Abrin A-chain (ABRA) inhibits protein synthesis by its N-glycosidase activity as well as induces apoptosis, but the molecular mechanism of ABRA-induced cell death has been obscure. Using an ABRA mutant that lacks N-glycosidase activity as bait in a yeast two-hybrid system, a 30-kDa antioxidant protein-1 (AOP-1) was found to be an ABRA(E164Q)-interacting protein. The interaction was further confirmed in vitro by a glutathione S-transferase pull-down assay. The colocalization of endogenous AOP-1 and exogenous ABRA proteins in the cell was demonstrated by confocal immunofluorescence. We also demonstrated that ABRA attenuates AOP-1 antioxidant activity in a dose-dependent manner and the intracellular level of reactive oxygen species (ROS) increases in ABR-treated cells. Moreover, ROS scavengers N-acetylcysteine and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl delayed programmed cell death. This indicates that ROS are important mediators of ABRA-induced apoptosis. When ectopically expressed, AOP-1 blocked the release of cytochrome c and prevented apoptosis in ABR-treated cells. These findings suggest that the binding of ABRA to AOP-1 promotes apoptosis by inhibiting the mitochondrial antioxidant protein AOP-1, resulting in the increase of intracellular ROS and the release of cytochrome c from the mitochondria to the cytosol, which activates caspase-9 and caspase-3.

Abrin (ABR) and ricin belong to the type II ribosome inactivating proteins which are heterodimeric glycoproteins that contain a toxophoric A-chain with protein synthesis inhibitory activity and a lectin B-chain that binds to β-galactose moieties on the cell membrane (1). The A-chain is transferred across the plasma membrane by the B-chain via endocytotic vesicles into cells (2). In addition to their ability to inhibit protein synthesis, recent studies have shown that these toxins are able to induce apoptosis (3–5). Baluna and colleagues (6, 7) have suggested that inhibition of protein synthesis and apoptosis may be mediated by different segments of the ricin A-chain molecule. Besides the inhibition of protein synthesis, it is an attractive hypothesis that ABRA adopts alternative molecular mechanisms to trigger apoptotic programs.

Apoptosis is a form of cell death that leads to elimination of excess or damaged cells. Apoptosis contributes to tissue homeostasis and embryonic development (8). A range of stimuli including DNA damage, growth factor withdrawal, anticancer drugs, and members of the tumor necrosis factor receptor family of death receptors can induce apoptotic signals (9–11). The apoptotic cascade in mammalian cells is a multistep process (12). In most cases, the apoptotic cascade is initiated by loss of integrity of the outer mitochondrial membrane accompanied by release of cytochrome c from the intermembrane space of mitochondria to cytosol. The cytosolic cytochrome c serves as a cofactor with the apoptotic protease activating factor (Apaf-1) to activate pro-caspase 9 (13–15). Caspase-9 then activates other caspases, which undermine the structural integrity of the cells by cleaving a key structural protein substrate (16).

The signaling events that affect the particular apoptotic mediators are currently a focus of intense study. Reactive oxygen species (ROS) participate in a wide variety of cellular functions, including cell proliferation, differentiation, and apoptosis (17, 18). Addition of ROS or depletion of cellular antioxidants induces apoptosis (17, 19, 20), and ROS are likely to act as signaling intermediates that are involved in the signal transduction mechanism for apoptosis (17, 21–23).

Elucidation of the mechanism that is involved in ABR-triggered apoptosis is important for the development of cancer chemotherapeutic agents. In order to identify ABRA-interacting proteins, the yeast two-hybrid system was used. Since ABRA inhibits the growth of yeast cells by its N-glycosidase activity, ABRA(E164Q) with a mutation of its N-glycosidase catalytic site was employed.

In this paper, we report on an ABRA(E164Q)-interacting protein, AOP-1, which is an antioxidant protein with two catalytic conserved cysteine residues and has 93.3% identity to SP-22 (24, 36), a mitochondria antioxidant protein. We show that ABRA can directly inhibit the antioxidant activity of AOP-1 in vitro and two antioxidants N-acetylcysteine (NAC) and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (4-OH-tempo) can delay ABR-induced apoptosis, suggesting that ABR may deplete cellular antioxidants. Moreover, the ectopic expression of AOP-1 prevents both mitochondrial cytochrome c...
release and phenotypic apoptosis in response to ABR. Taken together, our results support AOP-1 as one of the target molecules of ABR-induced apoptosis.

