Alzheimer's presenilin 1 gene expression in platelets and megakaryocytes

Identification of a novel splice variant

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Abstract The presenilin 1 (PS1) gene located on chromosome 14 has been linked with the majority of early-onset FAD. The normal biological role of PS1 as well as the mechanism by which mutations in PS1 cause FAD remains unknown. PS1 expression in platelets and the Dami megakaryocytic cell line was examined by Western blot analysis and RT-PCR. Using an anti-N-terminus PS1 antibody we detected PS1 immunoreactive bands of 44, 32 and 27 kDa in both cell types. After RT-PCR we observed that platelets and megakaryocytes carry at least four different PS1 transcripts. One of them is a novel PS1 splice variant that lacks the coding sequence for exon 10 resulting in a shorter 409 amino acid protein.

Key words: Alzheimer's disease; Presenilin; mRNA; Gene splicing; Chromosome 14; Platelet

1. Introduction

Alzheimer's disease is characterized pathologically by neuronal loss, glial proliferation and the deposition of amyloid in the form of senile or neuritic plaques, congophilic angiopathy and intraneural neurofibrillary tangles [1]. Amyloid fibrils in senile plaques and in cerebral vessels walls in AD are composed of a protein named amyloid β (Aβ), which is a fragment (39–44 residues) derived from the larger amyloid precursor protein βPP. When the βPP molecule is cleaved at different sites in the extracellular and transmembrane domains it generates soluble Aβ (sAβ). sAβ has been found in circulation in biological fluids and in the supernatant of cell cultures and it has been postulated to be the source of plaque amyloid in AD [2–5].

Missense mutations within or close to the Aβ region in the βPP gene localized in chromosome 21 have been found linked with some forms of FAD and hereditary cerebral hemorrhage with amyloidosis-Dutch type (HCHWA-D) [1], and current studies indicate that the polymorphism of the apo E gene (E4) on chromosome 19 is a genetic risk factor for familial and sporadic late onset AD. Recently, Sherrington and colleagues [6] have reported the cloning of a novel gene termed S182 or presenilin 1 (PS1) bearing missense mutations [6,7] associated with segregating early-onset FAD linked to chromosome 14. A number of mutations in PS1 have been all found associated with early-onset FAD (for review see [8]). In addition, two mutations were described in a similar gene (67% homologous to PS1), named STM-2, E5-1 or PS2 located on chromosome 1 [9,10]. The predicted amino acid sequence encoded in the longest open reading frame composed of 10 exons [7] reveals high similarities with the *Caernorhabditis elegans* protein SEL-12 [11]. SEL-12 facilitates LIN-12 mediated cell signalling via the Notch family of receptors during development of *C. elegans*. Protein homology has also been found with the *C. elegans* sperm integral membrane protein SPE-4, a membrane protein active in spermatogenesis as well as with the mammalian chromogranin A and the α-subunit of mammalian voltage-dependent calcium channel [6]. The primary structure of PS1 resembles that of an integral membrane protein such as a receptor, a channel protein or a structural membrane protein although its physiological function and role in AD pathogenesis are not known.

The expression of PS1 is not limited to neural tissue. Hybridization of the PS1 clone to northern blots detected two transcripts of approx. 3.0 and 7.0 kbp. Northern analysis, in situ hybridization, and immunohistochemical studies showed that presenilins are expressed in most regions of the brain and several peripheral tissues [6,12–15]. Different transcripts derived by alternative splicing of the PS1 gene have been described in brain, fibroblasts and leukocytes [7,10,16,17]. The analysis of the expression of the presenilins is an important step in the understanding of the physiological function and the role of these proteins in AD. Herein, we report the finding of PS1 transcripts in platelets and a megakaryocytic cell line by RT-PCR and Western blot analysis using a polyclonal anti-N-terminus PS1 antibody. A novel PS1 isoform found in platelets and megakaryocytes is also described.

