Inhibiting Amyloid Precursor Protein C-terminal Cleavage Promotes an Interaction with Presenilin 1*

Received for publication, March 28, 2000, and in revised form, May 1, 2000
Published, JBC Papers in Press, May 4, 2000, DOI 10.1074/jbc.C000208200

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Presenilin 1 (PS1) plays a pivotal role in the production of the amyloid-β protein, which is central to the pathogenesis of Alzheimer’s disease. It has been demonstrated that PS1 regulates the γ-secretase proteolysis of the amyloid precursor protein (APP) C-terminal fragment (APP-C100), which is the final step in amyloid-β protein production. The mechanism and detailed pathway of this PS1 activity has yet to be fully resolved, but it may be due to a presenilin-controlled trafficking of the APP fragment or possibly an inherent PS1 proteolytic activity. We have investigated the possibility of a direct interaction of PS1 and the APP-C100 within the high molecular mass presenilin complex. However, the APP-C100 is rapidly degraded, and if it forms, then any PS1-APP complex is likely to be very transitory. To circumvent this problem, we have utilized the protease inhibitor N-acetyl-leucyl-norleucinal (LLeL) and the lysosomotropic agent NH₄Cl, which inhibits the turnover of the APP-C100. Under these conditions, levels of the fragment increased appreciably, and as shown by gel-curl gradient analysis, the APP-C100 shifted to a higher molecular mass complex that overlapped with PS1. Immunoprecipitation studies demonstrated that a significant population of the APP-C100 co-precipitated with PS1. These findings suggest that PS1 may mediate the shuttling of APP fragments and/or facilitate their presentation for γ-secretase cleavage through a direct interaction.

Alzheimer’s disease (AD)† is characterized by extensive neuronal loss culminating in the presentation of dementia. One of the key histopathological elements of the disease is the abnormal deposition of amyloid plaques (for review, see Ref. 1). The major protein component of these plaques is an ~4-kDa peptide (ranging from 39 to 43 residues in length) termed the amyloid-β protein (Aβ) (2). Aβ is derived by proteolytic processing of the type 1 transmembrane amyloid precursor protein (APP). Aβ is released by the secretase proteases, which initially cleave APP at the N-terminal β-secretase site contained within the extracellular domain of the precursor. A β-secretase candidate gene has recently been independently identified by several groups (3–5). The residual 14-kDa C-terminal APP fragment containing Aβ, as well as the transmembrane and intracellular domains, is subsequently cleaved by a putative enzyme γ-secretase (6). The γ-secretase cleavage occurs at two principle sites to produce either the Aβ residues 1–40 (Aβ40) or a longer, more amyloidogenic form containing residues 1–42 (Aβ42) (7–9).

Presenilin 1 (PS1) accounts for the majority of inherited forms of early onset familial AD (for review, see Ref. 10). Although the precise function of PS1 and its relationship to AD have yet to be resolved, it has been clearly demonstrated that FAD-linked PS1 mutations result in a relative increase in the level of Aβ42 as compared with Aβ40 (11–14). These findings indicate that PS1 is intimately involved in Aβ production, which is further supported by the significant reduction in γ-secretase activity in PS1-ablated mice (15). In these mice, increased levels of the C-terminal APP fragment is observed that are accompanied by a virtual elimination of Aβ secretion.

It has recently been proposed that PS1 itself represents a potential γ-secretase based on studies involving the mutation of two predicted transmembrane aspartate residues at codons 257 or 385 (16). Substitutions at either site resulted in marked reduction in γ-secretase activity and Aβ production. It was subsequently hypothesized that these residues may be integral components within the catalytic site of an independent PS1 protease activity. Although this has yet to be substantiated, it is perhaps more plausible that PS1 is involved in the trafficking of the APP C-terminal fragment or may present substrates to a complex containing γ-secretase and possibly other components (17). One possibility is that PS1 modulates APP processing via a direct interaction between the two proteins, which has been observed by co-immunoprecipitation studies in transfected cells (18, 19). However, other groups have failed to replicate this finding (20, 21), and if it occurs, it has been suggested that only a small proportion of the total cellular PS1 and APP are found in such a complex (14). One possible explanation for these discrepancies is that the population of APP that contributes to the PS1-related Aβ processing is rapidly degraded, making it difficult to isolate (22, 23). This would be particularly true for the degradation pathway involving the
APP C-terminal fragment, which appears to be closely regulated by PS1.

