The Presenilin-2 Loop Peptide Perturbs Intracellular Ca\(^{2+}\) Homeostasis and Accelerates Apoptosis*

Chuanxi Cai§, Peihui Lin§, King-Ho Cheung¶, Na Li§, Christina Levchook§, Zui Pan§, Christopher Ferrante§, Gabrielle L. Boulianne#, J. Kevin Foskett¶, David Danielpour&, and Jianjie Ma§

From the §Department of Physiology and Biophysics, Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854, USA; # Program in Developmental Biology, The Hospital for Sick Children, University of Toronto, Toronto, Ontario, Canada M5G 1X8; ¶Department of Physiology, University of Pennsylvania, Philadelphia, PA 19104-6085; &Ireland Cancer Center, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH 44106, USA

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Address correspondence to: Dr. Jianjie Ma, Department of Physiology and Biophysics, Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854, USA; Tel. (732) 235-4494; Fax. (732) 235-4483; Email: maj2@umdnj.edu

In cells undergoing apoptosis, a 22 amino acid presenilin-2-loop peptide (PS2-LP, a.a. 308-329 in presenilin-2) is generated through cleavage of the carboxyl-terminal fragment of presenilin-2 by caspase-3. The impact of PS2-LP on the progression of apoptosis, however, is not known. Here we show that PS2-LP is a potent inducer of the mitochondrial-dependent cell death pathway when transduced as a fusion protein with HIV-TAT. Biochemical and functional studies demonstrate that TAT-PS2-LP can interact with the InsP\(_3\) receptor and activate Ca\(^{2+}\) release from the endoplasmic reticulum. These results indicate that PS2-LP-mediated alteration of intracellular Ca\(^{2+}\) homeostasis may be linked to the acceleration of apoptosis. Therefore, targeting the function of PS2-LP could provide a useful therapeutic tool for the treatment of cancer and degenerative diseases.

Eleven years ago, presenilin-1 and -2 (PS1 and PS2) were discovered through familial and molecular genetic studies that implicated their involvement in the etiology of Alzheimer’s disease (AD) (1). PS1 and PS2 proteins are composed of 467 and 448 amino acids, respectively, and share ~70% identity (2). They are both membrane proteins with eight predicted transmembrane (TM) domains and a large hydrophilic loop of approximately 120 amino acids between the sixth and seventh TM domains. PS1 and PS2 appear to be ubiquitously expressed throughout all tissues (1,3), and are the essential components of the \(\gamma\)-secretase complex that are involved in the processing of Notch receptor and amyloid-\(\beta\) precursor protein (A\(\beta\)) (4). Mutations in PS-1 and PS-2 can lead to increased formation of A\(\beta\)-42, a highly neurotoxic amyloid oligomer (5).

A characteristic feature of PS1 and PS2 is their proteolysis by an endogenous presenilinase, generating amino-terminal (NTF) and carboxyl-terminal fragments (CTF) within cells (6,7). Many studies have shown that both NTF and CTF are involved in regulating proliferation and death of cells (8,9). It is also known that in cells undergoing apoptosis, activation of caspase-3 leads to a specific cleavage of CTF, yielding a shorter form of CTF plus a 22 amino acid hydrophilic peptide (a.a. 308 to 329 in PS2) (10-12). The caspase-3 mediated cleavage of PS-2 has been linked to the execution process of apoptosis. For example, phosphorylation of PS2 at a caspase-recognition site (S330) that inhibited the cleavage of CTF by caspase-3 reduced the apoptotic activity of CTF (13). It is not known, however, how the 22 amino acid presenilin-2-loop peptide (PS2-LP) generated during the initial stage of apoptosis contributes to the execution of apoptosis.

Mutations in PS have been linked to alterations of intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]) homeostasis (14-16). Disturbances in [Ca\(^{2+}\)] homeostasis can lead to modification of a multitude of Ca\(^{2+}\)-dependent phenomena in cells and initiate cell death (17). In this study, we tested the hypothesis that PS2-LP, as an apoptosis by-product, can act as an enhancer of apoptosis by perturbing [Ca\(^{2+}\)] homeostasis. We found that recombinant PS2-LP coupled with a TAT-membrane penetrating peptide, can enter cells and induce apoptotic cell death. The pro-apoptotic effect of PS2-LP appears to correlate with its action on the intracellular Ca\(^{2+}\) release machinery at the endoplasmic reticulum (ER). Our data suggests that
perturbation of [Ca^{2+}], homeostasis can act as an amplifying factor contributing to the efficient execution of apoptosis.

