Vesicle-associated Membrane Protein of Arabidopsis Suppresses Bax-induced Apoptosis in Yeast Downstream of Oxidative Burst*

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Programmed cell death (PCD) in many systems is controlled by relative amounts of the apoptosis-regulating proteins Bax and Bcl-2 through homo- or heterodimerization. Here we show that Bax-induced PCD of yeast was suppressed by transformation with a vesicle-associated membrane protein from Arabidopsis (AtVAMP), which was isolated by screening a cDNA expression library against sugar-induced cell death in yeast. AtVAMP expression blocked Bax-induced PCD downstream of oxidative burst. AtVAMP also prevented H2O2-induced apoptosis in yeast and in Arabidopsis cells. Reduced oxidation of lipids and plasma membrane proteins was detected in the AtVAMP-transformed yeast, suggesting improved membrane repair. Inhibition of intracellular vesicle trafficking by brefeldin A induced apoptosis from a sublethal concentration of H2O2. No protection occurred by overexpression of the yeast homolog SCN2. However, efficient suppression of yeast PCD occurred by expression of a chimeric gene, composed of the conserved domains from yeast, fused to the variable N-terminal domain from Arabidopsis, resulting in exchange of the proline-rich N-terminal domain of SCN2 with a proline-poor Arabidopsis sequence. Our results suggest that intracellular vesicle traffic can regulate execution of apoptosis by affecting the rate of membrane recycling and that the proline-rich N-terminal domain of VAMP inhibited this process.

Programmed cell death is a major form of cell death in many organisms that is regulated by a genetically conserved program. PCD plays important roles in many diverse processes from development to stress responses. The function of PCD ranges from organismal sculpting by elimination of specific tissue areas (e.g. interdigit spaces in animals or xylem formation in plants) to removal of sublethally stressed cells. PCD is manifested by characteristic morphological and biochemical features (1). Although considerable differences in the mechanism of PCD exist among the kingdoms, recent studies show that many basic hallmarks of apoptosis are conserved among animal and plant cells (2, 3) and even in unicellular organisms, such as dinoflagellates (4) and yeast (5). Expression of a pro-apoptotic mammalian protein Bax in yeast or in plants, which lack the counteracting Bcl-2 gene, induced PCD with characteristics of apoptosis, such as nuclear condensation and DNA fragmentation and exposure of phosphatidylserine (5–7).

Yeasts are particularly suited for screening of heterologous genes by rescue from death-inducing stimuli (1), and a number of genes that suppress yeast cell death have been isolated from animal and plant cells (8). Recently, a homologue of glutathione S-transferase from tomato was isolated by screening yeast against Bax-induced PCD, which suppressed cell death by restoring mitochondrial function (9). In yeast, apoptosis can also be caused by sugar, a phenomenon termed sugar-induced cell death, whereby yeast cell death is triggered by transfer of resting (nondividing) cells from water to medium containing metabolized sugars but without a nitrogen source (10). We have screened an expression library of Arabidopsis cDNA (11) for genes that suppress sugar-induced cell death in yeast. Several genes were isolated, one of which had high homology to the family of eukaryotic synaptobrevins or VAMPs (vesicle-associated membrane protein), which belong to the v-SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors). Comparison with the Arabidopsis genome data base showed that the isolated gene corresponded to GenBank accession number AF025332 but was missing the first 74 amino acids. The highest homology of the isolated sequence (ΔNAtVAMP) was to VAMP7 gene from rat (41% identity and 64% similarity) and was therefore termed ΔNAtVAMP.

VAMPs, which are conserved from yeast to mammals, constitute the major vesicle-associated component and play an important role in vesicle docking through interaction with their counterpart t-SNAREs in the target membranes. The SNAREs, particularly VAMPs and syntaxins, are specifically localized throughout the secretory pathways, including the endoplasmic reticulum, the Golgi apparatus, the early endosomes, and the plasma membrane (12). Both v- and t-SNAREs contain α-helical coiled-coil domains that carry out the interaction between them. The target specificity of the vesicle is determined by the different SNAREs together with an associated family of Rab proteins (13). The VAMPs/synaptobrevins are composed of highly conserved C-terminal regions, which contain α-helix coils that participate in the v-SNARE binding to t-SNARE in the target membrane, and of a variable proline-rich N-terminal region that has been considered to be either dispensable or to exert an inhibitory effect on vesicle fusion (14, 15).

