Interaction of Alzheimer’s Presenilin-1 and Presenilin-2 with Bcl-X<sub>L</sub>

A POTENTIAL ROLE IN MODULATING THE THRESHOLD OF CELL DEATH*  

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The familial Alzheimer’s disease gene products, presenilin-1 and presenilin-2, have been reported to be functionally involved in amyloid precursor protein processing, notch receptor signaling, and programmed cell death or apoptosis. However, the molecular mechanisms by which presenilins regulate these processes remain unknown. With regard to the latter, we describe a molecular link between presenilins and the apoptotic pathway. Bcl-X<sub>L</sub>, an anti-apoptotic member of the Bcl-2 family was shown to interact with the carboxyl-terminal fragments of PS1 and PS2 by the yeast two-hybrid system. In vivo interaction analysis revealed that both PS2 and its naturally occurring carboxyl-terminal products, PS2short and PS2Cas, associated with Bcl-X<sub>L</sub>, whereas the caspase-3-generated amino-terminal PS2Ncas fragment did not. This interaction was corroborated by demonstrating that Bcl-X<sub>L</sub> and PS2 partially co-localized to sites of the vesicular transport system. Functional analysis revealed that presenilins can influence mitochondrial-dependent apoptotic activities, such as cytochrome c release and Bax-mediated apoptosis. Together, these data support a possible role of the Alzheimer’s presenilins in modulating the anti-apoptotic effects of Bcl-X<sub>L</sub>.  

Alzheimer’s disease, a progressive neurodegenerative disorder of late life, is characterized by deposition of β-amyloid plaques, accumulation of intracellular neurofibrillary tangles, and neuronal cell loss. Approximately 10% of Alzheimer’s disease cases are familial (FAD) and co-segregate with autosomal dominant inheritance. The majority of FAD is linked to mutations in genes encoding presenilin-1 (PS1) (3) and presenilin-2 (PS2) (4), which are highly penetrant and have been shown to influence amyloid precursor protein (APP) by increasing the production of the neurotoxic form of β-amyloid, β-amyloid-42/43 (6, 7).  

Physiologically, both PS1 and PS2 gene products are multipass membrane proteins consisting of 6–8 spanning regions with a large hydrophilic loop at the carboxyl terminus (8–11). Immunolocalization studies have demonstrated that these ubiquitously expressed molecules, primarily localized to the endoplasmic reticulum and the Golgi apparatus (9, 12), are also found on nuclear and plasma membranes (13, 14). At the amino acid level, these proteins are ~67% identical and exhibit homology to two Caenorhabditis elegans gene products, SEL-12 and HOP1, both of which facilitate notch receptor-mediated signaling, thus suggesting a role for presenilins in this process (15, 16).  

In addition to their roles in APP processing and notch receptor signaling, extensive evidence suggests presenilins involvement in programmed cell death (PCD) or apoptosis. ALG-3, a truncated mouse homologue of PS2, corresponding to the last 103 amino acids, rescues cells from T cell receptor-induced apoptosis by inhibiting Fas-mediated death signal (17). Overexpression of PS2 increases apoptosis induced by a number of apoptotic stimuli (18), whereas FAD-associated PS1 and PS2 mutations generate molecules with constitutive pro-apoptotic activity (19–21). Complementary studies have demonstrated that depletion of PS2 protein levels by antisense RNA protected cells against apoptosis induced by a number of cell death inducing apoptotic stimuli (18, 19). Strikingly, physiological ALG-3-like counterparts have also been identified, exhibiting similar anti-apoptotic properties. For example, PS2Cas, which represents a 119-amino acid carboxyl-terminal fragment of PS2 generated by caspase-3 cleavage (22–24), protects cells from various apoptotic stimuli (22). In addition, PS2short (PS2s), a molecule generated either by alternative transcription or by proteolysis, exhibits similar anti-apoptotic property (22).  

To characterize better the molecular mechanisms by which presenilins and the apoptotic machinery are linked, we have tried to identify proteins interacting with both PS1 and PS2. Here we show that Bcl-X<sub>L</sub>, an anti-apoptotic member of the Bcl-2 family of protein (25), associates with both PS1 and PS2. Moreover, we observed that presenilins can act upon the mitochondria by influencing cytochrome c release and by augmenting the proapoptotic effects of Bax, a Bcl-2 family member that opposes Bcl-X<sub>L</sub> function.  

