Developmental Regulation of Presenilin-1 Processing in the Brain Suggests a Role in Neuronal Differentiation

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Henrike Hartmann‡§, Jorge Busciglio‡, Karl-Heinz Baumann‡, Matthias Staufenbiel¶, and Bruce A. Yankner¶‡
From the ‡Department of Neurology, Harvard Medical School and Division of Neuroscience, The Children's Hospital, Boston, MA 02115 and ¶Preclinical Research, Sandoz Pharma Ltd., CH-4002 Basel, Switzerland

Most cases of early-onset familial Alzheimer’s disease are caused by mutations in the presenilin genes. Presenilin-1 (PS1) is subject to proteolytic cleavage resulting in the accumulation of N- and C-terminal fragments. In this report, we show that the proteolytic cleavage of PS1 is developmentally regulated in the brain. Low levels of full-length PS1 and higher levels of 30-kDa N-terminal and 20-kDa C-terminal fragments are identified at all developmental stages in the rat brain. However, in the adult brain, additional 36-kDa N-terminal and 14-kDa C-terminal fragments appear and become major PS1 species. Alternative N-terminal PS1 fragments also appear in the adult human brain, but are more heterogeneous than in the rat brain. The alternative PS1 fragments are not detected at significant levels in rat or human peripheral tissues that express PS1. The alternative cleavage of PS1 is also detected in primary cultures of rat hippocampal neurons, but not in astrocytes, and is induced by neuronal differentiation. Furthermore, alternative PS1 cleavage is detected in rat PC12 cells and human neuroblastoma SH-SY5Y cells following induction of neuronal differentiation. These results suggest that an alternative pathway of PS1 proteolytic processing is induced in the brain by neuronal differentiation. PS1 may therefore play an important role in brain development and neuronal function, which may relate to the brain-specific pathological effects of PS1 mutations.

Most cases of early-onset familial Alzheimer’s disease are caused by mutations in a new gene family, the presenilins (1, 2). More than 25 different inherited mutations have been identified in presenilin-1 (PS1), which is localized to chromosome 14. Two mutations have been identified in a highly homologous

protein, presenilin-2 (PS2), which is localized to chromosome 1. PS1 and PS2 are integral membrane proteins with a proposed structure of eight transmembrane domains (3, 4).

The presenilins are highly homologous to the Caenorhabditis elegans gene sel-12 (5), which acts to facilitate signaling through the Notch/lin-12 pathway, suggesting that the presenilins may play a role in the determination of cell fate during development. In particular, the importance of Notch signaling in the development of the central nervous system raises the possibility that presenilins may be directly involved in neuronal differentiation. The full-length PS1 protein undergoes proteolytic cleavage soon after synthesis so that the predominant PS1 species are N- and C-terminal fragments (6). The N- and C-terminal fragments may therefore be biologically active forms of PS1. In this report, we demonstrate that the proteolytic processing of PS1 is developmentally regulated in the brain. PS1 undergoes a major alternative proteolytic cleavage in the adult rat brain that is a minor component in the fetal brain. This alternative pathway of PS1 proteolysis is hardly detectable in non-neuronal tissues that express PS1. Alternative proteolytic cleavage of PS1 is also detected in primary rat hippocampal cultures and neuronal cell lines and is induced by neuronal differentiation. We suggest that the regulation of PS1 cleavage may play a role in brain development and neuronal function.

 EXPERIMENTAL PROCEDURES

Antibodies—The αPS1-N antibody is a rabbit antiserum raised against a synthetic peptide corresponding to residues 2–20 of PS1 (1) conjugated to keyhole limpet hemocyanin. αPS1-L is a rabbit antiserum raised against a bacterial glutathione S-transferase (GST) fusion protein containing residues 263–407 of human PS1 corresponding to the loop region following the predicted sixth transmembrane domain. The GST fusion protein was expressed in Escherichia coli, and the inclusion bodies were isolated and solubilized in 0.1% SDS and used for immunization of rabbits as described (7). Both antibodies recognize human and rat PS1, but not PS2 (8).

