Generation of Anti-apoptotic Presenilin-2 Polypeptides by Alternative Transcription, Proteolysis, and Caspase-3 Cleavage*

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PS2, the chromosome 1 familial Alzheimer’s disease gene, has been shown to be involved in programmed cell death by three complementary experimental approaches. Reduction of PS2 protein levels by antisense RNA protects from apoptosis, whereas overexpression of an Alzheimer’s PS2 mutant increases cell death induced by several stimuli. In addition, ALG-3, a truncated PS2 cDNA, encodes an artificial COOH-terminal PS2 segment that dominantly inhibits apoptosis. Here we describe a physiological COOH-terminal PS2 polypeptide (PS2s, Met398-314) generated by both an alternative PS2 transcript and proteolytic cleavage. We find that PS2s protects transfected cells from Fas- and tumor necrosis factor α (TNFα)-induced apoptosis. Furthermore, a similar anti-apoptotic COOH-terminal PS2 polypeptide (PS2Ccas) is generated by caspase-3 cleavage at Asp329. These results suggest that caspase-3 not only activates pro-apoptotic substrates but also generates a negative feedback signal in which PS2Ccas antagonizes the progression of cell death. Thus, whereas PS2 is required for apoptosis, PS2s and PS2Ccas oppose this process, and the balance between PS2 and these COOH-terminal fragments may dictate the cell fate.

Programmed cell death (PCD) is a biological process under genetic control that regulates the life span of different cell types and is required for normal development and survival of multicellular organisms. Disregulation of PCD can cause pathological processes such as neurodegenerative disorders, cancer, immunodeficiency, and auto immune diseases (1). Consequently, much interest has been focused on characterizing the molecular cascade that specifies cell death.

To study the biochemical events leading to apoptosis, we have used as a model system cell death induced by T cell receptor-triggering in a mouse T cell hybridoma (2, 3). In this experimental paradigm, Fas ligand is induced after T cell receptor stimulation, and the engagement of Fas by Fas ligand activates the cell death program (4–6). Using a functional selection strategy, called “death trap,” we have isolated several genes involved in apoptosis (7). ALG-3, one of the transcripts identified, was shown to be a truncated form of the chromosome 1 familial Alzheimer’s disease gene PS2 (8, 9). Expression of this partial PS2 cDNA rescued cells from T cell receptor-induced apoptosis by inhibiting Fas-mediated death signal. It was subsequently demonstrated that, whereas the full-length PS2 protein is involved in some forms of apoptosis (10, 11), ALG-3 codes for a COOH-terminal PS2 polypeptide that functions as a dominant negative mutant of PS2 (10). Through an unknown mechanism, ALG-3 interferes with the activity and/or activation of the cysteine proteases of the interleukin 1β converting enzyme/Ced-3 family (12) recently renamed caspasas, which are key components of PCD (13–16). Present in the cells as pro-enzymes, caspases are activated by proteolytic cleavage, and their activity is essential to carry on the apoptotic program (13, 14).

This evidence prompted us to investigate whether a natural ALG-3 equivalent existed. In the present study, we describe two physiological PS2 COOH-terminal polypeptides, PS2s and PS2Ccas, that oppose apoptosis induced through the death receptors Fas and TNF.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—Human embryonic 293T cells, human cervical cancer HeLa cells, and COS7 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Rabbit polyclonal antisera aPS-2n and aPS-2e (10), anti-Fas CH-11 and anti-caspase-3 monoclonal antibodies (Upstate Biotechnology, Inc.), anti-human poly(ADP-ribose) polymerase (PARP) antisemur (Enzyme System, Dublin, CA), recombinant human TNFα (Pharmingen), cyclohexamide (Sigma), ZDEVD-fmk and ZFA-fmk (Enzyme System) were used as described in the figure legends. Recombinant caspasas were produced as described previously (17).

cDNAs Cloning—PS2short was cloned from a mouse liver cDNA library (CLONTECH) screened with the ALG-3 fragment. The PS2s cDNA was cloned into the mammalian expression vector pcDNA3 (In-vitrogen). cDNA probes 1–5 (see Fig. 1a) were generated using the polymerase chain reaction and mouse PS2 cDNA as a template. Cloning of mouse PS1 was described previously (10). Human PS2 and caspase-3 cDNAs were cloned by polymerase chain reaction from a human brain cDNA library (CLONTECH) and inserted in pcDNAs. D329A, D326A, and D329A PS2 mutants were generated by in vitro site-directed mutagenesis using the human PS2 expression vector as a template. PS2Ncas and PS2Ccas human constructs were made using polymerase chain reaction. The reverse primer utilized to amplify PS2Ncas contained a stop codon downstream of the codon for Asp329, and the forward oligonucleotide for PS2Ccas had a consensus sequence for initiation of translation and an ATG upstream of the codon for Ser320. Protein kinase C δ (PKCδ) and PARP cDNAs were derived as described previously (17).

