Sanger et al. 1977) using Sequenase version 2.0.

Site-Directed Mutagenesis of cDNA by Overlap Extension

PCR amplification of wild-type cDNA was performed using two sets of primers consisting of a wild-type-derived and -mutated primer (B1/X and Y/B2, in table 1) (Ho et al. 1989). The resulting purified PCR products, identical with respect to the short DNA portion that contained the mutated oligonucleotide, served further as template for the second PCR using the nonmutated 3′ and 5′ flanking oligonucleotides (B1/B2) as primers (table 1). Similar cycling conditions were used for the first and second PCR of the overlap extension method using 30 cycles with 30 s denaturation at 95°C, 1 min annealing at 52°C, and 1.5 min extension at 72°C. The internal *Bst*EII-DNA portion of the wild-type DNA was substituted by the corresponding mutated DNA and finally ligated into the eukaryotic expression vector p91023(B) (Kaufman et al. 1989).

COS-1 Cell Expression

On the day before transfection, $\sim 5 \times 10^5$ COS-1 cells were plated on 21-cm^2 dishes. The transfections with DOTAP as lipofection reagent (Boehringer-Mannheim) were carried out by using 7 μg of pure plasmid DNA for each dish. During the initial 6-h period, the cells were maintained under OptiMEM medium (Gibco-BRL) without supplements. Forty-eight hours after the transfection the cells were harvested and assayed for acid sphingomyelinase activity, using [^{3}H] choline-labeled sphingomyelin (Quintern et al. 1989).

Sphingomyelinase Radiolabeling and Immunoprecipitation

Confluent cultured skin fibroblasts (25-cm^2 flasks) were starved for 1 h in a methionine-free DMEM medium containing 4% dialyzed FCS. Labeling was initiated by addition of 150 μCi of L-[^{35}S]-methionine in 1 ml (>3000 Ci/mmol). After varying pulse periods, chases were conducted by addition of L-methionine to a final concentration of 10 mM. Cell extracts were prepared with phosphate-buffered saline containing 1% Nonidet P-40, 10 mg/ml ovalbumin, 2 mM phenylmethylsulfonylfluoride (PMSF), 5 mM iodoacetamide, 2 mM EDTA, and 1 μg/ml leupeptin and pepstatin, each. Immunoprecipitation was performed using rabbit anti-ASM antiserum as described elsewhere (Hurwitz et al. 1994*b*). Immunoprecipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis using a 10% slab gel. The gels were impregnated with Amplify (Amersham) for fluorography.

Results

Identification of the Trp391Gly Mutation

The complete ASM-encoding cDNA segment, as well as parts of the 5′- and 3′-flanking regions of proband IV/6, were amplified and directly sequenced. This analysis revealed a novel mutation, a $T^{1171}\rightarrow G$ transversion (nucleotides counted from the A of the first putative initiation codon, ATG), resulting in a substitution of glycine (GGG) for normal tryptophan (TGG) at amino acid position 391 (Trp391Gly) (fig. 2). The remainder of the protein-coding sequence was entirely normal. Northern analysis of this patient did not show significant aberrations in quantity and size of mRNA, compared with normal control cells (data not shown). Sequence analysis of this region in the six other members of the family

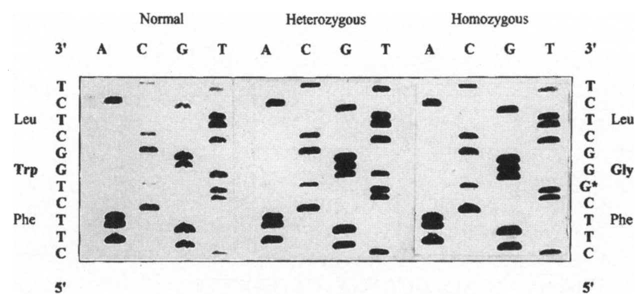

Figure 2 Mutation analysis of PCR amplified genomic DNA surrounding the point mutation of normal and an individual afflicted with Niemann-Pick disease. A single nucleotide transversion, T→G, at position 1171 is indicated by an asterisk (*). Probands expressing clinical manifestations were exclusively homoallelic (illustration represents DNA sequence derived from genomic DNA of proband IV/5, whereas two oligate heterozygotes were found to be carriers of the mutant allele.

