Identification of caspases that cleave presenilin-1 and presenilin-2

Five presenilin-1 (PS1) mutations do not alter the sensitivity of PS1 to caspases

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Abstract Mutations in the presenilin (PS) genes PS1 and PS2 are involved in Alzheimer's disease (AD). Recently, apoptosis-associated cleavage of PS proteins was identified. Here we demonstrate that PS1 as well as PS2 are substrates for different members of the caspase protein family. Remarkably, the caspases acting on PS1 could be subdivided in two groups. One group, containing caspase-8, -6 and -11, cleaved PS1 after residues $ENDD_{329}$ and to a lesser extent after residues $AQRD_{341}$. A second group consisting of caspase-3, -7 and -1 acted uniquely on $AQRD_{341}$. Importantly, these two cleavage sites were also recognized by caspases in the C-terminal PS1 fragment produced by constitutive proteolysis. In decreasing order of activity, caspase-8, -3, -1, -6 and -7 proteolysed PS2 at the recognition site $D_{326}SYD_{329}$. Caspase-8 and -3 exhibited the highest proteolytic activity on both PS1 and PS2. PS1 and PS2 were not hydrolyzed by caspase-2 and PS2 also not by caspase-11. None of five missense mutations affected the sensitivity of PS1 to caspase-mediated cleavage. This suggests that AD pathogenesis associated with PS1 missense mutations cannot be explained by a change in caspase-dependent processing.

Key words: Alzheimer's disease; Caspase; Cleavage; Presenilin; Substrate

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder of the central nervous system leading to dementia and ultimately to death. AD cases usually occur sporadically, although genetic factors play an important role in at least half of the cases. Three AD genes were identified causing early-onset familial AD (age at onset < 65 year): the amyloid precursor protein gene [1], the presenilin-1 gene (PS1) [2] and the presenilin-2 gene (PS2) [3,4]. The majority of the mutations are missense mutations and are found in PS1. In addition, apolipoprotein E-4 is a risk factor for AD [5,6].

The PS genes encode two homologous proteins (PS1/PS2) of approximately 450 amino acids having 67% similarity [2,4]. PS1/PS2 most likely have eight transmembrane domains and are located in the endoplasmic reticulum, to a lesser extent in the Golgi apparatus [7–10]. Two different types of proteolysis were identified for PS1/PS2. Constitutional, tightly regulated proteolysis occurs at and near residue M_{294} for PS1 by yet unknown endoproteases [11,12]. Constitutional proteolytic fragments were also described for PS2 [11–13]. Recently, an additional apoptosis-related proteolytic pathway was characterized [14,15]. Involvement of PS1/PS2 in apoptosis was first suggested by enhanced sensitivity to different apoptotic stimuli when PS2 was overexpressed [16,17] and by reduced sensitivity in the presence of an antisense PS2 construct [16,18]. In addition, apoptotic stimuli induced caspase-dependent cleavage of both PS1 and PS2 [14,15]. Peptide inhibitor studies strongly suggested that in mouse brain homogenates caspases other than caspase-3 execute PS1/PS2 cleavage [15]. The enhanced capacity of the PS2 (I141N) mutant to induce cell death [16,19] is accompanied by increased apoptosis-associated processing of the PS2 (I141N) mutant as compared to wild-type (wt) PS2 [14]. The effect of caspase-mediated cleavage of PS1/PS2 on apoptosis is still under investigation. Nevertheless, it was demonstrated that the C-terminal PS2 fragment antagonizes the progression of cell death [20], whereas full-length PS2 could be important for the induction of cell death [19]. Consequently, cleavage of PS2 may constitute a negative feedback signal in the pathway to cell death.

Caspases have been shown to be part of the cell death machinery [21,22]. To date, 10 human and nine murine members of the caspase family have been identified [22–26]. Caspases are cysteine proteases which specifically proteolyse after Asp residues [27,28]. The exact initiation mechanism of the caspase activation cascade is still largely unknown but once caspases are active, they are able to activate other caspases by cleavage after specific Asp residues. Activation of caspases finally results in the cleavage of specific caspase substrates [29]. Hence, the cell is prepared to die in a subtle and orchestrated way, which consists of a disassembly of critical molecular structures, permitting the condemned cell to vanish with a minimal disruption of surrounding tissues.

