European Journal of Human Genetics (1999) 7, 125–130

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ARTICLE

An Asn > Lys substitution in saposin B involving a conserved amino acidic residue and leading to the loss of the single N-glycosylation site in a patient with metachromatic leukodystrophy and normal arylsulphatase A activity

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Sphingolipid activator proteins are small glycoproteins required for the degradation of sphingolipids by specific lysosomal hydrolases. Four of them, called saposins, are encoded by the prosaposin gene, the product of which is proteolytically cleaved into the four mature saposin proteins (saposins A, B, C, D). One of these, saposin B, is necessary in the hydrolysis of sulphatide by arylsulphatase A where it presents the solubilised substrate to the enzyme. As an alternative to arylsulphatase A deficiency, deficiency of saposin B causes metachromatic leukodystrophy. We identified a previously undescribed mutation (N215K) in the prosaposin gene of a patient with metachromatic leukodystrophy but with normal arylsulphatase A activity and elevated sulphatide in urine. The mutation involves a highly conserved amino acidic residue and abolishes the only N-glycosylation site of saposin B.

Keywords: sphingolipid activator proteins; cerebroside sulphate activator; metachromatic leukodystrophy

Introduction

The lysosomal degradation of sphingolipids is operated by specific hydrolases, some of which require the presence of small non-enzymic glycoproteins called sphingolipid activator proteins.[1] Among them, the four saposins have a common precursor. The prosaposin gene (PSAP)[2–4] (cDNA sequence in GenBank, accession number D00422; genomic DNA sequence in GenBank, accession number M86181), encodes a polypeptide, glycosylated at five sites, which is transported to lysosomes where it is processed to produce the four mature saposins.[1] The physiological role of saposin A (sap-A) and saposin-D (sap-D) has not been completely elucidated. *In vitro* experiments suggest that sap-A stimulates glucosylceramidase and galactosylceramidase.[1] Sap-D seems to activate the hydrolysis of ceramide.[5,6] Saposin C (sap-C) and saposin B (sap-B)

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Received 8 June 1998; revised 25 August 1998; accepted 21 September 1998

have been more extensively characterised. Sap-C is essential for the degradation of glucosylceramide and galactosylceramide; deficiency of sap-C results in a variant form of Gaucher disease. Sap-B solubilises the glycolipid cerebroside sulphate which is then hydrolysed by arylsulphatase A (ASA), whose deficiency results in metachromatic leukodystrophy (MLD). However, this disease phenotype appears also, when functional sap-B is absent.[1]

A neurotrophic function of the precursor prosaposin protein itself has been elucidated,[7,8] and a role in ganglioside binding and transport has been hypothesized.[9]

Recently, the structure of a saposin-like protein, porcine NK-lysin, representative of a family of sequence related proteins sharing a possible common fold, has been determined, comprising five amphipathic α-helices, one at the centre, the others at the two opposite sides, folded into a single globular domain with three disulphide bonds.[10]

In this report we describe a new PSAP mutation causing the production of a nonfunctional sap-B protein in a patient with a late infantile MLD phenotype but with normal ASA activity and increased sulphatide excretion in urine. The mutation, for which the patient is homozygous, causes the loss of the single N-glycosylation site of sap-B and involves a highly conserved residue of sap-B.

Patient

The male patient (PV) was the second child of first cousins with no history of neurological disorders. At age 2 months an aortic coarctation was diagnosed and repaired. Psychomotor development was delayed. At about 2 years he presented walking difficulties, loss of fine motor skills and speech regression. Clinical examination at 3 years showed spasticity, axial hypotonia, depressed deep tendon reflexes and mental deterioration. Nerve conduction velocities were markedly slowed. MRI revealed a diffuse hyperintense signal in periventricular and subcortical white matter on T_2 weighted images. Ultrastructural analysis of a conjunctival biopsy showed inclusions suggestive of sulphatide deposits in macrophages and endothelial cells. The excretion of sulphatide in urine was elevated. The activity of arylsulphatase A measured with p-nitrocatecholsulphate as substrate was normal in leukocytes and cultured fibroblasts but a reduction in sulphatide degradation was observed in intact growing fibroblasts loaded with [^{14}C-stearic acid]-sulphatide. The patient continued to deteriorate and he is still alive at 5 years.

Materials and Methods

Sulphatide Loading Test

The sulphatide loading test was performed on fibroblasts from patient PV, from a late infantile MLD control and from five normal controls as described elsewhere.[11] Loading of cultured fibroblasts was performed with [^{14}C-stearic acid]-sulphatide.