MATERIALS AND METHODS  

Cells Lines and Reagents—Human kidney 293 T fibroblasts and COS-7 cells were grown in Dulbecco’s modified Eagle’s media supplemented with 10% fetal calf serum (Biofluids; Rockville MD) and glutamine (2 mM). The human Jurkat T-cell line was cultured in RPMI 1640 media containing 10% fetal calf serum (Biofluids), glutamine (2 mM), penicillin (100 units/ml), and gentamicin (10 μg/ml) (Life Technologies, Inc.). Cells were cultured at 37 °C in a 5% CO<sub>2</sub> humidifying chamber. Plasmids for the interactor trap were generously provided by Dr. Roger Brent (Harvard University, Boston), except for the pEG202-LexA-RFH17.3, −12 baits, and pG4−−B42-Cdi3 prey control plasmids, which were kindly provided by Dr. R. F. Finley (Wayne State University, Detroit, MI). The pDNA3-FLAG-Bcl-X<sub>L</sub> (Dr. G. Nunez, University of Michigan, Ann Arbor, MI) and the pSFFV-Bax (Dr. S. Korsmeyer, Washington University, St. Louis, MO) constructs were gifts. The pDNA3-FLAG-AIP1 has been previously described (26). PS1 and PS2 fragments were amplified by polymerase chain reaction and cloned into  

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†The abbreviations used are: FAD, familial Alzheimer’s disease; PCD, programmed cell death; APP, amyloid precursor protein; PS1, presenilin-1; PS2, presenilin-2; a.a., amino acid; PAGE, polyacrylamide gel electrophoresis; GFP, green fluorescent protein; mAb, monoclonal antibody; DTBP, dithiobispropionimide-2 HCl.
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**TABLE I** Yeast two-hybrid analysis reveals an interaction between PS2Cas and Bcl-X<sub>L</sub>

|                      | Day 3                        | Day 6                        |
|----------------------|------------------------------|------------------------------|
|          | Cdi3 | CL19 | Bcl-X<sub>L</sub> | Cdi3 | CL19 | Bcl-X<sub>L</sub> | Cdi3 | CL19 | Bcl-X<sub>L</sub> |
| β-Gal Growth        | −     | +     | −              | −     | +     | −              | −     | +     | −              |
|                 | RFHM 7.3 | +     | −              | −     | +     | +              |
|                 | RFHM 12 | +     | +              | +     | +     | +              |

pCdnA. Bcl-X<sub>L</sub> was cloned into the pEGFP-N1 construct (CLONTECH, Palo Alto, CA). The rabbit anti-human PS2n antibody was generated against the carboxyl-terminal region (a.a. 341–377) of the hydrophilic loop (22). The 2972 polyclonal antibody (27) was raised against the amino terminus (a.a. 2–81) of PS2 and was generously provided by Dr. C. Haass (University of Mannheim, Germany). The negative control mouse IgG and anti-human Bcl-X<sub>L</sub> monoclonal antibody (mAb) was purchased from Southern Biotechnology Inc. (Birmingham, AL).

**Yeast Two-hybrid System**—The carboxyl-terminal regions of PS1 (a.a. 264–467, PS1CT) and PS2 (a.a. 330–448, PS2Ccas) were fused to the LexA DNA-binding domain in pEG202. Full-length Bcl-X<sub>L</sub> cDNA was cloned into the galactose-inducible pGJ4-5 vector containing the B42-activation domain. The yeast two-hybrid system was performed as described previously (28). Briefly, the yeast strain RFT231 (MATa trp1Δ::hisGhis3ura3–1 trp2–3Lexop-2LEU2) (29) harboring either pEG202-LexA-PS1CT or PS2Cas and the lacZ reporter plasmid, pSH18–34, were transformed by the lithium acetate method with the pGJ4–5-B42-Bcl-X<sub>L</sub> construct. Presenilin and Bcl-X<sub>L</sub> interactions were assayed for growth on the basis of leucine prototrophy on Gal Ura His ‘Trp Leu’ plates. Subsequent testing for β-galactosidase activity was performed on Gal Ura His Trp plates containing 5-bromo-4-chloro-3-indolyl-dithiobispropionimidate (X-Gal) to detect expression of the β-galactosidase reporter gene.