In the present study, we demonstrate that PS1 is cleaved by multiple caspases at two different sites, whereas PS2 is proteolysed by multiple caspases at one site only, caspase-8 and caspase-3 being the most potent PS1/PS2-cleaving caspases. Additionally, we provide evidence that PS1 missense mutations do not alter the sensitivity of PS1 to caspase-mediated proteolysis.

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2. Materials and methods

2.1. Plasmid constructions

Human PS1/PS2 cDNAs were cloned downstream of the T7 RNA polymerase promoter in a pSG5 vector (Stratagene Cloning Systems, La Jolla, CA, USA) to result in pSG-PS1 [30] and pSG-PS2 plasmids, respectively [30]. Site-directed mutagenesis was applied to introduce five missense mutations in PS1, viz. Y115C, I143F, I143T, E318G or G384A (De Jonghe et al., in preparation). PS1 and PS2 caspase cleavage site mutants were constructed using a QuickChange site-directed mutagenesis kit (Stratagene Cloning Systems) according to the manufacturer's instructions. The two potential cleavage sites $ENDD_{329}$ and $AQRD_{341}$ of PS1 were mutated to ENAA and AQRA, respectively. For the first PS1 mutant (pSG-PS1M1), the primers TGTTGCAGAGAATGCTGCCGGCGGGTTCAGTGA and TCACTGAACCCGCCGGCAGCATTCTCTGCAACA were used. The primers GGAAGCCCAGAGGGCTAGCCATCTAGGGCCTC and GAGGCCCTAGATGGCTAGCCCTCTGGGCTTCC were used for the second PS1 mutant (pSG-PS1M2). All the PS1 constructs were devoid of the VRSQ-coding sequence. One potential caspase cleavage site of PS2, namely $D_{326}SYD_{329}$, was mutated to GSYA using the primers GGAGATGGAAGAAGGATCCTATGCTAGCTTTGGGGAGCCTT and AAGGCTCCCCAAAGCTAGCATAGGATCCTTCTTCCATCTCC.

2.2. In vitro transcription and translation

Coupled in vitro transcription/translation in the presence or absence of canine microsomal membranes was performed using a rabbit reticulocyte lysate-based TNT kit from Promega (Madison, WI, USA) according to the manufacturer's recommendations. The plasmids described above were applied as a template for T7 RNA polymerase.

2.3. Caspase cleavage assay

Since autoprocessing of murine caspases in bacteria occurs with variable efficiency, we chose to estimate the amount of active caspase in a purified preparation by computer quantification of the large subunit (~20 kDa) of mature recombinant caspase (M. Van de Craen, unpublished results). An amount of purified murine caspase containing 60 ng of large subunit (p20) was incubated with 2 μl of in vitro radiolabeled substrates in a total volume of 25 μl of 'caspase buffer' (50 mM HEPES pH 7.5, 10 mM DTT, 1 mM EDTA, 1 mM PMSF, 50 μM leupeptin and 20 μg/ml aprotinin) for 1.5 h at 37°C. The resulting cleavage products were analyzed on 12.5% SDS-PAGE. In order to avoid massive aggregation of PS1/PS2, the samples were not boiled before loading onto SDS-PAGE [31]. The caspase activity recorded on Ac-DEVD-AMC peptide caspase substrate (Peptide Institute, Osaka, Japan) was 11 726 U/mg for caspase-1, 42 000 U/mg for caspase-2, 8 004 500 U/mg for caspase-3, 568 180 U/mg for caspase-6, 312 800 U/mg for caspase-7, 911 950 U/mg for caspase-8, and 17 969 U/mg for caspase-11. One unit of purified caspase represents the amount of enzyme necessary to generate 1 pmol AMC/min from 50 μM Ac-DEVD-AMC tetrapeptide substrate at 30°C.

