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Correction of Sulfatide Metabolism after Transfer of Prosaposin cDNA to Cultured Cells from a Patient with SAP-1 Deficiency

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Summary

The lysosomal removal of the sulfate moiety from sulfatide requires the action of two proteins, arylsulfatase A and sphingolipid activator protein-1 (SAP-1). Recently, patients have been identified who have a variant form of metachromatic leukodystrophy which is characterized by mutations in the gene coding for SAP-1, which is also called "prosaposin." All of the mutations characterized in these patients result in (*a*) deficient mature SAP-1, as determined by immunoblotting after SDS-PAGE of tissue and cell extracts, and (*b*) decreased ability of cultured skin fibroblasts to metabolize endocytosed [^{14}C]-sulfatide. We now report the insertion of the full-length prosaposin cDNA into the Moloney murine leukemia virus–derived retroviral vector, pLJ, and the infection of cultured skin fibroblasts from a newly diagnosed and molecularly characterized patient with SAP-1 deficiency. The cultured cells infected with the prosaposin cDNA construct now show both production of normal levels of mature SAP-1 and completely normal metabolism of endocytosed [^{14}C]-sulfatide. These studies demonstrate that the virally transferred prosaposin cDNA is processed normally and is localized within lysosomes, where it is needed for interaction between sulfatide and arylsulfatase A. In addition, normal as well as mutant sequences can now be found by allele-specific oligonucleotide hybridization of PCR-amplified genomic DNA by using exonic sequences as primers.

Introduction

The lysosomal catabolism of most sphingolipids requires a specific lysosomal enzyme and a heat-stable protein we call "sphingolipid activator protein" (SAP). While the exact number of SAPs is not known, five have been clearly identified (Mehl and Jatzkewitz 1964; Ho and O'Brien 1971; Conzelmann and Sandhoff 1978; Furst et al. 1988; Morimoto et al. 1989). The activator of GM_2 ganglioside hydrolysis, which maps to chromosome 5, is deficient in patients with the AB variant form of GM_2 gangliosidosis (Conzelmann and Sandhoff 1978). The four others are coded for by one gene on chromosome 10 and arise via proteolytic cleavage of a precursor protein with a molecular weight of about 69,000 (Inui et al. 1985; Fujibayashi and Wenger 1986; O'Brien et al. 1988; Rorman and Grabowski 1989). Each of these four SAPs, consisting of about 80 amino acids, shares certain structural features such as location of glycosylation sites, cysteine residues, and proline residues. However, they do not have identical abilities to activate the enzymatic hydrolysis of sphingolipids (Wenger et al. 1982; Wenger and Inui 1984; Morimoto et al. 1988). Their exact mechanism of action have not been defined, but their physiologic importance has been confirmed by the finding of patients who have severe fatal genetic disorders caused by deficiencies of SAP. Patients with mutations in domain 2 (SAP-1) have symptoms related to their inability to metabolize sulfatide (Stevens et al. 1981; Hahn et al. 1982; Wenger et al. 1989). All of the mutations that have been characterized in patients (point mutations and a 33-nucleotide insertion) affect only this domain, leaving the others apparently functional (Kretz et al. 1990; Rafi et al. 1990; Zhang et al. 1990; Holtschmidt et al. 1991). These patients are characterized by early onset of neurological disease suggestive of a leukodystrophy, excretion of unde-

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graded sulfatide, inclusions in nerves classified as metachromatic in nature, normal arylsulfatase A, decreased ability to metabolize radiolabeled sulfatide taken up by cultured skin fibroblasts, and decreased levels of mature SAP-1, as determined by western blotting of leukocyte and fibroblast extracts (Stevens et al. 1981; Hahn et al. 1982; Inui et al. 1983; Fujibayashi et al. 1984; Wenger et al. 1989). Four patients studied in this laboratory survived until the second or third decade, and one died at 28 mo. Three patients with symptoms related to their inability to degrade glucosylceramide have been shown to have decreased levels of SAP-2 (domain 3 of the prosaposin mRNA) (Christomanou et al. 1986, 1988; Harzer et al. 1989).