In eukaryotic cells, vesicle trafficking is responsible for the delivery of proteins to organelles as well as membrane recycling, both of which are crucial processes for normal cell function. Membrane recycling is particularly important during oxidative stress, which causes damage to membrane compo-
Experimental Procedures

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Arabidopsis thaliana cell cultures, originally isolated by May and Leaver (24), were kept at 25 °C under constant light in Murashige and Skoog medium, pH 6.0, supplemented with 100 mg/liter myo-inositol, 0.4 mg/liter thiamine, 0.5 mg/liter α-naphthalenacetic acid, 0.05 mg/liter kinetin, and 3% sucrose. Cells were treated with H2O2 in 12-well culture dishes. Bax expression was confirmed by Western blotting (23) and by reisolation of the Bax clone and transformation of yeast cells. Yeast transformations were done by electroporation, and colonies were selected by plating on selective media. ROS were determined as described previously (5) except that cultures were incubated with the dye for 30 min.

RESULTS AND DISCUSSION

In animals, it was found that PCD is often controlled by relative amounts of the apoptosis-regulating proteins Bax and Bcl-2 through homo- or heterodimerization (19). An excess of Bax promotes apoptosis, while Bcl-2 suppresses it. Expression of Bax in yeast, which lacks Bcl-2, induces apoptosis via generation of oxidative stress (5, 21). To see whether in addition to suppressing sugar-induced cell death, the isolated plant gene (∆NAtVAMP7), could block Bax-induced apoptosis, yeast cells were transformed with Bax driven by a galactose-regulated promoter (Gal-Bax) and with ∆NAtVAMP7 driven by a constitutive (PGK) promoter. The double (Bax + ∆NAtVAMP7) transformants and the individual (∆NAtVAMP7 or Bax) transformants were inoculated into glucose- or galactose-containing media. All cells grew on glucose, but when transferred to galactose only the ∆NAtVAMP7-containing cells continued to grow (Fig. 1B).

Idential results were also obtained by replacing the isolated truncated ∆NAtVAMP7 with the full-length Arabidopsis gene, which included the missing 74 N-terminal amino acids (AtVAMP7). However, no protection was observed by overexpression of the yeast homolog SNC2 gene. To determine the region responsible for protection against Bax-induced apoptosis we constructed a chimeric gene in which the first 31 amino acids of SNC2 were exchanged with the remaining 63 N-terminal amino acids of the variable domain from Arabidopsis. This resulted in a gene that contained the conserved domains from yeast fused to the variable N-terminal domain from Arabidopsis (Fig. 1C). The conservative domain contains many α-helical domains and is thought to participate in protein-protein interactions during vesicle docking (14, 28), while the variable N-terminal region was shown to facilitate the repair of membranes (5, 11). Protein oxidation was assayed by the carbonyl method as described previously (27).
inducible GAL-Bax (Bax) or with GAL-Bax together with a constitutively expressed NAtVAMP7 (AtVAMP7) plasmid. A, growth of transformed yeast cells on glucose- and galactose-based media. Transformed cells were grown in glucose for 18 h, washed in sterile water, and resuspended in glucose- or galactose-supplemented media. Growth was estimated by measuring culture absorbance at 600 nm. Since growth rate is dependent on the medium composition, the results show growth relative to yeast cells transformed with the control plasmid pFL61 on glucose or galactose, respectively. B, the organization of the VAMP7 gene from Arabidopsis (white bar) and its homolog SNC2 gene from yeast (shaded bar). The swapped regions are indicated by their respective colors. Numbers indicate the amino acid residues. The black bar in the AIVAMP7 correlates to the amino acids that were missing from the originally isolated NAtVAMP7 gene. C, transformation of cells that carried Bax under the control of a galactose-dependent promoter (pYES2-Bax) with different VAMP constructs. Transformed cells were plated on glucose (left plate) or galactose (right plate). The clones are arranged in a clockwise direction in the following order (starting at the one o’clock position): AtVAMP7, NAtVAMP7, AtVAMP-SNC2, SNC2, empty vector (pRS424), pYES2-Bax (GAL-Bax), glu, glucose; gal, galactose.