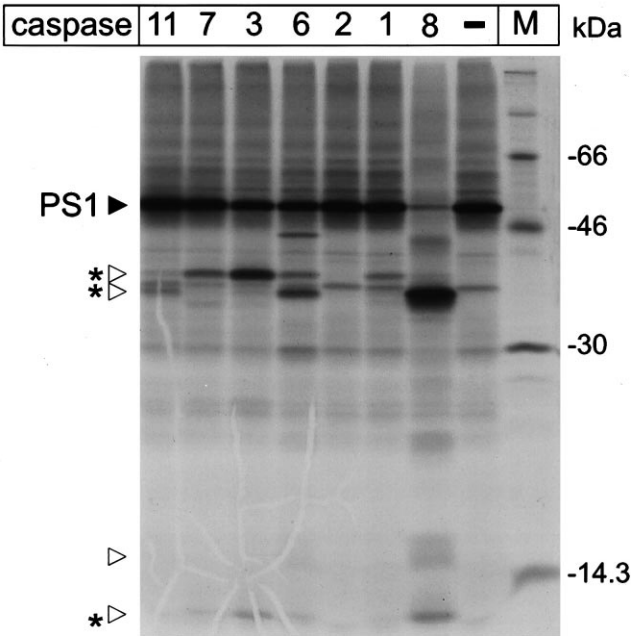


Fig. 1. In vitro cleavage of PS1 by purified caspases. An amount of murine caspase containing 60 ng of large subunit was incubated in 25 μl caspase buffer with 2 μl of [^{35}S]Met-labeled human PS1. Reaction products were separated on 12.5% SDS-PAGE and analyzed by autoradiography. Closed arrowhead, full-length PS1; open arrowheads, cleavage products. *, fragments migrating like alternative cleavage fragments previously observed in vivo [14,15,37,38].

3. Results

3.1. PS-1 is proteolysed at two cleavage sites (D_{329} and D_{341}) by multiple caspases

In order to test whether purified caspases are able to proteolyse human PS1, wt PS1 was labeled in vitro with [^{35}S]Met and incubated with seven different purified murine caspases (caspase-1, -2, -3, -6, -7, -8 or -11) in a 'caspase buffer'. Caspase-11 is considered to be the murine counterpart of human caspase-4 [24]. Caspase-mediated cleavage of PS1 resulted in ~40-kDa and ~13-kDa fragments for caspase-3, -7, -1 and -6, and in additional ~36-kDa and ~14.6-kDa fragments for caspase-8, -6, and -11 (Figs. 1 and 2). Caspase-2 did not proteolyse PS1. The caspases are mentioned in a decreasing

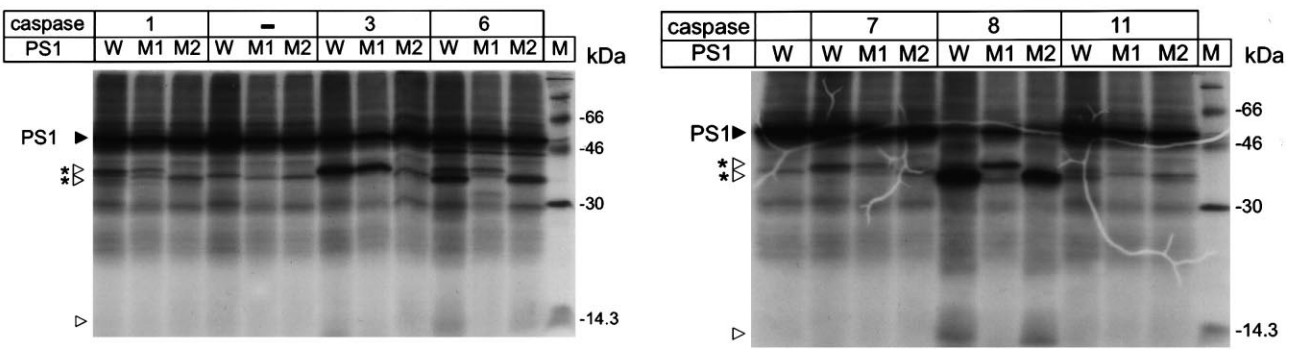


Fig. 2. In vitro cleavage of PS1 by purified caspases. An amount of murine caspase containing 60 ng of large subunit was incubated in 25 μl caspase buffer with 2 μl of [^{35}S]Met-labeled human PS1. Reaction products were separated on 12.5% SDS-PAGE and analyzed by autoradiography. Closed arrowhead, full-length PS1; open arrowheads, cleavage products. *, fragments migrating like alternative cleavage fragments previously observed in vivo [14,15,37,38].