**Transient Transfections and Immunoprecipitations**—2 × 10<sup>3</sup> 293 T cells were transfected by the CaPO<sub>4</sub> method, with 5 μg of each of the indicated constructs. Eight h post-transfection, cells were washed once in phosphate-buffered saline (Biofluids), plated in fresh media, and cultured overnight. Overexpressed proteins were cross-linked by resuspending cells in phosphate-buffered saline containing 10 μM dithiobispropionimidate-3,3′-dihydroxybenzamide (HCl) (Pierce) and incubated on ice for 40 min. Cells were washed and solubilized in 1% Nonidet P-40 (Calbiochem) lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and protease inhibitors including aprogin (1%), leupeptin (1 μM), pepstatin (2 μM) (Sigma), and 2,2′-diamino-4-aminoethylbenzenesulfonyl fluoride (2 μM) (ICN, Aurora, OH). After centrifugation (750 × g; 30 min; 4 °C), cell lysates were rotated with bovine serum albumin-preabsorbed anti-FLAG M2 affinity gel (Sigma), for 1 h at room temperature. Protein-bound beads were extensively washed, and immunobonded molecules were dissociated by the addition of Laemmli’s sample buffer (30) and resolved under reducing conditions (100 μM dithiothreitol) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12%). Proteins were blotted onto nitrocellulose (Schleicher & Schuell) and probed with anti-PS2 antibodies, followed by an anti-rabbit IgG-horseradish peroxidase antibody (Promega, Madison, WI) and visualized by enhanced chemiluminescence (ECL) (Pierce).

**Cell Transfections and Apoptosis Studies**—4 × 10<sup>4</sup> Jurkat cells (10%/ml) were transfected by electroporation with the indicated plasmids. All transfections were normalized with pCdnA (Invitrogen, Carlsbad, CA). Cells were allowed to recover by incubation for 1 h 30 min at 37 °C in a humidifying chamber. Cells were layered onto a Ficoll-Paque (Amersham Pharmacia Biotech) cushion and centrifuged (750 × g; 20 min; 25 °C). The cell layer at the interface was removed, washed, and placed into culture. Cell samples were taken at various times, and nuclei were stained by the addition of an equal volume of a 2× hypotonic propidium iodide solution (60 μg/ml). Cell death was assessed by measuring the percentage of fragmented DNA by flow cytometry (31).

For analysis of cytochrome release, 24–30 h after transfection, 293 T cells were resuspended in 500 μl of buffer A (20 mM Hepes, pH 7.5, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol) containing 250 mM sucrose and a mixture of protease inhibitors. Cells were homogenized with a glass Pyrex homogenizer; nuclei and broken cells were removed by centrifugation at 1,000 × g for 10 min at 4 °C. The resulting supernatant was subjected to 10,000 × g centrifugation for 20 min at 4 °C, and the pellet fraction, containing the mitochondria, was washed in buffer A/sucrose and solubilized in TNC buffer (10 mM Tris, pH 8.0, 0.5% Nonidet P-40, 5 mM CaCl<sub>2</sub>). The supernatant fraction was further centrifuged at 100,000 × g for 1 h at 4 °C to generate cytosol. Equal amount of lysate (10 μg) from each fraction was separated by SDS-PAGE (10%) and blotted onto nitrocellulose membranes and subsequently probed with either anti-cytochrome c (PharMingen, San Diego, CA) or anti-cytochrome oxidase subunit IV mAb (Molecular Probes, Eugene, OR). Confoocal Microscopy—COS-7 cells were plated at ~40% confluence the day before transfection. Transfections were carried out using a total of 1 μg of DNA by the Lipofectamine method (Life Technologies, Inc.) according to the manufacturer’s instructions. Twenty-four hours after transfection, cells were trypsinized and plated onto 15-mm round coverslips. Cells were fixed (2% paraformaldehyde), washed in phosphate-buffered saline, and incubated with the anti-PS2n antibody followed by a Texas Red-conjugated secondary antibody (Jackson Laboratories, West Grove, PA). Fluorescence analysis of GFP-tagged Bcl-X<sub>L</sub> and immunolabeled PS2 was performed on a Bio-Rad MRC1024 confocal microscope.