**EXPERIMENTAL PROCEDURES**

**Materials**—A Matchmaker Two-Hybrid System 2 kit was purchased from CLONTECH (Palo Alto, CA). Restriction endonucleases were obtained from New England Biolabs, Inc. (Beverly, MA). Chemicals for nucleotide autosequence analysis were purchased from PE Applied Biosystems (Foster, CA). T7u DNA polymerase, large scale RNA production system-T7, and rabbit reticulocyte lysate translation system, and the CspACASS assay system were purchased from Promega (Madison, WI). [35S]Methionine was obtained from PerkinElmer Life Sciences. DCFH-DA, Hoechst 33258, N-acetylcysteine, and 4-hydroxy-2,2,6,6-tetramethylpiperidiner-1-oxyl were purchased from Sigma. Glutathione-Sepharose 4B gel and pGEX-2T were obtained from Amer sham Pharmacia Biotech (Uppsala, Sweden). Abrin-a was purified from *Abras precatorius* as described (1). An in situ cell death detection kit was purchased from Roche Molecular Biochemicals GmbH (Mannheim, Germany). The protease inhibitor Cbz-Val-Ala-Asp(Ome)-fluoromethyl ketone (zVAD-fmk) was purchased from Kamiya Biomedical Co. (Seattle, WA). The fluorescent tetrapeptide protease substrate, Ac-LEHD-AMC, was obtained from Calbiochem (San Diego, CA). Anti-cytochrome c antibody (C-18) and anti-flag M5 antibody (1:1,000) were from PharMingen (San Diego, CA). Anti-α-tubulin antibody was purchased from Zymed Laboratories Inc. (San Francisco, CA). Anti-flag M5 antibody was purchased from Eastman Kodak Co. Chicken anti-DYKDDDK (flag) polyclonal antibody, and rhodamine-conjugated rabbit anti-mouse antibody, and rhodamine-conjugated rabbit anti-α-tubulin antibody were from Chemicon International (Temecula, CA).

**Cell Culture**—HeLa cells and 293 T cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.) under 5% CO2 at 37 °C. Yeast Two-hybrid System—Since wild type ABR is detrimental to yeast, mutant ABR/ΔABR(E164Q) cDNA was fused to pAS2-1 vector to screen a library cloned in yeast by transformation of 1.2 × 105 cells were analyzed, and the transformed yeast was selected on synthetic dropout agar plates lacking leucine, tryptophan, and histidine in the presence of 50 μl 3-amino-1,2,4-triazole (Sigma). Twenty-four colonies that developed color on filter paper were restreaked on selective plates to allow plasmid segregation and tested again for β-galactosidase activity. For quantitative data, the liquid culture assay using p-nitrophenyl-β-D-galactopyranoside as a substrate was performed, and the hydrolysis of the substrate was measured at A420. Two clones were specific for ABR/ΔABR(E164Q). Nucleotide sequence analysis of cDNA inserts was performed using a PE Applied Biosystems automated sequencer.

**In Vitro Binding Assay—**GST-ABR(ΔABR(E164Q)) fusion protein was expressed in *E. coli* and purified by affinity chromatography on glutathione-agarose as described (25). GST-ABR(E164Q)-tagged AOP-1 was prepared by using pBluescript-AOP-1 as a template with an RNA production system-T7 and rabbit reticulocyte lysate translation system as described by the supplier with modifications. The translation reaction contained 2 mM GTP and 2 mM MgCl2 in addition to the original formula. For the binding assay, BSA-washed glutathione-agarose beads were bound to GST-ABR/ΔABR(E164Q) or GST in buffer consisting of 10 mM NaHPO4, 150 mM NaCl, 2.7 mM KCl, 1.8 mM KH2PO4, 10 mM DTT, and 1% Triton X-100 for 1 h at 4 °C and then washed six times with the same buffer. After the GST- or GST-ABR/ΔABR(E164Q)-bound beads were incubated with [35S]labeled AOP-1 (50 μl of in vitro translated product) for 2 h at 4 °C, the reaction products were extensively washed with the same buffer and extracted with SDS sample buffer. The extracts were analyzed by SDS-PAGE and exposed to Kodak x-ray film.