MATERIALS AND METHODS

Materials - Synthetic PS-2 loop peptide (PS2-LP) and anti-PS2-LP polyclonal antibody were generated by the ProteinTech Group (Chicago, IL, USA). Ni-NTA columns were purchased from Qiagen, and Slide-A-Lyzer (MW 3.5 KD) and the FITC labeling Kit were purchased from Pierce. G-25 Sephadex gravity columns were obtained from Amersham Biosciences (Piscataway, NJ). The pET-28b-TAT vector (V2.1) was kindly provided by Dr. Steven F. Dowdy (18-20). Anti-cytochrome c monoclonal antibody and anti-β-actin monoclonal antibodies were purchased from BD Biosciences (Palo Alto, CA). Anti-manganese superoxide dismutase (anti-MnSOD) was purchased from Stressgen Biotechnologies (San Diego, CA). Anti-InsP3 receptor rabbit polyclonal antibody was purchased from Calbiochem (Darmstadt, Germany). Goat polyclonal antibody for SERCA2 was purchased from Santa Cruz Biotechnology, Inc. Cleaved caspase-3 (Asp175) (5A1) monoclonal antibody was purchased in bulk (CalBiochem, Carlsbad, CA). Cell culture - The NRP-154 rat prostate adenocarcinoma cell line (21) was grown in a humidified environment at 37°C and 5% CO_2 in DMEM/F12 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin (22).

Plasmids and protein purification - Oligonucleotides encoding the 22 amino acids of PS2-LP (500DPS SQG ALQ LPY DPE MEE DSY D320) (conserved region for rat, mouse and human) were synthesized as follows: sense oligo: 5'-CCC AGC TGG CCG TCG AAC CCG ACG ACT CCT CCG CCC AGC CCT CCC ACT ATG ACG GCA TGG AGT CTG AAT GAA-3'; anti-sense oligo: 5'-AGC TTT CAT TCA GAG TCC ATG CCG TCA TAG TCG GAG GGC TGG GCG GAG TAG TCG TCG GGT TCG AGC GGG AGC TGG GAG CT-3'. The oligos encoding the 22 amino acids of presenilin-1 loop peptide (320DPE AQI RVN VP LSP SQG ALQ LPY DPE MEE DSY D334) (PS1-LP) were synthesized as follows: sense oligo: 5'-CCG ACC CAG AAG CCC AAA GGA GGG TAC CCA AAA ACC CCA AGT ATA GCA CAC AAG GAA CAG AGA GGG AAT GAA-3'; anti-sense oligo: 5'-AGC TTT CAT TCC CTC TCT GTT CCT TGT GTG CTA TAC TTG GGG TTT TTG GGT ACC CTC CTT TGG GCT TCT GGG TCG GAG CT-3'. All the oligos contain the Sac I/Hind III restriction enzyme sites. After annealing, these oligos were cloned in-frame into the pET-28b-TAT vector (V2.1) between the Sac I and Hind III sites. The obtained plasmids, pTAT-PS2-LP, pTAT-PS1-LP and pTAT-PS2-SP, were used to transform E. coli strain HB101 that were subsequently screened by restriction analysis.

Positive recombinant clones were sequenced prior to transformation into the E. coli strain BL21(DE3) pLysS. Bacterial cultures were grown overnight and protein expression was induced by IPTG treatment for 4-6 hours followed by sonication in a buffer solution containing 100 mM NaH_2PO_4, 10 mM, Tris-HCl, 10 mM imidazole, 7 M urea, pH 8.0 (Buffer B-7M). The His-tagged fusion proteins were purified using Ni^{2+}-NTA agarose affinity column, through sequential wash with B-7M buffer and C-7M buffer containing 100 mM NaH_2PO_4, 10 mM imidazole, 7 M urea, pH 6.3, followed by the B-7M buffer and elution with a buffer containing 100 mM NaH_2PO_4, 10 mM Tris-HCl, 250 mM imidazole, 8 M urea, pH 4.5. The elution step was followed by dialysis against PBS using the Slide-A-Lyser dialysis cassette. The TAT-fusion proteins were then desalted on a PD-10 column into PBS, flash-frozen in 10% glycerol and stored at -80°C. FITC-labeled TAT fusion proteins were generated with fluorescein labeling according to standard protocols.

Caspase-3 activity assay – Enzymatic activity for caspase-3 was assayed using the EnzChek caspase-3 kit following the manufacturers protocol (23,24). Briefly, cells treated with 10 µM TAT-PS2-SP or TAT-PS2-LP for 3 hrs were harvested and resuspended in 50 µl × cell lysis buffer, followed by 3 cycles of freeze-and-thaw. 50 µl of the supernatant...
from each sample was incubated with 50 µl 2× substrate solution at room temperature for 30 min. Caspase-3 mediated cleavage of the substrate leads to increase in fluorescence, determined by the fluorescence measurement at excitation wavelength of 342 nm and emission wavelength of 441 nm. A standard curve using known amounts of AMC was used to convert fluorescent values to specific catalytic activity.