2. Materials and methods

2.1. Cell culture and platelet preparation

The Dami megakaryocytic cell line (ATCC no. CRL-9792) was grown in Iscove's modified Dulbecco's minimal essential media supplemented with 10% horse serum, 50 μg/ml of gentamicin, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 0.58 mg/ml of L-glutamine and maintained at 37°C in a 5% CO_2/95% air atmosphere. Cells in the log phase of growth were washed twice in PBS, pelleted, and processed either for isolation of cellular RNA or Western blotting analysis. Human platelets were isolated from whole blood as described [18]. Briefly, 40 ml of venous blood were collected and anticoagulated by sodium heparin solution. Blood was sedimented by centrifugation and the resultant platelet-rich plasma was collected. By differential centrifugation, platelets were harvested and washed three more times in 100 mmol/l NaCl, 8.5 mmol/l Tris, 8.5 mmol/l glucose, and 1 mmol/l Na_2EDTA, pH 7.4. The final platelet pellet was used for RNA isolation and Western blot analysis.

2.2. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNAs were isolated by the guanidine isothiocyanate method using Trizol LS (Gibco BRL) reagent. Reverse transcription of RNA (1 μg) extracted from cell line and platelets was performed with the first strand cDNA synthesis kit (Boehringer Mannheim)

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using the avian myeloblastosis virus (AMV) reverse transcriptase with the downstream primer 01R specific for PS1 (Table 1). PCR amplification of the first strand cDNA produced by reverse transcription was performed by introducing the upstream amplimer 01F in 1×PCR buffer (Perkin Elmer). Each PCR cycle consisting of a denaturation step (94°C, 1 min), an annealing step (40°C, 2 min), and an elongation step (72°C, 3 min) was repeated 30 times. For controls, RNA samples were pretreated with RNase A for 30 min at 37°C and subjected to RT-PCR; in these controls, no specific amplification was observed.

2.3. PCR amplification and DNA sequencing

10 μl of the products of the PS1 reaction were reamplified using oligonucleotide primers 02F and 02R for 30 cycles of 94°C for 1 min, 45°C for 1 min and, 72°C for 2 min The resulting PCR product was then digested with *Xmn*I (New England BioLabs) and *Dra*I (Gibco, BRL) following the manufacturer's conditions. In a different reaction, 10 μl of the PS1 cDNA were reamplified using oligonucleotides forward 05F and reverse O5R under the same conditions. After successful reamplification, the resulting PCR product was subcloned into the pCRII vector (TA cloning kit, Invitrogen). Recombinant plasmid DNA was isolated from seven clones and sequenced by the dideoxy chain termination method in both directions. To ensure specific amplification of platelet's RNA, RT-PCR reactions were performed using a set of oligonucleotide primers for the non-polymorphic domain of the beta chain gene of class II HLA-DR molecule (which is found predominantly on the surface of activated T-cells, B-cells, and antigen-presenting cells) [19] and in a different reaction, the same platelet's RNA was used in RT-PCR with synthetic oligonucleotides based on the sequence of the platelet membrane glycoprotein IIb (GPIIb) [20] as described by Gardella et al. [21]. PCR amplification using oligonucleotides 03F and 03R, was performed for 25 cycles of 30 s at 94°C, 30 s at 52°C, and 40 s at 72°C. Amplification products were resolved on 4% Methaphor (FMC) gel and visualized by ethidium bromide staining. Reamplification of PS1 cDNA was also performed with oligonucleotides 04F and 04R. PCR conditions of 94°C for 40 s, 45°C for 40 s and, 72°C for 1 min were repeated 30 times. PCR products were separated on 5% polyacrylamide gels, visualized by ethidium bromide staining and subcloned into the pCRII vector. Recombinant plasmid DNA was isolated from 10 clones and sequenced in both directions.

2.4. Western blotting of cell extracts

A peptide encompassing residues 48–70 of the PS1 molecule [6] was used to raise an anti-PS1 polyclonal antibody (PS1 (N48–70)). This antibody was produced by custom order at Research Genetics (Huntsville, AL). The serum was affinity purified using a CNBr-activated Sepharose column (Pharmacia, LKB) on which the HPLC purified synthetic peptide was coupled following the manufacturer's instructions. Isolated platelets and megakaryocytes were triturated in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% cholic acid, and 0.1% SDS) with protease inhibitors (complete, 1 μg/ml leupeptin, 0.1 μg/ml pepstatin, and 1 μg/ml L-1-chloro-3-[4-tosylamido]-7-amino-2-heptanone-HCl, all from Boehringer Mannheim) and cells extracts were analyzed in 16% Tris-Tricine SDS-PAGE, following by Western blotting with affinity purified PS1(N48–70) antibody (1:100). Controls included the use of pre-immune serum and pre-absorption of the purified antibody with the synthetic N48–70 peptide.