Fig. 1. Rescue of Bax-induced cell death by ectopic expression of Arabidopsis VAMP. Yeast cells were transformed with a galactose-inducible GAL-Bax (Bax) or with GAL-Bax together with a constitutively expressed NAtVAMP7 (Bax+At/VAMP) plasmid. A, growth of transformed yeast cells on glucose- and galactose-based media. Transformed cells were grown in glucose for 18 h, washed in sterile water, and resuspended in glucose- or galactose-supplemented media. Growth was estimated by measuring culture absorbance at 600 nm. Since growth rate is dependent on the medium composition, the results show growth relative to yeast cells transformed with the control plasmid pFL61 on glucose or galactose, respectively. B, the organization of the VAMP7 gene from Arabidopsis (white bar) and its homolog SNC2 gene from yeast (shaded bar). The swapped regions are indicated by their respective colors. Numbers indicate the amino acid residues. The black bar in the AIVAMP7 corresponds to the amino acids that were missing from the originally isolated NAtVAMP7 gene. C, transformation of cells that carried Bax under the control of a galactose-dependent promoter (pYES2-Bax) with different VAMP constructs. Transformed cells were plated on glucose (left plate) or galactose (right plate). The clones are arranged in a clockwise direction in the following order (starting at the one o’clock position): AtVAMP7, NAtVAMP7, AtVAMP-SNC2, SNC2, empty vector (pRS424), pYES2-Bax (GAL-Bax), glu, glucose; gal, galactose.

Importantly, while in the yeast SNC2 gene there are 5 prolines in the 32 residues of the variable region (4 of them within a single stretch of 8 amino acids), in the whole variable domain of the full-length Arabidopsis VAMP, there are 3 prolines within 130 amino acids (with none of them located close to each other).

The presence of Bax in the colonies was confirmed by restriction analysis of plasmids recovered from individual clones and grown in Escherichia coli (Fig. 2A). The recovered Bax-encoding plasmids were reintroduced into yeast cells. Colonies, which failed to grow on galactose-containing medium, were obtained on glucose plates (data not shown). Expression of Bax in the double (Bax+ΔNAtVAMP7) transformants was verified by Western blotting, which showed exclusive expression in the presence of galactose (Fig. 2B). To see whether Bax expression resulted in cell death or merely stopped yeast growth, 5-μl aliquots of culture were removed after 6 and 12 h and plated on a glucose-containing plate in sequential dilutions. Co-transformation with ΔNAtVAMP7 or with AtVAMP7 (not shown) rescued the extensive death observed in cells transformed with Bax alone. In cultures transformed with both plasmids colonies were established even after 10,000-fold dilution. This is in contrast to cultures that expressed only Bax, in which by 12 h the number of formed colonies was reduced already after 10–100-fold dilution, and almost no cells were rescued after 1,000-fold dilution (Fig. 2C).

Production of ROS has been demonstrated as one of the critical steps in many types of apoptosis (17), although in certain systems apoptosis occurs in the absence of ROS (31). In yeast, ROS generation was shown to have a key role in Bax-induced apoptosis (5). We were therefore interested to find out whether ectopic expression of AtVAMP7 interfered with the PCD process before or after ROS generation. To this end, yeast cells were transformed with Bax alone or with Bax together with ΔNAtVAMP7 and grown in a glucose-based medium to a mid-log phase, after which they were transferred to a galactose-based medium. ROS production was examined 6 and 12 h after changing the medium (5). No ROS were detected in cells in glucose-based medium, but substantial ROS accumulation was observed in Bax-producing cells, regardless of ΔNAtVAMP7 expression (Fig. 3A). The amount of ROS produced by yeast cells that were transformed with Bax alone was only slightly higher than by cells expressing both Bax and ΔNAtVAMP7 as determined by fluorescence measurements of an equal number of cells in a spectrofluorometer (Fig. 3B) or by integration of the signal intensity of the digital image within individual cells (data not shown). ROS induction was not a consequence of yeast transfer into the galactose-based medium since the cells transformed with a control plasmid (pFL61) did...
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Fig. 3. ROS production in yeast transformed with GAL-Bax alone (Bax) or together with pFL-ΔNAtVAMP7 (Bax+ΔVAMP). 

