Essential Role of Voltage-dependent Anion Channel in Various Forms of Apoptosis in Mammalian Cells

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Abstract. Through direct interaction with the voltage-dependent anion channel (VDAC), proapoptotic members of the Bcl-2 family such as Bax and Bak induce apoptogenic cytochrome *c* release in isolated mitochondria, whereas BH3-only proteins such as Bid and Bik do not directly target the VDAC to induce cytochrome *c* release. To investigate the biological significance of the VDAC for apoptosis in mammalian cells, we produced two kinds of anti-VDAC antibodies that inhibited VDAC activity. In isolated mitochondria, these antibodies prevented Bax-induced cytochrome *c* release and loss of the mitochondrial membrane potential ($\Delta\psi$), but not Bid-induced cytochrome *c* release. When microinjected into cells, these anti-VDAC antibodies, but not control antibodies, also prevented Bax-induced cytochrome *c* release and apoptosis, whereas the antibodies did not prevent Bid-induced apoptosis, indicating that the VDAC is essential for Bax-induced, but not Bid-induced, apoptogenic mitochondrial changes and apoptotic cell death. In addition, microinjection of these anti-VDAC antibodies significantly inhibited etoposide-, paclitaxel-, and staurosporine-induced apoptosis. Furthermore, we used these antibodies to show that Bax- and Bak-induced lysis of red blood cells was also mediated by the VDAC on plasma membrane. Taken together, our data provide evidence that the VDAC plays an essential role in apoptogenic cytochrome *c* release and apoptosis in mammalian cells.

Key words: VDAC • apoptosis • Bcl-2 • Bax • cytochrome *c*

Introduction

Apoptosis is a gene-regulated mechanism of cell death that is essential for elimination of unwanted cells in various biological systems in most metazoans. Apoptosis is driven by a family of cysteine proteases called caspases, which process various cellular proteins to execute apoptotic cell death (for review see Thornberry and Lazebnik, 1998). The Bcl-2 family of proteins are well-characterized regulators of apoptosis, consisting of three distinct subfamilies (for reviews see Adams and Cory, 1998; Green and Reed, 1998; Tsujimoto and Shimizu, 2000a,b). (a) The Bcl-2 subfamily contains antiapoptotic proteins, such as Bcl-2 and Bcl-x_L, which show sequence homology at the Bcl-2 homology (BH)[1] 1, BH2, and BH3 domains, as well as at the BH4 domain in most cases. (b) The Bax subfamily contains proapoptotic proteins, such as Bax and Bak, which show sequence homology at BH1, BH2, and BH3. (c) BH3-only proteins are another subfamily of proapoptotic proteins that only share homology at the BH3 domain, such as Bid, Bik, and Bim. It has been shown that in addition to BH1 and BH2, the BH4 domain is required for the antiapoptotic activity of Bcl-2 and Bcl-x_L, whereas the BH3 domain of proapoptotic proteins is essential, and sufficient, for proapoptotic activity (for reviews see Adams and Cory, 1998; Tsujimoto and Shimizu, 2000a,b). Many of the proapoptotic members of the Bcl-2 family (such as Bax, Bid, Bim, and Bad) are localized in the cytoplasm of living cells, but during apoptosis, translocate to the mitochondria through various modifications of these proteins, such as proteolytic cleavage and dephosphorylation, probably leading to conformational changes (for review see Tsujimoto, 1998).

Mitochondria play a crucial role in apoptosis by releasing several apoptogenic molecules, including cytochrome *c* and Smac/Diablo from the intermembrane space into the cytoplasm in response to a variety of death-promoting

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[1]*Abbreviations used in this paper:* ANT, adenine nucleotide translator; BH, Bcl-2 homology; $\Delta\psi$, mitochondrial membrane potential; GFP, green fluorescent protein; GPGH, glyceraldehyde 3-phosphate dehydrogenase; NRI, normal rabbit IgG; PT, permeability transition; rGFP, recombinant GFP; VDAC, voltage-dependent anion channel.

stimuli (for reviews see Adams and Cory, 1998; Green and Reed, 1998; Du et al., 2000; Tsujimoto and Shimizu, 2000a,b; Verhagen et al., 2000). Once in the cytoplasm, cytochrome *c* binds to Apaf-1, triggering oligomerization of the Apaf-1/cytochrome *c* complex that leads to recruitment and activation of a major apical caspase, caspase-9. In turn, caspase-9 activates various effector caspases such as caspase-3 (for review see Thornberry and Lazebnik, 1998). It has been shown that Bcl-2 family proteins regulate mitochondrial membrane permeability to control cytochrome *c* release: proapoptotic Bax, Bak, and BH3-only proteins like Bid and Bik induce cytochrome *c* release, whereas antiapoptotic Bcl-2 and Bcl-x_L prevent it (Eskes et al., 1998; Jürgensmeier et al., 1998; Marzo et al., 1998; Narita et al., 1998; Finucane et al., 1999; Pastorino et al., 1999; Shimizu and Tsujimoto, 2000).