**RESULTS**

**Analysis of PS2Cas and Bcl-X<sub>L** Interaction by the Yeast Two-hybrid System**—cDNA corresponding to the capase-3-generated carboxyl-terminal fragment of PS2Cas was fused to the LexA DNA-binding domain and used as a bait in the yeast two-hybrid system. This carboxyl-terminal region, which includes a portion of the large hydrophilic loop, was chosen as bait because (i) it is cytoplasmically exposed and therefore likely to interact with other molecules, (ii) it exists as a physiological fragment generated by proteolysis, and (iii) it inhibits some forms of PCD. Sequence analysis of a specific interactor obtained from the yeast two-hybrid hunt prompted us to investigate whether Bcl-X<sub>L</sub>, a member of the Bcl-2 family (25), interacts with PS2Cas. Table I summarizes the results of the interaction between PS2Cas and Bcl-X<sub>L</sub>. By day 3, as determined by both yeast growth and β-galactosidase activity, interaction between LexA-PS2Cas and B42-Bcl-X<sub>L</sub> was undetected, whereas growth and β-galactosidase activity for two different positive controls (LexA-PS2Cas and B42-cl.19; LexA-HM12 and B42-Cdi3) were observed. B42-cl.19 represents a novel molecule identified in the interactor trap, whereas the LexA-HM12 and B42-Cdi3 interaction has been previously described (32). Interestingly, by day 6, growth was observed by yeast containing LexA-PS2Cas and B42-Bcl-X<sub>L</sub>. Of importance, negative controls remained unchanged by this time. Taken together, this observation suggested the possibility that PS2 may interact with Bcl-X<sub>L</sub>.

**PS2 and Its Naturally Occurring Carboxyl-terminal Fragments Associate with Bcl-X<sub>L** in Vivo—To verify the interaction observed in yeast, we performed an in vivo interaction analysis. 293 T cells were transiently co-transfected with plasmids expressing either the negative control FLAG-AIP1 (26) or FLAG-
Bcl-X<sub>L</sub> products in combination with either PS2Ccas or the amino-terminal product of caspase-3 cleavage, PS2Ncas. Western blot analysis of protein complexes, initially chemically cross-linked in vivo and immunoprecipitated with mouse anti-FLAG antibodies, revealed that PS2Ncas, but not PS2Ncas, was co-precipitated by FLAG-Bcl-X<sub>L</sub>. (Fig. 1, A and B, respectively). In contrast, the FLAG-AIP1 negative control did not precipitate either PS2Ncas or PS2Ncas.

We then tested whether PS2 associated with Bcl-X<sub>L</sub>. PS2 typically runs as a prominent band of ~50 kDa, consistent with its predicted size, in addition to a high molecular mass smear due to aggregation. As shown in Fig. 2, PS2 was detected in FLAG-Bcl-X<sub>L</sub> but not in FLAG-AIP1 immunocomplexes. Overexpression studies of PS2 have been previously shown to result in the generation of PS2s, a proteolytically derived product of ~30 kDa (22, 23). As expected, a band migrating at ~30 kDa was observed in total cell lysates overexpressing PS2. Moreover, this cleaved product was also detected in FLAG-Bcl-X<sub>L</sub> but not in FLAG-AIP1 immunoprecipitating conditions. The observation that FLAG-Bcl-X<sub>L</sub> associated with the proteolytically generated PS2 s fragment prompted us to assess further this interaction. In these experiments, plasmids expressing FLAG-Bcl-X<sub>L</sub> and untagged PS2s were co-transfected in 293 T cells and analyzed for interactions. Fig. 3 confirms a specific interaction between FLAG-Bcl-X<sub>L</sub> and PS2s. Overall, these results demonstrate that Bcl-X<sub>L</sub> binds both full-length and its natural occurring carboxyl-terminal fragments of PS2 but not the amino-terminal PS2Ncas product and, thus, maps the site of Bcl-X<sub>L</sub> interaction to the carboxyl terminus of PS2.

The reducible chemical cross-linker DTBP was used in the above studies to assess the interactions between Bcl-X<sub>L</sub> and various forms of PS2. To rule out the possibility that DTBP captures two molecules only in close proximity and not in an actual association, we tested Bcl-X<sub>L</sub> and PS2s interactions without DTBP. Fig. 3 illustrates that FLAG-Bcl-X<sub>L</sub> readily precipitated PS2s in the absence of DTBP, as detected by the anti-PS2n antibody. Thus, these results argue against the possibility that these two molecules are only found in proximity and do not interact.