In the current study, we have examined the possibility that PS1 interacts directly with the αβ-containing, C-terminal fragment of APP. To circumvent the problems associated with rapid degradation, we have inhibited this pathway by using either the calpain 1 inhibitor, N-acetyl-leucyl-leucyl-norleucinal (LLnL), or the lysosomotropic agent ammonium chloride (NH₄Cl). Both of these agents have been previously shown to decrease the degradation of full-length PS1 and/or the APP-C100 (α- and β-secretase products, C99 and C83, collectively) (6, 24–26). Under these conditions, we have observed that the APP-C100 assembled into a higher molecular mass complex that overlapped with the PS1 endoproteolytic fragments. Immunoprecipitation of PS1 from treated cells resulted in the co-precipitation of a significant quantity of the APP-C100. Our findings suggest that PS1 may control αβ production through a direct interaction of the APP-C100 within the complex.

EXPERIMENTAL PROCEDURES

Immunoblotting and Co-precipitations—The human-specific PS1 monoclonal antibody, NT1, directed against residues 41–49 of PS1 was kindly provided by Dr. Paul Mathews (Nathan Kline Institute, Orangeburg, NY). A rabbit polyclonal antibody, Ab369, that was raised against a synthetic peptide encompassing the terminal 45 amino acid residues of the APP cytoplasmic domain was kindly provided by Dr. Sam Gandy (Nathan Kline Institute, Orangeburg, NY). To perform the immunoblotting experiments, cells were harvested with trypsin:EDTA, and the pellets were lysed in buffer containing 50 mM Tris-HCl, pH 7.6, containing 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride. Proteins were resolved on 4–10% Tris/glycine or 10–20% Tris/Tricine SDS polyacrylamide gels (NOVEX, San Diego, CA), transferred to nitrocellulose, and immune-reactive bands were visualized by ECL (Amersham Pharmacia Biotech) as described previously (17). For co-precipitation studies, PS1 was immunoprecipitated from treated and control cells using the NT1 antibody as described previously (27). PS1 and its associated proteins were separated by SDS polyacrylamide gel electrophoresis, and the presence of the APP C-terminal fragment was identified by probing with the antibody Ab369. For pre-absorption studies, 10 μg of a synthetic peptide encompassing residues 41–49 of PS1 was employed.

Cell Culture—Madin-Darby canine kidney (MDCK) cells, provided by Dr. Christian Haass (Ludwig Maximilians University, Munich, Germany) and human chorionic kidney (HEK 293) cells, provided by Dr. Dennis Selkoe (Harvard Medical School), were stably transfected with APPex. All cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum. Genetin (250 μg/ml) was included in the media for maintenance of the stably transfected lines. The protease inhibitor LLnL (Sigma), prepared in MeSO as a stock solution, was added to cells to a final concentration of 50 μM and incubated for 17 h at 37 °C. Control cells were incubated for identical times with media containing 1% MeSO in the absence of LLnL. In a comparable study, the APP-C100 degradation was inhibited by incubating the cells with 25 mM NH₄Cl for 17 h at 37 °C.

Glycerol Gradient Centrifugation—Glycerol gradient centrifugation was used as described previously (17). Fully confluent cells were washed with phosphate buffered saline, pH 7.4, prior to lysis with 1% digitonin lysis buffer (25 mM Hepes, pH 7.2, containing 150 mM NaCl, 2 mM dithiothreitol, 2 mM EDTA, 5 μg/ml each of chymostatin, pepstatin, leupeptin, and antipain). A clarified supernatant was layered onto a 10–40% (w/v) linear continuous glycerol gradient and centrifuged for 15 h at 35,000 rpm at 4 °C. Fractions were collected by upward displacement and assessed by immunoblotting to determine the presence of PS1 and the APP C-terminal fragment.