First-strand cDNA Synthesis

Total RNA was extracted from cultured fibroblasts of patient PV and of a normal control using the RNeasy total RNA kit (Qiagen).

Reverse transcriptase polymerase chain reaction (RT-PCR) was performed in a total volume of 20 μl on 100 ng of total RNA using 200 units of SuperScript Il RNase H – Reverse Transcriptase (BRL), 2 pmoles of primer PSAPLR: 5'-CCAATGCTGTGGTTTCTGCCAAGATGGAAT-3', 10 units of ribonuclease inhibitor (BRL) in 0.5 mM dNTPs, 50 mM Tris-HCI (pH 8.3), 75 mM KCI, 3 mM $MgCl_2$, 10 mM dithiothreitol (DTT). RNA and primer PSAPLR were heated at 70°C for 10 min, then dNTPs, DTT and buffer were added and the resulting solution was incubated at 42°C for 2 min. Finally, the ribonuclease inhibitor and the reverse transcriptase were added and the mixture obtained was incubated at 42°C for 50 min followed by a final stage at 70°C for 15 min to inactivate the reverse transcriptase. After first-strand cDNA synthesis, 2.2 units of ribonuclease H were added to obtain the degradation of the RNA complementary to the cDNA by incubating the mixture at 37°C for 20 min.

The first-strand cDNA was used for the amplification of the portion of the PSAP cDNA encoding sap-B (primers used: PSAPSF: 5'-ATCCCTCTCCTCCTCTACCCTC-3' and PSAPSR: 5'-GCTTCTTAATGGGCTCCACCAG-3') and of a fragment containing the whole PSAP open reading frame (ORF) (primers PSAPLF: 5'-CCGGCGCTATG-TACGCCCTCTTCCTCCTGG-3' and PSAPLR).

Amplification and Direct Sequencing of a PSAP cDNA Segment Containing the sap-B Encoding Region

To obtain the amplification of a portion of the PSAP cDNA containing the sap-B encoding region, 2 μl of first-strand cDNA were added, in a total volume of 25 μl, to 6.25 pmol of primers PSAPSF/PSAPSR in 0.2 mM dNTPs, 14 mM Tris-HCI, 56 mM KCI, 1.5 mM $MgCl_2$, 0.8 mM DTT, with 0.625 units of Taq Polymerase. Reaction parameters were 2 min of denaturation at 94°C, then 35 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, with a final extension at 72°C for 2 min. The expected 400 bp PCR product was sequenced by the Sanger method.

Amplification, Cloning and Automated Sequencing of a PSAP cDNA Segment Containing the Whole PSAP Open Reading Frame

To obtain the amplification of a fragment containing the whole PSAP ORF, 2 μl of first-strand cDNA were added, in a

total volume of 25µl, to 7.5 pmol of primers PSAPLF and PSAPLR, in 0.2 mM dNTPs, 14 mM Tris-HCI, 56 mM KCI, 1.5 mM $MgCl_2$, 0.8 mM DTT and 0.875 units of High Fidelity PCR enzyme mix (Boehringer). Reaction parameters were: 94°C for 5 min, then addition of enzyme mix, then 10 cycles at 94°C for 10 s, 63°C for 10 s, 72°C for 1 min 30 s, then 25 cycles at 94°C for 10 s, 63°C for 10 s, 72°C for 1 min 30 s with a delay of 10 s/cycle, then 10 min of elongation at 72°C. Reaction was performed in a 5330 Eppendorf mastercycler. The product obtained was 1632 bp long and comprised the whole ORF of the PSAP gene.

PCR products were cloned in pCR2.1 (Invitrogen) and transfected in *E. coli* INVαF' competent cells (Invitrogen). A PSAP allele from patient PV was identified when screening colonies by PCR using primers PSAPSF/PSAPSR (see above). A positive colony was isolated, plasmid DNA was purified and automated sequence analysis was performed using an ABI 373A DNA automated sequencer with dye terminator cycle sequencing kit (Applied Biosystems). Primers PSAPLF, PSAPSF, PSAP2F: 5'-GGAGGTGAC-CAAGCTGATTGAC-3', PSAPSR, PSAPLR and two vector primers (M13(-20) and M13rev) were used for the automated sequencing.