Cell Transfection and Survival Assay—HeLa and COS7 cells were transfected with lipofectAMINE (Life Technologies, Inc.) plus 2 μg of pcDNA3 expressing the indicated cDNAs, following the manufacturer’s indications. 293T cells were transfected with 2 μg of DNA by the calcium phosphate precipitation method. Cotransfections of PS2 constructs and caspase-3 cDNAs were done using 1 μg of each plasmid. In apoptosis experiments, a mixture of 0.2 μg of cytomegalovirus β-galactosidase (CLONTECH) with 2 μg of the listed vectors was transfected.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U57325.

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1 The abbreviations used are: PCD, programmed cell death; TNF, tumor necrosis factor; PARP, poly(ADP-ribose) polymerase.
24 h after transfection, cells were treated with TNF-α or anti-Fas antibody along with cyclohexamide. The β-galactosidase activity was visualized as described (10), and the live blue cells present in 20 fields were counted. The percent reduction corresponds to the ratio of treated versus untreated β-Gal1 cells for each individual transfection. The transfection efficiency was comparable in all samples.

Immunoprecipitation and Immunoblot Analysis—Cells were lysed in RIPA buffer plus the protease inhibitors aprotinin, pepstatin, and leupeptin (100 μg/ml, Sigma). Immunocomplexes were bound to protein A-Sepharose beads (Pharmacia Biotech Inc.). Modified 5× loading buffer (320 mM Tris, pH 6.8, 50% glycerol, 0.5% bromphenol blue, 10% SDS, 100 mM dithiothreitol, and 8 M urea), used to reduce aggregation of PS2, was added to immunoprecipitates or 10 μg of cell lysates. Samples were heated at 37 °C for 45 min, 75 °C for 5 min, and separated on a 12% polyacrylamide-SDS gel. In vitro labeled proteins were detected by x-ray exposure; unlabeled proteins were blotted onto nitrocellulose membranes (Gelman Science) and probed with the specified antibodies. Immunoblots were developed using the ECL System (Amersham Life Science, Inc.).

RESULTS AND DISCUSSION

The mouse PS2 gene is expressed as a ubiquitous 2.4-kilobase transcript coding for the full-length PS2 protein and a liver-specific 1.1-kilobase mRNA (7). This 1.1-kilobase mRNA, called PS2short (PS2s), contains the 3′ portion of PS2 and includes the artificial ALG-3 transcript (Fig. 1a). Sequence analysis of several PS2s cDNAs cloned from a mouse liver cDNA library revealed that PS2s encodes for the 151 COOH-
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PS2s is also generated by cleavage of the PS2 protein. a, total cell lysates from mouse thymus (T) and liver (L) were immunoprecipitated with αPS2c antisera and probed with αPS-2n antisera. The first lane contains extracts from COS7 cells transfected with PS2s. The broad signal between 50 and 36 kDa derives from the antisera used for immunoprecipitation. The endogenous PS2 protein (~50 kDa) is therefore not detectable. Aggregated forms of PS2 migrate above 64 kDa. b, Western blot analysis of COS7 cells transfected with PS2 cDNA using the αPS2n. Note that the majority of transfected PS2 migrates as high molecular mass forms. βTub., β tubulin.

FIG. 2. PS2s is also generated by cleavage of the PS2 protein. a, total cell lysates from mouse thymus (T) and liver (L) were immunoprecipitated with αPS2c antisera and probed with αPS-2n antisera. The first lane contains extracts from COS7 cells transfected with PS2s. The broad signal between 50 and 36 kDa derives from the antisera used for immunoprecipitation. The endogenous PS2 protein (~50 kDa) is therefore not detectable. Aggregated forms of PS2 migrate above 64 kDa. b, Western blot analysis of COS7 cells transfected with PS2 cDNA using the αPS2n. Note that the majority of transfected PS2 migrates as high molecular mass forms. βTub., β tubulin.

PS2s was translated into a PS2 COOH-terminal polypeptide named PS2s both in vitro and in transfected COS7 cells (Fig. 1d). The smaller and fainter band detected in COS7 cells transfected with PS2s could derive from usage of a downstream start codon.

As expected, a protein identical in size and immunoreactivity to transfected PS2s was found in liver (Fig. 2a). Surprisingly, a PS2s-like protein was also detected in cells transfected with the full-length PS2 cDNA (see COS7 cells in Fig. 2b and in 293 cells in Fig. 5) and in tissues that do not express the PS2 mRNA (see thymus (T) in Fig. 2a). This PS2s-like protein arises from proteolytic cleavage because an antibody specific for the NH2-terminal 20 amino acids of PS2 detects the corresponding NH2-terminal proteolytic fragment in transfected cells (not shown).

