Presenilins Are Processed by Caspase-type Proteases*

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Presenilin 1 (PS1) and presenilin 2 (PS2) are endoproteolytically processed in vivo and in cell transfectants to yield 27–35-kDa N-terminal and 15–24-kDa C-terminal fragments. We have studied the cleavage of PS1 and PS2 in transiently and stably transfected hamster kidney fragments. We have studied the cleavage of PS1 and PS2 yielding 27–35-kDa N-terminal and 15–24-kDa C-terminal chain reaction.

chloromethyl ketone; ER, endoplasmic reticulum; PCR, polymerase chain reaction.

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Experimental Procedures

Antibodies—Amino acids 1–80 of human PS2 (N terminus) and 270–387 of murine PS2 (large hydrophilic loop between transmembrane domains 6 and 7) were expressed in Escherichia coli as (NANP)19-fusion proteins and used for immunization of mice. PST20 is a monoclonal antibody specific for the hydrophilic N-terminal region of human and murine PS2. PST18, PST8, and PST10 react with the PS2 hydrophilic loop region, PST18 having preference for murine PS2. The rabbit antiserum 53217 is directed against the hydrophilic loop of PS2. The M2 is a commercially available anti-FLAG antibody (Kodak Scientific Imaging Systems Products).

Peptides—The tetrapeptide derivatives used in this study were obtained from the following sources: Ac-DSYD-AMC and Ac-DSYD-AMC from E. Kitas, Hoffmann-La Roche, Ac-DEVD-AMC from Alexis Corp., b-DEVD-CHO and Ac-YYAD-dbmk from Bachem AG, b-DSYD-CHO and b-AQRD-CHO from SynPept Corp.

Cloning and Expression of Mouse and Human Presenilins—Mouse and human PS1 and PS2 cDNAs were amplified by PCR from a mouse brain cDNA library in pcDNA1 and a human brain cDNA library in pAC2 (CLONTECH) and inserted into pcDNA3 as BamHI/XbaI fragments. FLAG epitopes (DYKDDDDK) were added at the C terminus using appropriate primers and PCR amplification. The respective PS1 and PS2 fragments were cloned into pcDNA3 (Invitrogen) and pSVF1 (Life Technologies, Inc.) for stable expression in mouse Neuro2A and transient expression in BHK21, respectively. BHK cells were transfected by electroporation (20 μg of RNA per 107 cells) and harvested 20–24 h later. For stable tetracycline-controlled expression in SK-N-MC cells, mouse PS2-FLAG and mouse PS2-N141I-FLAG were placed under control of the tTA-dependent promoter and cloned into
Isolation of the C-terminal Fragment and Sequencing—Five 10^6 BHK cells transfected with Ps2-FLAG or Ps1-FLAG constructs were washed in 5 mL Hepes, pH 7.2, 250 mM sucrose, 1 mM EDTA, 10 mM benzamidine, 100 units/mL aprtinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin A, 1 mM phenylmethylsulfonyl fluoride (breaking buffer) and mechanically disrupted in a mini-bead-beater using 0.1-mm glass beads. After removing nuclei and large cell debris by low speed centrifugation a crude membrane fraction was collected at 100,000 × g for 45 min and resuspended in breaking buffer. The suspension was adjusted to 0.5% SDS and incubated for 10 min at 37 °C. The solution was then diluted 1.5 with 1.25 × RIPA buffer, not containing SDS (25 mM Tris, pH 7.4, 171 mM NaCl, 12.5% glycerol, 0.625% sodium deoxycholate, 1.25% Triton X-100, 2.5 mM EDTA), and clarified by centrifugation at 100,000 × g for 30 min. The clear supernatant was applied to an M2 anti-FLAG immunoaffinity column (0.5-mL bed volume) equilibrated in 1 × RIPA buffer. After extensive washing with 1 × RIPA buffer and phosphate-buffered saline containing 0.1% octyl glucoside the column was eluted with 100 mM glycine, pH 2.6, 0.1% octyl glucoside. Fractions containing the C-terminal fragment were concentrated, subjected to SDS-PAGE, and electrophoretically transferred to an Immobilon-PSQ membrane (Millipore). The membrane was stained with 0.1% Amido Black in 45% methanol, 7% acetic acid for 10 min, and the band corresponding to the Ps2 fragment was cut out and N-terminally sequenced by Edman degradation in an ABI 490C LC sequencer. The same procedure was applied to isolate human Ps2 from ~10^8 Neuro-2a cells except that a mixture of immobilized antibodies PstS, PstT, and PstS18 was used to affinity purify the C-terminal fragment.