**Transfection of HeLa Cells with AOP-1 cDNA—**A 768-base pair cDNA containing the entire coding region of AOP-1 and a flag tag at its C terminus was subcloned into a mammalian expression vector, and the plasmid purification was prepared using a Qiagen Maxi Kit (Groningen, Netherlands). The mammalian expression vector pCDNA3-AOP-1 using LipofectAMINE reagent according to the manufacturer’s instructions. The LipofectAMINE DNA complexes were left on the cells for 5 h, and then the transfection mixtures were removed and replaced with normal growth medium to grow for 24 h.

**Preparation of Subcellular Fractions and Immunoblotting—**Cells were washed twice with PBS, and the pellet was suspended in 0.5 ml of buffer containing 20 mM Hepes, pH 7.4, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, protease inhibitors (Complete Mixture, Roche Molecular Biochemicals), and 250 mM sucrose. The cells were homogenized by 40 strokes in a Dounce homogenizer. The homogenates were centrifuged at 500 × g for 10 min at 4 °C to remove nuclei and unbroken cells, and the supernatant was re-centrifuged at 8,000 × g for 10 min at 4 °C to pellet the cytosol fraction. The supernatant was centrifuged at 20,000 × g for 30 min at 4 °C, and the supematant was referred to as the cytosolic fraction. Samples containing 40 μg of protein were subjected to 12% SDS-PAGE and electroblotted on a polyvinylidine difluoride membrane. The membrane was probed with anti-cytochrome c monoclonal antibody 7H8.2C12 at a dilution of 1:4,000, mouse anti-α-tubulin antibody at a dilution of 1:1,000, and anti-α-tubulin antibody at a dilution of 1:2,000 in the buffer followed by horseradish peroxidase-conjugated goat anti-mouse IgG. Antigen-antibody complexes were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech).

**Immunocytochemistry and Confocal Microscopy—**HeLa cells were grown on glass coverslips for 24 h in DMEM containing 10% FBS. Cells were transfected with pcDNA3-AOP-1 as described previously. Apoptosis was induced by ABR treatment. The reaction was terminated by washing the culture cells three times with PBS, followed by fixation in freshly prepared 4% paraformaldehyde in PBS for 12 min. The fixed cells were washed three times in PBS, followed by permeabilization in 0.2% Triton X-100 in PBS for 2 min. The cells were then blocked for 30 min with 10% FBS (X100) in PBS, followed by a 1-h incubation with mouse monoclonal antibody against cytochrome c (6H2.B4) at a dilution of 1:1,000 and chicken polyclonal antibody against DYKDDDK(flag) at a dilution of 1:500. The cells were washed four times at 10 min each in blocking buffer followed by a 1-h incubation with a fluorescein-conjugated goat anti-mouse antibody for cytochrome c and a rhodamine-conjugated rabbit anti-chicken antibody for flag. After extensive washing, slides were mounted and examined under a Zeiss Axioskop inverted microscope with a 100× oil objective lens.

To assess colocalization of AOP-1 with ABR in HeLa cells, uptake experiments with ABR and immunofluorescence for detecting endogenous AOP-1 were performed. Cells were incubated in serum-free medium containing 10 μg/ml FITC-ABR for 30 min at 4 °C and then warmed at 37 °C for 45 min. The treated cells were placed back on ice rinsed with 0.1 M galactose to remove surface-bound FITC-ABR, fixed, permeabilized, and stained for AOP-1 using rabbit polyclonal antibody and a rhodamine-conjugated goat anti-rabbit antibody. Samples were imaged on a confocal laser scanning microscope (Leica TCS SP2), with a 100× oil objective lens.

**Measurement of Apoptosis—**Apoptotic cell death was examined by the terminal deoxynucleotidyl transferase-catalyzed deoxyuridine triphosphate (dUTP)- nick end labeling (TUNEL) method as described by the supplier (Roche Molecular Biochemicals). Samples (10⁴ events) were analyzed with a Becton-Dickinson FACSCalibur, and the distribution of cells was determined.

To assess DNA ladder formation, HeLa cells (1 × 10⁶) were treated with 0.1 M DCFH-DA for 24 h at 37 °C. The DNA was digested overnight at 37 °C in 0.5 mg/ml proteinase K and 0.5% sarcosyl in PBS, treated with 10 μg of RNase A for 1 h at 37 °C, then gently extracted with phenol and chloroform, and analyzed on 1.5% agarose gel.