RESULTS AND DISCUSSION

Inhibiting Degradation of the APP C-terminal Fragment—Initial experiments were undertaken to determine the effects of LLnL and NH₄Cl on PS1 and APP-C100 turnover. Cell lines were transfected with human APPex or doubly transfected with APPex and PS1 containing the wild-type or L392V mutant sequence. Treatment with 50 μM LLnL resulted in an increased level of the APP-C100 fragments but did not significantly affect the turnover of full-length APP (Fig. 1A, lanes 2 and 4). This effect of LLnL has been previously documented, and at this concentration it significantly reduces the levels of both AB42 and AB43 (7, 26, 28). In contrast, similar LLnL treatment resulted in marked stabilization of the full-length PS1, whereas no changes were observed for the PS1 N-terminal fragment (NTF) (Fig. 1A, lanes 2 and 4). This is likely because of the considerable differences between the rapid, proteasome-mediated degradation of the full-length PS1 as compared with the extremely long half-life of the endoproteolytic fragments (22, 23). Treatment of cells stably expressing clinical PS1 mutants such as the L392V displayed a similar response for the full-length protein and its fragments (data not shown). These observations are consistent with comparable studies on LLnL (calpain 1) inhibition of PS1 degradation (25, 29). In stably transfected cells expressing higher levels of PS1, the stabilization effect of LLnL was even more pronounced as shown by the significantly higher increase in the immunoreactive band for the full-length protein (Fig. 1A, lane 4). This is likely because of the considerable differences between the rapid, proteasome-mediated degradation of the full-length PS1 as compared with the extremely long half-life of the endoproteolytic fragments (22, 23). Treatment of cells stably expressing clinical PS1 mutants such as the L392V displayed a similar response for the full-length protein and its fragments (data not shown). These observations are consistent with previous studies showing that alkalization of intracellular compartments alters the turnover of APP and its proteolytic fragments (30, 31). However, this treatment did not have any significant effect on the relative levels of either full-length PS1 or its endoproteolytic fragments. This was shown by Western blotting of cells stably expressing either wild-type or the L392V mutant PS1 protein (Fig. 1B, lanes 2 and 4). Taken together, the data indicate that, in these cells, LLnL and NH₄Cl can effectively inhibit the degradation of the APP-C100 fragment. Because our ultimate goal is to investigate whether any direct interaction of these APP fragments occurs with PS1, we have utilized these agents to metabolically freeze the lysosomal/endosomal pathway to overcome the problems associated with isolating this potentially unstable intermediate in the γ-secretase pathway.

Formation of High Molecular Mass Complexes of PS1 and APP Fragments—We have previously demonstrated by glycerol gradient fractionation that PS1 undergoes a maturation proc-
ess with the formation of a high molecular mass complex (17, 32). The holoprotein exists as a possibly immature and rapidly degraded complex with an apparent molecular mass of ~150 kDa. Following endoproteolytic cleavage, the N and C termini remain associated by assembly into a much larger complex (200–400 kDa), which may be due to the recruitment of functional ligands such as β-catenin. It is also possible that PS1, given its relationship to APP processing, may also form a complex with this molecule or its degradative by-products.

Similar glycerol gradient fractionation techniques were used to examine the distribution of APP-C100 fragments under normal conditions and after treatment with LLnL. Separation of digitonin extracts from HEK 293 cells stably transfected with APP and PS1 demonstrated that the APP fragments were found primarily in the lighter fractions ranging from 20–200 kDa (Fig. 2A). These overlapped to some extent with both the PS1 holoprotein and its endoproteolytic fragments. Following treatment with 50 μM LLnL, the relative intensity of the APP-C100 was increased when compared with the unfractionated samples (Fig. 2B). These also overlapped with the PS1 NTF, but the APP-C100 also populated a much larger range of higher molecular masses extending to ~400 kDa. In addition, the fractions exhibiting the highest levels of APP-C100 coincided with those containing the PS1 fragments. This was particularly true for the higher molecular mass complex (e.g. fraction 6 in Fig. 2B), which contained the larger complex of the PS1 fragments. These observations suggest that inhibiting the degradation of the APP C-terminal fragments result in the formation of these larger complexes. One possible explanation is that the APP-C100 fragments are associated with a number of other proteins as has been shown, for example, by the binding of Hsc-73 to APP upon treatment with LLnL (33). In the case of PS1, this also raises the interesting potential for a direct interaction of these proteins in the APP processing pathway.