In the present paper, we describe the transfer of the full-length cDNA for the SAP-1 precursor, called "prosaposin," in a retroviral vector into cultured skin fibroblasts of a newly diagnosed and molecularly characterized patient with SAP-1 deficiency. The success of this transfer was documented both by detection of mature SAP-1 in the cells after SDS-PAGE and immunological staining and by complete correction of sulfatide catabolism after its uptake from the medium in our loading test.

Subject and Methods

Subject

Skin fibroblasts were grown from a 10-year-old child of Mexican ancestry who was thought to have metachromatic leukodystrophy, because of evidence of peripheral neuropathy and weakness, with deteriorating mental capacities, for at least 4 years. Biochemical studies showed normal arylsulfatase A activity in our laboratory and one other, but the urine tests showed excretion of excess sulfatide. Cultured skin fibroblasts were given [^{14}C]-stearic acid–labeled sulfatide for 1 and 3 d in our standard loading test (Kudoh and Wenger 1982). Only about 12% of the sulfatide was metabolized to other products after 3 d (compared with an average of 80% in controls). No mature SAP-1 could be detected after SDS-PAGE of a cell extract followed by visualization with our monospecific, polyclonal antibody (Inui and Wenger 1984). This confirmed the diagnosis of metachromatic leukodystrophy caused by SAP-1 deficiency. Sequencing of the cDNA from this patient revealed that he was homozygous for a single nucleotide change (C-to-T at position 650, counting from the A of the ATG initiation codon) (authors' unpublished data). This mutation is identical to that found in two other unrelated Mexican siblings (Kretz et al. 1990; Rafi et al. 1990). This mutation changes the codon for threonine to one for isoleucine and eliminates the only glycosylation site in the mature SAP-1 domain, therefore exposing a neighboring arginine residue to proteolytic cleavage leading to unstable mature SAP-1.

Generation of Full-Length Prosaposin cDNA

A lambda gt11 cDNA library, derived from mRNA of human skin fibroblasts, was screened using probes of 900 and 700 bp from the 5′ and 3′ ends of prosaposin cDNA. A full-length cDNA starting six nucleotides before the initiation codon to the poly-A tail was subcloned into pBluescript SK + / − (Stratagene, La-Jolla, CA) and then was sequenced. Some differences from the initially published sequence (Dewji et al. 1987) were found. Our sequence agrees with that published by others (Nakano et al. 1989; Rorman and Grabowski 1989), except that we did not have the extra nine nucleotides that can occur by alternative splicing (Zhang et al. 1991). In order to clone this cDNA into the *Sal*I site of pLJ retroviral vector (obtained from Allen Korman), PCR primers I and II, presented in table 1, were utilized. Amplification was performed at 94°C for 1 min, 52°C for 1 min, and 72°C for 3 min, for 30 cycles on a Coy thermal cycler. Reagents and materials for the amplification were purchased from Perkin Elmer Cetus (Emeryville, CA).

Plasmid Construction

The full-length prosaposin cDNA with *Sal*I restriction sites was digested with *Sal*I and ligated into the unique *Sal*I site of Moloney murine leukemia virus–derived retroviral vector, pLJ (Korman et al. 1987) (fig. 1). The construct was used to transform *Escherichia coli* HB101 cells. Clones representing both the sense (pLJ-S-12) and antisense (pLJ-S-13) orientations (with respect to the 5′ long-terminal repeat) were screened on kanamycin-containing agar plates.

Production of Infectious, Helper-free Recombinant Retroviruses

The retroviral vector, pLJ, and constructed plasmids, pLJ-S-12 and pLJ-S-13, were transfected into the amphotropic packaging cell line, Ψ-CRIP (Danos and Mulligan 1988) (obtained from Richard Mulligan), by using the calcium phosphate method described by Graham and van der Eb (1973) and modified by Wigler et al. (1979). After transfection, neomycin-resistant colonies were selected with G418 (400 μg/ml) and subcloned. The medium from these se-