Moreover, PS2 processing has also been reported recently by others (18, 19). Since the two COOH-terminal fragments generated by either proteolysis or the PS2s transcript have the same gel mobility and pattern of immunoreactivity, we will refer to both polypeptides as PS2s. Thus, PS2s can be generated by both alternative transcription and proteolytic cleavage.

Interestingly, the chromosome 14 familial Alzheimer’s disease protein PS1 (20), which shares structural and amino acid similarity with PS2 (67% identity), also undergoes proteolytic processing (21). PS1 cleavage occurs mainly at Met291 (22), which corresponds to Met298 of PS2, suggesting that both PS1 and PS2 are cleaved by the same or closely related protease(s).

Since PS2s is a COOH-terminal fragment of PS2 and potentially encompasses the inhibitory domain found in ALG-3, we tested whether it could confer resistance to cell death. HeLa cells were transfected with various PS2 constructs, and cell death was induced with TNFα or an anti-Fas antibody. Like ALG-3, PS2s partially protects transfected cells from cell death induced by both stimuli (Fig. 3).

An additional ~18-kDa COOH-terminal PS2 fragment was noticed in thymic lysates (see PS2Cas in Fig. 2a). This was of particular interest because ~90% of developing T cells undergo cell death during differentiation in the thymus. To determine whether this 18-kDa PS2 polypeptide was specifically produced during apoptosis, HeLa cells were induced to die by TNFα or Fas engagement. Both stimuli induced the appearance of the ~18-kDa COOH-terminal PS2 polypeptide (Fig. 4a). The same treatments also resulted in activation of caspase-3, which is present in the cell as a proenzyme and is activated by proteolysis during apoptosis (23–25) and cleavage of its physiological substrate PARP (Fig. 4a).

To determine whether PS2 cleavage was dependent on caspase activity, HeLa cells were treated with TNFα together
with the specific caspases inhibitor ZDEVD-fmk. As shown in Fig. 4, PS2 cleavage was inhibited by ZDEVD-fmk, whereas no effect was seen with the irrelevant reagent ZFA-fmk (Fig. 1a). These results demonstrate that the 18-kDa PS2 fragment is produced during PCD and that PS2 cleavage requires caspase activity.

In looking at the amino acids sequence of PS2, we noticed a putative caspase-3 consensus cleavage sequence (26–28) (Asp326-Ser-Tyr-Asp329) (Fig. 1c). Cleavage of PS2 at the predicted Asp329-Ser330 site would give rise to a COOH-terminal segment compatible with the 18-kDa PS2 polypeptide generated during PCD. To address whether PS2 is directly cleaved by caspases, we tested seven recombinant caspases in an in vitro cleavage assay using in vitro translated, recombinant proteins as substrates. PS2 was cleaved only by caspase-3 (Fig. 5a) to generate two fragments of ~40 and ~18 kDa (Fig. 5b). This cleavage was specific for PS2 since the highly homologous Alzheimer's disease protein PS1, which lacks the putative caspase-3 cleavage site, was not processed (Fig. 5c). Caspase-3 cleaved PS2 and the two known substrates PARP and PKCd (17) with comparable efficiency in vitro, as shown in the dose titration (Fig. 5c). The ~18-kDa fragment was immunoprecipitated by the aPS2c antiserum (Fig. 5d), indicating that it represented the COOH-terminal PS2 cleavage product. Hence, we will refer to the 18- and 40-kDa clipped fragments as PS2Cas and PS2Ncas, respectively. If cleavage occurred at the predicted Asp329-Ser330 (Fig. 1c), PS2Ncas and PS2Cas would consist of Met1-Asp329 and Ser330-Ile448, respectively. Two constructs encoding for these portions of PS2 were translated into PS2 polypeptides that co-migrated with the cleavage products

Fig. 5. PS2 is cleaved by caspase-3 at the predicted Asp-Ser site. a, in vitro metabolically labeled PS2 and PS1 proteins were incubated for 30 min at room temperature with recombinant human caspase-1 (5 μg/ml), -2 (75 μg/ml), -4 (5 μg/ml), -5 (5 μg/ml), -3 (2.5 μg/ml), -6 (5 μg/ml), -7 (5 μg/ml), caspase buffer (m), or nothing (–). b, human and mouse PS2 are cleaved by caspase-3 with the same efficiency. c, comparison of PS2, PKCδ, and PARP cleavage by caspase-3. PS2Cas is poorly visible in the experiments shown in panels a–c, because PS2 was labeled with [35S]methionine (the only amino acid that can be labeled is Met438). FL, full-length; CF, cleavage fragment. d, cleavage of PS2 proteins by recombinant human caspase-3 (5 μg/ml). I.P. indicates immunoprecipitation with the aPS2c antiserum of the cleavage products of the PS2 wild type (W.T.) protein. PS2Cas is much more visible in panel d as compared with panels a–c, because proteins were labeled with 3H-leucine.