Association of the APP C-Terminal Fragment and PS1—To establish the presence of PS1 and APP-C100 in these complexes, we undertook co-immunoprecipitation experiments that involved the use of a human-specific monoclonal antibody (NT1) directed toward the PS1 N-terminal. Isolated presenilin complexes from control and treated cells were immunoprecipitated with NT1 antibody, and any co-precipitating APP-C100 was identified using an antibody raised to its cytoplasmic tail (Ab369). In untreated HEK 293 cells stably expressing human APP695 and endogenous PS1, no APP-C100 was detected in the immunoprecipitated PS1 fraction (Fig. 3A, lane 1). Only non-specific, high molecular mass bands were observed that corresponded to IgG as demonstrated by the control immunoprecipitation where the antibody was omitted (Fig. 3A, lane 3). However, under these native conditions the PS1 complex remained functionally intact and contained other proteins such as β-catenin (data not shown; Ref. 17). The lack of a detectable amount of the APP-C100 that may be associated with PS1 may be due to either the low levels of the APP fragment and/or the rapidity of its degradation and therefore transient interaction with the presenilin complex.

Freezing of APP-C100 degradation by LLnL inhibition was used to stabilize its interaction with other proteins in its degradation pathway. The observed increase in its apparent molecular mass suggests that this can be achieved (Fig. 2B). In an effort to characterize the complex further, we used a PS1-specific antibody. Under these conditions, an identical immunoprecipitation with the PS1 NTF antibody followed by immunoblotting with the APP-C100 antibody revealed the presence of a significant level of the APP-C100 fragment (Fig. 3A, lane 2). The specificity of this precipitated band was shown by preabsorption of the antibody with the corresponding peptide antigen. This completely eliminated the immunoreactive band (Fig. 3A, lane 4). Identical results were obtained in an independent MDCK cell line expressing the same human APP695 species (Fig. 3A, lanes 5 and 6). These observations clearly support a direct interaction of PS1 and the APP fragment that is involved in amyloid-β processing.

It could be argued that increased levels of PS1 and APP-C100 due to LLnL treatment might result in a nonspecific association between these two molecules. In support of this argument a marked increase in immunoprecipitated full-length PS1 was observed when cells were treated with LLnL (Fig. 3D, lanes 3 and 6). However, the increased PS1 that was precipitated may also result in the formation of other unrelated complexes. To address this question, APP-C100 was selectively increased by treatment with the alkalizing agent NH4Cl, which inhibits the lysosomal/endosomal pathway. This pathway has
been shown to play a role in the processing of the APP-C100 fragment to generate Aβ (30, 31). As with LLnL, the NH_4Cl treatment increases the cellular level of the APP-C100 fragment (Fig. 1). Cells stably expressing wild-type or mutant PS1, as well as human APP, were treated with NH_4Cl, and the PS1 complex was isolated by immunoprecipitation. In the absence of NH_4Cl corresponding to low levels of APP-C100, no co-immunoprecipitation of the fragments was observed (Fig. 3B, lanes 1 and 3). However, the association of PS1 and APP-C100 was observed following incubation in NH_4Cl (Fig. 3B, lanes 2 and 4). Similar to the LLnL study, the specificity of this complex was demonstrated by the preabsorption of the PS1 antibody with its peptide antigen (Fig. 3B, lane 5). Immunoprecipitation with the anti-APP CTF antibody (Ab369) and probing with the PS1 antibody NT1 resulted in a significant co-precipitation of the two proteins (Fig. 3C). The PS1-APP-C100 interaction was enhanced by treatment with LLnL or NH_4Cl (Fig. 3C). The specificity of this complex was demonstrated by the lack of PS1 present after precipitating with an unrelated antibody (Fig. 3C, lane 7). The amount of PS1 relative to APP-C100 within the precipitated complex was similar in the NH_4Cl-treated cells, suggesting that the interaction was not simply due to elevated PS1 levels (Fig. 3D, lanes 1, 2, 4, and 5). These findings indicate that under independent conditions that alter both the degradation of APP and the production of Aβ, both of which are PS1-linked pathways, the association of PS1 with the APP-C100 fragment was observed.