3. Results

We amplified PS1 cDNA by RT-PCR from platelets and the Dami megakaryocytic cell line using oligonucleotide primers 01F and 01R. The resulting PCR product contained full-length PS1 cDNA (1885 bp) (not shown). After amplification, PCR products were characterized by polyacrylamide gel electrophoresis. Besides noting it expected size, reamplified fragments using oligonucleotides 02F and 02R (573 bp) (Fig. 1A, lane 2) were digested with *Xmn*I and *Dra*I restriction enzymes (Fig. 1A, lanes 3,4, respectively). The resulting DNA fragments correspond exactly to the predicted size of these restriction enzyme-digested fragments (132 and 441 bp for *Xmn*I and 204 and 369 bp for *Dra*I). PS1 cDNA reamplified using oligonucleotides 05F and O5R (651 bp) was subcloned and sequenced in both directions. The complete sequence of the subcloned cDNA fragments showed 100% identity to that of the PS1 molecule (GenBank accession no. L42110 and [22]). To ensure specific amplification of platelet's RNA, RT-PCR reactions were performed using oligonucleotide primers specific either for the class II HLA-DR molecule (which is found predominantly on the surface of activated T-cells, B-cells, and antigen presenting cells) or for the platelet membrane glycoprotein IIb molecule (GPIIb). PCR amplification of platelet's RNA for HLA-DR was negative (even after a second round of reamplification). On the other hand, RT-PCR for platelet's GPIIb produces an expected fragment of 809 bp (Fig. 1B, lane 2) establishing the uncontaminated nature of the RNA used in these experiments [21]. Similarly, RNA isolated from the Dami megakaryocytic cell line produced a strong signal for GPIIb (Fig. 1B, lane 3) and was negative for HLA-DR. RT-PCR using megakaryocyte's RNA and PS1 oligonucleotides primers 02F and 02R in the same manner described for platelet's RNA gave a PCR product of 573 bp (Fig. 1B, lane 4) that was then digested with *Xmn*I and *Dra*I as described before (Fig. 1B, lanes 5,6, respectively).

Alternative splicing of exon 3 [7] was analyzed using oligonucleotides 03F and 03R. After reamplification, PCR products from platelets and megakaryocytes were resolved on 4% metaphor agarose showing two bands of 146 and 134 bp (Fig. 2A, lanes 2,3). The difference of 12 bp is due to alternative splicing of the sequence VRSQ (codons 26–29) (Fig. 2B). A similar pattern of expression was observed in platelets, the Dami megakaryocytic cell line and two neuroblastoma cell lines NTera-2/cL.D1 and SH-SY5Y (Fig. 2A, lanes 4,5). After PCR amplification of PS1 cDNAs with oligonucleotides 04F and 04R a PCR product of 584 bp representing full-length PS1 was found to constitute the majority of

Table 1
Sequence and position of oligonucleotide primers

Primer	Sequence	Exon
01F	5′-ATC GGG ATC CTC ACA TCG GAA ACA AAA CAG-3′	2
01R	5′-CTA AGC GGC CGC ACC TCG TCC CTC AAA TCT-3′	12
02F	5′-GAG CAA GAT GAG GAA GAA GAT-3′	4
02R	5′-TGA AAT CAC AGC CAA GAT GAG-3′	7
03F	5′-GAC AAC CAC CTG AGC AAT-3′	3
03R	5′-CTC ATC TTG CTC CAC CAC CTG-3′	4
04F	5′-ATC TCC GGC AGG CAT ATC T-3′	7
04R	5′-ATA ATC TAG AAC TAT ATG GCT ACG AA-3′	11
05F	5′-GAT TTA GTG GCT GTT TTG TG-3′	8
05R	5′-ATC AGC GGC CGC TAA CCG CAA ATA TGC-3′	12