The viability of cells was assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were preincubated with NAC or 4-OH-tempo for 2 h, followed by treatment with 5 ng/ml ABR for 15 h. The medium was replaced by 500 μl of MTT solution (1 mg/ml in DMEM supplemented with 10% FBS) and further incubated for 1 h at 37 °C. The well was then washed three times with PBS, and ethanol was added to the well. The absorbance at 570 nm was measured by an enzyme-linked immunosorbent assay reader.

**Measurement of Intracellular ROS—**Levels of intracellular ROS were measured as described (26) with minor modifications. Briefly, HeLa cells (1 × 10⁶/ml) were treated with 100 ng/ml ABR for various periods of time and they were incubated with 10 μM DCFH-DA in the dark for 30 min before the cells were harvested. The cells were washed with PBS and analyzed (excitation, 488 nm; emission, 515–540 nm) on a Becton-Dickinson FACSCalibur. The fluorescence intensity of AOP-1 Antioxidant Activity—The primers, 5′-GGAGATTCCATATGGGCACTCCTGGCAGCAGCATGCA-3′ and 5′-CCGGATCTTACTCTGATTTACCTGAAAA-3′, were designed to PCR amplify a 558-base pair fragment from pGAD10-AOP-1. The amplified DNA fragment encoding the amino acids 62–256 of AOP-1 was subcloned in-frame to the Ndel/BamHI sites of the bacterial expression vector pET-15b. The expression plasmid was transformed into bacteria.
ABR Induces Apoptosis by Inactivating AOP-1

Yeast strain Y190 was transformed with various combinations of plasmids and β-galactosidase units were determined by the liquid culture assay.

<table>
<thead>
<tr>
<th>Protein fused to GAL4 domain</th>
<th>β-Galactosidase units</th>
<th>LacZ phenotype</th>
</tr>
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<tbody>
<tr>
<td>pVA3a</td>
<td>pTD1–1b</td>
<td>Blue</td>
</tr>
<tr>
<td>pAS2–1-ABRA(E164Q)</td>
<td>pGAD10-AOP-1</td>
<td>White</td>
</tr>
<tr>
<td>None</td>
<td>pGAD10-AOP-1</td>
<td>White</td>
</tr>
<tr>
<td>pAS2–1-ABRA(E164Q)</td>
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<td>Blue</td>
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Table I: Two-hybrid interaction assay between ABRA and AOP-1 constructs

The thiol-specific antioxidant activity of AOP-1 was measured by the method described by Kim et al. (27) with slight modifications. Assays were performed in a 50-μl reaction mixture containing 0.5 unit of glutamine synthetase, 10 mM DTT, 12 μM FeCl3, 100 mM Hepes (pH 7.4), with re-AOP-1 (0.06 mg/ml) and various amounts of ABRA(E164Q) and were incubated at 30 °C. At various time periods, aliquots (10 μl) were removed to assay the residual glutamine synthetase activity.

Measurement of DEVDase and LEHDase Activities—The CaspACE® assay system was used according to the instructions of the manufacturer. Briefly, cytosol extracts (50 μg) were incubated with 245 μl of reaction buffer containing 80 μl of caspase buffer, 5 μl of MeSO, and 10 mM DTG for 30 min at 30 °C and then Ac-DEVD-AMC or Ac-LEHD-AFC was added at a final concentration of 50 μM. The reaction mixtures were incubated for 1 h at 30 °C. Cleavage of fluorogenic substrate was quantitated by using a fluorescence spectrophotometer (F-4500, Hitachi) at 360/460 nm or 400/505 nm.

Results

Interaction of AOP-1 with ABRA(E164Q)—To identify proteins interacting with ABRA, a yeast two-hybrid system was employed. Two clones that specifically interacted with ABRA(E164Q) were confirmed by a colony-lift β-galactosidase filter assay and were quantified by a liquid culture assay (Table I). Sequencing of these cDNA clones identified two major transcripts, which consisted of an open reading frame encoding 256 amino acids (Fig. 1). Computer analysis using NCBIBLASTN (28) revealed that the open reading frame was identical to human protein AOP-1 (24). Data base searches revealed that AOP-1 shared 93.3% identity with SP-22, a bovine mitochondrial thioredoxin-dependent peroxide reductase, and the mitochondrial targeting signal (29) (Fig. 1). The mitochondria targeting signal was composed of 20–60 amino acid residues with abundant positive charges, no negative charges, and frequent hydroxylated residues, and they were rich in hydrophobic residues (30–32). The sequence upstream of the cleavage site was coincident with the specificity of two processing peptidases for mitochondrial proteins. The amino acid sequences RX↓[F/L/I][X][T/S][G]↓ were cleaved sequentially in two steps by mitochondrial processing peptidase and mitochondrial intermediate peptidase (33). The data showed a high probability that AOP-1 was localized in the mitochondria.