The mechanism by which PS1 controls Aβ processing has not been completely resolved, but it may regulate APP-C100 trafficking and/or presentation of this fragment to γ-secretase(s). All PS1 mutations studied to date lead to an increased generation of Aβ_{42} with little or no change in Aβ_{40} levels (11–14). This may be the result of two unique proteases as has been suggested by some studies (8, 34). Alternative evidence has been presented suggesting these γ-secretase activities are localized in different compartments. Peraus et al. (35) provided evidence for γ-secretase activity in the early endosomes resulting in the generation of Aβ peptides that correspond mainly to the Aβ_{40} species. On the other hand, Aβ_{42} had been localized to the endoplasmic reticulum and the trans-Golgi network (36–39).

It has been shown by sucrose gradient fractionation that under normal conditions APP C-terminal fragments co-distributed with PS1 NTF and CTF in the endoplasmic reticulum and the Golgi compartments (40). This finding raises the possibility that PS1 is involved in the trafficking of the APP-C100 from the endosomal/lysosomal organelles to these compartments for Aβ production. Some circumstantial evidence for this has been provided by the recent finding of an interaction between the hydrophilic loop of PS1 and Rab11 (41). Rab11 is a small GTPase belonging to the RAS-related superfamily involved in vesicular transport especially in the endocytic pathway (42). Alternatively, molecular chaperones could be involved in trafficking proteins from compartment to compartment. Evidence in support of this notion is demonstrated by the co-distribution of APP-C100 and PS1 (both NTF and CTF) with receptor-associated protein (RAP) (40). RAP is a molecular chaperone assisting membrane proteins in their passage through the precipitate relative to the APP-C100 fragment, the PS1 immunoprecipitation was probed for PS1 using the polyclonal antibody Ab14 (D). Similar amounts of PS1 were present in the untreated and NH_4Cl-treated precipitates (lanes 1, 2, 4, and 5), which were increased markedly after LLnL treatment (lanes 3 and 6). In addition, a very low amount was precipitated, followed by preabsorption of the PS1 antibody with the N-terminal peptide antigen, further demonstrating the specificity of the PS1-C100 interaction (lane 7). FL, full-length.
early secretory pathway between the endoplasmic reticulum and the Golgi apparatus (43). RAP has been shown to interact with megalin, a member of the low-density lipoprotein receptor gene family and has been shown to facilitate endocytic trafficking (44). Taken together it is conceivable that mutations in PS1 may direct the APP-C100 to a compartment containing an active $\alpha_4\beta_2$ $\gamma$-secretase. Likewise, native PS1 could transport APP-C100 to the compartment where the $\alpha_4\beta_2$-generating protease is more active. This trafficking could be performed either through vesicular transport or molecular chaperones.

In summary, our findings provide strong evidence for a direct interaction between PS1 and the C100 fragment of APP. Demonstration of this direct interaction was enhanced by metabolically freezing either APP-C100 or both PS1 holoprotein and APP-C100 degradation by employment of the lysosomotropic agent NH$_4$Cl and the proteasomal inhibitor LlNl, respectively. These approaches should greatly facilitate future studies of this nature directed at understanding the mechanism by which mutant PS1 selectively regulates APP metabolism to produce A$\beta$.

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