Fig. 6. 293T cells were transfected with the indicated vectors, and cell lysates were analyzed for expression of PS2 polypeptides with the aPS2n antiserum. Only a small fraction of the full-length PS2 protein runs according to its molecular mass (~50 kDa), due to aggregation. An antibody specific for β-tubulin was used to allow normalization to the amount of protein loaded on each lane. Like PS2s, PS2Cas has an apparent molecular mass (~18 kDa) higher than the expected one (13.5 kDa). α-Tub, α-tubulin.

PS2Cas and PS2Ncas (Fig. 5d). Caspases have an absolute requirement for aspartic acid at position P1 of their substrate and a less stringent one for the amino acid at position P4 (26–28). PS2 mutants in which positions P1 (Asp239, mutant D239A) or P4 (Asp326, mutant D326A) were substituted with an alanine were made. Whereas the D326A mutant was a very poor substrate for caspase-3 and cleavage was detectable only after longer exposure (not shown), D329A was not processed at all (Fig. 5d). Mutation of an unrelated aspartic acid (D320A)
did not affect PS2 cleavage (Fig. 5d). Thus, caspase-3 cleaves PS2 at the predicted Asp<sup>329</sup>-Ser<sup>330</sup> site.

To verify whether the 18-kDa COOH-terminal PS2 fragment generated in vivo during apoptosis was identical to PS2Ccas, 293T cells were transfected with wild type PS2, mutant D329A, and PS2Ccas human cDNAs alone or together with a plasmid coding for caspase-3. PS2 was processed in cells cotransfected with caspase-3, and the 18-kDa COOH-terminal PS2 polypeptide co-migrated with transfected PS2Ccas (Fig. 6). Moreover, the PS2 mutant D329A, which is expressed at similar levels of wild type PS2, was not cleaved in cells coexpressing caspase-3 (Fig. 6). Together, these results indicate that PS2 is a physiological substrate of caspase-3.

PS2Ccas includes ALG-3 and is included in PS2s (Fig. 1c), raising the interesting possibility that it could have an anti-apoptotic function. Expression of PS2Ccas in HeLa cells conferred partial protection from cell death triggered by the Fas and TNF receptor molecules (Fig. 7). The D329A mutant, which cannot be cleaved to generate PS2Ccas, appeared to increase the number of dead cells in some experiments (Fig. 7, second panel), consistent with the lack of a negative feedback. However, to properly address this point, the reproducibility of this effect must be tested, and cells that do not express the wild type PS2 protein should be used. Thus, caspase-3 cleavage generates a proteolytic fragment, PS2Ccas, that can inhibit PCD.

In this study, we have identified two physiological PS2 COOH-terminal polypeptides, PS2s and PS2Ccas, that oppose apoptosis induced through the death receptors Fas and TNF. PS2s is derived by two distinct processes: one is by translation of an alternative short PS2 transcript, expressed in mouse liver and human placenta<sup>2</sup>; the other is by proteolysis, mediated by a still unknown protease, of the PS2 protein. The latter would lead to inactivation of a protein critical for cell death and concomitantly generate an anti-apoptotic fragment of the same protein. Consequently, both the alternative transcript and PS2 proteolysis can modulate the PS2/PS2s balance and, ultimately, provide a means for the cell to regulate its susceptibility to death.

An additional anti-apoptotic PS2 COOH-terminal protein termed PS2Ccas is produced during apoptosis by caspase-3 cleavage of PS2. Of note, the highly homologous Alzheimer’s disease protein PS1 was not cleaved. After submission of this paper, it was reported that PS1 is cleaved during apoptosis, and that this cleavage is blocked by inhibitors of caspases (29). Based on these observations, the authors claimed that PS1 is a substrate of a caspase-3 family member. The discrepancy between our data and these findings could indicate that either PS1 is cleaved by a caspase other than those assayed or that PS1 cleavage in an in vitro assay is such that the caspase cleavage site is not accessible. On the other hand, the conclusion that PS1 is cleaved by caspases might be inaccurate. An alternative interpretation of their data could be that PS1 is cleaved by a different protease whose activation during programmed cell death is dependent on caspases. Thus, further experiments should be performed to distinguish among these possibilities.

Interestingly, both caspase-3 and PS2 play a critical role in the central nervous system. Caspase-3 is essential for cell death in the brain (30), and mutations in PS2, which generate a molecule with enhanced basal apoptotic activity (11), are associated with early onset familial Alzheimer’s disease (8), a disease characterized by extensive neurodegeneration. Our observations provide further evidence for the involvement of PS2 in programmed cell death and suggest that caspase-3, which has been shown to proteolytically activate the pro-apoptotic functions of a number of substrates (13, 14, 31–33), can also initiate a negative feedback signal.

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