Expression of Recombinant Caspase-3—The active form of human caspase-3 was amplified by PCR from Ser^22 to the end of the coding region and inserted as a BamHI fragment into pET15b (Novagen). Transformants in *E. coli* BL21(DE3)pLysS (Novagen) were grown in 5 mL of LB medium, induced at A_{600} = 0.6 with 0.2 mM isoprropyl-1-thio-β-D-galactopyranoside for 2 h before extracts were prepared by sonification in 0.5% Nonidet P-40, 20 mM Hepes (pH 7.4), 100 mM NaCl (21). Caspase Activity Assay—5 μg of *E. coli* extract containing recombinant caspase-3 or 0.5 mg of mouse brain homogenate were incubated with 20 μM aminonethylcoumarin (AMC) (AMC) tetrapeptide substrates in 100 mM Hepes, pH 7.0, 5 mM dithiothreitol, 0.5 mM EDTA, 0.1% CHAPS, 20% glycerol at 25 °C. Total volume was 1 mL. The release of AMC as a function of time was measured in a fluorescence spectrophotometer using 380 nm and 460 nm as excitation and emission wavelengths, respectively. Background reagent fluorescence was determined and subtracted. The relative fluorescence intensities were correlated with AMC concentrations by standard curve titrations. In the inhibition experiments the release of AMC from Ac-DSYD-AMC was measured in the presence of different concentrations of the tetrapeptide aldehydes after incubation for 3 h (*E. coli* extract) or 4.5 h (brain homogenate) at 25 °C.

RESULTS AND DISCUSSION

Analysis of Ps2 Processing Pattern—We have studied the expression and processing of murine and human Ps2 and Ps1 constructs tagged with a C-terminal FLAG epitope in transient and stable transfectants. Immunoblot experiments with the M2 anti-FLAG antibody confirmed expression of full-length murine Ps2 in Semliki Forest virus (SFV) transfected BHK cells migrating at around 55 kDa and the presence of a major C-terminal fragment at around 18 kDa (Fig. 1A). This fragment was recognized by the monoclonal antibody PstT18 directed against the C terminus of Ps2. Using monoclonal antibody PST20 directed against the N terminus of Ps2, a major N-terminal fragment migrating at ~34 kDa and a weaker band at ~31 kDa were detected by immunoblotting. The 18-kDa C-terminal fragment together with a double band at 22 and 24 kDa were detected using the rabbit antiserum 53217. The poor but occasionally visible immunoreactivity of the 22- and 24-kDa double band with the anti-FLAG and PstT18 antibodies probably reflects a rather low abundance of these fragments. The processing pattern of murine Ps2 carrying the FAD mutant N141I was similar to that of wild-type Ps2 as revealed by the antiserum 53217. Likewise, processing of human Ps2 lacking the C-terminal FLAG epitope (mPs2 IP) or wild-type murine Ps2 with C-terminal FLAG (mPs2F) were grown in the presence or absence of 1 μg/mL tetracycline (tet) for 3 days, and extracts were analyzed by immunoblotting using the M2 anti-FLAG antibody. The arrows indicate the 18-kDa and, as a faint band, the 22-kDa C-terminal fragments. Numbers on top indicate different clones; C represents a vector control.

FIG. 1. Processing of Ps2 in transient and stable cell transfectants. Panel A, different Ps2 constructs in a Semliki Forest virus vector were expressed in BHK cells and immunoblotted onto nitrocellulose membranes. mPs2F, murine Ps2 with a C-terminal FLAG epitope; mPs2 IP, murine Ps2 with the N141I FAD mutation and C-terminal FLAG; hPs2, human Ps2 without a FLAG epitope. A control vector with a cDNA insert coding for the green fluorescent protein (GFP) was included as a negative control. The blot was probed with various antibodies as indicated and described in the text. Panel B, SK-N-MC cells harboring a pterREP vector with murine Ps2 with the N141I mutation and C-terminal FLAG epitope (mPs2 IP) or wild-type murine Ps2 with C-terminal FLAG (mPs2F) were grown in the presence or absence of 1 μg/mL tetracycline (tet) for 2 and 4 days, and extracts were analyzed by immunoblotting using the M2 anti-FLAG antibody. The arrows indicate the 18-kDa and, as a faint band, the 22-kDa C-terminal fragments. Numbers on top indicate different clones; C represents a vector control.

