Characterization of human presenilin 1 using N-terminal specific monoclonal antibodies: Evidence that Alzheimer mutations affect proteolytic processing

Marc Mercken[a,*], Hiroshi Takahashi[a], Toshiyuki Honda[b], Kazuki Sato[a], Miyuki Murayama[a], Yuko Nakazato[a], Kaori Noguchi[a], Kasutomo Imahori[a], Akihiko Takashima[a]

[a] *Mitsubishi Kasei Institute of Life Sciences, 11 Minamiooya, Machida-shi, Tokyo 194, Japan*
[b] *Yokohama Research Center, Mitsubishi Chemical Corporation, Yokohama 227, Japan*

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Abstract The majority of cases of early-onset familial Alzheimer disease are caused by mutations in the recently identified presenilin 1 (PS1) gene, located on chromosome 14. PS1, a 467 amino acid protein, is predicted to be an integral membrane protein containing seven putative transmembrane domains and a large hydrophilic loop between the sixth and seventh membrane-spanning domain. We produced 7 monoclonal antibodies that react with 3 non-overlapping epitopes on the N-terminal hydrophilic tail of PS1. The monoclonal antibodies can detect the full-size PS1 at M_r 47 000 and a more abundant M_r 28 000 product in membrane extracts from human brain and human cell lines. PC12 cells transiently transfected with PS1 constructs containing two different Alzheimer mutations fail to generate the 28 kDa degradation product in contrast to PC12 cells transfected with wild-type PS1. Our results indicate that missense mutations in this form of familial Alzheimer disease may act via a mechanism of impaired proteolytic processing of PS1.

Key words: Alzheimer disease; Chromosome 14; Presenilin; Proteolysis; Monoclonal antibody

1. Introduction

The deposition of β/A4-amyloid in the brain parenchyma and the presence of neurofibrillary degeneration in brain regions important for cognitive functions are typical for familial as well as sporadic forms of Alzheimer disease [1]. The biochemical processes leading to these lesions are, however, not well understood.

To date, four different genes have been implicated to play a role in the disease process. The first gene identified in early-onset familial Alzheimer was the β/A4-amyloid protein precursor (APP) [2] and mutations in the APP gene account for less than 5% of the early-onset familial cases of AD. The APP gene is located on chromosome 21 and the observation that virtually all individuals with Down's syndrome acquire AD neuropathology in later life provides compelling evidence for a central role of this protein in the disease process of all Alzheimer patients. Another genetic factor identified is the inheritance of specific apolipoprotein E (apoE) alleles which determine the age of onset in both sporadic and late-onset familial Alzheimer disease [3]. The mechanism through which the apoE4 allele lowers the age of onset is still unknown. Mutations in two other genes, recently identified, account for the majority of the cases of early-onset familial Alzheimer disease (for review see [4]). These two novel genes, named presenilin 1 (PS1) and presenilin 2 (PS2), are located on chromosome 14 and chromosome 1, respectively, and are highly homologous (67%). Sequence analysis predicts integral membrane proteins, that contain seven putative transmembrane domains, a short hydrophilic amino- and carboxyl-terminal tail, and a large hydrophilic loop between the sixth and seventh membrane-spanning domain. All of the reported mutations that result in early-onset Alzheimer's disease are missense mutations, 24 in PS1 [5–11] and two in PS2 [12,13], or mutations that affect the splicing without affecting the coding of the protein [14] and this has led to the hypothesis that they may result in a gain of (mis)function. This view is also consistent with a recent report describing that an intron polymorphism in the PS1 gene is related to approx. 20% of the cases of late-onset Alzheimer disease [15].

The high similarity in the pathology observed in familial and sporadic forms of Alzheimer's disease indicates converging pathological mechanisms and clarification of the (mal)function of the presenilins will most likely provide important clues for the disease process, not only in familial, but also in sporadic Alzheimer's disease. In this study we produced a series of mono- and polyclonal antibodies and started to investigate the metabolism of normal and mutated PS1. We demonstrate that in vivo PS1 is expressed as a 47 kDa protein. Monoclonal antibodies, directed against amino-terminal epitopes of PS1, also detect an abundant 28 kDa degradation product in extracts from human brain and human cell lines. We transfected PC12 cells with wild-type human PS1 and two constructs containing Alzheimer mutations and observed striking differences in the proteolytic processing of PS1. Cells transfected with mutated PS1 showed a highly reduced capacity to generate the 28 kDa product.

Taken together, our results indicate that in vivo selective proteolysis of PS1 generates an abundant 28 kDa degradation product and that this process is affected by the mutations that cause Alzheimer's disease.