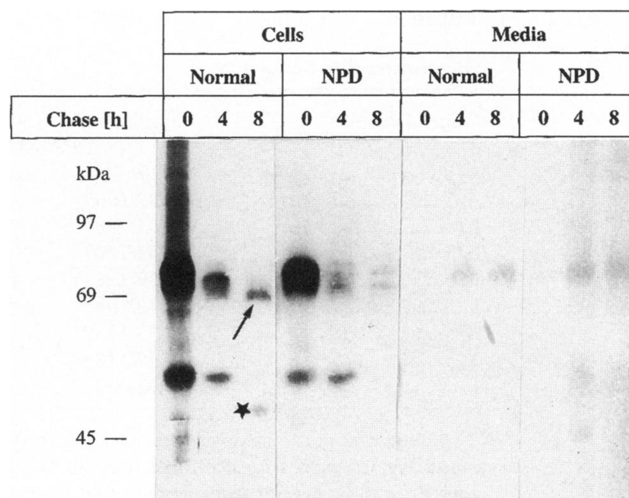

Figure 3 Immunoprecipitation of cell homogenate of ^{35}S-methionine labeled normal and Niemann-Pick fibroblasts was performed after a 1-h pulse period and additional chase periods of 4 and 8 h. The prevalent lysosomal form of mature ASM (70 kDa), which contains most of enzymatic activity, is illustrated by an arrow. This enzyme form, as well as its subsequent processed product of 50 kDa (shown by a star), were not detected in the ASM-deficient (NPD) cells. Immunoprecipitation of culture medium of metabolically labeled normal and Niemann-Pick fibroblasts shows normal biosynthesis and targeting of ASM precursor and early processing products (72-kDa form and 57-kDa form). Neither the normal nor mutant cells secrete into the medium the mature lysosomal enzyme, which accounts for most of the catalytic activity.

studied indicated that all individuals carried at least one Trp391Gly allele. Only the sequence containing this mutation was present in this region of the genome in the affected probands III/1, IV/4, IV/5, and IV/6 and their affected cousin, patient IV/3. The healthy husband of patient III/1 and her unaffected son carried only one allele with this mutation, in good accordance with intermediate sphingomyelinase activities (data not shown) and the obligate heterozygote status of these two individuals.

Expression of the Trp391Gly Mutant Allele in COS-1 Cells

The effect of the nucleotide substitution on the catalytic activity of the resulting protein was assessed by transient expression in COS-1 cells of cDNA containing the T^{1171}→G transversion generated by the overlap extension method (pASM391). As shown in table 2, sphingomyelinase activity measured in COS-1 cell homogenates transfected with the mutagenized DNA by an in vitro detergent-containing assay showed a residual activity of 6%–10% compared with that in wild-type transfectants. This level of residual activity appeared higher than the 1%–2% values found in cultured fibroblasts from the homozygous patients (Sperl et al. 1994; present study).

Table 2

Transient Expression of Acid Sphingomyelinase in COS-1 Cells

Source	Acid Sphingomyelinase Activity (nmol/h/mg homogenate protein ± SD)
COS-1 cells	10.5 ± 1.4
p91023 (B)	10.1 ± 0.7
pASMas (antisense)	8.0 ± 1.1
pASMs (sense)	68.2 ± 4.6
pASM391as (antisense)	8.4 ± 0.9
pASM391s (sense)	15.4 ± 1.6

NOTE.—The values represent the mean of three independent experiments, each using a different batch of recombinant DNA.

Metabolic Labeling and Immunoprecipitation

Pulse-chase studies (1-h pulse and 2- or 6-h chase) revealed that the 75-kDa ASM form that lacks significant catalytic activity (Ferlinz et al. 1994; Hurwitz et al. 1994*b*) was present in similar amounts in the mutant and control fibroblasts both in cellular extracts as well as in the medium (fig. 3). In mutant cells, the early proteolytic processing, which partially converts the proproform to the 72-kDa precursor and the 57-kDa form, was also found to be normal. However, no mature lysosomal ASM including the 70-kDa and the 50-kDa form, which normally account for most of the enzyme activity, could be detected in the mutant cells (fig. 3).

Discussion

Mutations in at least one allele have been reported to date in nearly 50 patients with Niemann-Pick disease, all of whom belong to the classical A or B phenotypes.

A majority of the type A patients so far studied were of Ashkenazi Jewish descent. In that population, two major (R496L and L302P) and one minor (fsP330) mutations accounted for 65% of the 52 tested alleles (Levran et al. 1991*a*, 1992, 1993*b*). The seven additional mutations identified in six other families with type A patients appear sporadic (Ferlinz et al. 1991; Takahashi et al. 1992*b*; Levran et al. 1993*a*). Only two mutations associated with typical type B Niemann-Pick disease have so far been reported. A three-base deletion, R(608), very common (26/30 alleles) in patients from Northern Africa (Vanier et al. 1993), may also be found in Ashkenazi Jewish type B patients (Levran et al. 1991*b*), and more generally in patients from the Mediterranean region (M. T. Vanier, S. Duthel, and R. Rousson, unpublished data). A homoallelic S436R substitution was found in the only Japanese type B patient studied so far (Takahashi et al. 1992*a*).