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period, an increase in the 34-kDa N-terminal fragment was clearly observed in immunoprecipitations with the PST20 antibody (data not shown). Together, these experiments proved the processing of PS2 into N- and C-terminal fragments in transient and stable expression systems and suggested a slow proteolytic processing of the full-length protein to yield the processing products.

Determination of the Proteolytic Cleavage Site(s)—To determine the sites of proteolytic cleavage, the 18-kDa C-terminal fragment of murine PS2-FLAG was isolated from a BHK cell lysate by M2 anti-FLAG affinity chromatography and SDSPAGE. N-terminal sequencing of the fragment indicated cleavage between Asp329/Ser330 (Table I). In a second independent experiment an additional minor sequence was determined starting at Ser327 and representing about 5% of the material that started at Ser329. Cleavage of PS2 between Asp329 and Ser330 results in two processing products with predicted molecular masses of 37 kDa and 14 kDa (including the FLAG epitope) which is in reasonable agreement with the observed mobilities on SDS-PAGE/immunoblots. An 18-kDa C-terminal fragment was also isolated from mouse Neuro-2a cells stably expressing the human PS2 cDNA under the control of the cytomegalovirus promoter by immunofluorescence chromatography using a mixture of PST8, PST10, and PST18 antibodies. The fragment was shown to start at the same N terminus as its mouse homologue (Table I).

A similar approach was chosen to map the cleavage site in PS1. We isolated and expressed the murine PS1 cDNA, purified its 15-kDa C-terminal fragment from BHK cell lysates, and determined the proteolytic processing site (Table I). The N-terminal residue of this fragment corresponded to cleavage between Asp345/Ser346 mapped by us and, moreover, bear no similarities to caspase-type cleavage sites. This indicates that PS1 can be processed at multiple sites and by presumably different proteases. For PS2 we also have identified larger C-terminal fragments of about 22 and 24 kDa (see Fig. 1A). While the exact identity of these fragments remains to be determined, they clearly contain the C-terminal domain of PS2 and correspond in size to the larger C-terminal fragment of PS1. Thus, there may be alternate processing pathways also for PS2 with possibly different functional properties.

Cleavage Site Inhibitors—Substrate mimetics of naturally occurring caspase cleavage sites have been described which act as specific caspase inhibitors in vitro and in vivo (25, 27). We tested the activity of a caspase-3-type inhibitor (Ac-DEVD-CHO) and of a caspase-1-type inhibitor (Ac-YVAD-dbmk) on PS2 and PS1 processing in BHK cells transfected with SFV constructs. The caspase-3-selective inhibitor reduced the accumulation of the 18-kDa C-terminal fragment of PS2 almost completely in a concentration-dependent manner, whereas the caspase-1-selective inhibitor had only a minor effect (Fig. 2A). The processing of PS1 was also affected by both types of inhibitors although the extent of inhibition was less pronounced (Fig. 2B). The clear inhibition of PS2 and PS1 processing by the general serine and cysteine protease inhibitor TLCK suggests a common pathway of PS1 and PS2 fragmentation. TLCK may interfere with serine-type proteases acting upstream in a cascade of proteolytic events which finally leads to the activation of caspases selective for PS2 and PS1.

Cleavage Site Mutations—The cleavage site in murine PS2 was further characterized by analyzing the effects of mutations around the cleavage site upon expression in the SFV/BHK cell system. Deletion of residues 326–331 completely abolished processing as did the mutation of the P1 residue Asp329 → Arg (Fig. 2C). Mutant Asp329 → Asn showed some residual activity whereas mutations of Ser327 → Phe or Tyr328 → Ala had little effect on PS2 processing (not shown). The mutants thus confirmed the importance of an aspartic acid residue in the P1 and, to a lesser extent, in the P4 position for PS2 processing. Interestingly, the caspase cleavage site mutations did not induce a significant increase of the 22–24-kDa PS2 fragments strongly suggesting independent processing pathways. The slightly smaller size of the caspase-independent processing product of the Δ326–331 PS2 mutant might reflect a subtle structural change or a secondary modification induced by the deletion.