Mitochondria isolation - Mitochondria were isolated from NRP-154 cells following the protocol of Unkila et al (25). Briefly, after treatment with TAT-PS2-LP or TAT-PS2-SP, NRP-154 cells were washed three times with ice-cold PBS. The cell pellet was suspended in a solution containing (in mM) 250 sucrose, 20 HEPES, 1 DTT, 10 KCl, 1 EDTA, 1 EGTA, 1.5 MgCl2, and a cocktail of protease inhibitors at 4°C. Cells were then homogenized with 20 strokes in a 22 gauge syringe and centrifuged at 800 × g for 10 min to remove intact cells and nuclei. Mitochondria were pelleted by centrifugation at 15,000 × g for 10 min at 4°C, and resuspended in RIPA buffer (150 mM NaCl, 5 mM EDTA, 1% NP40, 20 mM Tris-HCl, pH 7.5). For each sample, 30 µg total proteins from the cytosolic and mitochondrial fraction were used for SDS-PAGE and Western blot with anti-cytochrome C antibody, also with anti-β-actin and anti-manganese superoxide dismutase (MnSOD) as loading controls for cytosol and mitochondria, respectively (26).

Ca²⁺ measurements - 50-70% confluent NRP-154 cells were loaded with 2 µM fura-2-AM for 45 min at 37°C, in a balanced salt solution (BSS) (in mM): 140 NaCl, 2.8 KCl, 2 CaCl₂, 2 MgCl₂, 10 HEPES, pH 7.2). Following wash out of fura-2-AM from the culture medium, cells were resuspended in BSS buffer, and incubated with 10 µM TAT-PS2-SP, TAT-PS1-LP or TAT-PS2-LP, respectively, for 30 min at 37°C. To measure the ATP-induced Ca²⁺ release from the ER, cells were then resuspended in BSS buffer containing 0 Ca and 0.5 mM EGTA, and transferred to a cuvette system attached to the PTI fluorometer (Photon Technology International, Monmouth Junction, NJ). 0.5 mM ATP was added to the cell suspension solution within 45 s after resuspension in the Ca²⁺ free BSS solution. The ATP-induced Ca²⁺ release was assayed through ratiometric excitation of fura-2 at excitation wavelength of 340 nm and 380 nm, according to our published protocols (27).

Western blot and co-immunoprecipitation - After treatment with TAT-PS2-SP or TAT-PS2-SP, NRP-154 cells were harvested and lysed in ice-cold RIPA buffer (150 mM NaCl, 5 mM EDTA, 1% NP40, 20 mM Tris-HCl, pH 7.5) in the presence of a cocktail of protease inhibitors. The cell lysates were centrifuged at 12,000 rpm for 10 min at 4°C, and supernatants were mixed with Laemmli sample buffer. 20 µg of protein were separated on a 4-12% SDS-PAGE. The proteins were transferred to a polyvinylidene difluoride membrane and blotted with primary antibody and secondary horseradish peroxidase antibody. Peroxidase activity was developed with ECL.

Co-immunoprecipitation assays of TAT-PS2-SP, TAT-PS2-LP, InsP₃R and SERCA2 were performed as follows. Lysates of NRP-154 cells treated with TAT-PS2-SP or TAT-PS2-LP was resuspended in 0.5 ml modified RIPA buffer plus protease inhibitors. 500 µg of whole cell lysate was incubated overnight with 5 µg polyclonal anti-His-tag, anti-InsP₃R or anti-SERCA2 antibody. As a negative control, 5 µg of pre-immune rabbit IgG was used. The immuno-complexes were collected on protein G-Sepharose beads by incubating for 2 hrs and washed four times with RIPA buffer. The beads were resuspended in 30 µl of 2x concentrated Laemmli electrophoresis buffer, separated by SDS-PAGE and transferred onto PVDF membranes for the detection of His-tagged TAT-PS2-SP and TAT-PS2-LP, InsP₃R and SERCA2.

Confocal fluorescence imaging – Detection of TAT-PS2-LP or TAT-PS2-SP inside NRP-154 cells was performed using fluorescence imaging of FITC that is conjugated with the various membrane-penetrating peptides. For assays of cell death, cells were treated with propidium iodide (PI) (1 µg/ml) and visualized by confocal fluorescence microscopy using a Zeiss 510-META system.