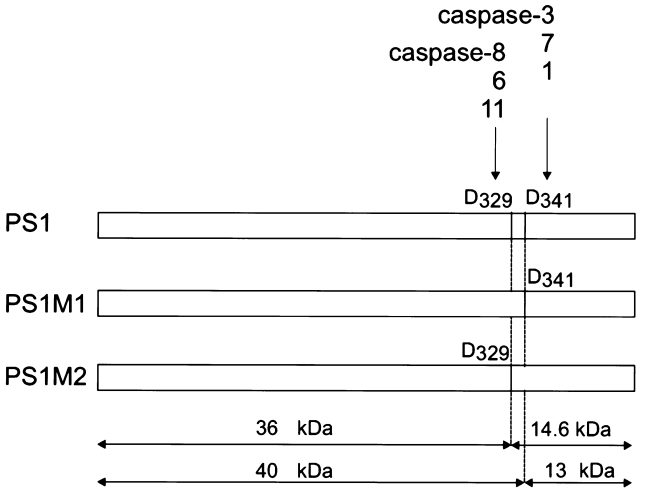


Fig. 3. Schematic representation of PS1, the mutants PS1M1 and PS1M2, as well as the predicted cleavage products.

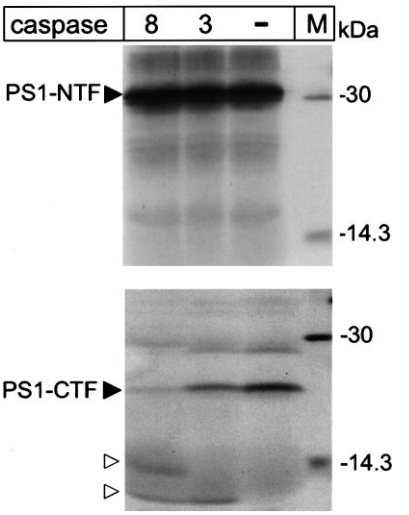


Fig. 4. Incubation of caspase-3 or caspase-8 with radiolabeled PS1-NTF or PS1-CTF fragments. Constitutional cleavage of PS1 at and near M_{294} results in an N-terminal fragment (PS1-NTF) and a C-terminal fragment (PS1-CTF) that are predominantly present in brain tissue. Caspase-3 as well as caspase-8 were incubated under standard conditions with radiolabeled PS1-NTF (amino acids 1–295) or PS1-CTF (amino acids 291–476). Symbols, see Fig. 1.

order of activity on PS1. In mammalian cells, the alternative PS1 cleavage fragment migrates on SDS-PAGE like a molecule of ~14 kDa [14].

It was demonstrated by N-terminal sequencing that the C-terminal fragment generated under apoptotic conditions is due to proteolytic cleavage between D_{341} and S_{342} [15]. However, examination of the primary amino acid sequence of PS1 revealed that another potential caspase cleavage site, viz. $ENDD_{329}$, is present 12 amino acids N-terminally from D_{341}. In order to investigate whether both $ENDD_{329}$ and $AQRD_{341}$ sites are targets for caspase-mediated cleavage, these sites were mutated to $ENAA_{329}$ (PS1 mutant 1 or PS1M1) and $AQRA_{341}$ (PS1 mutant 2 or PS1M2), respectively. Caspase-3 was still able to hydrolyze PS1M1 but not PS1M2 (Figs. 2 and 3). The same pattern, but less pronounced, was observed with caspase-7 and -1. These results indicate that caspase-3, -7 and -1 cleaved PS1 at the $AQRD_{341}$ site (Fig. 1). On the other hand, caspase-8 vigorously proteolysed PS1M2 to the 36-kDa fragment, whereas the 40-kDa band was absent. As expected, the 36-kDa band was not produced by the PS1M1 mutant but a weak 40-kDa fragment was still observed (2). The same pattern, but less pronounced, was obtained with caspase-6 and -11. These data reveal that caspase-8, -6 and -11 use both caspase cleavage sites but prefer the $ENDD_{329}$ site (Fig. 1). The faint 44-kDa band obtained with caspase-6, and to a lesser extent with caspase-8, is of unknown origin.