A. ROS accumulation was assessed 12 h after resuspension of cells in galactose (Gal) or glucose (Glu)-based media by staining the cultures with 2,7-dihydrodichlorofluorescein (5). Cells were photographed under fluorescent or bright field conditions. Representative fields are shown.

B. Quantitative measurements of ROS accumulation in Bax-expressing transformants. Yeast cells were harvested after 12 h, adjusted to an equal concentration of cells (5 × 10^6), and stained with 2,7-dihydrodichlorofluorescein for 30 min. ROS-dependent fluorescence was measured in a fluorometer.

not accumulate ROS after transfer to galactose.

The above results indicated that the protection provided by ΔNAtVAMP7 occurred downstream of Bax-induced oxidative stress. We therefore examined the ability of ΔNAtVAMP7 to protect yeast from oxidative stress produced by the direct addition of H_2O_2. Earlier work, which was reproduced by us, showed that 3–5 mM H_2O_2 induced a typical PCD in yeast (5). When compared with control cells, the ΔNAtVAMP7 transformants grew much better after treatment with 3 mM H_2O_2 (Fig. 4A). No protection was observed from a necrogenic dose of 9 mM H_2O_2. The increased viability of the ΔNAtVAMP7 transformants was confirmed by rescue-plating cells into fresh medium. Aliquots were removed 12 h after H_2O_2 treatment and plated on agar in a series of 10-fold dilutions. Cells that were transformed with the control vector produced a small number of colonies after the first round of dilution, and no colonies were seen after 100-fold dilution, indicating extensive cell death in the culture exposed to 3 mM H_2O_2 (Fig. 4B). ΔNAtVAMP7 transformants, however, formed a substantial amount of colonies after a 100-fold dilution, and a small number of colonies was seen even after a 1,000-fold dilution. Since VAMP is not expected to directly affect ROS detoxification, these results indicate that the ectopic expression of ΔNAtVAMP7 succeeded in interfering with the execution of ROS-induced PCD.

Protection against oxidative stress-induced PCD by ectopic expression of AtVAMP7 was also tested in plant cells. To this end, AtVAMP7 was cloned into a binary expression vector under the control of a constitutively expressed cauliflower mosaic virus (CaMV) 35S promoter and introduced into Arabidopsis cells via Agrobacterium tumefaciens-mediated transformation (26, 32). Expression of the gene was verified by Northern blotting (Fig. 4C, inset). The cell culture was treated with H_2O_2 at a concentration that induces PCD (20, 33). The ectopic expression of AtVAMP7 reduced cell death from 76% in cultures transformed with a control vector to 46%, indicating efficient suppression of PCD in the homologous system as well (Fig. 4C).

The actual protection was even higher since the procedure of A. tumefaciens-mediated transformation of suspension-cultured plant cells results in transient expression of the transgene in 60–70% of cells (25, 26).

Since VAMPs are known to function in intracellular vesicle transport (14, 34), we examined the involvement of vesicle trafficking in H_2O_2-induced PCD. Intracellular vesicular traffic was blocked with a specific inhibitor, brefeldin A (BFA), which causes reversible dissolution of the Golgi complex in many cells, including yeast (35). Yeast cells were treated with a sublethal dose of H_2O_2 (see Fig. 4) in the presence or absence of BFA. Pretreatment of culture with BFA followed by the addition of a mild dose of 1.5 mM H_2O_2 strongly reduced yeast growth (Fig. 5A). The growth reduction was associated with increased cell death as evaluated by parallel measurements of propidium iodide staining, which tests the plasma membrane integrity (Fig. 5B). The collapse of plasma membrane occurs late in the apoptotic program and constitutes the terminal stage of PCD (36, 37). Overexpression of ΔNAtVAMP7 diminished the effect of BFA, supporting the involvement of vesicular trafficking in yeast cell death and implying that AtVAMP7 and BFA operate in the same pathway. The ΔNAtVAMP7 transformants also showed improved survival when assayed by culture dilution and colony growth on plates (Fig. 5C).