Table 1

Oligonucleotides Used for PCR Amplification and ASO Hybridization

Number, Orientation	Sequence[a]
I, Sense	5′-TGCAGTCGACGCTATGTACGCCCTCTTCCT-3′
II, Antisense	5′-TGCAGTCGACTTCTGGCTAACAGAATTTTATTGTT-3′
III, Sense	5′-TCACTGCAGTCCTCTTAAGTTGCAAACCT-3′
IV, Antisense	5′-ACGTGAATTCACTCCCAGGCCCAGAACAT-3′
V, Sense	5′-GTCGACTGCAGATGGTGACTGACATCCAGA-3′
VI, Antisense	5′-GGCATGGCCGACATATGCAA-3′
VII, Normal (N)	5′-CCAACTCCACCTTTGTCCA-3′
VIII, Mutant (M)	5′-CCAACTCCATCTTTGTCCA-3′

[a] Underlined sequences are complementary to prosaposin sequences; overlined sequences are restriction-endonuclease cutting sites; underlined nucleotides in VII and VIII show the site of the mutation in this patient.

lected cells was collected after 12 h, filtered through a 0.45-μm membrane (Millipore Corp, Bedford, MA), and used for infection of cultured skin fibroblasts of the patient.

Infection of Cultured Skin Fibroblasts from the Patient, and Confirmation of Insertion

Lightly confluent fibroblasts from the patient were infected with the filtered medium in the presence of polybrene (8 μg/ml) for 12–16 h. The cells were screened for neomycin resistance in the presence of G418 (400 μg/ml). Expanded clonal populations of G418-resistant cells were screened for (*a*) the presence of SAP-1 by using our monospecific antibody, which detects mature SAP-1 after SDS-PAGE and transfer to nitrocellulose (Inui and Wenger 1984), and (*b*) their ability to metabolize endocytosed [^{14}C]-stearic acid–labeled sulfatide for 1 and 3 d, by using our published method (Kudoh and Wenger 1982). In addition, the

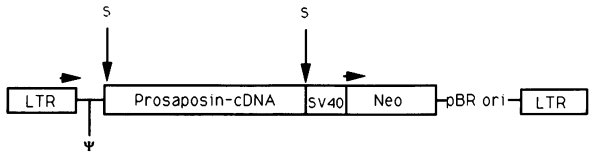

Figure 1 Schematic representation of the retroviral vector pLJ containing the human prosaposin cDNA. The prosaposin cDNA was cloned into a *SalI* (S) site in the vector. Transcription of the prosaposin is promoted by Moloney murine leukemia virus long-terminal repeat (LTR), and the neomycin (Neo)-resistant gene is promoted by SV40 promoter sequences. The arrowheads indicate the direction of transcription. Ψ = Viral packaging signal.

presence in the patient's cells of the normal DNA sequence (conferred by the retroviral construct) as well as the mutated sequence were examined by specific PCR amplification using primers generated from both intronic sequences (III and IV in table 1) and exonic sequences (V and VI in table 1), followed by allele-specific oligonucleotide (ASO) hybridization using probes (VII and VIII in table 1) and conditions reported elsewhere by our laboratory (Rafi et al. 1990).

Results

Since the initial isolation of a partial cDNA clone for SAP-1 (Dewji et al. 1986), a number of reports have described the isolation of full-length clones and the interesting observation that this gene codes for four putative SAPs (Furst et al. 1988; O'Brien et al. 1988; Nakano et al. 1989; Rorman and Grabowski 1989). We independently isolated a full-length cDNA clone from a human skin fibroblast library. This clone began six nucleotides prior to the ATG initiation codon and included 21 nucleotides of the poly-A tail. PCR amplification of this clone was accomplished using the two primers (I and II) listed in table 1 and included *SalI* restriction sites at both ends for cloning into the retroviral vector pLJ (fig. 1). Recombinant viral construct with the cDNA insert in both orientations was obtained after restriction-digest analysis and was used to transfect Ψ-CRIP packaging cell line. The Ψ-CRIP cells were transfected with the vector alone and with the insert in the sense and antisense directions. G418-resistant colonies were obtained without

difficulty, and the filtered medium from these cells was used to infect the cultured cells of the patient. G418-resistant cells were obtained and subcloned.

