Presenilin 1 Protein Directly Interacts with Bcl-2*

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Presenilin proteins are involved in familial Alzheimer’s disease, a neurodegenerative disorder characterized by massive death of neurons. We describe a direct interaction between presenilin 1 (PS1) and Bcl-2, a key factor in the regulation of apoptosis, by yeast two-hybrid interaction system, by co-immunoprecipitation, and by cross-linking experiments. Our data show that PS1 and Bcl-2 assemble into a macromolecular complex, and that they are released from this complex in response to an apoptotic stimulus induced by staurosporine. The results support the idea of cross-talk between these two proteins during apoptosis.

Mutations of the presenilin (PS1) genes account up to 50% of all familial Alzheimer’s disease cases (1–3). The homologous presenilin proteins are characterized by several transmembrane domains and a large hydrophilic loop that faces the cytosol (4, 5). PS holoproteins are rapidly processed in two stable NH2- and COOH-terminal fragments that assemble into heterodimers (6). These are localized within the endoplasmic reticulum, the Golgi apparatus, and the nuclear envelope (7, 8). Potential functions of presenilins include regulation of β-amyloid precursor protein processing (9–11), intracellular trafficking, regulation of transport (12, 13), regulation of intracellular calcium homeostasis (14, 15), stabilization of the cytoskeleton (16, 17), and participation in the Wg/Wnt signaling pathway (18). Presenilins can also sensitize cells to apoptotic stimuli leading to programmed cell death (19–23), raising the hypothesis that PS interfere with specific steps of the apoptotic cascade. Apoptosis is strongly inhibited by Bcl-2, the founding member of a large family of proteins involved in the regulation of neuronal apoptosis including the survival motor neuron protein (SMN) are known to modulate Bcl-2 function by binding it (34, 35). To test the hypothesis that PS1 can bind Bcl-2, we performed yeast two-hybrid interaction system, cross-linking experiments, and co-immunoprecipitation assays in different cell lines, in the presence or the absence of an apoptotic stimulus. Our results demonstrated binding between the two proteins. These data provide new insights in biological functions of presenilins and their possible role in the pathogenesis of Alzheimer disease (AD).

EXPERIMENTAL PROCEDURES
cDNA Cloning—Alfa Bcl-2 cDNA was amplified by reverse transcription-PCR from total RNA prepared from SY5Y cells subjected to overnight treatment with 100 nM phorbol 12-myristate 13-acetate. PCR products were cloned in pCR3.1 expression vector by TA cloning (Invitrogen).

Human full-length PS1 cDNA was amplified by reverse transcription-PCR from total RNA prepared from HeLa cells, cloned in pCR2.1 expression vector by TA cloning. Correct cDNA sequences were confirmed by automated DNA sequencing.

Yeast Two-hybrid Screening—pCR2.1-PS1 and pCR2.1-Bcl-2 cDNAs were subcloned into the Gal4 binding domain of pGBT9. pCR3.1-Bcl-2 was digested with EcoRI-SalI, and the resulting Bcl-2 cDNA fragment was subcloned into the Gal4 binding domain of pGAD24. The correct insertions and sequences of both cDNAs were confirmed by automatic sequencing. Bait pGBT9 and prey pGAD24 plasmids were introduced into CG 1945 yeast cells either alone or together by using standard lithium acetate protocol. The expression of fusion proteins was confirmed by Western blotting with antibodies against PS1, the Gal4 binding domain, or Bcl-2. Transformed yeasts were plated on histidine-deficient selective medium in the presence of 25 mg ml−1 3-amino-1,2,3-triazol (3-AT). Synthesis of β-galactosidase was subsequently tested by filter assay (CLONTECH).

Antibodies—The monoclonal antibody 124 (mAb 124) directed against residues 41–54 of Bcl-2 protein was purchased from Upstate Biotechnology Inc. A polyclonal antiserum (Bcl-2 Δ21) directed against residues 1–205 of Bcl-2 protein was purchased from Santa Cruz Biotechnology Inc. Monoclonal antibody anti-cytochrome c and anti-cytochrome oxidase subunit II; 3-AT, 3-amino-1,2,3-triazol; PCR, polymerase chain reaction; Tricine, N-tris(hydroxymethyl)methyglycine; TUNEL, terminal dUTP nick-end labeling.