Tissue and Cell Extraction—Cortex, hippocampus, and cerebellum from fetal (E18), adult (3 month), and aged (24 month) Harlan Sprague Dawley rats were homogenized in 2× SDS sample buffer (4% SDS, 20% glycerol, 0.1 M Tris-HCl, pH 6.8) supplemented with protease inhibitors (Complete®, Boehringer Mannheim). Human fetal cortex was obtained from 15–17 weeks gestation abortuses and immediately homogenized in 2× sample buffer. Fresh intraoperative biopsy material from adult human temporal cortex was processed in the same way. Frozen adult human cortical tissue was obtained from the Kathleen Price Bryan Brain Bank at Duke University Medical Center. The post-mortem intervals (PMI) varied from 1 to 12 h. Gray matter from the temporal cortex was dissected and homogenized in 2× sample buffer containing protease inhibitors. Post-mortem autopsy tissue from adult human heart, liver, kidney, and spleen (PMI 10 h) was homogenized under identical conditions. The homogenates were sheared with a 22-gauge needle and centrifuged at 14,000 × g for 20 min at 4 °C. The protein concentration of the supernatant was determined using the Bio-Rad protein assay kit.

Western Blotting—15 μg of protein was loaded per lane and separated by 4–20% or 12% polyacrylamide gel electrophoresis (PAGE) in 2× sample buffer (4% SDS, 20% glycerol, 5% β-mercaptoethanol, 0.1 M Tris-HCl, pH 6.8) without prior heating and then electrotransferred to polyvinylidene difluoride membrane. Western blotting was performed with antibodies αPS1-N (1:3000) and αPS1-L (1:1000) following incubation with peroxidase-conjugated anti-rabbit IgG and detection by enhanced chemiluminescence (Amersham Corp.). For preabsorption electrophoresis—DMEM, Dulbecco’s modified Eagle’s medium; α-MEM, α-minimum Eagle’s medium.

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controls, αPS1-N was incubated with the cognate peptide (1 μl of antiseraum/6 μg of peptide) and αPS1-L was incubated with the GST-fusion protein (1 μl of antiseraum/50 ng of GST-fusion protein) as described previously (10). To inhibit immunoreactivity with GST, incubation with αPS1-L were performed in the presence of 10 μg/ml GST.

Cell Culture—Primary cultures of rat hippocampal neurons were established from embryonic day 18 (E18) fetuses and plated on poly-l-lysine-coated dishes in DMEM containing 10% supplemented calf serum (HyClone) as described previously (9). One day after plating, the medium was changed to DMEM containing N2 supplements (Life Technologies, Inc.). Cells were harvested at 1, 3, 7, and 14 days after plating. Enriched astrocyte cultures were prepared from E18 rat cortex as described previously (10).

PC12 cells were maintained on collagen-coated dishes in DMEM containing 10% horse serum (Life Technologies, Inc.) and 5% fetal calf serum supplemented with glutamine. For differentiation, PC12 cells were grown in the presence of NGF (100 ng/ml) under low serum conditions (0.5% fetal calf serum). Human SH-SY5Y neuroblastoma cells were maintained in α-MEM (Life Technologies, Inc.) containing 10% fetal calf serum. For differentiation, SH-SY5Y cells were grown in α-MEM containing N2 supplements in the presence of retinoic acid (1 μM) and phosphor 12-myristate 13-acetate (16 nM). Cells were harvested in 2 × sample buffer containing protease inhibitors at different stages of differentiation and processed for Western blotting as described above.