2. Materials and methods

2.1. Antibody production

All synthetic peptides were coupled to Imject keyhole limpet hemocyanin (Pierce) before immunization. Hybridomas MKAD3.1–MKAD3.7 were isolated after immunization of Balb/c mice with a synthetic peptide containing the amino acid residues 21–59 (N-peptide) of human presenilin 1 [5]. Fusion and culture procedures were as described elsewhere [16]. Polyclonal rabbit antisera were obtained after immunization with synthetic peptides spanning residues 299–313 (serum PS1-M) and 453–467 (C-peptide, serum PS1-C).

*Corresponding author. Fax (81) (427) 246316.

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2.2. Epitope mapping

Overlapping octapeptides for epitope mapping were synthesized on polypropylene pins arranged in a microtiter plate format using the Multipin Peptide Synthesis System (Chiron Mimotopes). Antibody reactivity with octapeptides was determined by ELISA using tetramethylbenzidine (Boehringer) as substrate and absorbances were read at 450 nm. Readings more than 3 times higher than background were considered as positive signals.

2.3. Constructs

Full-length *PS1* DNA was amplified by RT-PCR from human brain, inserted in pGEM-T (Promega) and, after subcloning, sequenced using the ThermoSequenase fluorescent labeled primer cycle sequencing kit (Amersham). The clone lacks the 4 amino acids (VRSQ) which are encoded by the last 12 base pairs of exon 3. Alzheimer mutations $Met^{146}Val$ and $Ala^{246}Glu$ were introduced by PCR-based site-directed mutagenesis and confirmed by sequencing using the same method.

2.4. GST-PS1 fusion protein purification

Full-size *PS1* cDNA was subcloned into pGEX-4T-1 (Pharmacia). GST-PS1 fusion protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h at 37°C. After harvesting, cells were resuspended in 50 mM Tris, pH 7.4, 150 mM NaCl (Tris-Sal) containing 1% Triton X-100, 5 mM DTT, 5 mM EDTA, 1 mM Pefabloc, 5 μg/ml pepstatin, 10 μg/ml leupeptin and lysed by sonication twice for 1 min with a Sonifier Cell Disruptor (Heat Systems-Ultrasonics) using a microprobe. Lysates were cleared by centrifugation, filtered and loaded overnight at 4°C on a glutathione-agarose column (Sigma). GST-PS1 fusion proteins were eluted by adding 15 mM reduced glutathione (Sigma) to the buffer.

2.5. PS1 immunoaffinity purification

Monoclonal antibody MKAD3.3 was purified from culture supernatant on protein G (Pharmacia) and coupled to CNBr-activated Sepharose 4B (Pharmacia). Membrane extracts were prepared as described below and loaded overnight at 4°C. After washing with 10 column volumes Tris-Sal, 1% Triton X-100, proteins were eluted in 0.1 M glycine, 0.1% Triton X-100, pH. 2.7 and the pH of the fractions was neutralized immediately with 1 M Tris, pH 9.0 (50 μl/ml).

2.6. Preparation of membrane fractions

Membrane fractions from human cell lines and adult human brain (neocortex) were prepared as follows. Homogenates, prepared with 50 mM Tris, pH 7.4, 150 mM NaCl (Tris-Sal) were centrifuged at $12000 \times g$, pellets were extracted with Tris-Sal containing 1% Triton X-100, recentrifuged at $12000 \times g$, and the supernatant collected. Protease inhibitor cocktail was added to all buffers (1 mM Pefabloc, 5 μg/ml pepstatin, 2.5 mM EDTA, 10 μg/ml leupeptin).

2.7. Transfections

For transfection, wild-type and mutant *PS1* cDNA was subcloned in pEF321 [17]. PC12 cells were transfected using LipofectAMINE (GibcoBRL) and total cell extracts were prepared 72 h later by lysis in modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, protease inhibitors as above). The lysates were centrifuged in a microcentrifuge at 15000 rpm at 4°C, the supernatant was collected and equal amounts of protein were separated in SDS-PAGE and immunoblotted as described in Section 2.9.

2.8. Mammalian cell cultures

Human SH-SY neuroblastoma cells and 293 kidney cells were cultured in DMEM containing 4.5 mg/ml D-glucose (GibcoBRL) supplemented with 10% FBS (fetal bovine serum) at 37°C in a 5% CO_2 atmosphere. Rat PC12 cells were cultured in DMEM containing 4.5 mg/ml D-glucose (GibcoBRL) supplemented with 5% FBS and 5% HS (horse serum) at 37°C in a 10% CO_2 incubator. Gentamicin sulfate at 84 mg/l was added in all media to prevent bacterial growth.