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chromosome oxidase subunit II (COII) were purchased, respectively, from PharMingen and Molecular Probes.

The polyclonal antisera R4318 was raised against the keyhole limpet hemocyanin-coupled peptide VQFMDQKAFHQFYI-C, corresponding to positions 453-467 of the carboxyl terminus of PS1. R4318 precipitated the full-length PS1 from the nonionic detergent-soluble cellular fraction; the immunoreactive signal of the R4318 antibody was preadsorbed by the corresponding uncoupled peptide (data not shown).

The polyclonal rabbit antisera R9713 was raised against the keyhole limpet hemocyanin-coupled peptide SQDTVAEDDGGFSEEWEAQ-C corresponding to residues 324–343, and representing the NH₂ terminus of the putative caspase cleavage site. For immunoblotting, R9713 was affinity-purified by adsorption to the corresponding antigenic peptide immobilized to N-hydroxysuccinimide (NHS) HTrap columns (Amersham Pharmacia Biotech). On immunoblots, R9713 detected the full-length PS1 and the PS1-CTF as 48- and 21-kDa bands, respectively, that were absorbed by preincubation with the peptide used for immunizations. Cellular amounts of this protein clearly increased upon transfection of the cells with PS1 expression constructs.

**Protein Extracts, Immunoprecipitation, and Western Blotting**—Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed with 2 mg/mM potassium phosphate, pH 7.6, 0.1% Triton X-100 in the presence of a protease inhibitor mixture. Lysates were centrifuged at 60,000 × g for 20 min, and protein concentrations in the supernatant fluids were determined by BCA assay (Pierce). The lysates were preclarified by incubaion with protein G-Sepharose (Amersham Pharmacia Biotech) for 2 h at room temperature. Immunoprecipitations were done at room temperature by incubating overnight 200 μg of proteins with primary antibodies, or with the preimmune R4318 serum previously absorbed by the corresponding uncoupled peptide (data not shown).

**Cross-linking of Protein Extracts**—Cells were lysed in 2 mM potassium phosphate, pH 7.6, in the presence of protease inhibitors. NHS and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (hydrochloride) were freshly prepared in Me₂SO and MES, respectively, and were added to cell lysates at a final concentration of 2 mM. The cross-linking reaction was done on ice and by twice adding fresh linkers after 10 min. The reaction was stopped after 20 min with 9 mM glycine. Samples were solubilized with 0.1% Triton X-100 and centrifuged at 60,000 × g for 20 min.

**Tissue Culture, Stable Transformations, Experimental Treatments, and Reagents**—H9 human lymphoblastic cells were grown in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 1 mM l-glutamine, and with 10% fetal calf serum (Life Technologies, Inc.). Penicillin (100 units/ml), streptomycin (100 μg/ml), and 1 mM EDTA, 200 μg/ml phenylmethylsulfonyl fluoride, 1 mM NaCl, 1 mM EDTA, 200 mM phenylmethylsulfonyl fluoride, 1 mM l-glutamine, 20 μg/ml leupeptin, 1 μg/ml pepstatin A, 5 mM dithiothreitol) and homogenized in a glass–glass homogenizer using the loose pestle (25 strokes). The homogenate was incubated 10 min on ice and then centrifuged at 60,000 × g for 20 min at 4 °C to generate the soluble cytosolic fraction (supernatant). The pellet was reconstituted in buffer B (Buffer A + 1% Nonidet P-40), homogenized using a tight pestle (25 strokes), incubated on ice for 30 min, and centrifuged at 60,000 × g for 20 min at 4 °C. The supernatant was the particulate or membrane fraction. Loading of the samples was normalized for the total content of cellular proteins determined separately with Bradford or BCA method and homogenized in a glass–glass homogenizer using the loose pestle (25 strokes).