Electrophysiology - Spodoptera frugiperda (Sf9) cells were grown and maintained as describe (28). Nuclei (5-10 µM diameter) were isolated and selected for electrophysiology based on unique morphology. For patch clamp measurements of InsP₃R channel activity, the pipette solution contained (in mM) 140 KCl, 0.5 Na₂ATP, 10 HEPES, pH 7.3, 1 µM [Ca²⁺] and 33 nM InsP₃. All solutions were carefully buffered to desired free [Ca²⁺] (29), which was confirmed by fluorometry. Single channel data were acquired as described (29). Segments of current traces exhibiting a single InsP₃R channel were used for determination of open probability (Pₒ), and dwell time analyses by QuB software (30).
of Ni²⁺-affinity columns, we found that abundant of these recombinant fusion proteins. Through the use of control purpose (Fig. 1A) (6,7,11). To test the cellular function of PS2-LP, we attempted to express the PS2-LP peptide by transfection of cDNA plasmids into cultured cells. However, multiple trials have failed to result in detectable expression of PS2-LP in these cells, e.g. NRP-154, Hela, or HEK 293 cells. This is not surprising, because small peptide molecules are frequently vulnerable to degradation by cellular quality control mechanisms.

As an alternative approach to test the in vivo function of PS2-LP, we took advantage of the well-established HIV-mediated method for delivery of synthetic peptides into cells (19,20). Extensive studies have shown that an 11-amino acid TAT peptide “YGRKKRRQRRR” can enable peptides of various sizes to penetrate the cell membrane. Recently, Dr. Dowdy’s group developed the bacterial expression vector pHis-TAT for production of recombinant TAT-fusion proteins (18,19), and has kindly provided us with this plasmid. Using the pHis-TAT plasmid, we have generated the following three constructs: TAT-PS2-LP, TAT-PS1-LP and TAT-PS2-SP. The TAT-PS1-LP and TAT-PS2-SP constructs were used for comparative studies and for control purpose (Fig. 1B).

We used the BL21 E. coli strain for expression of these recombinant fusion proteins. Through the use of Ni²⁺-affinity columns, we found that abundant amount of TAT-PS1-LP, TAT-PS2-LP and TAT-PS2-SP proteins could be purified to homogeneity (Fig. 1C). Based on gel electrophoresis and Western blot, the molecular size of the peptide appeared to be ~7 kDa, as expected from the predicted molecular size for TAT-PS1-LP, TAT-PS2-LP or TAT-PS2-SP.

To analyze the ability of these TAT-fusion proteins to penetrate into NRP-154 cells, two independent assays were employed. First, we used immunoblot to detect the presence of TAT-PS2-LP and TAP-PS2-SP inside NRP-154 cells after brief incubation in the culture medium. For this purpose, we have generated a polyclonal antibody against the presenilin-2 loop peptide. This antibody could recognize the synthetic PS2-LP, CTF and the full-length PS2 (not shown). As shown in Fig. 1D, TAT-PS2-LP could efficiently enter NRP-154 cells by 30 min incubation with either 1 µM or 10 µM TAT-PS2-LP. Under these conditions, the cells retained TAT-PS2-LP expression for 3 hours after the fusion protein was washed out of the medium.

Second, we used fluorescence microscopy to detect the presence of FITC conjugated TAT-PS2-LP inside individual NRP-154 cells (Fig. 1E). Clearly, green-fluorescent cells could be observed following 30 min incubation with 5 µM TAT-PS2-LP, revealing the effective membrane penetrating capability of the TAT-PS2-LP peptide into NRP-154 cells.

**RESULTS**

**Expression and purification of TAT fusion proteins and delivery of TAT-PS2-LP into NRP-154 cells** - Previous studies have shown that PS2-LP could be generated via double cleavage of PS-2 by presenilinase and caspase-3 (Fig. 1A) (6,7,11). To test the cellular function of PS2-LP, we attempted to express the PS2-LP peptide by transfection of cDNA plasmids into cultured cells. However, multiple trials have failed to result in detectable expression of PS2-LP in these cells, e.g. NRP-154, Hela, or HEK 293 cells. This is not surprising, because small peptide molecules are frequently vulnerable to degradation by cellular quality control mechanisms. As an alternative approach to test the in vivo function of PS2-LP, we took advantage of the well-established HIV-mediated method for delivery of synthetic peptides into cells (19,20). Extensive studies have shown that an 11-amino acid TAT peptide “YGRKKRRQRRR” can enable peptides of various sizes to penetrate the cell membrane. Recently, Dr. Dowdy’s group developed the bacterial expression vector pHis-TAT for production of recombinant TAT-fusion proteins (18,19), and has kindly provided us with this plasmid. Using the pHis-TAT plasmid, we have generated the following three constructs: TAT-PS2-LP, TAT-PS1-LP and TAT-PS2-SP. The TAT-PS1-LP and TAT-PS2-SP constructs were used for comparative studies and for control purpose (Fig. 1B).