2.9. Western blot analysis

Proteins were separated in 10% SDS-PAGE and transferred to nitrocellulose membranes (Schleicher and Schuell). Samples were not heated before loading except when indicated. When applied, heating was for 10 min at 100°C. Membranes were blocked by 10% milk in PBS, 0.1% Tween 20. Primary antibodies were diluted in 5% milk in PBS, 0.05% Tween 20 (hybridoma supernatant 1:5 and rabbit serum 1:1000) and incubated overnight at 4°C or 2 h at room temperature. Monoclonal antibodies were detected with rabbit anti-mouse Ig peroxidase conjugate (Zymed) (Fig. 2, Fig. 3 (pre, post and eluate fractions from brain), and Fig. 4) or biotinylated goat anti-mouse IgG followed by streptavidin-peroxidase conjugate (Amersham) (Fig. 3, lanes 1–10 from brain and lanes 1–8 and pre, post and eluate fractions from SH-SY). Rabbit polyclonals were developed with rabbit anti-mouse Ig peroxidase conjugate (Zymed). Peroxidase was detected by ECL (Amersham), except in Fig. 2a, lanes 2 and 4–6 where diaminobenzidine (Sigma) was used as a substrate.

In the preabsorption experiments 200 μl of the diluted antibody was preincubated with 100 μl of the synthetic peptides at 250 μg/ml for 4 h at room temperature.

3. Results

3.1. Production of mono- and polyclonal antibodies against presenilin 1

Seven IgG monoclonal antibodies, termed MKAD3.1–MKAD3.7, were isolated after immunization with a synthetic peptide, containing residues 21–59 of the hydrophilic amino-terminus of human PS1. Epitope mapping, using the multipin method, showed that the antibodies can be divided in three groups according to their reactivity with 3 different non-overlapping epitopes (Fig. 1b). One monoclonal antibody, MKAD3.7, reacts with amino acid residues 27–32. This epitope contains part of the VRSQ sequence known to undergo alternative splicing in vivo [6]. The two other epitopes contain amino acid residues 36–39, recognized by both MKAD3.2 and MKAD3.6 and amino acid residues 45–48 recognized by MKAD3.1 and MKAD3.3–MKAD3.5.

We confirmed the specificity of the antibodies by their reactivity with bacterially expressed glutathione-*S*-transferase fusion proteins of full-size presenilin 1 (GST-PS1) (Fig. 2a). MKAD3.7 was the only monoclonal antibody that showed no reaction with the purified GST-PS1 fusion protein. This was not unexpected since our recombinant construct lacks the alternatively spliced VRSQ sequence which is part of the MKAD3.7 epitope. Preincubation of the monoclonal antibodies with the synthetic peptide used for immunization, but not with control peptide, eliminates the binding to the GST-PS1 fusion protein (Fig. 2a and data not shown). The GST-PS1 fusion protein was highly sensitive to aggregation when heated in SDS-PAGE sample buffer, a known characteristic of other hydrophobic integral membrane proteins [18] and also observed by others [19] for in vitro translated PS1.

We also raised two different polyclonal rabbit antisera, named PS1-M and PS1-C, directed against synthetic peptides of regions in the large hydrophilic loop (PS1-M) and the C terminus (PS1-C) of PS1 (Fig. 1a). The reactivity of both antisera with full-size presenilin 1 was also confirmed using bacterially expressed glutathione-*S*-transferase fusion proteins (Fig. 2a) and this binding can be inhibited specifically by preincubation with the synthetic peptide used for immunization (data not shown).

3.2. Presenilin 1 protein expression in human cell lines and human brain

The monoclonal antibodies were subsequently used to characterize PS1 protein in membrane extracts from human SH-SY neuroblastoma cells, human 293 kidney cells and human brain (Fig. 2b). In all three extracts the antibodies MKAD3.3 and MKAD3.7 were able to detect a band at M_r 47000,