the amplified material in platelets (Fig. 3A, lane 2) and megakaryocytes (not shown). Amplification of PS1 cDNA from neuroblastoma cell lines produced a similar signal (Fig. 3A, lanes 5,6). Alternative splicing of exon 8 (codons 257–289) previously reported in leukocytes [10] was observed to constitute a very small percentage of PS1 transcripts in platelets and the Dami cell line. After reamplification of PS1 cDNA using oligonucleotides primers (04F and 04R) we observed a faint band of 485 bp in polyacrylamide gels. This band was cut out the gel and reamplified under the same conditions (Fig. 3A, lane 3). After cloning, this PCR product was identified by DNA sequencing as the alternative splicing PS1 form lacking exon 8 (99 bp) (Fig. 3B). Another faint band of lower size (410 bp) present in amplification products from platelets and megakaryocytes was cut out from the gel and reamplified as described before (Fig. 3A, lane 4). Sequence analysis revealed that this amplified PCR product represents a novel alternative splicing form of PS1, lacking exon 10 (174 bp). As a result, exon 10 (codons 319–376) is spliced out, without altering the reading frame of the rest of the PS1 molecule (Fig. 3C).

We prepared a specific anti-N-terminus antiserum against a synthetic peptide (N48–70) derived from the protein sequence of the PS1 molecule. Serum antibodies were affinity purified against the peptide and used in Western blot experiments. The predicted molecular mass of PS1 is ~52 kDa, but it has been recently reported that in vitro translated PS1 migrates as a protein of ~43 kDa in SDS-polyacrylamide gels [16]. A ~50 kDa band can be seen in megakaryocytes and platelets with an intensity dependent on the boiling time in loading buffer previous to electrophoresis. Higher molecular mass bands of ~80 and 100 kDa can also be detected as a function of boiling time as has previously been described [16]. Our N-terminus antibody also recognized a protein of 44 kDa in platelets and megakaryocytes (Fig. 4) and in neuroblastoma cell lines (data not shown). Staining was abolished when the antibody was pre-incubated with the synthetic peptide. Two lower molecular mass bands of ~27 and 32 kDa can also be seen in both cell types (Fig. 4, lanes 1,2) and in neuroblastoma cell lines (not shown).

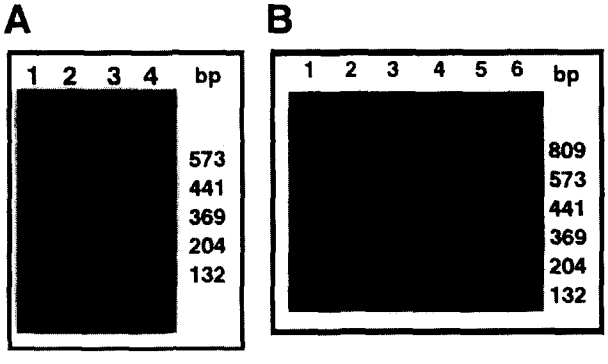

Fig. 1. Ethidium bromide stained 5% polyacrylamide gel (PAGE). (A) 573 bp PCR product of amplification from platelets (lane 2) and restriction enzyme analysis of the same fragment. *Xmn*I (lane 3) digestion results in fragments of 132 and 441 bp. *Dra*I digestion (lane 4) produces fragments of 204 and 369 bp. Lane 1: BioMarker Low (Bio Ventures, Inc.) molecular weight marker. (B) RT-PCR products (809 bp) of amplification of platelets (lane 2) and megakaryocytes (lane 3) RNA for GPIIb. Reamplification of PS1 cDNA from megakaryocytes results in a band of 573 bp (lane 4). Restriction enzyme analysis results in the same pattern described in (A) for *Xmn*I (lane 5) and *Dra*I (lane 6). Lane 1: BioMarker Low.