The effects of ΔNAtVAMP7 and BFA implied that the vesicle trafficking system may be involved in the execution of yeast PCD. One possible mechanism for the anti-PCD mode of action of AtVAMP could be increased repair of oxidatively damaged membranes, which is crucial for cell viability. VAMP7 was indeed shown to function in vesicle fusion to the plasma membrane (38).

To examine the changes in the plasma membrane condition during oxidative stress, we analyzed lipid and protein oxidation in control and ΔNAtVAMP7-expressing cells. Both cultures were treated with a PCD-inducing dose of 5 mM H_2O_2, and the amount of lipid hydroperoxides was analyzed 2 h later, which is ~4 h before the first cells begin to show loss of membrane integrity (Ref. 5 and our own results). ΔNAtVAMP7 expression reduced the amount of lipid hydroperoxides by about 2.5-fold (Fig. 6A). ΔNAtVAMP7 also reduced the degree of membrane protein oxidation after 5, 30, and 120 min of H_2O_2 treatment. While in control cells protein oxidation increased during this period, due to the slow clearance of H_2O_2 from the culture (the half-life of 1 mM H_2O_2 in the yeast cultures was measured to be 30 min), in the ΔNAtVAMP7 transformants the protein oxidation was lower, and the level decreased further during the incubation period (Fig. 6B). These results are in agreement with the suppression of yeast PCD from Bax- or H_2O_2-triggered death by improved repair of oxidative damage by overexpressing a tomato glutathione S-transferase homologue (9).

To study the effect of ΔNAtVAMP7 expression on the exchange of plasma membrane proteins we analyzed changes in protein trafficking to the plasma membrane. Newly synthesized proteins were labeled with a radioactive mixture of [35S]cysteine and [35S]methionine that was added together with a PCD-inducing dose of H_2O_2 for 2 h. Samples were normalized according to equal label incorporation, and the membrane proteins were separated from the cytosol by first incubating the protoplasts with N-hydroxysuccinimide-biotin and then collecting the biotin-coupled proteins with avidin-coated beads. In the control cells H_2O_2 treatment almost completely stopped the incorporation of new proteins into the plasma membrane, but in the ΔNAtVAMP7 transformants protein export was essentially unaffected (Fig. 7A). The greatly diminished incorporation of the label into the plasma membrane was not a consequence of reduced protein synthesis as indicated by equal labeling of the cytosolic proteins (Fig. 7B).

In eukaryotic cells, proteins are transported to organelles by a series of membrane transport steps, which are regulated by specific interactions between syntaxins and v-SNAREs. Interestingly, VAMP7 has been implicated in vesicle trafficking specifically to the apical plasma membrane of epithelial cells.
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Fig. 4. Protection from H$_2$O$_2$-induced PCD by expression of ΔNAtnVAMP7. A, yeast cells transformed with ΔNAtnVAMP7 or with the control vector were grown on glucose and treated with 1, 3, or 9 mM H$_2$O$_2$. Culture growth (expressed as percentage of growth relative to untreated culture) was measured 21 h later. In the culture treated with 9 mM H$_2$O$_2$ growth was negative due to cell death. B, rescue analysis. Cells transformed with ΔNAtnVAMP7 or with the control vector were treated with 3 mM H$_2$O$_2$ in a glucose-based medium. After 12 h 5-μl aliquots were withdrawn and plated on agar for another 21 h at serial 10-fold dilutions (numbers on top). C, Arabidopsis cells were transformed with A. tumefaciens-carrying plasmids expressing the AtVAMP7 or β-glucuronidase (pBI121, control) genes under the control of cauliflower mosaic virus (CaMV) 35S promoter. Cultures were treated with 8 mM H$_2$O$_2$, and cell death was assayed 18 h later by Evans blue staining. 100% cell death was estimated from parallel cultures after boiling for 5 min. In untreated cultures the cell death was 5% as estimated from the microscopic analysis of Evan’s blue-positive cells. Inset, Northern blotting of RNA from transformed cultures. Total RNA (40 μg) was prepared 48 h after transformation and probed with the AtVAMP7 gene.