**Interaction between Presenilin 1 and Bcl-2**—In order to test the PS1/Bcl-2 interaction in vivo, we set up a yeast two-hybrid interaction system. Full-length PS1, PS1 hydrophilic loop and full-length Bcl-2 were expressed in yeast strain CG-1945 as fusion proteins with a Gal4 DNA binding domain and a Gal4 transcription activation domain, respectively. Whereas growth of yeasts expressing both Bcl-2 or PS1 alone was either undetectable or limited, cells harboring both fusion proteins were able to grow in the absence of histidine (Fig. 1, lanes 1–3). In contrast to the interaction with full-length PS1, we did not observe any interaction when the PS1 hydrophilic loop was expressed (data not shown). Positive control yeasts transformed with pVA31 and pTD1 grew at higher rates (Fig. 1, lane 4).

**Western Blot Analysis**—COS-7 cells expressing the full-length Bcl-2 were expressed in yeast strain CG-1945 as fusion proteins with a Gal4 DNA binding domain and a Gal4 transcription activation domain, respectively. Whereas growth of yeasts expressing both Bcl-2 or PS1 alone was either undetectable or limited, cells harboring both fusion proteins were able to grow in the absence of histidine (Fig. 1, lanes 1–3). In contrast to the interaction with full-length PS1, we did not observe any interaction when the PS1 hydrophilic loop was expressed (data not shown). Positive control yeasts transformed with pVA31 and pTD1 grew at higher rates (Fig. 1, lane 4). The transactivation of the second reporter gene, lacZ, was tested in a filter X-gal assay. Whereas the positive control colonies started to turn blue after 2 h, none of the other transformed yeasts expressed lacZ, even after overnight incubations with X-gal.

**RESULTS**

Interaction of PS1 with Bcl-2 in Yeast—In order to test the PS1/Bcl-2 interaction in vivo, we set up a yeast two-hybrid interaction system. Full-length PS1, PS1 hydrophilic loop and full-length Bcl-2 were expressed in yeast strain CG-1945 as fusion proteins with a Gal4 DNA binding domain and a Gal4 transcription activation domain, respectively. Whereas growth of yeasts expressing either Bcl-2 or PS1 alone was either undetectable or limited, cells harboring both fusion proteins were able to grow in the absence of histidine (Fig. 1, lanes 1–3). In contrast to the interaction with full-length PS1, we did not observe any interaction when the PS1 hydrophilic loop was expressed (data not shown). Positive control yeasts transformed with pVA31 and pTD1 grew at higher rates (Fig. 1, lane 4). The transactivation of the second reporter gene, lacZ, was tested in a filter X-gal assay. Whereas the positive control colonies started to turn blue after 2 h, none of the other transformed yeasts expressed lacZ, even after overnight incubations with X-gal.
Co-immunoprecipitation of PS1 with Bcl-2 Protein in Human Cell Lines—In order to determine whether Bcl-2 and PS1 interact in human cell lines, we used co-immunoprecipitation assays of proteins extracted from H9 lymphoblastic and H4 neuroglioma cell lines. Proteins immunoprecipitated by mAb 124 were analyzed by Western blot with the PS1 polyclonal antiserum R4318 and analyzed by Western blot with Bcl-2 mAb 124. In native cells (lane 3), in proteins immunoprecipitated with preimmune R4318 serum (lane 2), only unspecific signal (~29 kDa) was detected.

Cross-linking Reaction of PS1 and Bcl-2—Cross-linking of cell lysates of H4 cells reduced the electrophoretic mobility of the Bcl-2 immunoreactive signal in SDS gels (Fig. 3A), whereas cross-linked PS1 proteins migrated with a smear above 200 kDa (Fig. 3B). Analysis of Bcl-2 immunoprecipitation after cross-linking presented the same shift observed in protein extracts, with a Bcl-2 recovery by mAb 124 comparable in treated and untreated samples (Fig. 3A, lanes 3 and 4). Cross-linkers did affect PS1 co-immunoprecipitation with Bcl-2, as evidenced by weaker signals at ~50 kDa, and by a concomitant signal at high molecular mass range of the blots (Fig. 3B).