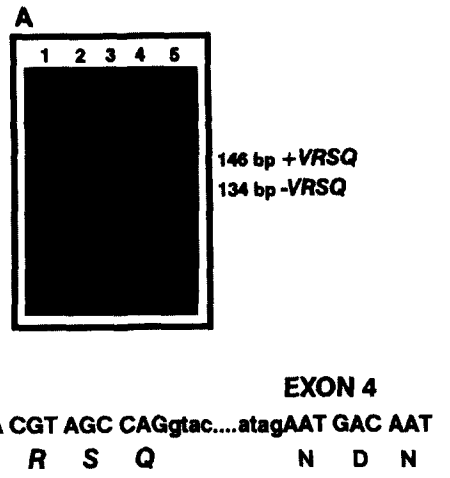

Fig. 2. (A) PCR amplification of PS1 cDNA from platelets (lane 2), megakaryocytes (lane 3), NT2 cells (lane 4), and SHSY5Y (lane 5) cell line shows amplified fragments of 134 and 146 bp. Lane 1: BioMarker Low. (B) Intron/exon structure of alternatively spliced exon 3 [7]. The predicted amino acids are positioned below the nucleotide sequence in the middle of their respective codons. Exons are in capitals while the flanking introns are in lower-case letters.

4. Discussion

Platelets are responsible for up to 90% of the anti-Aβ immunoreactivity in whole blood [23], representing a probable source of sAβ. After the discovery by linkage analysis of the relationship between the majority of the cases of early-onset FAD and the PS1 gene [6] we decided to investigate whether platelets also express PS1 protein.

We found evidence of PS1 transcription in both platelets and megakaryocytes by specific RT-PCR. Although full-length PS1 represents the major transcript, we identified other PCR products. We observed in platelets and megakaryocytes the presence of alternative splicing of exon 3 as described by Clark et al. [7]. The importance of these isoforms should be considered since the sequence VRSQ (VRXQ motif) may be part of two potential phosphorylation sites for casein kinase II and protein kinase C in the PS1 molecule [7]. Both isoforms are present in almost equimolecular amounts in platelets, megakaryocytes and neural cell lines whereas brain and lymphoblasts have a different pattern with the shorter isoform representing more than 60% ([24] and unpublished observation). A shorter mRNA derived by alternative splicing of exon 8 (codons 257–289) is present in low amounts in both platelets and megakaryocytes. This represents an isoform of 434 amino acids that has been reported to be more prominent in leukocytes than in brain [10]. Exon 8 contains a hot-spot for PS1 mutations with exons 5 and 8 together accounting for approx. 65% of all PS1 mutations reported so far. We also found a novel splice variant that splices out exon 10 (174 bp). The translated polypeptide lacks the sequence between codons 319 and 373 of the full-length PS1 that forms part of the acidic hydrophilic loop between the predicted transmembrane domains VI and VII [6]. So far, no mutations have been reported in this exon, but another isoform has been described where the insertion of an additional exon (92 bp) after exon 9 results in a frame shift with a stop codon that generates a PS1 protein (I-374) of 374 amino acids [16]. In our case, the alternative splicing phenomenon of exon 10 does not alter the

reading frame, producing a shorter isoform of 409 amino acids. Another splice variant that lacks exon 10 and part of exon 11 (involving part of the predicted loop and the transmembrane domain VII) and codon 321 changes from glutamic to aspartic acid was recently described as a member of a number of different PS1 isoforms [17].

The Western blotting data using our polyclonal anti-N-terminus antibody shows a band of 44 kDa that can be detected in platelets and megakaryocytes (also seen in neuroblastoma cell lines), indicating that the PS1 gene product is expressed not exclusively in cell of neural origin. Beside full-length PS1, we observed immunoreactive bands of lower molecular weight representing proteolytic fragments or the presence of shorter isoforms of PS1. We found an immunoreactive band of ~32 kDa that corresponds approximately to the molecular mass described for I-374 by Sahara et al. [16]. A lower molecular mass band observed at ~27 kDa suggests proteolytic cleavage. Interestingly, we have found an immunoreactive band of approximately the same molecular mass (~27 kDa) that is labeled by our anti-N-terminus PS1 antibody present in both normal and AD cerebrospinal fluid as well as a C-terminal immunoreactive band of ~17 kDa in brain tissue [25]. The biochemical characterization of these molecules is currently in progress.