Restriction Analysis on PCR Products from Genomic DNA

An amplification from genomic DNA was performed using primers PSAPClaF: 5'-TAGGAACTGGTCAG-CAAGCTGC-3' and PSAPClaR: 5'-CCAAGGCCTGGA-CAAAGATCGA-3'. The second, carrier of mismatching positions (underlined), produces a ClaI restriction site in PCR products obtained from alleles carrying the described mutation. Since it was not possible to obtain the direct amplification of the PSAPClaF/PSAPClaR fragment, a nested-PCR procedure was followed. Primers PSAP4F: 5'-GGGTTCAAGCGATCTGCCTGCC-3' and PSAP4R: 5'-CTCCCAGGCCCAGAACATCCCC-3' were used to get the amplification of a 477 bp fragment containing the PSAP-ClaF/PSAPClaR tract. 6.25 pmoles of each primer were mixed in a total volume of 25 µl with 50 ng of genomic DNA in 0.2 mM dNTPs, 10 mM Tris-HCI (pH 8.3), 50 mM KCI, 1.5 mM $MgCl_2$. Reaction parameters were 94°C for 2 min, then 35 cycles of 94°C for 40 s, 59°C for 40 s, 72°C for 40 s, followed by 2 min of elongation at 72°C. 0.1 µl of this PCR product was used for a subsequent amplification using primers PSAPClaF/PSAPClaR. Amplification was performed in the same previously described conditions except for the reaction parameters that were 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, with a final step of 2 min at 72°C. The 200 bp PCR product was ClaI-digested for 1 h at 37°C. In the presence of the described mutation the 200 bp product is cut into two fragments of 177 bp and 23 bp.

Results

Arylsulphatase A activity of fibroblasts from patient PV measured *in vitro* was in the normal range, in contrast with his clinical phenotype and with the presence of elevated sulphatides in urine. This fact suggested a possible sap-B deficiency. To investigate this hypothesis, a sulphatide loading test[11] was performed on fibroblasts from patient PV, from a late infantile metachromatic leukodystrophy control, and from five normal controls. A high level of cerebroside sulphate was found compared with normal controls and low levels of galactosyl ceramide and of phosphatidylserine, -inositol, -choline gave additional evidence of a sap-B deficiency (Figure 1).

To identify the molecular defect at the DNA level, we performed direct sequencing of the RT-PCR product obtained with primers PSAPSF/PSAPSR, inclusive of the PSAP gene region encoding sap-B. Sequence analysis led to identification of a C > A transversion causing an Asn > Lys substitution at position 215 of the PSAP cDNA (Figure 2). This mutation abolishes an N-glycosylation site, the only one in sap-B. Moreover, the substituted Asn residue is highly conserved among human saposins and among saposins B from different species (Figure 3). Mutation was confirmed on genomic DNA by a nested PCR-RFLP method. Restriction analysis confirmed not only the presence of the C > A transversion causing the described Asn > Lys substitution, but also showed that patient PV was homozygous for this mutation (Figure 4).

RT-PCR performed with primers PSAPLF/PSAPLR produced the expected 1632 bp long product (Figure 5). No aberrant transcript caused by the mutation was identified. Automated sequencing of the cloned PSALF/PSAPLR fragment excluded the presence of other molecular alterations in the PSAP ORF carrying mutation N215K.

Discussion

The described PSAP mutation (N215K) is likely to be the cause of a sap-B deficiency responsible of the MLD

	CS	GC	C	PE	PS, PI, PC
Patient PV	64	1.0	4.0	6.3	21
LI-MLD control	87	0.0	3.4	2.1	5.8
Normal controls (5)	16-37	2.1-7.3	4.8-9.0	5.9-13	37-54

Figure 1 *Sulphatide loading test performed on fibroblasts from the patient, from a late infantile MLD control and from five normal controls. Loading of cultured fibroblasts was performed with [^{14}C-stearic acid]-sulphatide. Percentages of incorporated lipid radioactivity as found in lipid metabolites in fibroblasts after 3 days are indicated.*
CS: cerebroside sulfate; GC: galactosyl ceramide; C: ceramide; PE: phosphatidylethanolamine; PS: phosphatidylserine; PI: phosphatidylinositol; PC: phosphatidylcholine.