Initial studies were done to detect the presence of mature SAP-1 in the patient's cells, which had been undetectable before infection. In figure 2 is shown the western blot of fibroblast extracts from the patient with the vector alone (lane 3) and with pLJ-S-12, the vector containing the insert in the sense direction (lane 2). The cells with no vector and those with a vector containing the insert in the antisense direction showed no staining with antibody, exactly as in lane 3 (data not shown). The region that stained with our antibody corresponds to fully processed SAP-1, migrating with an estimated molecular weight of 8,000–9,000.

This patient has been shown to have a single nucleotide change in the SAP-1 coding region (domain 2). In order to detect the presence of both the normal sequence, conferred by the construct, and the pre-existing mutant sequence, ASO hybridization was performed on PCR-amplified genomic DNA by using two sets of primers. One set of primers, III and IV (table 1), representing newly obtained sequences from introns (authors' unpublished data) spanning the mutation site, resulted in the detection of only one mutant sequence in the patient with or without a vector containing the insert in the correct orientation (fig. 3*A*). When primers V and VI (table 1), representing exonic sequences, were utilized, the cells from the patient infected with pLJ-S-12 showed hybridization to oligonucleotide probes for the normal and mutant alleles (fig. 3*B*). These studies clearly document the presence of the normal allele in the patient's cells, proving that the vector containing cDNA is in the correct orientation, and confirm the preliminary studies showing mature SAP-1 in these cells.

In order to determine whether this biochemically processed SAP-1 derived from the construct was localized within the lysosomes and was functional, sulfatide loading was performed. As can be seen in figure 4, very little of the endocytosed [^{14}C]-sulfatide was metabolized to other compounds in the cells from the patient infected with pLJ alone (lane 2). However, when pLJ-S-12 was used, most of the [^{14}C]-sulfatide was metabolized to other compounds (lane 3). As the [^{14}C]-sulfatide is radiolabeled in the fatty-acid moiety, the degradation of sulfatide continues until the fatty acid is cleaved by ceramidase and until it leaves the lysosome where it is utilized in the synthesis of phospholipids, cholesterol ester, and specific glycolipids. There is no synthesis of sulfatide in cultured skin fibroblasts. To quantitate the degradation of sulfatide, all of the areas of radioactivity were scraped from the plate and counted. As shown in figure 5, the cells from the patient with the vector alone metabolized only about 10% of the sulfatide after 3 d. With the vector in the sense orientation (pLJ-S-12), about 85% was metabolized after 3 d. This is clearly within the normal range. The cells from the patient given the vector containing the insert in the antisense orientation showed results identical to those shown by the cells without treatment (data not shown).

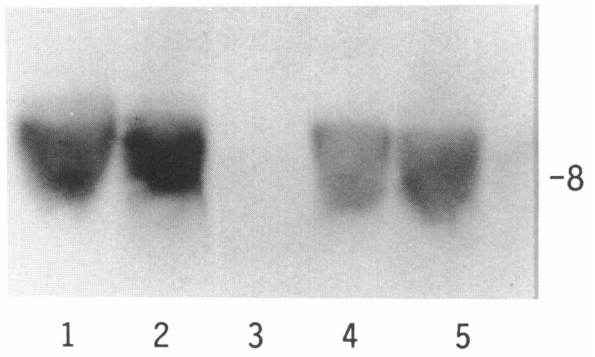

Figure 2 Immunoblotting of cultured skin fibroblast extracts by using anti-SAP-1 rabbit antiserum. Samples (190–200 μg) of protein from cell extracts were lyophilized and subjected to SDS-PAGE on a 15% acrylamide gel, according to a method described by Inui and Wenger (1984). Lanes 1, 4, and 5, Extracts from control cell lines. Lane 2, Extract from the patient infected with pLJ-S-12. Lane 3, Extract from the patient infected with pLJ alone. The numeral "8" on the right indicates the region where proteins having a molecular weight of 8,000 would migrate.