RESULTS

Developmental Regulation of PS1 Cleavage in the Rat Brain—To characterize the developmental regulation of PS1 expression and proteolytic cleavage in the rat brain, we examined PS1 processing in the cortex, hippocampus, and cerebellum of fetal, adult, and aged rat brain. The lysates were analyzed by Western blotting using antibodies against the N terminus of PS1 (αPS1-N) and against the hydrophilic loop after the sixth predicted transmembrane domain (αPS1-L). Both antibodies have previously been shown to exhibit specificity for PS1 in transfected and untransfected cell lines and are not immunoreactive with PS2, as determined by overexpression of PS1 and PS2 in COS cells (8). In the fetal rat brain, the major PS1 species recognized by αPS1-N was a 30-kDa N-terminal fragment that was expressed at similar levels in the cortex, hippocampus and cerebellum. Low levels of full-length PS1, migrating at about 50 kDa, were also detected. The 30-kDa N-terminal fragment in brain co-migrated with the predominant N-terminal fragment in undifferentiated neuroblastoma SH-SY5Y cells (Fig. 1A), and in other neuronal and non-neuronal cell lines (8), and was detected at similar levels in the fetal, adult, and aged rat brain. However, in the adult rat brain, an additional major N-terminal fragment appeared at 36 kDa that was present at only very low levels in the fetal brain and was not detected in cultured cell lines (Fig. 1A). This fragment was detected at slightly higher levels in cortex and hippocampus than in cerebellum. Preabsorption of the αPS1-N antibody with its cognate peptide abolished immunoreactivity with both N-terminal fragments and the full-length 50-kDa species, indicating antibody specificity (Fig. 1A, last lane). Western blot analysis of brain homogenates with αPS1-L, which is directed against the large PS1 C-terminal loop, showed a 20-kDa C-terminal fragment that was constitutively expressed at similar levels at all developmental stages. An additional 14-kDa C-terminal fragment appeared at very high levels in the adult and aged brain, but was detected at very low levels in the fetal brain (Fig. 1A). Preincubation of αPS1-L with the PS1 loop fusion protein inhibited immunoreactivity of the antibody with the 20- and 14-kDa bands, indicating specificity for PS1 (Fig. 1A). Incubation of the antibody with the GST component of the fusion protein had no effect.

The alternative 36-kDa N-terminal and 14-kDa C-terminal fragments together add up to the approximate molecular weight of full-length PS1, suggesting that these fragments are formed by proteolytic cleavage of PS1. To determine whether the alternative fragments could be generated by phosphorylation, the brain homogenates were incubated with alkaline phosphatase under conditions that effectively dephosphorylate tau. Phosphatase treatment did not alter the migration pattern of any of the PS1 fragments, indicating that the alternative PS1 fragments are unlikely to be generated by differential phosphorylation (data not shown). In addition, it has recently been shown that the presenilins are not glycosylated or sulfated (11). Thus, the difference in electrophoretic migration between the constitutive and the alternative PS1 fragments is unlikely to be the result of post-translational modification. Taken together, these results suggest that PS1 is subject to alternative proteolytic cleavage in the adult rat brain.

Brain-specific Cleavage of PS1—To determine whether the alternative cleavage of PS1 is specific for the brain, we analyzed homogenates of different peripheral tissues from the adult rat. Western blot analysis with αPS1-N demonstrated that the constitutive 30-kDa species is the predominant PS1 N-terminal fragment in the heart, liver, kidney, and spleen of the adult rat (Fig. 1B). The alternative 36-kDa N-terminal fragment identified in brain could not be detected at significant levels in peripheral tissues. Thus, alternative cleavage of PS1 is specifically increased in the brain.