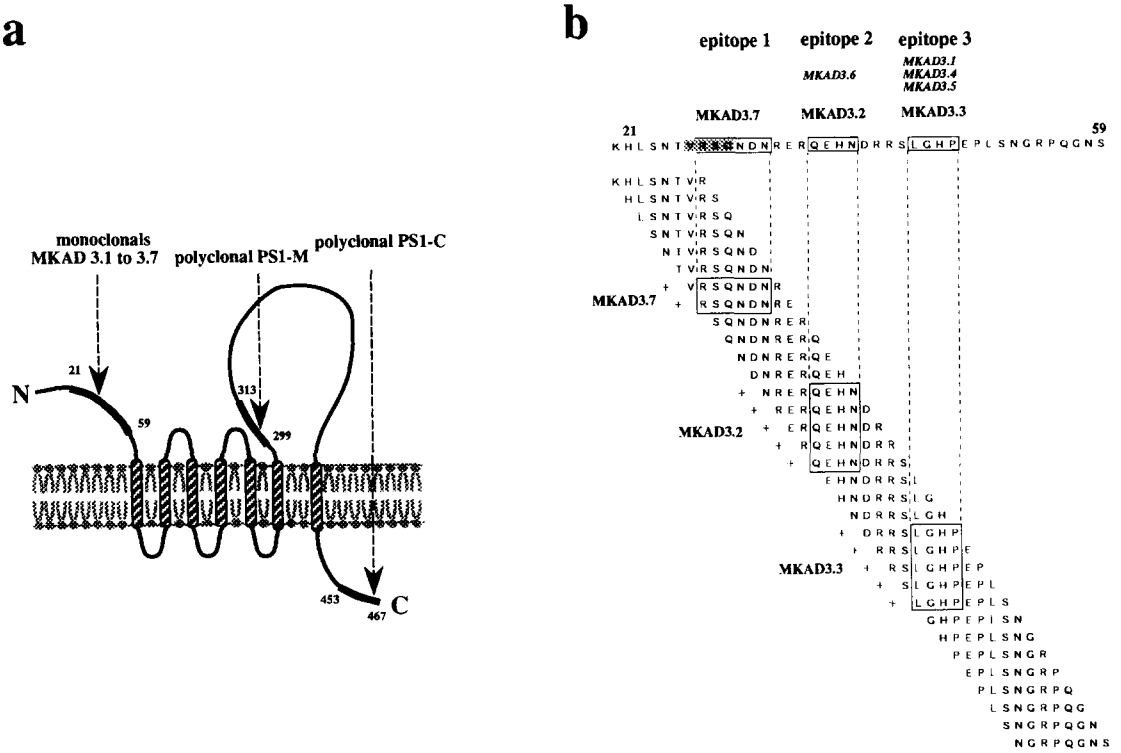

Fig. 1. Production of monoclonal and polyclonal antibodies against presenilin 1 (PS1). (a) Model of the putative seven transmembrane structure of PS1. Thick lines indicate the location of the peptide sequences used for immunization in the production of monoclonal antibodies (N-terminal, amino acids 21–59) and polyclonal antibodies (hydrophilic loop, amino acids 299–313 (PS1-M); C-terminal, amino acids 453–467 (PS1-C)). (b) Epitope mapping of monoclonal antibodies by use of the multipin method. The antibodies are divided into 3 groups according to 3 non-overlapping epitopes. The positive reaction of a representative monoclonal antibody of each group with individual octapeptides is indicated by + and the corresponding epitope is indicated as a boxed sequence. The shaded area represents a site which is subject to alternative splicing in vivo.

which we believe corresponds to full-size PS1. The faster migration than the M_r 52000 calculated from the DNA sequence is not surprising for a highly hydrophobic protein [20] and has also been reported for PS1 by others [19]. The appearance as a sharp single band may indicate the lack of extensive posttranslational modifications. A second and stronger signal, detected with all monoclonal antibodies, was observed at approx. 28 kDa. Both signals are not detected when the sample is heated before loading, a property shared with the GST-PS1 fusion protein. In the extract from post-mortem human brain we detected, in addition to the 28 kDa band, a small amount of products larger than 28 kDa but smaller than 47 kDa. They may represent non-specific post-mortem degradation of the full-size 47 kDa protein, most likely to occur primarily within the exposed hydrophilic loop. In contrast to post-mortem brain, the human cell-lines contain almost exclusively the 47 and 28 kDa products. For this reason we believe that, in vivo, PS1 undergoes selective cleavage at or near the beginning of the hydrophilic loop.

3.3. *Immunoaffinity purification of PS1 from human brain and SH-SY cells*

To further characterize these products, we prepared an affinity column using the monoclonal antibody MKAD3.3 and purified PS1 from SH-SY cells and from human brain (Fig. 3). We obtained highly enriched fractions containing both the 47 and the 28 kDa proteins. The purified proteins were evaluated by immunoblot with all seven amino-terminal specific monoclonal antibodies and with the two polyclonal rabbit antisera raised against regions in the large hydrophilic loop and the C terminus of PS1. As expected, the 47 kDa protein is recognized not only by all seven monoclonal antibodies but also by both polyclonal antisera, confirming its identity as full-size PS1. The 28 kDa band, however, is only seen after staining with the amino-terminal specific monoclonal antibodies and is not reactive with the antisera raised against the hydrophilic loop and the carboxyl-terminus of PS1. The apparent molecular weight and the pattern of mono- and polyclonal antibody reactivity are in agreement with the previously mentioned proposition that, in vivo, PS1 is processed by specific cleavage at or near the beginning of the hydrophilic loop.