As conventional cleavage of PS1 at and near M_{294} by unknown proteases results in an N-terminal fragment (PS1-NTF) and a C-terminal fragment (PS1-CTF) that are predominantly present in brain tissue [11,12,32], it is also important to examine these fragments for their sensitivity to caspase cleavage. Fig. 4 shows that PS1-NTF (ranging from amino acids 1 to 295) was not cleaved by caspase-8 or caspase-3. However, caspase-8 recognized both caspase cleavage sites in PS1-CTF (ranging from amino acids 291 to 476), whereas caspase-3 proteolysed PS1-CTF at one site (Fig. 4).

3.2. PS-2 is proteolysed by several caspases at one site (D_{329})

Incubation of PS2 with different concentrations of caspases showed that PS2, in contrast to PS1, was cleaved at the same

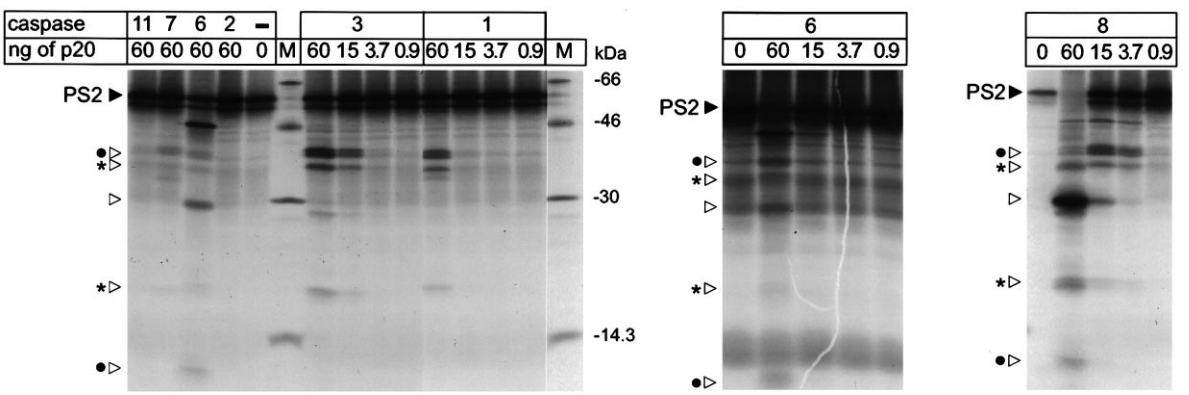


Fig. 5. In vitro cleavage of PS2 by purified caspases. An amount of purified murine caspases was incubated in 25 μl caspase buffer with in vitro transcribed and translated human PS2. Among those caspases that easily cleave PS2, several enzyme concentrations were used. Closed arrowhead, full-length PS2; open arrowheads, cleavage products. *, fragments migrating like alternative cleavage fragments previously observed in vivo [14,15]. ●, bands migrating as theoretically expected (see Fig. 7).

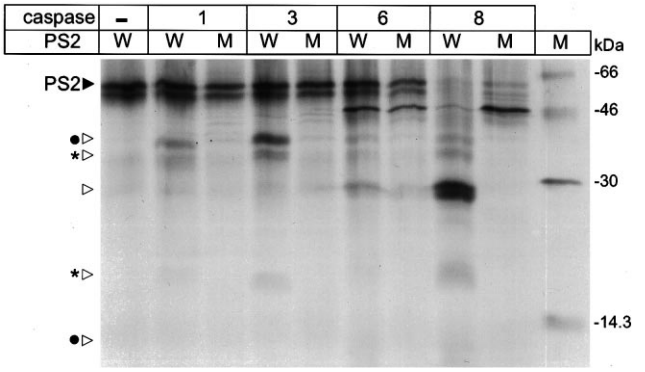


Fig. 6. In vitro cleavage of the PS2 caspase cleavage site mutant. PS2 was mutated at one potential caspase cleavage site ($D_{326}SYD_{329}$) to generate PS2M. Both PS2M (M) and wt PS2 (W) were incubated under standard conditions with different caspases. Symbols, see Fig. 5.