Finally, we addressed whether endogenous PS2 could associate with Bcl-X<sub>L</sub>. Cell lysates derived from 293 T cells transiently transfected with the indicated plasmids were immobilized with the anti-PS2n antibody. Analysis of Bcl-X<sub>L</sub> and AIP1 expression levels in total lysates were detected with the anti-FLAG antibody (left panel). Immunoblot analysis of PS2 in total cell lysates and of anti-FLAG immunoprecipitations were probed with the anti-PS2n antibody (right panel). Note that in the right panel a longer exposure time was used to rule out the possibility of detecting low level interactions. PS2Ncas migrates at ~18 kDa, whereas the unaggregated form of PS2Ncas runs at ~40 kDa. I.P., immunoprecipitation; w.b., Western blot analysis.

**Fig. 1.** Bcl-X<sub>L</sub> interacts with the caspase-3-generated carboxyl-terminal fragment, PS2Ccas, but not with the amino-terminal product, PS2Ncas. 293 T cells were co-transfected with 5 μg of either FLAG-AIP1 negative control or FLAG-Bcl-X<sub>L</sub> constructs in combination with 5 μg of either PS2Ccas or PS2Ncas plasmids. Western blot analysis of immunoprecipitated protein complexes was performed with antiseraum against either the carboxyl-terminal (PS2n) or amino-terminal (2972) portions of PS2. A, Bcl-X<sub>L</sub> interacts with PS2Ccas. Analysis of Bcl-X<sub>L</sub> and AIP1 expression levels in total cell lysates probed with an anti-FLAG antibody (left panel). Analysis of PS2Ncas expression levels in total cell lysates and of anti-FLAG immunoprecipitations immunoblotted with anti-PS2n antibody (right panel). Asterisks denote unknown protein bands. B, PS2Ncas is not co-immunoprecipitated by Bcl-X<sub>L</sub>. Bcl-X<sub>L</sub> and AIP1 expression levels in total cell lysates were detected by anti-FLAG antibody (left panel). Western blot analysis of PS2Ncas in total cell lysates and of anti-FLAG immunoprecipitations were probed with the 2972 anti-PS2 antibody (right panel). Note that the proteolytically generated PS2 s fragment is detected in both AIP1 and Bcl-X<sub>L</sub> containing total cell lysates and under immunoprecipitating conditions detected only with FLAG-Bcl-X<sub>L</sub>. Note that a longer exposure time was required to detect PS2s. PS2s represent a fragment of ~30 kDa.

**Fig. 2.** Bcl-X<sub>L</sub> associates with PS2 and the proteolytically derived PS2s fragment. Lysates from 293 T cells transiently transfected with the indicated plasmids were immobilized with the anti-PS2n antibody. Analysis of Bcl-X<sub>L</sub> and AIP1 expression levels in total lysates were detected with the anti-FLAG antibody (left panel). Immunoblot analysis of PS2 in total cell lysates and of anti-FLAG immunoprecipitations was probed with the anti-PS2n antibody (right panel). Note that the proteolytically generated PS2 s fragment is detected in both AIP1 and Bcl-X<sub>L</sub> containing total cell lysates and under immunoprecipitating conditions detected only with FLAG-Bcl-X<sub>L</sub>. Note that a longer exposure time was required to detect PS2s. PS2s represent a fragment of ~30 kDa.
Interaction of Alzheimer's Presenilins with Bcl-\(X_L\)

The above studies establish an in vivo interaction between Bcl-\(X_L\) and presenilins. As a step toward validating the relevance of this association, co-localization studies were carried out. COS-7 cells were co-transfected with GFP-tagged Bcl-\(X_L\) and untagged PS2 expression constructs, and 24 h after transfection, cells were analyzed by confocal microscopy. Single color analysis of PS2 (Texas Red; Fig. 5, left panel) and GFP-Bcl-\(X_L\) (Fig. 5, right panel) co-transfectants revealed that the expressed proteins appeared similar in their localization patterns, reminiscent of the vesicular transport system (Fig. 5).

**Assessment of PS1CT and Bcl-\(X_L\) Interactions by the Yeast Two-hybrid System**—PS1 is both structurally and functionally homologous to PS2. They display ~67% homology at the amino acid level, exhibit similar membrane topologies, and are endoproteolytically processed in a similar fashion (23, 33–35). PS1 and PS2 also participate in APP processing, notch receptor signaling, and PCD. Given these similarities, we investigated whether PS1 also associated with Bcl-\(X_L\) in the yeast two-hybrid system. By using the carboxyl-terminal portion of PS1CT, which included the entire hydrophilic loop, we obtained results similar to those found with LexA-PS2Cas. As compared with the positive control interaction of LexA-PS1CT and B42-cl.69, growth and \(\beta\)-galactosidase activity was delayed, not appearing until day 6 (Table II). B42-cl.69 was originally identified as a LexA-PS1CT interactor in a yeast two-hybrid screen; however, for these studies, it was used as a positive control. Thus, like LexA-PS2Cas, LexA-PS1CT binds Bcl-\(X_L\).