3.4. *Wild-type and mutant presenilin 1 in transfected pc12 cells*

Next, we transfected rat PC12 cells with wild-type human PS1 and two constructs, containing the $Met^{146}Val$ and $Ala^{246}Glu$ Alzheimer mutations (Fig. 4). The endogenous rat PS1 is not recognized by monoclonal antibody MKAD3.3 (Fig. 4, lane 3) which enabled us to confirm that human PS1 is expressed as a 47 kDa protein. This experiment also clearly shows that a 28 kDa proteolytic product is generated in living cells after transfection with full-size human PS1 (Fig. 4, lanes 2,5) and argues against the possibility that the 28 kDa product is generated by alternative splicing or alternative translation initiation. In contrast, PC12 cells transfected with constructs containing the Alzheimer mutations produce little or no 28 kDa protein (Fig. 4, lanes 1,4), indicating that these mutations alter the proteolytic processing of PS1 in the transfected cells.

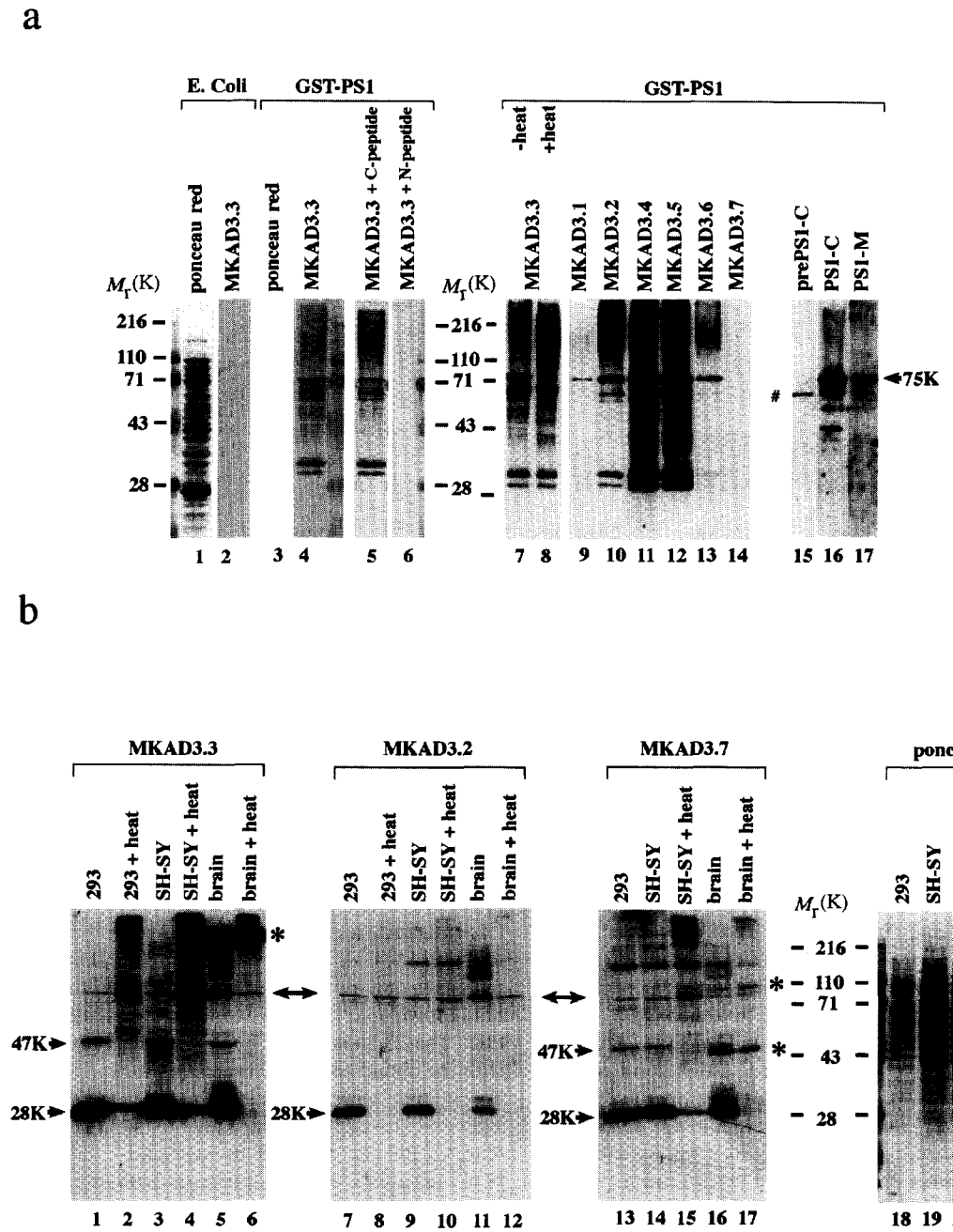

Fig. 2. Confirmation of antibody specificity and detection of PS1 in human brain and cell lines. (a) Reactivity of different antisera with bacterially expressed glutathione-*S*-transferase fusion proteins of full-size presenilin 1 (GST-PS1). No signal was detected in total cell-lysates from non-transformed *E. coli* cells (lane 2). The amount GST-PS1 loaded in lanes 3–17 is below the detection limit for ponceau red protein staining (lane 3). GST-PS1 is immunostained at M_r 75000 (75K), degradation products and aggregation are also observed. In lanes 5 and 6 the antibody was preincubated with control peptide (C-peptide) or the peptide used for immunization (N-peptide). The 75K band aggregates when the sample is heated before loading (lane 8). All monoclonal and polyclonal antisera, except MKAD3.7 (lane 14) and preimmune rabbit serum (prePS1-C, lane 15), react at 75K. Note the non-PS1 reactivity of the preimmune serum at approx. M_r 65000 (#). (b) Detection of heat-sensitive PS1 a 47 and 28 kDa in membrane extracts from human cell lines and human brain with 3 different monoclonal antibodies. Note the cross-reaction with non-heat-sensitive proteins for MKAD3.3 and MKAD3.7 (*). These bands are not present in affinity-purified products (Fig. 3) and probably represent non-specific binding arising upon denaturation. Bands indicated by (↔) are reactive with the secondary antibody. Prestained molecular weight markers (GibcoBRL) were used.

4. Discussion

Presenilins are becoming increasingly important for our understanding of the biochemical processes that lead to Alzheimer's disease. Antibodies to PS1 are reactive with plaques in all amyloid-β-related amyloidoses [21], whether or not they were linked to chromosome 14 and a recent report [15] indicates a genetic association between an intronic polymorphism in the PS1 gene and late-onset Alzheimer's disease. The converging mechanisms that lead different genotypes to similar Alzheimer's disease pathology may therefore all include presenilins in their pathway.