To confirm that AOP-1 did indeed bind to ABRA(E164Q), human AOP-1 was transcribed and translated in vitro. We used GST-ABRA(E164Q) linked to glutathione-Sepharose beads to examine the interactions between ABRA(E164Q) and AOP-1. After incubation with 35S-labeled AOP-1, these GST-ABRA(E164Q) beads (or the control bait, GST beads) were pelleted and washed extensively. The proteins which remained bound tightly to GST or to GST-ABRA(E164Q) were resolved by SDS-PAGE. The 35S-labeled AOP-1 was visualized by autoradiography. The AOP-1 protein was bound specifically by the GST-ABRA(E164Q) (Fig. 2). Taken together, these data suggest that AOP-1 is an ABRA(E164Q)-interacting protein.

AOP-1 Localized in the Mitochondria—To determine whether AOP-1 localizes in the mitochondria as TP-22 does, we examined the localization of AOP-1 within the cells and compared it with the distribution of α-tubulin. We constructed a plasmid clone pcDNA5-AOP-1 that was designed to express AOP-1 with a flag tag at the C-terminal end (AOP-1-flag). This plasmid DNA was transfected into HeLa cells, and the distribution of AOP-1-flag was detected by subcellular fractionation and Western blotting. As shown in Fig. 3A, the subcellular localization of AOP-1-flag was found in the mitochondria, and this indicated that AOP-1 was distinctly separated from α-tubulin. To further confirm the mitochondrial localization of AOP-1, immunofluorescence staining was carried out by using an anti-cytochrome c antibody (a well characterized mitochondrial marker). In HeLa cells, the anti-cytochrome c antibody gave a punctate staining pattern characteristic of mitochondrial localization and the AOP-1-flag colocalized with cytochrome c (Fig. 3B). The same results were obtained by rabbit anti-AOP-1 polyclonal antibody to detect endogenous AOP-1 (data not shown). We conclude that AOP-1 localizes in the mitochondria.

Colocalization of AOP-1 and ABR—The ability of AOP-1 and ABR to associate in vivo would indicate a possible role for this interaction in ABR-induced apoptosis. We studied the intracellular localization of AOP-1 and ABR by confocal microscopy of HeLa cells, which were treated with FITC-conjugated ABR and stained for endogenous AOP-1. In Fig. 4, green fluorescence indicated the endocytotic FITC-ABR (left panel) and red fluorescence showed the mitochondrial punctate staining of AOP-1 (center panel). As shown in the right panel, there was colocalization of AOP-1 and ABR in yellow (white arrows). The data suggest that ABR interacts with AOP-1 in vivo.

Induction of Apoptosis and Endogenous ROS by ABR—The typical laddering pattern of fragmented DNA associated with apoptosis appeared after exposure of cells to 100 ng/ml ABR (Fig. 5A). The nuclei of HeLa cells treated with ABR and then stained with Hoechst 33258 showed that chromatin condensation was produced in apoptotic cells (data not shown). These results are consistent with previous studies (34) and show that abrin is able to trigger programmed cell death.

To determine whether the level of ROS in ABR-treated cells was increased, we used FACS analysis with DCFH-DA, which is a nonpolar compound that readily diffuses into cells and is hydrolyzed to DCFH by esterase. When DCFH is oxidized within the cell, it becomes a highly fluorescent 2′, 7′-dichlorofluorescein. The fluorescence of untreated HeLa cells represented the endogenous ROS, and 300 μM H2O2 treatment was used as a positive control (Fig. 5B). The intracellular ROS was increased 2-fold at 4 h after ABR treatment. The results suggest that the level of ROS is increased at the early phase in ABR-treated cells.
ABR or GST. GST fusion protein and 35S-labeled interacting protein green fluorescence center panel antibody (cdria using an anti-cytochrome and monoclonal anti-cytochrome pcDNA3-AOP-1-flag were stained for flag using an anti-flag antibody bmetal procedures.

SDS-PAGE. were recovered on glutathione-agarose and analyzed on 12%\%

deepth: 605.0x786.0

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Identical aligned residues are shaded in black, and similar aligned residues are shaded in gray. Squares represent two catalytic cysteine residues (Cys^47 and Cys^168) (24), and residue numbers are indicated on the right.