Fig. 5. Potentiation of oxidative stress-induced PCD by inhibitor of vesicle transport, brefeldin A. A, yeast cells were preincubated with 1 μg/ml BFA (white bars) or with water (black bars) for 30 min and then treated with 1.5 mM H$_2$O$_2$. Growth was measured by the increase in culture absorbance after 18 h. B, H$_2$O$_2$-induced cell death. Control and ΔNAtnVAMP7-transformed cells were treated as in A, and cell death was assayed by propidium iodide staining. The degree of cell death was analyzed by fluorescent microscopy (100 cells, n = 5) and also measured in a fluorometer (not shown) with similar results. 15 mM H$_2$O$_2$ was used for a positive control, which produced >95% dead cells. C, cell viability of control and ΔNAtnVAMP7-transformed cells was assayed by removing 5-μl aliquots from the cultures treated as in A for 12 h after H$_2$O$_2$ addition and plating on selective media. Numbers on top show 10-fold serial dilutions.

Fig. 6. The effect of ΔNAtnVAMP7 on H$_2$O$_2$-dependent membrane damage. Control and ΔNAtnVAMP7-transformed yeast cells were treated with 5 mM H$_2$O$_2$. A, lipid hydroperoxides were assayed 2 h after treatment. B, membrane protein oxidation was assayed 5, 30, and 120 min after H$_2$O$_2$ addition. (39).

Fig. 7. Incorporation of new proteins into plasma membrane. Yeast protoplasts were treated with 5 mM H$_2$O$_2$ together with 35S-labeled cysteine and methionine for 2 h. After washing, the samples were adjusted to contain an equal amount of radioactivity and were processed in parallel. The membrane proteins were isolated by first incubating the intact protoplasts with N-hydroxysuccinimide-biotin followed by binding to avidin beads. After washing, the samples were separated by SDS-polyacrylamide gel electrophoresis and subjected to autoradiography. A shows the plasma membrane proteins released from the avidin beads by boiling in a denaturing sample buffer, and B shows the unbound (cytosolic) proteins synthesized during the incubation period.

antibodies suggested that VAMP7 is important for membrane trafficking events (12). In light of the above observations, the increased protein delivery to the plasma membrane (Fig. 7) strongly suggests that AtVAMP7 assisted plasma membrane repair.

Very little data exists in the literature on the link between vesicle traffic and apoptosis. Caspase-mediated cleavage of kinectin, which is the membrane anchor for microtubule-based vesicle movement, is a general feature of tumor necrosis factor-α, Fas-, or radiation-induced apoptosis in human cells and results in disruption of membrane trafficking (41). It has also been shown that perturbations in vesicular traffic could affect capacitative calcium entry, resulting in apoptosis (42). Inhibition of vesicle fusions to membranes by the inactivation of an essential component of endosome fusion, Rabaptin-5, has been reported in several models of apoptosis (43). Moreover, it was shown that Rabaptin-5 is cleaved specifically by caspase-3, which causes a subsequent decrease in endosome fusion, thus directly affecting the membrane recycling (44). Bel-2 expression prevented the cleavage of Rabaptin-5 and the inhibition of endosome fusion as well as the subsequent apoptosis, suggesting that membrane recycling is involved in the late stages of apoptosis (43).

Interestingly, a mutant yeast strain, KFY437, that is deficient in the CDC48 gene, which controls the homotypic fusion
of endoplasmic reticulum-derived vesicles, exhibits a spontaneous apoptotic phenotype, which is partially suppressed by ROS scavengers (45). Spontaneous apoptosis also occurs in Drosophila mutants in the small-minded (smid) gene, which is the animal homolog of CDC48 (46). Severe lethality of vesicle trafficking mutants also occurs in A. thaliana, which possesses an especially large arsenal of v- and t-SNAREs (47). These observations are in line with the results presented here and suggest that the intracellular vesicle trafficking is closely connected with PCD. Hence, perturbations in trafficking can induce apoptosis, while enhanced trafficking can inhibit apoptosis in an indirect way by increased membrane recycling.

In summary, our results show that apoptosis induced by Bax or by oxidative stress is a dynamic process during which there is a progressive deterioration of membrane integrity, leading to apoptosis, that could be prevented by improved recycling of the membrane components through overexpression of one of the vesicle docking proteins. Since oxidative stress is closely associated with many signaling events, including the induction of apoptosis in many systems (16, 48, 49), suppression of PCD by a molecule not directly involved with ROS detoxification offers an attractive tool for halting apoptosis.

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