site(s) by most caspases (Figs. 5 and 7). Using the highest concentration of caspase-8 (60 ng p20 per 25 μl), PS2 was proteolysed to a major ~29-kDa fragment and minor bands of approximately 35, 19 and 12 kDa (Fig. 5). The full-length PS2 was entirely degraded. Using lower amounts of caspase-8, the ~29-, ~19- and ~12-kDa products gradually disappeared but the ~35-kDa fragments showed up more clearly. The latter picture was also observed when the highest concentration of caspase-3 or -1 (60 ng p20 per 25 μl) was used. In mammalian cells, the alternative PS2 cleavage fragments migrate on SDS-PAGE like molecules of ~34 and ~20 kDa [14]. In conclusion, caspase-8, -3, -1, -6 and -7 (in decreasing order of activity) proteolysed PS2 at the same site(s). Caspase-11 and -2 exhibited no proteolytic activity on PS2.

Loetscher et al. [15] demonstrated by N-terminal sequencing that apoptosis-related cleavage of ectopically overexpressed murine and human PS2 took place at a cleavage recognition site $D_{326}SYD_{329}$ enclosing two different Asp residues. In order to show that this site is actually cleaved by caspases, we constructed a PS2 mutant protein (PS2M) where $D_{326}SYD_{329}$ was substituted for $G_{326}SYA_{329}$. Then the caspases displaying clear proteolytic activity on PS2 were incubated with wt PS2 or PS2M. Fig. 6 demonstrates that PS2M was not processed by any caspase. This indicates that all proteolytic fragments derived from full-length PS2 are the result of cleavage at the $D_{326}SYD_{329}$ sequence and that this site is the major caspase target site. Note that the appearance of the 29-kDa band of wt PS2 cannot be explained by potential additional caspase cleavage sites (Fig. 7). Presumably, this band is a result of aggregation of hydrophobic domains [31].

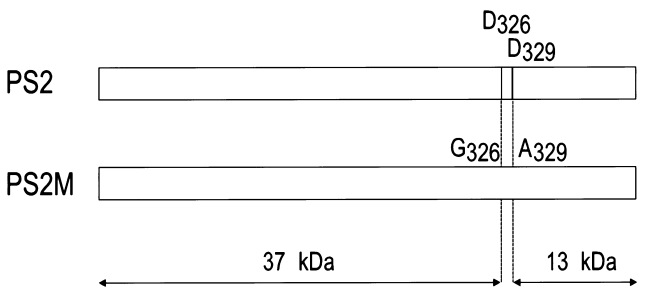
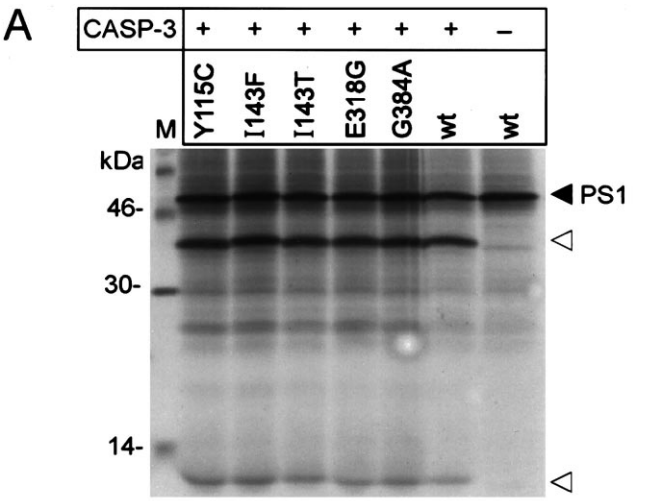


Fig. 7. Schematic representation of PS2, PS2M and the predicted cleavage products.