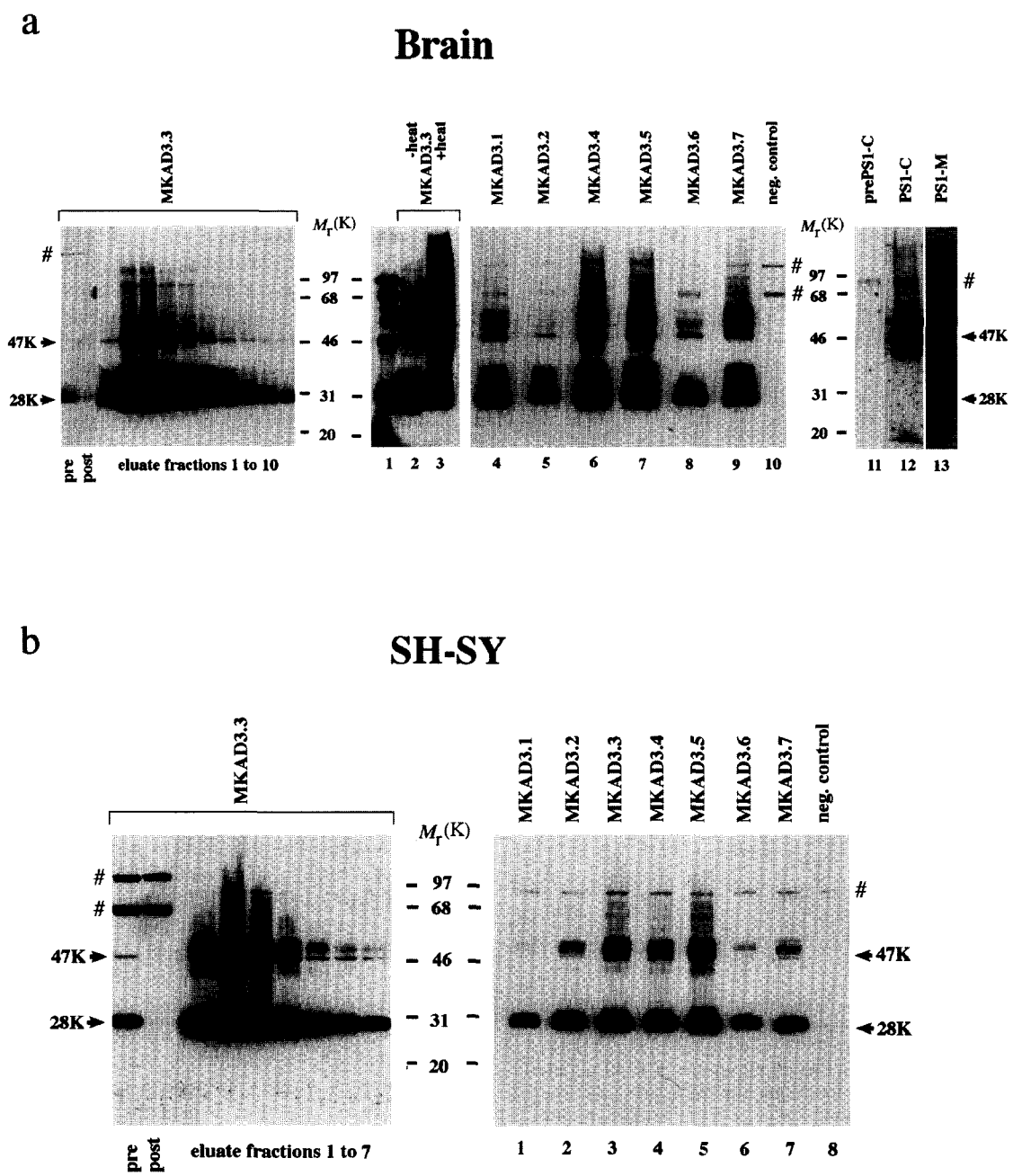

Fig. 3. MKAD3.3 immunoaffinity purification of PS1 from human brain and SH-SY cells. Purification profiles show extracts before loading (pre), the flow-through fraction (post), and purified fractions (eluate). The eluate fractions contain highly enriched concentrations of 47 and 28 kDa proteins. The top fraction was tested with different monoclonal and polyclonal antibodies. Bands caused by the secondary antisera are indicated by (#). (a) Human brain. Polyclonal antisera PS1-M and PS1-C do not recognize the 28K product (lane 12,13) indicating that the proteolytic cleavage is located before or at the beginning of the hydrophilic loop. Lane 10 is without primary antibody and lane 11 is with preimmune rabbit serum (prePS1-C). Biotinylated molecular weight markers (Amersham) were used (lane 1). Note that all purified fractions (eluate fractions, lanes 1,3–9) contain reactive products at approx. 48–65 kDa, which are not detected with the polyclonal antibodies and probably represent aggregated 28 kDa product. (b) SH-SY neuroblastoma cells. Lane 8 is without primary antibody.