Presenilin Influence on Mitochondrial Associated PCD Activities—Because presenilins can interact with Bcl-\(X_L\) and have been previously shown to sensitize cells to apoptotic stimuli, it is plausible that presenilins can modulate PCD through its interactions with Bcl-\(X_L\). Bcl-\(X_L\) has been shown to promote cell survival by preventing cytochrome \(c\) redistribution, a known downstream activator of apoptosis (36–38), from the mitochondria to the cytosol. Thus, one way by which presenilins could exert their influence on the anti-apoptotic functions of Bcl-\(X_L\) could be through regulating cytochrome \(c\) release. To this end, we overexpressed either PS2 or the FAD mutant PS2 (N141D) (PS2mut) in 293 T cells, isolated either cytosolic or mitochondrial fractions and assayed for the presence of cytochrome \(c\) (Fig. 6). As expected, overexpression of PS2 alone had a negligible effect on cytochrome \(c\) release (Fig. 6, left two lanes). On the other hand, PS2mut, which has been previously demonstrated to increase the basal levels of apoptosis, clearly induced mitochondrial release of cytochrome \(c\) (Fig. 6, right two lanes). We excluded the possibility of cytochrome \(c\) contamination in our cytosol preparations because the mitochondrial specific marker, cytochrome oxidase subunit IV (COX), was confined solely to the mitochondrial compartment. As stated above, PS2 is a natural occurring carboxyl-terminal fragment of full length that opposes the apoptotic effects of PS2 and thereby acts as a dominant negative. Thus, one possible mechanism by which the effects of PS2 on cytochrome \(c\) release could be attenuated may be due to the generation of the anti-apoptotic PS2as fragment. To test this, we overexpressed PS2mut in combination with PS2s, and we determined whether PS2s could impede the inducing effects of PS2mut on cytochrome \(c\) release. As shown in Fig. 6 (middle two lanes), overexpression of PS2s significantly lowered the amount of cytochrome \(c\) released into the cytosol by PS2mut, as only modest levels of cytochrome \(c\) were observed. The likelihood that this phenomenon was due to a toxic effect generated by our overexpression system was ruled out since neither PS2 alone nor the combination of PS2mut and PS2s induced cytochrome \(c\) release. Regardless of whether these effects are direct or indirect, these results demonstrate that PS2 can act at the level of the mitochondria by influencing cytochrome \(c\) release.

To substantiate further the biological relevance of a presenilin/Bcl-\(X_L\) association, we investigated the influence of PS1 and PS2 on Bax-mediated cell death. Bax, like Bcl-\(X_L\), is a member of the Bcl-2 family but functions in promoting apoptosis (39). Upon treatment with various apoptotic stimuli, Bax translocates to the mitochondria, induces cytochrome \(c\) release, and changes mitochondrial transition potential (37, 38, 40). These pro-apoptotic effects can be abrogated by the presence of Bcl-\(X_L\) (37, 38). Based on our present and previous studies, we would predict that presenilins could enhance the pro-apoptotic effects of Bax, possibly through its association with Bcl-\(X_L\). To test this, we transfected Jurkat cells with either Bax (15 \(\mu\)g) alone or with increasing amounts of PS1 (10–30 \(\mu\)g) and subsequently analyzed for cell death at various time points. As measured by DNA fragmentation, Fig. 7A shows that by 7 h, transfection of Bax alone...
PS1CT and Bcl-X<sub>L</sub> interaction by the yeast two-hybrid system

|          | Day 3 |          | Day 6 |
|----------|-------|----------|-------|
|          | Cdi3  | CL69     | Bcl-X<sub>L</sub> | Cdi3  | CL69   | Bcl-X<sub>L</sub> |
|          | β-Gal | Growth   | β-Gal | Growth | β-Gal | Growth | β-Gal | Growth | β-Gal | Growth |
| PS1CT    | −     | −        | +     | +      | −     | −      | +     | +      | −     | −      |
| RF11.7.3 | −     | −        | +     | +      | +     | +      | −     | −      | +     | +      |
| RFHM 12  | −     | −        | +     | +      | −     | −      | +     | +      | −     | −      |