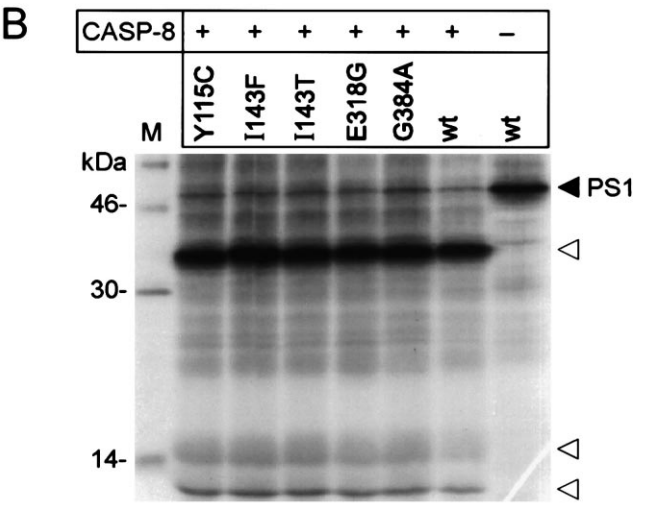


Fig. 8. PS1 missense mutations do not affect PS1 cleavage by caspases. PS1 mutants and wt PS1 were in vitro transcribed and translated in rabbit reticulocyte lysates in the presence of canine microsomal membranes. Incorporation of PS1 proteins into the membranes was confirmed by centrifugation. Wt or mutant PS1 proteins were incubated with 60 ng of p20 subunit of caspase-3 (A) or caspase-8 (B) in a total volume of 25 μl of caspase activation buffer for 1.5 h at 37°C. The resulting cleavage products (open arrowheads) were separated on 12.5% SDS-PAGE and visualized by autoradiography.

However, when caspase-6 or -8 were incubated with PS2, an extra ~46-kDa band was observed (Figs. 5 and 6). This band is reminiscent of the faint 44-kDa PS1 band obtained with caspase-6 or caspase-8 and is of unknown origin.

3.3. *PS1 mutations have no direct effect on PS1 proteolysis by caspases*

We tested in vitro whether missense mutations in PS1, observed in AD cases, altered the sensitivity to caspases. In order to allow potential, subtle structural changes of the PS1 mutants, translation was performed in the presence of canine microsomal membranes. The latter technique was used previously to determine the transmembrane domain structure of PS1 [33]. The PS1 mutants Y115C, I143F, I143T, E318G and G384A [34,35] were equally well translated; centrifugation was applied to confirm incorporation

of the PS1 proteins into the membranes (data not shown). Both caspase-3 and caspase-8 were chosen as the enzymatic source, since the latter cleaved wt PS1 most efficiently at either one of the two cleavage sites (Fig. 1). Fig. 8 indicates that none of the tested PS1 mutants exhibited a different proteolytic sensitivity to the enzymatic activity of caspase-8 or caspase-3. Also at limiting enzyme concentrations caspase-dependent cleavage was not increased (data not shown). These in vitro data demonstrate that the five AD mutations did not influence the sensitivity of PS1 to caspase-dependent proteolysis. It should be noted that incorporation of wt PS1 into membranes (Fig. 8) did not change the accessibility of the caspase cleavage sites as compared to non-incorporated PS1 (Fig. 1).

4. Discussion

The physiological relevance of caspase-mediated PS1/PS2 cleavage has already been suggested by staurosporine and etoposide-induced apoptosis of H4 neuroglioma cells [14]. However, it was not clear which members of the caspase family were capable of performing this alternative PS1/PS2 processing. This report focuses on alternative proteolysis of PS1 and PS2 using recombinant murine caspases and in vitro translated human PS1/PS2. Human and murine PS1/PS2 have a very high degree of similarity, viz. 94%. The $AQRD_{341}$ and $D_{326}SYD_{329}$ cleavage sites are conserved between man and mouse in PS1 and PS2, respectively. However, the $ENDD_{329}G$ site in human PS1 is changed to a $GNDD_{329}G$ site in murine PS1. Nevertheless, overexpression of human PS1 and human PS2 in mouse Neuro2a cells [15] or in human H4 neuroglioma cells [14] resulted in identical apoptosis-related cleavage products in both cell lines, indicating that identical proteolysis occurred. Moreover, overexpression of human or murine PS2 in hamster BHK21 cells also resulted in the same cleavage patterns [15].