The present study identified the first mutation in patients with an intermediate "subacute" phenotype (Spence and Callahan 1989). All of the five patients studied appeared to be homoallelic, on the basis of the sequencing data on genomic DNA. Although the data themselves do not rigorously exclude a heteroallelic state of the W391G mutation on one allele and a major deletion on the other, careful pedigree analysis and other pieces of evidence indicate that such a possibility is highly unlikely. The W391G mutation was present not only in all of the five patients but also in two obligate carrier individuals, the husband of an affected mother, and one of her offsprings. One of her affected children showed normal size and quantity of sphingomyelinase mRNA, a finding that provides support for the claim that this child is homoallelic for the W391G mutation. The distribution of the W391G mutation in the pedigree (fig. 1) indicates that four reportedly unrelated families contributed this mutation. All patients exhibited the same atypical intermediate phenotype. These findings collectively indicate that the W391G mutation occurs in high frequency in the Serbian population and that the mutation is directly associated with this atypical clinical presentation. A study is underway to determine whether other unrelated patients with a similar phenotype may have the same mutation.

When transiently expressed in COS-1 cells, the mutant cDNA, W391G, exhibited an apparently higher residual activity (6%–10% of control) than that in intact cultured fibroblasts from patients (1%–2%). This result suggested that the mutation may not directly affect the catalytic activity of the enzyme protein but that the deficiency of sphingomyelinase activity may be due to abnormal processing, transport, and/or stability of the mutant enzyme. Recent availability (Ferlinz et al. 1994; Hurwitz et al. 1994*b*) of high-affinity, precipitating anti-ASM antiserum finally enabled us to perform immunoprecipitation studies on metabolically labeled cultured fibroblasts. As described elsewhere, COS-1 cells transfected with wild-type cDNA (Ferlinz et al. 1994), as well as normal fibroblasts (Hurwitz et al. 1994*b*), generate mature forms of ASM from a common catalytically inactive precursor of 75 kDa. Maturation of the normal enzyme is achieved mainly by a stepwise mechanism: proteolytic cleavage of the nascent polypeptide results in a 72-kDa proform inside the endoplasmic reticulum (ER)/Golgi complex, which, after sorting for the lysosomal pathway, is further processed to the mature ASM of 70 kDa within ~6 h after biosynthesis. The half-life of the 70-kDa form, to which most of the catalytic activity of ASM can be attributed, is >12 h. Subsequent degradation finally generates a protein of ~50 kDa. Besides this regular maturation event, the ASM precursor is also partially cleaved early inside the ER/Golgi complex, yielding variable but low amounts of a 57-kDa form of ASM. In the present study, patients' fibroblasts failed to generate either the 70-kDa or 50-kDa form of ASM (fig. 3). Immunoprecipitation of ASM protein from the culture medium showed secretion of minor amounts of the precursor at the same level as in the medium of normal fibroblasts (fig. 3). This finding indicates that, in contrast to I-cell disease fibroblasts, where a large amount of the ASM precursor is secreted (Hurwitz et al. 1994*b*), newly synthesized ASM in the patient cells is targeted to the lysosomes. On the other hand, secretion of the mutant ASM precursor into the culture medium argues against possible misfolding of the protein followed by precipitation and degradation inside the ER. Moreover, the early processing, which results in formation of the 57-kDa form, points to proper biosynthesis and transport. Collectively, these data strongly suggest that the mutant ASM is correctly folded, sorted, and presumably processed to mature lysosomal enzyme but is rapidly degraded inside the acidic compartments due to structural alterations as the result of the point mutation. If overexpressed ASM in COS-1 cells overwhelms the limited proteolytic capacity in the lysosome, significant amounts of the mature mutant protein may escape rapid and complete hydrolysis. This would explain the different residual activities in cultured fibroblasts and transfected COS-1 cells. Similar abnormalities of ASM processing have recently been reported in normal fibroblasts treated with desipramine (Hurwitz et al. 1994*a*). Addition of tricyclic antidepressants to cultured cells is known to affect severely the activity of ASM but not of most other lysosomal enzymes (Albouz et al. 1981). This effect can be prevented by preincubating the cells 18–24 h with leupeptin (Vanier et al. 1986), shown to inhibit conversion of the 70-kDa into the 50-kDa form of ASM (Hurwitz et al. 1994*a*). Addition of

leupeptin for ≤1 week to the mutant Niemann-Pick fibroblasts had only minimal effect on in vitro sphingomyelinase activities (residual activity remained <1% of normal). No restoration of activity could be demonstrated in desipramine-treated cells after 18-h leupeptin addition (R. Zeitouni and M. T. Vanier, unpublished data), either, suggesting that proteolytic degradation of ASM is a more complex process. Further experiments on ASM metabolism using various human cell types will, it is hoped, provide more insight into this potential regulatory mechanism.

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