TABLE II

FIG. 5. Bcl-X<sub>L</sub> and PS2 partially co-localize. COS-7 cells were co-transfected by the LipofectAMINE method with a total of 1 μg of GFP-Bcl-X<sub>L</sub> and PS2 constructs. 24 h after transfection, cells were harvested, fixed, and plated onto coverslips. Immunofluorescence of overexpressed PS2 was performed by incubation of the cells with the anti-PS2n antibody followed by a Texas-Red-conjugated secondary antibody. Fluorescence analysis of either PS2 (left panel), Bcl-X<sub>L</sub> (right panel), or the combination (middle panel) was performed on a Bio-Rad MRC1024 confocal microscope. Note that the staining pattern of PS2 and Bcl-X<sub>L</sub> appears to co-localize to the vesicular transport system, possibly the endoplasmic reticulum.

FIG. 6. FAD PS2mut (N141I) induced cytochrome c release, whereas PS2s impairs this effect. 293 T cells were transfected with either PS2 alone (left), FAD PS2mut (N141I) alone (right), or the combination of FAD PS2mut (N141I) and PS2s (middle). Cytosolic (c) or mitochondrial (m) fractions were isolated and run on a 10% SDS-PAGE gel (10 μg/lane) and assayed for the appearance of cytochrome c (Cyt.C) by immunoblotting with an anti-cytochrome c antibody. The FAD PS2mut (N141I) but not PS2s induced release of cytochrome c from the mitochondria, whereas PS2s impaired this effect. The middle and lower (longer exposure) panels show negligible mitochondrial contamination of mitochondrial specific marker, COX, in the cytosol.

resulted in an ~27% increase in cell death compared with the vector only control, whereas PS1 (30 μg) alone produced minimal effects. In contrast, Bax and PS1 co-transfectants augmented Bax-induced PCD in a dose- and temporal-dependent manner. By 7 h, maximal effects were observed, with 30 μg of PS1 resulting in a significant increase in DNA fragmentation (1.7-fold) as compared with cells containing only Bax. Albeit to a lesser degree, increases PCD were seen with 10 and 20 μg of PS1. As expected, the addition of Bcl-X<sub>L</sub> (10 μg) abrogated the PS1-enhancing effects of Bax-mediated apoptosis. We also investigated the effects of PS2 on Bax-induced apoptosis. Similar to PS1, PS2 enhanced Bax-induced apoptosis in a time-dependent fashion. As compared with Bax only, a 2-fold increase in DNA fragmentation was seen in Bax and PS2 co-transfectants by 9 and 20 h (Fig. 7B).

The overexpression of Bax by itself resulted in significant levels of PCD (25–30%). In order to ascertain whether presenilins can enhance apoptosis under conditions where the effects of Bax are minimal, we repeated the above studies with an amount of Bax (10 μg) that resulted in low levels of PCD (8%). These studies were performed in triplicate and assayed at various times. As a function of time, a steady increase in cell death was observed by either PS1 or PS2 on Bax-mediated PCD, reaching approximately a 4-fold increase by 20 h (Fig. 7C). Thus, these studies demonstrate that presenilins can modulate mitochondrial dependent PCD events, such as the pro-apoptotic effects of Bax, and thereby lower the threshold to cell suicide. Furthermore, these findings are also consistent with the notion that a presenilin and Bcl-X<sub>L</sub> interaction may be involved in modulating the pro-apoptotic effects of Bax.

DISCUSSION

We have identified a molecular link between FAD presenilins and the PCD pathway. Bcl-X<sub>L</sub>, and an anti-apoptotic member of the Bcl-2 family was shown to interact with full-length and naturally occurring carboxyl-terminal products of presenilins. This interaction was demonstrated in both the interactor trap system and in vivo. Similar studies have confirmed an association between PS1 and Bcl-2.4 Although presenilin and Bcl-X<sub>L</sub> interactions were readily detected in 293 T cells, interactions in yeast appeared weak, as yeast growth and β-galactosidase activity was not visualized until day 6, compared with positive control interactions, which appeared by day 3. This discrepancy could be accounted for by differences in the sensitivities of the two biological systems. Alternatively, presenilin

4 A. Alberici, D. Moratto, L. Benussi, L. Gasparini, R. Ghidoni, L. Benerini Gatta, D. Finazzi, G. B. Frisoni, M. Tabucchi, R. Nitsch, J. H. Growdon, and G. Binetti, submitted for publication.
and Bcl-X₇L interactions in 293 T cells could be reinforced by an endogenous molecule(s), such as an adaptor protein. Regardless, our results establish a molecular connection between presenilins and the apoptotic pathway and, as such, predict a possible role of presenilins in facilitating cell death by modulating Bcl-X₇L activity.