We demonstrated that purified caspases are able to exert proteolytic activity in vitro both on PS1 and PS2. Moreover, we were able to confirm the cleavage sites $AQRD_{341}$ for PS1 and $D_{326}SYD_{329}$ for PS2 [15] as actual caspase cleavage sites. For PS1 a second caspase cleavage site $ENDD_{329}$ was identified, which lies 12 amino acids N-terminally from $AQRD_{341}$. The existence of this proteolytic site has also been demonstrated in vivo by Steiner et al. [36]. Remarkably, based on the preference of caspases for a particular cleavage site in PS1, the caspases could be divided in two groups. One group, consisting of caspase-8, -6 and -11, predominantly used (in decreasing order of proteolytic efficiency) the $ENDD_{329}$ cleavage site. When this site was removed by mutagenesis, these caspases still recognized the $AQRD_{341}$ site. The second group, comprising caspase-3, -7 and -1, proteolysed (in decreasing order of importance) the $AQRD_{341}$ site but not the $ENND_{329}$ site. In native cell lines and in brains, little or no full-length PS1 is detected. In these cells PS1 undergoes a highly regulated processing at or near M_{294} by unknown endoproteases, which results in the generation of two stable fragments referred to as PS1-NTF and PS1-CTF [11,12]. PS1-NTF was no substrate for caspase-8 and -3, whereas PS1-CTF was clearly cleaved by caspase-8 and -3. This demonstrates that not only the full-length PS1 gene product but also PS1-CTF is a direct substrate for caspases and strengthens the physiological relevance of caspase-mediated cleavage of PS1. The situation for PS2 is less complex than for PS1. Caspase-8 as well as caspase-3, -1, -6 and -7 proteolysed, in decreasing order of efficiency, PS2 at the cleavage site $D_{326}SYD_{329}$ [15]. Caspase-2 and -11 were not active on PS2.

It was previously reported that only caspase-3 is important for PS2 cleavage and that none of the caspases hydrolyze PS1 [20]. However, our data do not point in that direction, since not only PS2 but also PS1 was a substrate for multiple caspases. Our observations are in agreement with various reports on alternative cleavage of PS2 as well as of PS1 obtained in cultured cells and brain homogenates [14,15,37–39]. Our data on PS1 cleavage are also strengthened by the fact that the PS1 cleavage site identified in cultured cells ($AQRD_{341}$) is indeed a target for caspases [15] and that conventional PS1-CTF is directly recognized by caspases (Fig. 4). Peptide studies predicted that also proteases other than caspase-3 are involved in PS1 and PS2 cleavage by brain homogenates [15]. In agreement with this observation, we demonstrate that both caspase-8 and caspase-3 cleave PS1 and PS2, as well as several other caspases.

Although highly speculative, even the weak cleavage of PS1/PS2 by caspase-1 can be physiologically important. In the course of neuronal differentiation of nerve growth factor-treated PC12 cells, a gradual enhancement of alternative PS1 cleavage, resulting in 36-kDa and 14-kDa fragments, was detected [37]. Since nerve growth factor rather prevents PC12 cells from dying, one may reason that proteases other than caspases may be implicated in this alternative PS1 cleavage. It is known that treatment of PC12 cells with nerve growth factor also induces mature interleukin-1β secretion [40], suggesting that caspase-1 is operating in these cells. Consequently, sustained action of less efficiently PS1-cleaving caspases, such as proinflammatory and highly inducible caspase-1, might be responsible for the alternative PS1 cleavage in differentiating PC12 cells. But proteolysis by unknown proteases cannot be excluded.

Five PS1 mutations found in AD cases, viz. Y115C, I143F, I143T, E318G and G384A, did not influence the sensitivity of PS1 to caspase activity in vitro, providing evidence that PS1 missense mutations do not result in AD pathogenesis because of altered caspase cleavage. This is in contrast to huntingtin, the protein mutated in Huntington's chorea, another neurodegenerative disease of the central nervous system [41]. Huntingtin becomes a more efficient substrate for caspases when a polyglutamine tract is extended [42].

In conclusion, both PS1 and PS2 are directly cleaved by multiple members of the caspase family, particularly caspase-8 and -3. PS1 has two caspase cleavage sites, whereas PS2 has only one. Although the biological relevance of caspase-mediated PS1 cleavage is strengthened by the fact that PS1-CTF is also a direct substrate for caspases, the pathological role of PS1 missense mutations in AD is unlikely to be explained by an enhanced susceptibility to caspase-mediated cleavage.

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