In Vitro Caspase Activity Assay—The proteolytic activity of caspase-type proteases can be assayed in vitro by fluorogenic

| Construct | Expression system | C-terminal fragment | Sequence determined |
|-----------|-------------------|---------------------|---------------------|
| mPS2-FLAG | SFV/BHK           | 18 kDa              | D-S-Y-D-X-F-G-E-P-S-Y-P-E-A (3 pmol) |
| hPS2      | Neuro-2a          | 18                  | D-S-Y-D-S-F-G-E-P-S-Y-P-E-A (10 pmol) |
| mPS1-FLAG | SFV/BHK           | 15                  | A-Q-R-D-S-H-L-G-P-H-R-s-T-P-E-S (5 pmol) |

Note: The sequence determined by sequencing of the C-terminal fragments is indicated in bold. The arrows indicate the peptide bonds hydrolyzed by the caspase-type protease.
peptide substrates. We have tested homogenates of mouse brains for proteolytic activity on fluorogenic substrates based on the PS2 and PS1 cleavage sites. The strongest endopeptidase activity was measured with the caspase-3-specific substrate DEVD-AMC followed by the PS2-peptide DSYD-AMC. We also observed a weak but significant activity on the PS1 substrate peptide AQRD-AMC (Fig. 3A). Thus, caspase-type activities are present in mouse brain which also accept, in addition to the caspase-3 substrate, peptide substrates derived from both PS2 and PS1. Caspase substrate peptides, if derivatized with an aldehydic or dimethylbenzoyloxymethyl ketone function at the C-terminal Asp, act as potent caspase inhibitors. Indeed, the aldehyde derivatives of all three tetrapeptides used here were found to inhibit DSYD-AMC cleavage by mouse brain homogenates in a concentration-dependent fashion, the most potent inhibitor being the caspase-3 inhibitor followed by the PS2- and PS1-derived peptides (Fig. 3C).

Several caspases, including caspase-3, show activity when expressed in E. coli as an N-terminally truncated protein (21). To further substantiate the role of caspases in PS processing and to characterize the caspase-type activity found in mouse brain we inserted the human caspase-3 cDNA (from Ser29 to the end of its open reading frame) into a pET15 vector and prepared crude cell lysates from selected transformants. The lysates were assayed for activity on the fluorogenic peptides based on the PS1 or PS2 cleavage sites and on the prototype caspase-3 substrate peptide to confirm expression of a functional enzyme. We observed a time-dependent cleavage activity with the PS2-derived substrates whereas, in contrast to the mouse brain homogenate, no activity was measured with the PS1 peptide (Fig. 3B). Inhibition of recombinant caspase-3 activity was most pronounced with the caspase-3-type inhibitor DEVD-aldehyde with an IC50 of about 2 nM (Fig. 3D). The same inhibitor was 50–100 times less efficient in inhibiting the endopeptidase(s) from mouse brain indicating that the caspase involved in the hydrolysis of the PS2-derived substrate might be different from caspase-3. Although the PS1-derived tetrapeptide AQRD-AMC was not hydrolyzed by recombinant caspase-3, the corresponding aldehyde derivative was capable of inhibiting DSYD-AMC cleavage by caspase-3 similar to the DSYD-aldehyde analogue (Fig. 3D). This apparent discrepancy might reflect improper binding and processing of the AQRD-AMC substrate peptide at the active site of caspase-3, whereas the inhibitory function of the aldehyde derivative is maintained by forming a thiohemiacetal with the catalytic cysteine.

In conclusion, our results demonstrate that PS2 and PS1 are substrates for caspase-type proteases. Alternate processing by non-caspase-type proteases yields fragments of different sizes. Hartmann et al. (28) recently reported constitutive expression of a 20-kDa C-terminal fragment of rat PS1 in all tissues and stages of development. A second C-terminal fragment of 14 kDa was observed in the brain of adult and aged animals which corresponds in size to the caspase-generated PS1 fragment described here. At least two C-terminal PS2 fragments of about...
21 kDa and 18 kDa were detected in mouse tissues indicating alternative processing pathways for PS2 also in vivo.\(^3\) A C-terminal PS2 fragment of about 10 kDa protected T-cells against apoptotic challenge whereas overexpression of PS2 restored their sensitivity (15, 16). Cleavage of PS2 by a caspase-type protease generating a similar fragment suggests a role of PS2 processing in the cellular response to apoptotic signals. Elucidating the significance of presenilin processing will help to understand apoptotic or neurodegenerative phenomena in Alzheimer’s disease.\(^4\)

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\(^3\) H. Loetscher, unpublished data.

\(^4\) Processing of presenilins by caspase-type proteases was also reported by R. E. Tanzi at the *17th Blankenese Conference on Neurodegeneration*, Hamburg, Germany, June 29–July 3, 1997.
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