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**TAT-PS2-LP triggers apoptosis in NRP-154 cells** - Continuous monitoring of FITC-labeled NRP-154 cells demonstrated a potent cytotoxic effect of TAT-PS2-LP (Fig. 2A). At 30 min after incubation of 5 µM TAT-PS2-LP with NRP-154 cells, most of the green-fluorescent cells are healthy, as illustrated by the negative staining with PI. By 9 hrs, the majority of green-fluorescent cells were PI positive, illustrating that they were dead or dying. The pronounced cell death phenomenon was not observed in cells incubated with TAT-PS2-SP at either 30 min or 9 hrs incubation times.

Quantitative analysis showed that greater then 90% of FITC-labeled TAT-PS2-LP cells were dead at 9 hrs, whereas less than 10% of FITC-labeled TAT-PS2-SP cells suffered such fate (Fig. 2B). These results indicate that TAP-PS2-LP has a potent cytotoxic activity in NRP-154 cells.

**TAT-PS2-LP causes release of cytochrome c from mitochondria and activation of caspase-3** - To distinguish the nature of TAT-PS2-LP-induced cell death, e.g. apoptosis or necrosis, we performed the following two sets of experiments. First, we examined whether treatment of cells with TAT-PS2-LP could lead to activation of caspase-3, a marker for apoptosis. As shown in Fig. 3A, activation of caspase-3 was observed in NRP-154 cells treated with TAT-PS2-LP (10 µM), but not with TAT-PS2-SP. In addition, 3 hours after washing out the TAT-PS2-LP, the degree of caspase-3 activation was higher compared with that at 30 min, consistent with the cytotoxic effect of TAT-PS2-LP.

Western blot analyses using the polyclonal anti-PS2-LP antibody showed that prolonged incubation of cells with TAT-PS2-LP led to maintenance of the integrity of the peptide inside the cell, since the apparent molecular size of the
TAT-PS2-LP protein did not change, and there were no degradation products detected. For quantitative assay of the caspase-3 enzymatic activity, we used the EnzChek caspase-3 kit purchased from Molecular Probes. As shown in Fig. 3B, pre-treatment of cells with TAT-PS2-LP significantly increased the caspase-3 activity, an effect that was not observed with treatment of cells with TAT-PS2-SP.

Second, we assayed whether TAT-PS2-LP could trigger the release of cytochrome c from mitochondria, another marker for cells undergoing apoptosis. As shown in Fig. 3C, treatment of NRP-154 cells with TAT-PS2-LP induced significant release of cytochrome c from mitochondria into the cytosol. In contrast, TAT-PS2-SP had negligible effect on cytochrome c release as compared to the control. Furthermore, no release of other mitochondrial proteins such as manganese superoxide dimutase (MnSOD) (26) was detectable in the same samples (Fig. 3C).

Together, our data demonstrate that TAT-PS2-LP triggers mitochondria-dependent cascade of cell death in NRP-154 cells.

**TAT-PS2-LP reduces intracellular Ca^{2+} content in NRP-154 cells** - Previous studies (14,31,32) have shown that PS mutations are associated with alterations of intracellular Ca^{2+} homeostasis. Changes in intracellular Ca^{2+} homeostasis have also been linked to the initiation and amplification of apoptosis (33-35). To test whether PS2-LP mediated apoptosis is related to changes in intracellular Ca^{2+} stores, we measured the amount of Ca^{2+} inside the ER, by activating the InsP_{3}-receptor-mediated Ca^{2+} release pathway using 500 µM ATP in the absence of extracellular Ca^{2+}.

Pre-treatment of NRP-154 cells with 10 µM TAT-PS2-LP (30 min) significantly reduced the amount of Ca^{2+} released from the ER by ATP, an effect that was not seen with the TAT-PS2-SP protein (Fig. 4A). Moreover, compared to TAT-PS2-LP, pre-treatment of cells with TAT-PS1-LP did not alter the ER Ca^{2+} storage (Fig. 4B). Since the PS1-LP and PS2-LP do not show conservation in primary amino acid sequence, this result suggests that PS2-LP may have a specific effect on intracellular Ca^{2+} homeostasis. The reduction of ER Ca^{2+} store may reflect two possibilities, either by altering Ca^{2+} uptake or Ca^{2+} release across the ER membrane.