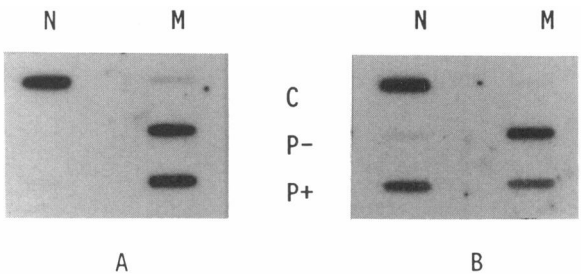

Figure 3 *A*, ASO hybridization of genomic DNA amplified using intronic primers. An aliquot of the 240-nucleotide product was slot-blotted on nitrocellulose and was hybridized with normal (N) and mutant (M) probes according to a method described by Rafi et al. (1990). *B*, Sample of genomic DNA amplified using exonic primers, and aliquot of the 120-nt product that was treated as above. Row C, DNA from a control person. Row P−, DNA from the patient's cells with no vector. Row P+, DNA from the patient's cells infected with pLJ-S-12.

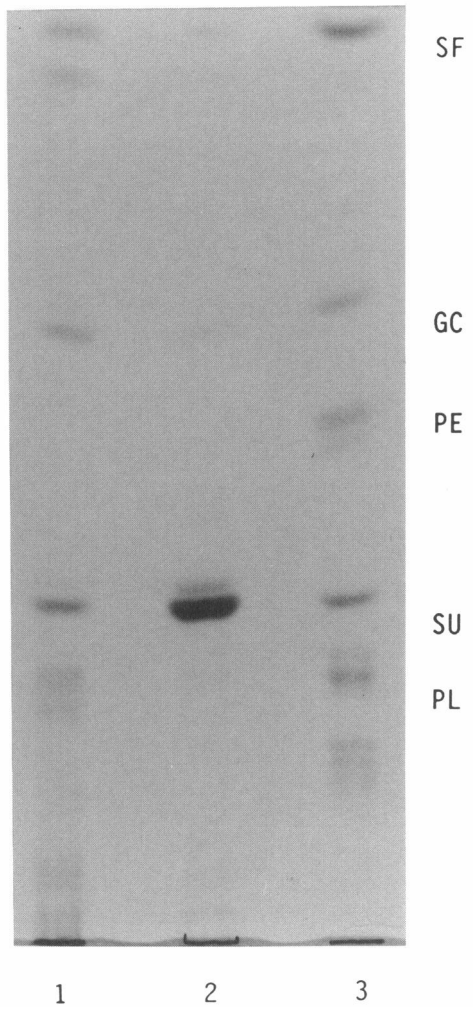

Figure 4 Autoradiogram of a thin-layer chromatography plate showing radiolabeled lipids after [^{14}C]-sulfatide loading for 3 d. Lane 1, Control cell line. Lane 2, Patient's cells infected with the pLJ vector alone. Lane 3, Patient's cells infected with pLJ-S-12. The areas of radioactivity include the solvent front (SF), galactosylceramide (GC), phosphatidylethanolamine (PE), sulfatide (SU), and other phospholipids (PL). The solvent was chloroform:methanol:water (72:28:4, by volume).