Although presenilins, by themselves, do not promote PCD, accumulating evidence suggests a role of presenilins in sensitizing cells to apoptosis induced by diverse stimuli. For example, PS2 has been shown to sensitize cells to apoptotic death induced by trophic factor withdrawal and amyloid β-peptide (17, 19, 21) or, in the case of FAD presenilin mutants, increases the basal apoptotic activity of cells (19, 20, 41). However, the molecular mechanisms by which presenilins “prime” cells for death remain unknown. In this regard, presenilins could modulate PCD by regulating Bcl-X₇L activity.

Although presenilins, by themselves, do not promote PCD, accumulating evidence suggests a role of presenilins in sensitizing cells to apoptosis induced by diverse stimuli. For example, PS2 has been shown to sensitize cells to apoptotic death induced by trophic factor withdrawal and amyloid β-peptide (17, 19, 21) or, in the case of FAD presenilin mutants, increases the basal apoptotic activity of cells (19, 20, 41). However, the molecular mechanisms by which presenilins “prime” cells for death remain unknown. In this regard, presenilins could modulate PCD by regulating Bcl-X₇L activity. Our in vitro functional data are consistent with such a view. In these studies, we asked whether presenilin overexpression sensitizes cells to cell suicide induced by Bax, a pro-apoptotic Bcl-2 family member that is blocked by Bcl-X₇L. Our results clearly show that Bax-induced apoptosis was significantly enhanced by presenilins in a dose- and temporal-dependent manner, whereas overexpression of Bcl-X₇L abrogated this effect. Because Bax imposes its effects at the level of the mitochondria by inducing cytochrome c release, presenilins may therefore exert their effects at this level, possibly by indirectly regulating the activity of Bax through the control of Bcl-X₇L. In accordance, we showed that PS2mut, which has intrinsic apoptotic activity, induced the release of cytochrome c, whereas the anti-apoptotic PS2 fragments diminished this effect. Because they are not mutually exclusive, presenilins could also act further downstream on the apoptosome (42). This complex consists of the C. elegans Ced-4 homolog, Apaf-1, caspase-9, and cytosolic cytochrome c (43). Apaf-1 activation by cytochrome c triggers caspase-9 activation and eventual cell death (43). Bcl-X₇L can block this process (44), as it has been shown that Bcl-X₇L forms a ternary complex with Apaf-1 and caspase-9 (44, 45). In this scenario, the enhancing effects of presenilins on Bax-induced cell death may result from disrupting the influence of Bcl-X₇L on the apoptosome. Since fluctuations in the ratios of pro- and anti-apoptotic Bcl-2 family members have been proposed to be critical for cell survival or suicide decisions (39), perturbations could obviously have detrimental influences on the cell death program.

How could FAD presenilin mutants enhance basal apoptotic activity in this context? Such mutations could generate molecules with an increased propensity to regulate negatively the anti-apoptotic effects of Bcl-X₇L. Thus, presenilin FAD mutants could exacerbate the cell suicide program by further repressing inhibitory mechanisms. Our current studies and those of Guo and colleagues (20), which demonstrated that the enhanced apoptotic effects by PS1 mutants on nerve growth factor withdrawal could be attenuated by Bcl-2, are compatible with this view.

Finally, how could this model be applied to the pathogenesis of Alzheimer’s disease? Presenilin FAD is an autosomal dominant disorder, as all somatic cells carry the presenilin muta-
tion. Yet the pathological features of this syndrome are confined to the brain and selectively affect certain neurons (46). Unlike injured peripheral cells, most damaged neurons cannot be replaced by newly generated cells. Therefore, even a slight imbalance in favor of apoptosis could eventually progress to neuron cell loss seen in FAD. Accordingly, our investigations would suggest that presenilin FAD mutants may be involved in lowering the threshold to cell suicide by impairing anti-apoptotic mechanisms, resulting in cumulative neuronal damage.

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