**TAT-PS2-LP interacts with the InsP_{3}R and regulates its channel activity** - To understand the mechanisms underlying the TAT-PS2-LP-mediated changes in [Ca^{2+}], homeostasis, we first examined the possible interactions between TAT-PS2-LP and InsP_{3}R or SERCA2, through co-IP assays. As shown in Fig. 5A, IP of TAT peptides with anti-His antibody could pull-down InsP_{3}R from cells treated with TAT-PS2-LP but not from cells treated with TAT-PS2-SP (left panel); and IP with anti-Insp_{3}R antibody could pull-down TAT-PS2-LP but not TAT-PS2-SP (right panel). This interaction appears to be specific since we did not observe any interaction between TAT-PS2-LP and SERCA2 (lower panel). These results indicate that TAT-PS2-LP can interact with Insp_{3}R, but not with the SERCA2, raising the possibility that PS2-LP may affect the activity of Insp_{3}R.

We next explored the functional consequences of the interaction of TAT-PS2-LP with the Insp_{3}R, by performing patch clamp studies of endogenous Insp_{3}R channels in their native membrane environment (28,29,36) (Fig. 5B). The pipette solution contained 1 µM free Ca^{2+} and 33 nM Insp_{3}, agonist conditions that activate the channel sub-maximally (open probability, P_{o} = 0.15 ± 0.04). As shown in Fig. 5B, addition of 0.5 µM or 2.5 µM TAT-PS2-LP to the pipette solution enhanced P_{o} by ~100% (P_{o} = 0.25 ± 0.04 and 0.39 ± 0.04, respectively; n = 5 each). In contrast, 0.5 µM or 2.5 µM TAT-PS2-SP had no significant effects on channel P_{o} (n = 5). Kinetic analyses demonstrated that stimulation of channel P_{o} by TAT-PS2-LP was associated with reduced channel closed time, whereas channel open time was not affected (Fig. 5B). The number of channels activated by Insp_{3} (N_{A}) was also enhanced in the presence of TAT-PS2-LP (Fig. 5B). The product N_{A}P_{o}, a measure of the total Insp_{3}-mediated flux, was enhanced by nearly 3-fold and over 4-fold by 0.5 µM or 2.5 µM TAT-PS2-LP, respectively (Fig. 5B).

These data demonstrate that the interaction of TAT-PS2-LP with the Insp_{3}R leads to a marked activation of channel gating in the presence of sub-saturating concentrations of Insp_{3}. These results are consistent with our findings that TAT-PS2-LP decreases the ER Ca^{2+} store.

**DISCUSSION**

In the present study, we have used the TAT protein-penetrating transduction system to investigate the possible involvement of PS2-LP in cell apoptosis and [Ca^{2+}], homeostasis. The advantage of this system results from the fact that it is technically challenging to use DNA transfection to introduce small peptides into living cells (37,38), as these peptides are often
unstable and susceptible to cellular degradation. TAT-mediated protein transduction occurs in a rapid, concentration-dependent fashion that is independent of receptors and transporters. This technology has been used to introduce proteins ranging in size from 15 to 120 kDa into a wide variety of human and murine cells (38). To determine whether this method could be used to deliver PS2-LP into cultured cells, we generated the recombinant TAT-PS2-LP fusion protein, plus the necessary control constructs. We found that transduction of TAT-PS2-LP, but not TAT-PS2-SP, into NRP-154 cells, induced the release of cytochrome c from mitochondria, increased the activity of caspase-3, and triggered cell apoptosis. We also observed that TAT-PS2-LP-treated cells exhibited significantly reduced Ca2+ storage inside the ER, but TAT-PS1-LP and TAT-PS2-SP did not exhibit such effect. TAT-PS2-LP interacts with the InsP3R and increases its Ca2+ release channel activity. These results provide the first physiological evidence for PS2-LP in regulating intracellular Ca2+ homeostasis and cell apoptosis.

A number of previous studies have explored the role of PS2 and its derivatives in cell apoptosis. For example, PS2 has been shown to function as a pro-apoptotic effector (39-44). Over-expression of the CTF of PS-2 has been shown to increase Aβ recovery and to decrease cell viability, by augmenting caspase-3 activity (45). Our results showed that PS2-LP, as a by-product of caspase-3 cleavage of CTF, could act as a potent amplifier of apoptosis.

Previous studies have suggested that PS1 and PS2 also play different roles in cell death. Specifically, overexpression of PS2 in PC12 cells led to increased cell death in response to a variety of apoptotic stimuli (46,47). Expression of a PS2 mutation associated with AD led to increased levels of apoptosis at both basal and stimulated conditions (48). On the other hand, the role of PS1 in cell death is less clear. Overexpression of L286V PS1 mutant but not wild-type PS1 resulted in increased susceptibility to cell death induced by trophic factor withdrawal and Ab-mediated neurotoxicity (49), whereas a separate study showed that overexpression of A246E mutant in PS1 did not enhance apoptosis (50). These studies are consistent with our data indicating that PS2-LP, but not PS1-LP, could perturb intracellular Ca2+ homeostasis and accelerate cell death.