FIG. 1. Alignment of amino acid sequences of AOP-1 and SP-22. The sequence upstream of the cleavage sites coincides with the specificity of two processing peptidases for mitochondrial proteins (33). The vertical arrows indicate the processing sites in mitochondria. Identical aligned residues are shaded in black, and similar aligned residues are shaded in gray. Squares represent two catalytic cysteine residues (Cys^47 and Cys^168) (24), and residue numbers are indicated on the right.

FIG. 2. Interaction of AOP-1 with ABRA(E164Q). The input of lysate containing 1 µl of in vitro translation (IVT) 35S-labeled AOP-1 is shown in the left lane. 35S-Labeled AOP-1 was incubated with GST-ABR or GST. GST fusion protein and 35S-labeled interacting protein were recovered on glutathione-agarose and analyzed on 12% SDS-PAGE.

FIG. 3. Subcellular localization of AOP-1. A, AOP-1 is localized in the mitochondria. Immunoblotting was performed with anti-flag, and anti-α-tubulin antibodies and performed as described under “Experimental procedures.” B, confocal microscopy. The cells transfected with pcDNA3-AOP-1-flag were stained for flag using an anti-flag antibody and monoclonal anti-cytochrome C antibody with a rhodamine-conjugated secondary antibody (red fluorescence, left panel) and for mitochondria using an anti-cytochrome C antibody with FITC-labeled secondary antibody (green fluorescence, center panel). The overlay of both images is shown in the right panel.

Since increases of ROS generation in ABR-treated cells are the early sign of the ABR toxicity, it is suggested that ROS are important mediators of ABR-induced cell death. To test whether ROS levels play a role in mediating the death signal of ABR, two antioxidants NAC and 4-OH-tempo, which is known to be localized into the mitochondria, were used, and the results showed that the antioxidants inhibited ABR-induced apoptosis in a dose-dependent manner (Fig. 5C). These data further demonstrate that the generation of ROS plays an important role in the induction of apoptosis by ABR.

ABRA(E164Q) Inhibited the AOP-1 Antioxidant Activity in Vitro—To further examine whether ROS are important mediators of ABR-induced cell death, we tested the effects of re-ABRA(E164Q) on the antioxidant activity of recombinant AOP-1 in vitro. The antioxidant activity of re-AOP-1 was detected by monitoring the ability of the protein to inhibit the inactivation of glutamine synthetase using a DTT/Fe^3+/O_2 metal-catalyzed oxidation system (27). Various amounts of ABRA(E164Q) were added to the reaction mixture, and the protection value of AOP-1 (0.06 mg/ml) was decreased from 81% protection (AOP-1 alone) to 32% (molar ratio: AOP-1: ABRA(E164Q) = 1:2; Fig. 6). The inhibitory effects of ABRA(E164Q) on AOP-1 antioxidant activity were dose-dependent. The results indicate that ABRA(E164Q) not only specifically interacts with AOP-1, but also directly inhibits the antioxidant activity of AOP-1, which may elevate the level of ROS, followed by cell death.

ABR Induced the Translocation of Mitochondrial Cytochrome C into the Cytosol and the Activation of Caspases—To study whether the release of cytochrome C from the mitochondria to the cytosol is involved in ABR-triggered apoptosis, the amount of cytochrome C in the cytosol was determined by immunoblot analysis. Untreated cells did not contain any detectable amounts of cytochrome C in the cytosol, whereas the level of cytosolic cytochrome C was increased significantly at 4 h after ABR treatment (Fig. 7A). The results suggest that ABR induces apoptosis by releasing cytochrome C from the mitochondria to the cytosol. The intracellular distribution of cytochrome C in HeLa cells was further examined by immunofluorescence staining. Treatment of HeLa cells with ABR for 4 h induced the loss of mitochondrial cytochrome C staining (Fig. 7B). Arrows indicated that cells with ABR treatment have more diffuse cytochrome C immunostaining. These findings indicate that ABR-triggered apoptosis is through the release of cytochrome C.

To assay the role of caspases in ABR-mediated apoptosis, zVAD-fmk, a broad spectrum inhibitor of mammalian caspases, was preincubated with HeLa cells and then the cells were treated with ABR for 24 h. FACS analysis with TUNEL indicated that zVAD-fmk prevented cell death in a dose-dependent manner, and 80 µM zVAD-fmk inhibited about 50% of cell death caused by ABR treatment (Fig. 8A). The results indicate that caspases are involved in ABR-triggered apoptosis.