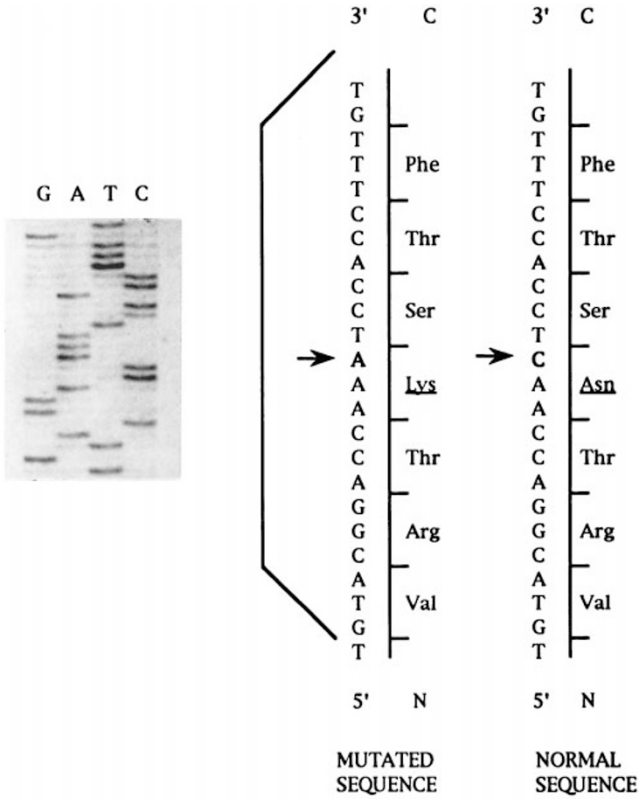

Figure 2 *Sequence analysis showing the N215K mutation. The sequence gel inset shows the mutated sequence identified in patient PV. Arrows indicate the C > A transversion causing the Asn > Lys substitution. The normal sequence is also shown.*

(A)

Sap-A	TAAGDMLKDNAT EEEILVY	12-30
Sap-B	TDIQTAVRTNSTFVQALVEH	12-31
Sap-C	KEVTKLIDNNKT EKEILDA	13-31
Sap-D	GYLDRNLEKNST KQEILAA	13-31
	* *	

(B)

Human	TDIQTAVRTNSTFVQALVEH	12-31
Mouse	SDVQTAVKTNSSFIQGFVDH	12-31
Rat	TDIQTAVRTNSSFVQGLVDH	12-31
Porcine	TDLQNAVRTNSTFVEALVNH	12-31
Chicken	TDVQEAVRTNATFVKSLVAH	12-31
	* * ** ** * * *	

Figure 3 *Alignment of amino acid sequences of saposins. Portions of saposins aligned are indicated. Amino acids identical in the homologous proteins are marked with *. The conserved asparagine residue in sap-B (N at position 21, underlined) is mutated to lysine in the present patient.* **A***) Alignment of human saposins A, B, C, D.* **B***) Alignment of human[3], mouse[20], rat[21], porcine[22] and chicken[23] saposins B. The conserved asparagine residue involved in mutation N215K is at position 21 in human sap-B.*

phenotype observed in patient PV, who is homozygous for the mutation, as could be suspected from the consanguinity of his parents.

Mutation N215K, which involves a residue probably located between the two α-helices regions,[10] is particularly interesting for two reasons: first, the mutation causes the loss of the only N-glycosylation site of sap-B; second, the mutation abolishes a conserved Asn residue (Asn21 in sap-B).

About the loss of the N-glycosylation site, it has been shown that deglycosylated sap-B stimulates the enzymatic hydrolysis of sulphatide by ASA to the same extent as native sap-B, it binds sulphatide just like native sap-B, and is as sensitive to proteolytic digestion.[12] However, deglycosylated and native sap-B refold in a qualitatively different way.[12] Therefore a defect in folding caused by absence of the N-glycosylation site can be hypothesised.

Concerning the Asn21 residue, it is conserved among the four human saposins and among saposins B from different species (Figure 3). This fact suggests a very

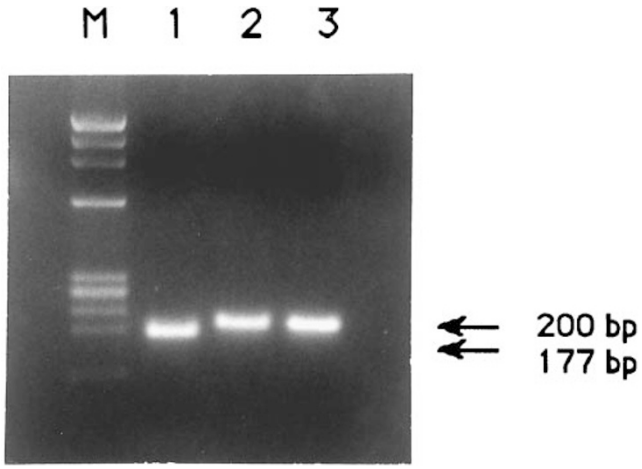

Figure 4 *PCR-RFLP identifying the N215K mutation. Amplification from genomic DNA of a fragment of the prosaposin gene encompassing the site of the N215K mutation. In the presence of the mutation, the 200 bp product is ClaI-digested into two fragments of 177 bp and 23 bp.*
Lane M: ΦX 174 DNA HaeIII digested; lane 1: patient PV; lanes 2 and 3: normal controls.