Regulation of PS1 Cleavage by Neuronal Differentiation—To determine whether the developmental change in PS1 cleavage in the brain is related to neuronal differentiation, we examined the proteolytic cleavage of PS1 in primary hippocampal cultures derived from E18 rat embryos. These cultures are highly enriched in neurons and exhibit a defined time course of neuronal differentiation (12). Cells were harvested at increasing time intervals after plating that corresponded to more advanced stages of neuronal differentiation. Western blot analysis with αPS1-N showed that the level of the constitutive 30-
kDa N-terminal fragment was constant for 7 days in culture and then decreased by day 14 (Fig. 2A). A similar pattern of expression was observed for the constitutive 20-kDa C-terminal fragment. In contrast, the alternative 36-kDa N-terminal and 14-kDa C-terminal fragments showed a marked dependence on neuronal differentiation. These PS1 fragments were present at very low levels immediately after plating, but gradually increased at later time points corresponding to increased neuronal differentiation (Fig. 2A). The alternative cleavage products generated in culture co-migrated with the alternative fragments identified in the adult rat brain (Fig. 2A).

To confirm that the alternative fragments were specifically produced in neurons, we examined highly enriched astrocyte cultures. The composition of the cultures was confirmed by Western blot analysis for neuron-specific tau and astrocyte-specific GFAP (data not shown). Astrocytes did not show alternative cleavage of PS1 after 14 days in culture (Fig. 2A). Thus, the alternative proteolytic cleavage of PS1 is neuron-specific and is induced by neuronal differentiation.

To further confirm that alternative cleavage of PS1 is induced by neuronal differentiation, we investigated PS1 cleavage in rat PC12 cells during the course of differentiation induced by NGF. Similar to the situation in primary rat hippocampal cultures, the levels of the constitutive 30-kDa N-terminal and 20-kDa C-terminal fragments did not change significantly over time, whereas the levels of the alternative 36-kDa N-terminal and 14-kDa C-terminal fragment gradually increased during the course of differentiation. The alternative fragments generated in differentiated PC12 cells co-migrated with the alternative fragments in the adult rat brain (Fig. 2B).

Regulation of PS1 Cleavage in the Human Brain—To determine whether the alternative cleavage of PS1 detected in the rat brain also occurs in the human brain, we examined the PS1 cleavage pattern in human fetal and adult cortex. Western blot analysis of cortical tissue homogenates with aPS1-N showed a major 30-kDa N-terminal PS1 fragment in both fetal and adult human cortex (Fig. 3A). In the adult human cortex, an additional ladder of alternative N-terminal PS1 fragments was detected migrating between 30 and 40 kDa (Fig. 3A). This heterogeneous pattern of N-terminal fragments is specific for the human brain. Lanes: cts, adult cortex; he, heart; li, liver; ki, kidney; sp, spleen; preab, same tissues blotted with aPS1-N preabsorbed with the cognate peptide. C, Western blot analysis of cell lysates of human SH-SY5Y neuroblastoma cells with aPS1-N. Cells were differentiated with retinoic acid for the indicated number of days. Note the induction of heterogenous N-terminal PS1 fragments during the course of differentiation (bracket).

![Fig. 2. Induction of alternative PS1 cleavage by neuronal differentiation.](attachment:fig2.png)

![Fig. 3. Developmental regulation of PS1 cleavage in the human brain.](attachment:fig3.png)
Developmental Regulation of PS1 Processing in the Brain

fragments was not detected in the adult human cortex (Fig. 3A, bottom), possibly due to decreased stability of the alternative C-terminal fragments.

To determine whether the human brain exhibits a unique pattern of PS1 cleavage, as found in the rat (Fig. 1B), we examined PS1 processing in several peripheral human tissues. The generation of heterogenous N-terminal PS1 fragments was detected only in the human brain. A discrete 30-kDa N-terminal fragment was detected in the heart, liver, kidney, and spleen (Fig. 3B). To determine whether the heterogenous pattern of PS1 N-terminal fragments is induced by neuronal differentiation, we investigated PS1 cleavage in human SH-SY5Y neuroblastoma cells during the course of differentiation. Differentiation of SH-SY5Y neuroblastoma cells with retinoic acid resulted in the generation of a heterogenous pattern of N-terminal PS1 fragments (Fig. 3C), similar to the pattern found in the adult human cortex. Thus, the formation of alternative N-terminal fragments is induced by neuronal differentiation. These results suggest that proteolytic cleavage of PS1 is developmentally regulated in both the rat and human brain, although the cleavage pattern exhibits species specificity.