Identification of Stable Interactions of PS1 and Bcl-2 in Bcl-2-transfected H4 Cells—Reciprocal experiments carried out in H9 and H4 cell lysates with different PS1 polyclonal antisera failed to co-immunoprecipitate proteins recognized by mAb 124 (Fig. 4; data shown for H4 cell and R4318 polyclonal antiserum). To demonstrate immunoprecipitations of Bcl-2 with PS1, we stably overexpressed Bcl-2 in H4 neuroglioma cells; protein extracts (10 μg) from native H4 and stable transfected H4 cells were loaded onto 4–20% Tris-Tricine gel electrophoresis, blotted, and analyzed with polyclonal antiserum Bcl-2 mAb 124 and analyzed by Western blot with R4318 polyclonal antiserum; PS1 co-immunoprecipitation was similar in native H4 and H4-transfected cells (A, lanes 4 and 6). Bcl-2 protein was co-immunoprecipitated (co-IPP) by two different PS1 polyclonal antisera, R4318 and R9713 by using protein extracts (Prot. Extr.) from Bcl-2-overexpressing cells. Co-immunoprecipitated Bcl-2 were detected as signals at 26 kDa (B, lanes 3 and 4); in washed R4318-conjugated beads (B, lane 1) and in proteins immunoprecipitated (IP) by preimmune R4318 serum (B, lane 2), only unspecific signals, probably due to the IgG (~29 kDa), were detected. Pre-adsorption of mAb 124 with its cognate peptide abolished 26-kDa Bcl-2-related signals (B, lanes 8–10), while unspecific bands remained unchanged (B, lane 7).

Co-immunoprecipitation analysis after cross-linking reaction. Lysates from stable transfected H4 cells were incubated twice for 10 min with NHS and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride linkers, then immunoprecipitated with Bcl-2 mAb 124 after solubilization with 0.1% Triton X-100. Protein extracts (Prot. Extr.) treated with or without cross-linkers (CL) (A, lanes 1 and 2). The corresponding immunoprecipitates (A, IPP, lanes 3 and 4) were analyzed by Western blot with Bcl-2 polyclonal antibody Δ21. Protein extracts treated with or without cross-linkers (B, lanes 1 and 2). The corresponding co-immunoprecipitations (B, co-IPP, lanes 3 and 4) were analyzed by Western blot with R4318 anti-PS1 antiserum.

Fig. 3. PS1 co-immunoprecipitation analysis after cross-linking reaction. Lysates from stable transfected H4 cells were incubated twice for 10 min with NHS and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride linkers, then immunoprecipitated with Bcl-2 mAb 124 after solubilization with 0.1% Triton X-100. Protein extracts (Prot. Extr.) treated with or without cross-linkers (CL) (A, lanes 1 and 2). The corresponding immunoprecipitates (A, IPP, lanes 3 and 4) were analyzed by Western blot with Bcl-2 polyclonal antibody Δ21. Protein extracts treated with or without cross-linkers (B, lanes 1 and 2). The corresponding co-immunoprecipitations (B, co-IPP, lanes 3 and 4) were analyzed by Western blot with R4318 anti-PS1 antiserum.

Co-immunoprecipitation of PS1 with Bcl-2 Protein in Human Cell Lines—In order to determine whether Bcl-2 and PS1 interact in human cell lines, we used co-immunoprecipitation assays of proteins extracted from H9 lymphoblastic and H4 neuroglioma cell lines. Proteins immunoprecipitated by mAb 124 were analyzed by Western blot with the PS1 polyclonal antiserum R4318, which recognized ~50-kDa full-length PS1 both in H9 and in H4 cell lysates. Full-length PS1 was co-immunoprecipitated with Bcl-2 in both cell lines (Fig. 2, A and B).