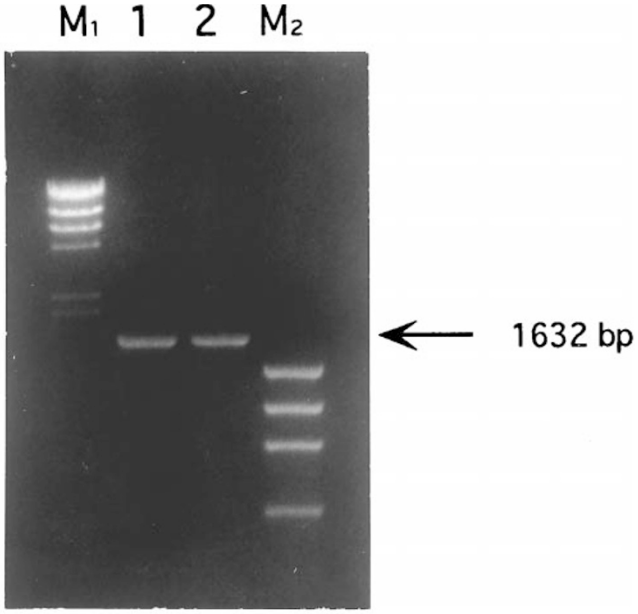

Figure 5 *Long-template PCR: a product comprehensive of the whole ORF. Amplification by RT-PCR of a cDNA fragment containing the whole prosaposin ORF from total RNA.*
Lane M_1: Λ DNA HindIII digested; lane 1: patient PV; lane 2: normal control; lane M_2: ΦX 174 DNA HaeIII digested.

important role for this residue in the biological activity of the protein.

Probably both these characteristics of the N215K mutation, the consequent loss of the glycosylation site and the involvement of a conserved residue, co-operate in producing a non-functional sap-B protein.

Two other mutations causing the loss of the same N-glycosylation site have been identified, one involving the same Asn21 residue (mutation N215H)[13] the other involving a different residue (Thr23) (mutation T217I).[14,15]

Mutation N215H has been identified in a patient described as a 4-year-old child with MLD.[13] Mutation T217I has been found in two siblings with a probably milder MLD phenotype than in patient PV, showing first symptoms at 4.5 and 6 years of age respectively,[16] whilst our patient showed walking difficulties at 2 years and mental retardation at 3. Interestingly, the Thr23 residue is conserved in human, porcine and chicken sap-B, but is not conserved in mouse or rat sap-B, whilst the Asn21 residue is conserved in the saposins B from all these five species (Figure 3). A severe form of the disease can be expected in consequence of a mutation involving such a highly conserved residue.

No aberrant transcript due to the N215K mutation has been identified. Therefore the regularly transcribed mRNA must encode a mutated prosaposin protein. We can speculate that point mutation N215K cannot affect sorting, nor the subsequent processing of the protein, thus leading to the production of normal sap-A, sap-C, sap-D and mutated sap-B. In the case of mutation N215H, which involves the same residue of mutation N215K, the four saposins were detected by immunochemical methods.[13] Since no anti-saposin antibodies were available for our study, we could not confirm a positive immunoreactivity for the products of the N215K mutated prosaposin but, with the results of the N215H mutated case, positive immunoreactivity also of sap-B with N215K mutation is very likely.

We can exclude the concept that N215K mutated prosaposin protein can be defective as a neurotrophic factor:[7,8] in fact, the portion of the protein involved in this role lies in the sap-C region. To date five other MLD-causing sap-B mutations have been identified in the *PSAP* gene.[13–15,17–19] All patients carrying these mutations are homozygotes for their particular molecular defect. The low number of MLD-causing PSAP mutations identified to date could also be ascribed to a possible underestimation of the disease at the diagnostic level.

Acknowledgements

This work was supported by Italian Telethon grant C.20. Cell lines were obtained from the collection 'Cell lines and DNA bank from patients affected by genetic disease' supported by Italian Telethon.

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