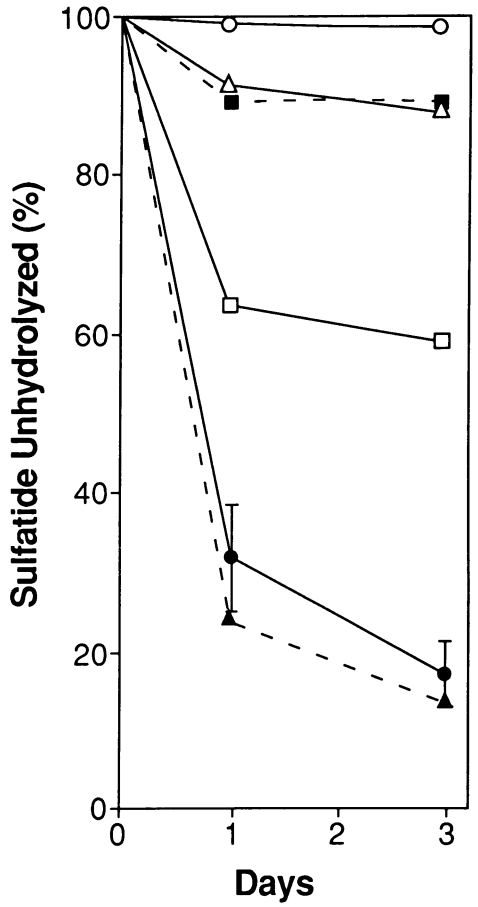

Figure 5 Percentage of endocytosed [^{14}C]-sulfatide left unhydrolyzed after 1 and 3 d. In each lane the areas corresponding to radiolabeled lipids were scraped with a razor and were counted according to a method described by Kudoh and Wenger (1982). ■– – –■, = Patient's cells infected with vector pLJ alone; ▲– – –▲ = patient's cells infected with pLJ-S-12; ○–○ = cells from a patient affected with late infantile MLD; △–△ = cells from another unrelated patient with MLD caused by SAP-1 deficiency; □–□ = cells from a patient with adult MLD; and ●–● = cells from seven control cell lines (± SD).

Discussion

The degradation of sulfatide, a major sphingolipid component of peripheral and central nervous system myelin, requires two proteins, arylsulfatase A and SAP-1. Mutations in either of the genes that code for these proteins can result in defective sulfatide catabolism, accumulation of metachromatic storage granules, severe mental retardation, and death. While most patients with metachromatic leukodystrophy have mutations in the gene coding for arylsulfatase A, some have mutations in the gene coding for SAP-1. The newly discovered patient in this study had a single nucleotide change at position 650, which changes the codon for threonine to one for isoleucine and eliminates the only glycosylation site in the SAP-1 domain. This mutation has been described elsewhere (Kretz et al. 1990; Rafi et al. 1990). Studies of biosynthesis and processing showed the production of unstable SAP-1 in the cells of the previously identified patients (Fujibayashi and Wenger 1986).

In the present paper we have described (*a*) the isolation of a full-length cDNA transcript for prosaposin

containing *SalI* cloning sites at the termini, (*b*) the insertion, into the *SalI* site, of the Moloney murine leukemia virus–derived retroviral vector, pLJ, and (*c*) the infection of cultured skin fibroblasts from this patient. The use of a retroviral vector to transfer a cDNA to human cells has several advantages over other methods. These include a high rate of DNA uptake by infection, integration into the host genome, ability to initiate a high level of transcription of the inserted sequence, and the presence of a selectable marker to insure infection of the host cells, and substantiation of recent experiments demonstrating the application of retroviral mediated gene transfer for clinical trials.

The cells from this patient that were infected with the full-length cDNA construct were shown to produce normal levels of mature SAP-1, which were readily detectable after standard extraction with hypotonic lysis followed by SDS-PAGE of a suitable aliquot and by immunochemical staining, as we reported elsewhere (Inui and Wenger 1984). Although exact quantitation was not performed, it is obvious from figure 2 that the cells produce a high level of mature SAP-1, which was not visible in extracts of the untreated cells, cells with vector alone, or cells with a vector-containing insert in the antisense orientation. The proof that mature SAP-1 was processed normally and able to enter the lysosomes was demonstrated by the results of the [^{14}C]-sulfatide loading test (figs. 4 and 5). It is clear that cells containing pLJ-S-12 were able to metabolize endocytosed [^{14}C]-sulfatide normally, thereby correcting the defective sulfatide metabolism found in cultured cells from these patients. The degree of correction is almost identical to that found by this laboratory when cells from another SAP-1-deficient patient were given mature SAP-1 directly in the medium along with [^{14}C]-sulfatide (Inui et al. 1983). Although sulfatide is not produced by cultured skin fibroblasts, it is readily endocytosed from the medium and is rapidly available to the action of arylsulfatase A, a lysosomal enzyme. It is now clear that mutations in the prosaposin gene cause sulfatide accumulation, and this can be prevented by giving cells the normal cDNA in a retroviral vector.

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