Identification of Stable Interactions of PS1 and Bcl-2 in Bcl-2-transfected H4 Cells—Reciprocal experiments carried out in H9 and H4 cell lysates with different PS1 polyclonal antisera failed to co-immunoprecipitate proteins recognized by mAb 124 (Fig. 4; data shown for H4 cell and R4318 polyclonal antiserum). To demonstrate immunoprecipitations of Bcl-2 with PS1, we stably overexpressed Bcl-2 in H4 neuroglioma cells; protein extracts (10 μg) from native H4 and stable transfected H4 cells were loaded onto 4–20% Tris-Tricine gel electrophoresis, blotted, and analyzed with polyclonal antiserum Bcl-2 mAb 124 and analyzed by Western blot with R4318 polyclonal antiserum; PS1 co-immunoprecipitation was similar in native H4 and H4-transfected cells (A, lanes 4 and 6). Bcl-2 protein was co-immunoprecipitated (co-IPP) by two different PS1 polyclonal antisera, R4318 and R9713 by using protein extracts (Prot. Extr.) from Bcl-2-overexpressing cells. Co-immunoprecipitated Bcl-2 were detected as signals at 26 kDa (B, lanes 3 and 4); in washed R4318-conjugated beads (B, lane 1) and in proteins immunoprecipitated (IP) by preimmune R4318 serum (B, lane 2), only unspecific signals, probably due to the IgG (~29 kDa), were detected. Pre-adsorption of mAb 124 with its cognate peptide abolished 26-kDa Bcl-2-related signals (B, lanes 8–10), while unspecific bands remained unchanged (B, lane 7).
different PS1 polyclonal antisera R4318 and R9713 clearly co-immunoprecipitated Bcl-2 only in transfected cells (Fig. 5B, lanes 3 and 4). In related control experiments, Bcl-2 immunoreactive signals were absent in R4318- and R9713-conjugated beads as well as in proteins precipitated with the R4318 pre-immune serum (Fig. 5B, lanes 1 and 2). To confirm that the 26-kDa immunoreactive product detected by mAb 124 corresponded to Bcl-2, we preadsorbed the signal with the corresponding peptide. Preadsorption eliminated the 26-kDa Bcl-2-related signal, while 29-kDa signal, caused by cross-reactivity to IgG light chains (Fig. 5B, lane 7), remained unchanged.

Effects of Staurosporine-induced Apoptosis on PS1/Bcl-2 Interaction and Mitochondrial Events Associated to PS1/Bcl-2—Overnight exposure to 100 nM staurosporine in serum-deficient media induced apoptosis in roughly 40% of stably Bcl-2-transfected H4 cells, as indicated by TUNEL assay (Fig. 6, A and B). Western blots showed that immunoreactive signals of Bcl-2 protein increased in lysates from cells exposed to staurosporine (Fig. 7A, lanes 1 and 2) and that levels of full-length PS1 did not change staurosporine (Fig. 7B, lanes 1 and 2). Despite increased Bcl-2 levels in protein extracts, its recovery by immunoprecipitation following staurosporine exposure was slightly lower as compared with control samples (Fig. 7A, lanes 3 and 4); and apoptosis strongly reduced co-immunoprecipitated full-length PS1 (Fig. 5B, lanes 3 and 4).

In order to determine at which step of the apoptotic cascade PS1 and Bcl-2 interact each other, we transfected COS-7 cells with PS1 and Bcl-2 expression constructs and we assessed cytochrome c release from mitochondria. Transient transfections of PS1 increased the release of cytochrome c; this increase was significantly attenuated by co-transfection with Bcl-2 (Fig. 8). To rule out the possibility that cytosolic preparations were contaminated with, we determined the mitochondrial marker of COII in the subcellular fractions; COII was present only in the membrane fraction (data not shown).

**DISCUSSION**

In this study we present evidence from yeast-two hybrid system, from co-immunoprecipitations, and from cross-linking assays that PS1 interacts with Bcl-2. First, full-length PS1, but not the PS1 hydrophilic loop, used as bait, transactivated a histidine reporter gene in the presence of a Bcl-2-Gal4 activation domain fusion protein. The absence of lacZ induction in the same system could be due either to weakness and instability of PS1 and Bcl-2 fusion complex in yeast or to the requirement for a third interaction partner. The yeast two-hybrid system was successfully used to identify interaction between PS1 and β-amyloid precursor protein (37), filamin (16), and β-catenin (38). Together, these data suggest multiple interaction partners of PS1, and possibly, promiscuity of their functional consequences.