A recent study showed, by using in situ hybridization techniques, that presenilin expression in brain is almost exclusively restricted to neuronal populations [22]. In vitro translation and transfection experiments in that study have shown that recombinant FLAG-tagged proteins of PS1 and PS2 appear in SDS-PAGE at approximate M_r 50 000 but no differences were observed in the migration patterns for wild-type or mutant proteins.

In our study we produced antibodies against different regions of presenilin 1 in order to investigate in more detail the in vivo metabolism of this protein. The use of highly specific and sensitive monoclonal antibodies allowed us to detect PS1 in membrane extract from human cell lines and human brain. The reactivity of the monoclonal antibodies with a M_r 47 000 product is as expected for the full-size PS1 and probably corresponds to the bands at approximate M_r 50 000 observed by others [19] after in vitro expression. These authors and others [19] also report higher molecular weight smears which are

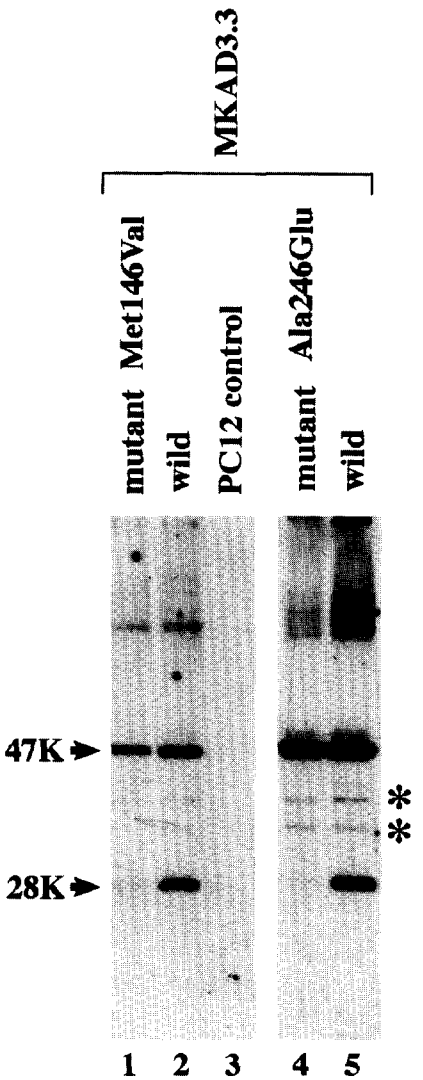

Fig. 4. Western blot using the monoclonal antibody MKAD3.3 showing transfection of PC12 cells with human wild-type PS1, human mutant PS1 or LipofectAMINE only (control). Cells transfected with two different Alzheimer mutants produce 47 kDa full-size PS1 but not the 28 kDa degradation product (lanes 1,4). The bands indicated by (*) are products which are not affected by the mutations.