These experiments demonstrate that the proteolytic cleavage of PS1 is developmentally regulated in the brain. Alternative cleavage of PS1 in the adult rat brain results in the appearance of brain-specific N- and C-terminal PS1 fragments. The generation of these alternative cleavage products can also be demonstrated in primary rat hippocampal cultures and is neuron-specific. Furthermore, the time course of production of the alternative fragments in hippocampal cultures, in PC12 cells and in SH-SY5Y neuroblastoma cells suggests that their generation is related to neuronal differentiation. A role of PS1 in neuronal differentiation would be consistent with recent findings demonstrating that PS1 is present in all neuritic compartments and in growth cones of differentiating neurons (8).

Consistent with previous findings in various tissues and cell lines (6), we identified 30-kDa N-terminal and 20-kDa C-terminal constitutive cleavage products as the predominant forms of PS1 in the adult rat brain. However, in the adult brain, high levels of the alternative PS1 fragments accumulate, whereas the level of constitutive fragments is not significantly changed. Although it cannot be excluded that the alternative fragments are derived from an alternatively spliced form of PS1 (1, 15), the concomitant increase in size of the N- and C-terminal fragments indicates the presence of a new proteolytic cleavage site.

Examination of the human cortex also demonstrated developmental regulation of PS1 processing with the appearance of new PS1 N-terminal fragments in the adult cortex. However, in contrast to the discrete 36-kDa N-terminal band in the adult rat brain, a ladder of new PS1-N-terminal fragments appeared in the adult human brain. This ladder was detected in fresh brain biopsy material, as well as in post-mortem autopsy material, indicating that these fragments are generated in vivo and not as a result of post-mortem autolysis. Moreover, the production of a similar heterogenous pattern of PS1 N-terminal fragments in differentiated human neuroblastoma cells in culture suggests that alternative cleavage of PS1 in the human brain is induced by neuronal differentiation. The lack of detection of alternative C-terminal fragments in the adult human cortex may be due to decreased stability or loss of the epitope recognized by our PS1 loop antibody.

The sequences of mouse, rat, and human PS1 are highly homologous, but vary significantly in two regions, at the N terminus and between amino acids 319 and 330 in the large cytoplasmic loop. It has been suggested that the constitutive cleavage site of PS1 is between amino acids 290 and 300 (16), a region that is identical in mouse, rat, and human PS1 (15). However, alternative cleavage of PS1 in the variable region around amino acid 320 could give rise to the longer 36-kDa N-terminal fragment in the rat brain and may result in the generation of different N-terminal fragments in the human cortex. It is also possible that different proteases are involved in the cleavage of PS1 in the rat and human brain. Attempts to directly sequence the alternative fragments have thus far been hampered by the low levels of PS1 fragments produced in the brain and in culture and the propensity of PS1 fragments to form insoluble aggregates.

The observation that PS1 processing is regulated by neuronal differentiation is intriguing in view of a potential role of PS1 in a signaling pathway that regulates cell fate decisions during development. Presenilins exhibit a high degree of homology to the C. elegans gene sel-12, which may be involved in signaling in the Notch/lin-12 pathway (5). The functional relationship of PS1 and sel-12 is supported by the finding that PS1 can rescue sel-12 loss of function mutations in C. elegans (13). Thus, PS1 may play a role in development, consistent with PS1 mRNA expression in the embryonic neuraxis and in developing organs (6, 14, 15). Differential proteolysis may be a mechanism for modifying PS1 function or activity during brain development. The regulation of PS1 proteolysis by neuronal differentiation suggests that PS1 may play a fundamental role in brain development and neuronal function.

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