We co-immunoprecipitated PS1 with a Bcl-2 antibody from human H9 lymphoblastic and H4 neuroglioma cells. Cross-linkers did neither interfere sensibly with the Bcl-2 immunoreactive signals in proteins extract nor with its recovery by mAb 124. In the presence of cross-linkers, PS1 proteins were associated with a high molecular mass complex over 200 kDa. This may be due to self-aggregation or to complex formation with other putative partners. Co-immunoprecipitation of Bcl-2 and PS1 by mAb 124 was significantly affected by cross-linkers by shifting the ~50-kDa full-length PS1 to a signal with a molecular mass higher than 200 kDa.

Reciprocal co-immunoprecipitation of Bcl-2 with a PS1 antibody was demonstrated in H4 cells stably overexpressing Bcl-2. Co-immunoprecipitation of PS1 with Bcl-2 was similar in transfected and untransfected cells. Our data suggest that the interaction between the two proteins occurs via the formation of a macromolecular complex, rather than a 1:1 stoichiometric association. Apparently, increasing amounts of Bcl-2 in transfected cells can facilitate its aggregation into this macromolec-
ular complex. This hypothesis is further supported by the known formation of both Bcl-2 homodimers and heterodimers with other members of the Bcl-2 family (39, 40). Alternate interpretations include the possibility that high cellular concentrations of Bcl-2 may modify the intracellular milieu to the effect that interactions of Bcl-2 with its partners are favored. The stably transfected H4 cells used in this study were more resistant to apoptotic stimuli as compared with the native, untransfected cells (data not shown). These data confirm reports from other Bcl-2-overexpressing cell lines in which apoptosis resistance was attributed to higher control of cellular calcium homeostasis (41, 42). It is further known that PS proteins interact with calcium-binding proteins (14, 15), and PS1 mutants sensitize to apoptosis through a pathway that modulates intracellular calcium ions homeostasis (32). If maintenance of intracellular calcium concentration is a physiological function shared by PS1 and Bcl-2, we can hypothesize that Bcl-2 overexpression may stabilize their reciprocal association.

The broad inhibitor of protein kinases, staurosporine, induces apoptosis in various cell lines by mediating a sustained increase of intracellular calcium ion concentrations (43). In our experiments, staurosporine increased cellular levels of Bcl-2 protein that were not followed by subsequent increase of immunoprecipitated Bcl-2 by mAb 124, suggesting the possibility that Bcl-2 may be sequestered in complexes not recognized by mAb 124 antibody. Full-length PS1 immunoreactivity was unchanged by apoptosis, and instead apoptosis promoted the dissociation of PS1 from Bcl-2, as demonstrated by reduced co-immunoprecipitation of PS1 with mAb 124. Thus, reversible association of Bcl-2 and PS1 could account for an anti-apoptotic role played by the two proteins. Moreover, redistribution of cytochrome c, one of the best known downstream activators of apoptotic cascade, could be one of the crucial events influenced by PS1/Bcl-2 interaction; the interaction of PS proteins with Bcl-xL, another anti-apoptotic member of Bcl-2 family and the subsequent change in cytochrome c release, further support this idea.

A similar synergism exists in the interaction of Bcl-2 and SMN. Mutations of SMN cause spinal muscular atrophy, a neurodegenerative disease characterized by neuronal apoptosis. Our data are consistent with similar interactions between PS1 and Bcl-2. In analogy to the role of SMN in muscular atrophy, defective interaction of PS1 with Bcl-2 may underlie the pathophysiology leading to AD. PS1 could thus have a role in apoptosis by providing a part of Bcl-2 protein macromolecu-

\[ L. D'Adamio, personal communication. \]
structures as DEF may alter the native folding of different bodies, Huntington's disease (47), prion disease (48, 49), and marks of many neurodegenerative conditions including fronto-DEF. Insoluble intracellular filamentous inclusions are hall-

sights to a common pathway by which distinct proteins are involved in neurodegeneration.

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