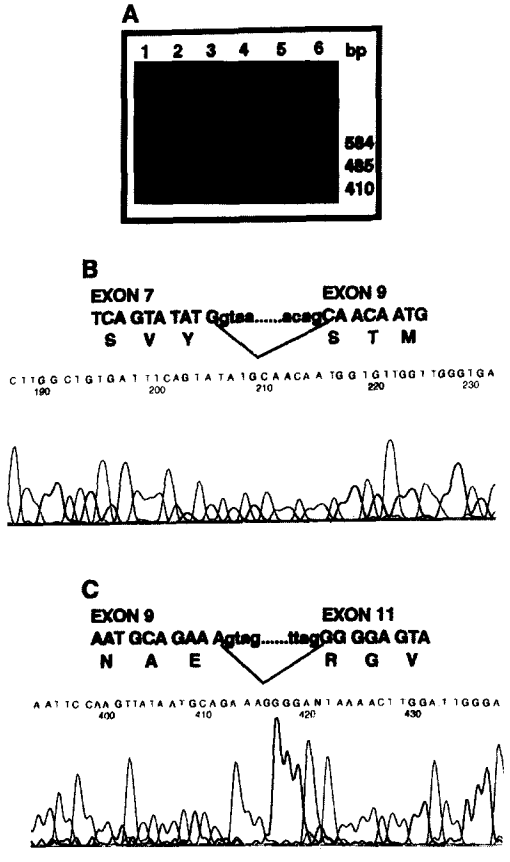

Fig. 3. 5% polyacrylamide gel showing products of amplification (A) from platelets representing full length (lane 2), alternative splicing of exon 8 (lane 3), and exon 10 (lane 4). Lanes 5 and 6 show the results of amplification in the same conditions for NTera-2/cL.D1 and SH-SY5Y cell lines, respectively. Lane 1: 123 bp DNA marker (Gibco, BRL). DNA sequence analysis indicates that exon 7 was spliced to exon 9 (B) and that exon 10 was spliced out (C).

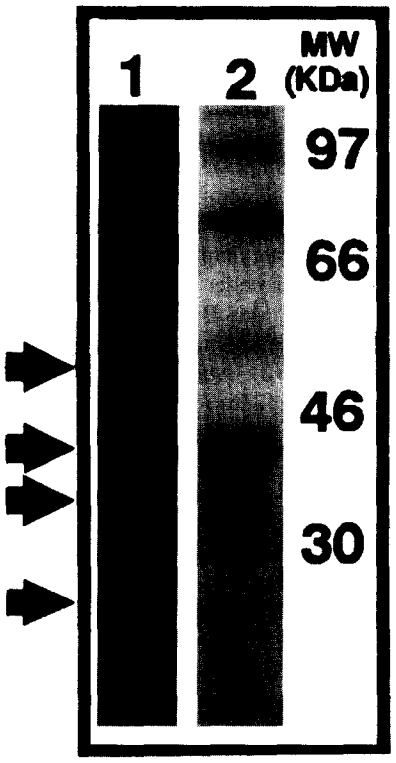

Fig. 4. Western blot of cell extracts run on 16% Tris-Tricine SDS-PAGE from megakaryocytes (lane 1) and platelets (lane 2). After blocking overnight with 5% non-fat dry milk in PBS-T, primary antibody (PS1 (N48–70)) was added diluted 1:100 in PBS-T and incubated for 2 h at room temperature. Secondary antibody was peroxidase-conjugated anti-IgG (Dako) diluted 1:5000. Fluorograms were prepared with an ECL Western blotting kit (Amersham) according to the manufacter's specifications.

PS1 has been linked to most of the cases of early-onset FAD and has also been reported to be present in senile plaques of sporadic and chromosome 14 linked AD cases [26] as well as in cerebrovascular Aβ deposits of AD patients [25]. The present study provides direct evidence that platelets have transcripts that encode the PS1 protein. Furthermore, examination of the Dami megakaryocytic cell line reveals that these cells also have PS1 mRNA and express PS1 protein, indicating that PS1 transcription occurs in cells of the thrombocytic lineage. So far, the expression profile of PS1 and βPP seems to be similar, with both genes being ubiquitously expressed in neural and non-neural tissues. Additional experiments will be necessary to determine the function of PS1 and its relationship to βPP and how PS1 is involved in the pathogenesis of AD.

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