generated after extensive exposure to SDS, a characteristic for highly hydrophobic membrane proteins [18]. Our monoclonal antibodies detect similar smears, with loss of 47 kDa detection, when the samples are heated before loading. In all extracts tested, the 47 kDa signal was minor compared to a much more abundant signal at 28 kDa. This 28 kDa product was detected by 7 monoclonal antibodies against 3 non-overlapping epitopes, hereby virtually excluding the possibility that it is the result of crossreactivity and not related to PS1. In some experiments this 28 kDa band appeared as a closely spaced doublet (unpublished results) of which the origin is uncertain but which may indicate the presence of the isoform missing the alternatively spliced VRSQ sequence encoded by the last 12 base pairs of exon 3. Affinity-purified 28 kDa products showed no reactivity with 2 polyclonal antisera directed against the first part of the large hydrophilic loop and the carboxyl-terminus of PS1. It is unlikely that the 28 kDa product we observed is the truncated form, presenilin I-374, that was recently described [22], since presenilin I-374 is not expressed in brain. In addition, the absence of reactivity with our polyclonal antiserum PS1-M, directed against the loop region, also indicates that 28 kDa is different from presenilin I-374. The possibility that the 28 kDa product in the different human cell-lines and in human brain is generated by alternative splicing or cross-reaction with another member of the presenilin gene family cannot be ruled out completely, but the observation that expression of human PS1 cDNA in PC12 cells, where there is no such possibility, leads to a similar 28 kDa product strongly suggests otherwise. The fact that the N-terminal sequence of PS1 is not well conserved may explain why the MKAD3.3 antibody shows no cross-reactivity with endogenous rat PS1 in PC12 cells and also argues against the possibility that in human cells the detection of the 28 kDa band is caused by cross-reaction with other members of the presenilin family. Thus, we can conclude that in vivo an amino-terminal polypeptide of 28 kDa, approx. 300 amino acids, is generated by cleavage of PS1 and that the proteolytic site is located at or near the beginning of the large hydrophilic loop.

This region of the protein contains a hotspot for Alzheimer mutations [4] and it has been reported that deletion of exon 9 (bp 857–942) can cause early-onset Alzheimer disease [14]. These genetic findings point to the possible importance for this part of the protein in the pathological processes leading to Alzheimer disease and encouraged us to study the effect of more remote mutations on proteolysis at this location. Our results in transfected PC12 cells indeed indicate that the Alzheimer mutations at $Met^{146}Val$ and $Ala^{246}Glu$ inhibit the proteolysis that generates this 28 kDa product. In contrast, expression of wild-type PS1 under the same experimental conditions does lead to a 28 kDa fragment.

Clearly, more mutations need to be tested to confirm this relationship but from the above results it becomes tempting to speculate that the proposed gain of function of mutated PS1 in Alzheimer's disease is a consequence of interference with the normal proteolytic turn-over, resulting in excessive activity of the protein. This can happen in a direct way by a change in the sequence of a major proteolytic site. One such site, as shown above, is located at or near the beginning of the large hydrophilic loop, a known hot spot for Alzheimer mutations. Proteolysis at this site can also be affected indirectly through the induction of conformational changes. This scenario would apply for the mutations tested here. The fact that the mutations tested, although located at more amino-terminal positions, do affect the proteolysis of PS1 at a site presumed to be located in exon 8 or 9 only accentuates the possible importance of this region. The accessibility for the possibly membrane associated protease that cleaves PS1 may be altered by small changes in the anchoring of PS1 in the membrane. The different mutations can be interfering with this process to a different extent resulting in a characteristic age of onset in the affected family.

Mutations may also affect the proteolysis of PS1 by other mechanisms. The altered protein can be misrouted and simply never reach the location in the cell where it is normally cleaved. The abundant and selective appearance of the 28 kDa product in vivo however is likely to reflect a physiological process in some way related to the function of PS1, which could include the release of active C-terminal fragments.

These hypotheses remain to be tested but the differences observed in the processing of wild-type and mutated PS1 in PC12 cells likely reflect similar metabolic events in the adult brain. Understanding of the possible relationship between this phenomenon and the processes that lead to β-amyloid deposition and neurofibrillary degeneration may inspire to novel therapeutic approaches for Alzheimer's disease.

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