To assess the involvement of caspase-9 and caspase-3, acti-
vation of caspases was evaluated by the cleavage of the fluorogenic substrates, Ac-LEHD-AFC and Ac-DEVD-AMC. After treatment of HeLa cells with ABR for 4 h, caspase-9 and caspase-3 activities were increased to 4- and 10-fold, respectively (Fig. 8, B and C). Caspase-1 activity was not increased after ABR treatment (data not shown). These results demonstrate that ABR-triggered apoptosis is through the release of cytochrome c and the activation of caspase-9, which in turn activates caspase-3.

AOP-1 Attenuates Apoptosis and Cytochrome c Release—As described above, the antioxidant activity of AOP-1 was inhibited by ABRA in vitro and antioxidants such as NAC and 4-OH-tempo delayed ABR-induced cell death. We examined whether ectopic expressed AOP-1 could attenuate the apoptosis induced by ABR. The anti-apoptotic activity of AOP-1 was assayed by FACS analysis. When HeLa cells were transiently transfected with 3 μg of AOP-1 plasmid before the treatment with 100 ng/ml ABR for 24 h, cell death decreased from 79% to 41% (Fig. 9A). These results suggest that the overexpression of AOP-1 can attenuate ABR-induced apoptotic cell death.
To investigate whether the overexpression of AOP-1 is capable of regulating the release of cytochrome c from the mitochondria in ABR-treated cells, we performed Western blot analysis. As shown in Fig. 9B, HeLa cells transfected with various amounts of AOP-1 plasmid were treated with ABR and the release of cytochrome c from the mitochondria was decreased in a dose-dependent manner. The results indicate that overexpression of AOP-1 can inhibit the translocation of cytochrome c from the mitochondria to the cytosol in ABR-treated cells.

To further confirm the inhibitory activity of AOP-1 on the translocation of cytochrome c, immunofluorescence staining of HeLa cells with anti-cytochrome c and anti-AOP-1 antibodies was carried out. ABR induced the accumulation of cells with diffuse cytochrome c staining, which was significantly inhibited by AOP-1 (Fig. 9C). Arrows indicated that non-trans-
Finally, apoptosis occurs. ROS, the release of cytochrome by ABRA, the redox balance is disrupted, followed by the generation of ABR-treated cells. Once the antioxidant ability of AOP-1 is blocked, programmed cell death involves at least two pathways. One is the inhibition of protein synthesis by its N-glycosidase activity to influence unknown cellular factors, and the other is the alteration of the function of mitochondria by specific interaction with AOP-1. Overexpression of AOP-1 prevents cytochrome c (Cyt. c) release and attenuates apoptosis in ABR-treated cells. Once the antioxidant ability of AOP-1 is blocked by ABRA, the redox balance is disrupted, followed by the generation of ROS, the release of cytochrome c, and activation of the caspase cascade. Finally, apoptosis occurs.

fig. 9. effects of AOP-1 on attenuation of apoptosis and the release of cytochrome c from the mitochondria into the cytoplasm in ABR-treated cells. A. FACS analysis of ABR-triggered apoptosis in HeLa cells and HeLa/AOP-1 cells. HeLa cells were transiently transfected with 3 μg of AOP-1 plasmids and then treated with 100 ng/ml ABR for 24 h. Quantification of the DNA fragmentation was performed by the TUNEL method, and the details are described under "Experimental Procedures." B, immunoblot analysis. HeLa cells transfected with various amounts of AOP-1 plasmids were treated with 100 ng/ml ABR. Western blot was performed as described previously. C, confocal microscopy. HeLa cells transfected with AOP-1-flag and treated with 100 ng/ml ABR for 5 h were stained and experiments performed as described previously. The red fluorescence (left panel) represents AOP-1 transfected cells, and the green fluorescence (center panel) represents cytochrome c staining. The overlay of both images is shown in the right panel. The non-transfected cells with more diffuse cytosolic cytochrome c immunostaining are indicated by arrows.

fig. 10. The model of ABR-triggered apoptosis. ABR-induced programmed cell death involves at least two pathways. One is the inhibition of protein synthesis by its N-glycosidase activity to influence unknown cellular factors, and the other is the alteration of the function of mitochondria by specific interaction with AOP-1. Overexpression of AOP-1 prevents cytochrome c (Cyt. c) release and attenuates apoptosis in ABR-treated cells. Once the antioxidant ability of AOP-1 is blocked by ABRA, the redox balance is disrupted, followed by the generation of ROS, the release of cytochrome c, and activation of the caspase cascade. Finally, apoptosis occurs.