In summary, our data demonstrate that PS2-LP, as a by-product of apoptosis, can provide a potent feed-forward mechanism to accelerate the apoptosis process. Since altered intracellular Ca2+ homeostasis is associated with the cellular function of PS2-LP, we suspect that this small peptide molecule may play important roles in the overall cell growth and death processes. Targeting PS2-LP may thus offer an attractive therapeutic approach for cancer-related human diseases.

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FOOTNOTES

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The abbreviations used are: Aβ, amyloid-β precursor protein; ER, endoplasmic reticulum; AD, Alzheimer’s disease; FITC, Fluorescein isothiocyanate; InsP3R, inositol 1,4,5-trisphosphate receptor; IP, immunoprecipitation; PI, propidium iodide; PS2-LP, presenilin-2 loop peptide; PS2-SP, presenilin-2 scrambled loop peptide; PS1-LP, presenilin-1 loop peptide; SERCA, sarco(endo)plasmic reticulum Ca2+-ATPase.

FIGURE LEGENDS

Fig. 1. Purification from E. coli and delivery of TAT-PS2-LP into NRP-154 cells. A. Schematic diagram illustrates that PS2-LP is generated from presenilin-2 via double cleavage by presenilinase and caspase-3. B. A membrane penetrating peptide, TAT, was fused to the amino-terminal end of PS1-LP and PS2-LP, to facilitate the delivery of fusion proteins into cells. As a control, TAT-PS2-SP fusion protein containing the scrambled PS2-LP peptide was also generated. C. Using a modified TAT-fusion protein purification method, large amounts of TAT-PS2-LP, and TAT-PS2-SP, could be expressed and purified from E. coli. Highly purified proteins were separated on a 4-12% SDS-PAGE. For unknown reason, the yield of TAT-PS1-LP is low. D. Penetration of TAT-PS2-LP into NRP-154 cells was analyzed by immunoblot using our custom-made anti-PS2-LP antibody. 30 min of incubation of cells with either 1 µM or 10 µM TAT-PS2-LP led to detectable amount of TAT-PS2-LP in the cell lysate. The TAT-PS2-LP protein is retained inside the NRP-154 cells at 3 hrs after washout of the peptide from the culture medium. β-actin was used as loading control. The Western blot is representative of three other experiments. E. Confocal microscopic image revealed accumulation of FITC-labeled TAT-PS2-LP protein inside the NRP-154 cells (right panel). The picture was taken at 30 min after incubation of 5 µM FITC labeled TAT-PS2-LP with NRP-154 cells.

Fig. 2. TAT-PS2-LP displays potent cyto-toxic effect in NRP-154 cells. A. PI staining was used as an assay of cell death. FITC fluorescence measures the presence of TAT-PS2-LP and TAT-PS2-SP inside NRP-154 cells. Thirty minutes after incubation of NRP-154 cells with 5 µM TAT-PS2-LP or TAT-PS2-SP, FITC positive cells are all healthy, as indicated by the absence of PI staining (top panels). 9 hrs later, majority of the TAT-PS2-LP
labeled cells became positive for PI staining, whereas TAT-PS2-SP labeled cells remained negative for PI staining. The images were representative of 12 other experiments. B. Statistical result for cell survival after treatment with TAT-PS2-LP or TAT-PS2-SP at indicated time points.

**Fig. 3. TAT-PS2-LP causes release of cytochrome c from mitochondria and activation of caspase-3.** A. Activation of caspase-3 was observed in NRP-154 cells treated with TAT-PS2-LP (10 µM), but not with TAT-PS2-SP. Notice that 3 hrs after washout of TAT-PS2-LP, the degree of caspase-3 activation is higher compared with that at 30 min, even though the intracellular content of TAT-PS2-LP declined with time. Western blot with anti-PS2-LP was performed with a polyclonal antibody against the synthetic PS2-LP peptide. B. Quantitative assay for caspase-3 enzymatic activity was performed using the EnzChek kit (see Materials and Methods). Incubation of cells with TAT-PS2-LP (10 µM, 3 hrs) led to significant activation of caspase-3, an effect that was not observed with TAT-PS2-SP. C. Treatment of NRP-154 cells with TAT-PS2-LP induces significant release of cytochrome c from mitochondria into the cytosol. Compared with the control, TAT-PS2-SP is less effective in triggering cytochrome c release. β-actin and MnSOD were used as loading control for cytosol and mitochondrial pellet, respectively. The Western blots were representative of three other experiments.