Discussion

In this report, we have identified a novel ABR-interacting protein, AOP-1, which was originally reported to be a thiol-specific antioxidant protein in vitro (35), but its physiological function was unclear. Analysis of the amino acid sequence has revealed that the N-terminal 61 amino acids have characteristics of signals that target translocation of proteins into mitochondria. By Western blot analysis and immunocytochemistry (Fig. 3), we have shown that AOP-1 is located in the mitochondria and not in the cytoplasm. AOP-1 is 93.3% identical (97.4% similar) to SP-22, which has been reported to be one of the antioxidant proteins located in the mitochondria (29). Both of them conserve the two critical motifs found in all human peroxide reductases (the two cysteine-containing segments Cys(FFYPLDFTVCPTEI) and Cys(HEVGCPA)). These cysteine motifs have been suggested as being important for the catalysis of peroxides (36, 37). Proteins imported into the mitochondria are usually cleaved by proteases, thereby losing their N-terminal signal sequence. The resulting recombinant AOP-1 protein showed antioxidant activity (Fig. 6). These results suggest that AOP-1 can scavenge ROS as SP-22 does by cooperating with mitochondrial thioredoxin and can protect mitochondrial components from the action of superoxide anions or hydrogen peroxide (38, 39). It will be interesting to determine whether there are other factors cooperating with AOP-1 to function as an antioxidant complex in the mitochondria.

Mitochondria play a key role in the regulation of apoptosis. The oxidant stress triggers mitochondrial permeability transition pore opening by oxidizing thiol groups in the pore protein (40, 41). Thus, the mitochondrial redox potential is important for regulating the opening of mitochondrial permeability transition pore, which in turn allows the release of cytochrome c and then induction of caspase-dependent apoptosis (42–44). Interestingly, ABR induces cells to generate ROS and the antioxidants NAC and 4-OH-tempo can delay ABR-caused cell death. The overexpression of AOP-1 can attenuate the apoptosis and block the release of cytochrome c in ABR-treated cells (Fig. 9). These data indicate that AOP-1 functions in the protection of cells from apoptosis and may be implicated as an endogenous regulator of apoptosis.

We have shown that recombinant ABRA(E164Q) inactivates the antioxidant activity of AOP-1 in vitro (Fig. 6). Hence, we speculate that ABRA interacts with AOP-1 and inactivates the antioxidant activity of AOP-1, which may initiate apoptosis by a pathway distinct from inhibition of protein synthesis. Notably, zVAD-fmk, a broad spectrum caspase inhibitor, was unable to completely abrogate apoptosis caused by ABR. One explanation for this is that the activation of caspase is not the only pathway involved in apoptosis. The stress caused by the inhibition of protein synthesis partially contributes to ABR-triggered apoptosis. It has recently been demonstrated that the
segment of the ricin A-chain (SVTLADVTNAY) linked to antibodies can induce the apoptosis of human vein endothelial cells (7). The data suggested that the ricin A-chain-mediated inhibition of protein synthesis and apoptosis might be exerted by different motifs of the ricin A-chain molecule.

In summary, we propose the model shown in Fig. 10. The binding of ABR/AE164Q to AOP-1 can shield the sulphhydryl groups and result in the inhibition of the antioxidant activity. As a consequence of the loss of AOP-1 antioxidant activity, the mitochondrial redox potential is shifted to a more oxidized state and the mitochondria generate large amounts of ROS and release cytochrome c, which in turn activates caspases to trigger apoptosis.

ABR or immunotoxins have a dose-limiting toxicity in vascular leak syndrome in mammals and humans (45–50). Since the antioxidants NAC and 4-OH-temarin significantly delay the apoptosis induced by ABR, it is suggested that antioxidants can be employed in reducing the toxicity of immunotoxins and hence improve the efficiency of cancer chemotherapy.

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REFERENCES

20. J. Biol. Chem. 274, 23076–23080
Abrin Triggers Cell Death by Inactivating a Thiol-specific Antioxidant Protein
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