**Fig. 4. TAT-PS2-LP reduces intracellular Ca²⁺ content in NRP-154 cells.** A. Representative traces for Ca²⁺ measurement. To measure the amount of Ca²⁺ inside the ER, 500 µM ATP was used to activate the InsP₃-receptor-mediated Ca²⁺ release pathway (Control). Pretreatment of NRP-154 cells with 10 µM TAT-PS2-LP (30 min) significantly reduced the amount of ATP-induced Ca²⁺ release (+PS2-LP), an effect that was not seen with TAT-PS2-SP (+PS2-SP). In contrast, pretreatment of cells with TAT-PS1-LP did not alter ER Ca²⁺ storage (+PS1-LP). B. Statistical result for ATP-induced release of Ca²⁺ from ER, in NRP-154 cells, and after treatment TAT-PS2-SP, TAT-PS2-LP and TAT-PS1-LP. * indicates p <0.01.

**Fig. 5. Functional interaction between TAT-PS2-LP and the InsP₃R.** A. Co-IP of TAT-PS2-SP, TAT-PS2-LP and InsP₃R from NRP-154 cells transduced with 10 µM TAT-PS2-SP and TAT-PS2-LP for 30 min at 37°C. Left panel represents IP with anti-His-tag antibody, Right panel represents IP with anti-InsP₃R antibody. Whole cell lysates were used as positive control. IP with pre-immune IgG from rabbit was used as negative control. Lower panel illustrates lack of interaction between PS2-LP and SERCA2. B. Representative single channel current traces of InsP₃R obtained from patch clamp recording of isolated Sf9 nuclei. Holding potential was +20 mV. Arrows indicate closed state of the channel. The pipette solution contained 1 µM Ca²⁺ and 33 nM InsP₃ and no PS2-LP peptide (control), 0.5 µM or 2.5 µM TAT-PS2-LP, or 0.5 µM or 2.5 µM TAT-PS2-SP. Averaged data from multiple experiments (n = 5) with mean open probability (Pₒ), mean channel open time (τₒ) and mean channel closed time (τc), number of activated channels (Nₐ), and the product NₐPₒ, a measure of the total InsP₃-activated ER flux, are shown. * p < 0.01.
Figure 1

A

Presenilinase → Caspase-3

PS2-LP

B

YGRKKRRQRRR

6×His

TAT

PS2-LP

PS2-SP

PS1-LP

C

Marker PS2-SP PS2-LP PS1-LP

D

1 μM 10 μM

30 min 3 hr 30 min 3 hr

PS2-LP

β-actin

E
Figure 2

A  
| Transmission | FITC | PI staining |
|--------------|------|-------------|
| PS2-SP       |      |             |
| PS2-LP       |      |             |

30 min

9 hr

B

Cell survival percentage (%) vs. Time (hours)

- + PS2-SP
- + PS2-LP
Figure 3

A

|       | 30 min | 3 h     |
|-------|--------|---------|
|       | PS2-SP | PS2-LP  |
|       | PS2-SP | PS2-LP  |

- Caspase-3 (Cleaved)
- Anti-PS2-LP
- Anti-His
- Actin

B

- Caspase-3 activity
  - Control
  - +PS2-SP
  - +PS2-LP
  
- n = 6

C

- Cytosol
  - Control
  - +PS2-SP
  - +PS2-LP

- Mitochondria
  - Control
  - +PS2-SP
  - +PS2-LP

- Cyto C
- β-actin
- MnSOD
Figure 4

A

Control
+PS2-SP
+PS1-LP
+PS2-LP
ATP

B

| Group          | n  | ΔF (F₃₄₀/F₃₈₀) |
|----------------|----|----------------|
| Control        | 20 | 0.8 ± 0.1      |
| +PS2-SP        | 20 | 1.2 ± 0.2      |
| +PS2-LP        | 8  | 0.4 ± 0.1      |
| +PS1-LP        | 8  | 0.8 ± 0.1      |

* denotes significance compared to control.
Figure 5

A

| Lysate | IgG | +PS2-SP | +PS2-LP |
|--------|-----|---------|---------|
| α-His             | α-His             |
| α-IP₃R           | α-IP₃R           |

B

Control

Control

PS2-SP (0.5 µM)

PS2-SP (2.5 µM)

PS2-LP (0.5 µM)

PS2-LP (2.5 µM)

$P_o$

$\tau_o$

$\tau_c$

$N_A$

$N_A P_o$
The presenilin-2 loop peptide perturbs intracellular Ca\textsuperscript{2+} homeostasis and accelerates apoptosis

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