Recently, we have shown that Bax/Bak and Bcl-x_L, but not Bik and Bid, can bind directly to the voltage-dependent anion channel (VDAC) and modulate its activity (Shimizu et al., 1999, 2000a,b; Shimizu and Tsujimoto, 2000). The VDAC is a mitochondrial outer membrane channel, which usually functions as the pathway for the movement of various substances in and out of the mitochondria (for review see Colombini, 1989), and is considered to be a component of the oligoprotein permeability transition (PT) pore complex that plays a role in the PT (for reviews see Bernardi et al., 1994; Zoratti and Szabó, 1995). Our biochemical and electrophysical studies have shown that Bax and Bak enhance VDAC activity so that cytochrome *c* passes through the channel, whereas Bcl-x_L closes the VDAC (Shimizu et al., 1999, 2000a,b; Shimizu and Tsujimoto, 2000). We have also shown that nonfunctional mutants of Bax and Bcl-x_L lose their effect on VDAC activity (Shimizu et al., 1999, 2000a,b). Furthermore, Bax/Bak induces apoptotic mitochondrial changes, including cytochrome *c* release and mitochondrial membrane potential ($\Delta\psi$) loss, in mitochondria isolated from wild-type yeast, but not VDAC1-deficient yeast (Shimizu et al., 1999). We have also shown that Bax expression induces cytochrome *c* release in wild-type yeast cells, but not in VDAC1-deficient yeast cells (Shimizu et al., 2000c). Based on these findings, we have proposed that the VDAC plays an essential role in Bax/Bak-induced apoptotic mitochondrial changes and thus in the process of apoptosis in mammalian cells (Shimizu et al., 1999, 2000a,b), although no direct evidence has been available. Other models for apoptogenic cytochrome *c* release have also been proposed. Cytochrome *c* release might be mediated by physical rupture of outer membrane resulting from mitochondrial swelling (Vander Heiden et al., 1997) or destabilization of membrane induced by Bax as well as tBid (Basanez et al., 1999; Kudla et al., 2000). Alternatively, Bax and Bak form oligomer channels in membrane that are permeable to cytochrome *c*, and tBid facilitates the insertion into the mitochondrial membrane or oligomerization of Bax and Bak (Eskes et al., 2000; Saito et al., 2000; Wei et al., 2000).

Microinjection of specific antibodies into cells has proven very useful to analyze the biological functions of particular proteins (Imamoto et al., 1995; Hieda et al., 1999). Therefore, we generated anti-VDAC antibodies that blocked VDAC activity, and showed that Bax-induced, but not Bid-induced, cytochrome *c* release and apoptosis were significantly inhibited by these antibodies, and that these antibodies also significantly inhibited etoposide-, paclitaxel-, and staurosporine-induced apoptosis. These results provide evidence that the VDAC plays an essential role in apoptotic mitochondrial changes and apoptosis in mammalian cells.

Materials and Methods

Chemicals

A monoclonal antibody for pigeon denatured cytochrome *c* (65981A used for Western blot analysis) and an antibody for pigeon native cytochrome *c* (65971A used for immunostaining), both of which cross-reacted with human and rat cytochrome *c*, were purchased from BD PharMingen. An anti–human VDAC monoclonal antibody (31HL) and an anti–human Bax polyclonal antibody (N20) were obtained from Calbiochem and Santa Cruz Biotechnology, Inc., respectively. An anti-Tom20 polyclonal antibody was kindly provided by Prof. K. Mihara (Kyushu University, Fukuoka, Japan). [^{14}C]sucrose and [^{3}H]glucose were purchased from Amersham Pharmacia Biotech. Other chemicals were obtained from Wako Biochemicals.

Protein Purification

Recombinant human Bax (rBax) was expressed as a His-tagged protein in *Escherichia coli* strain XL1-blue using the Xpress System (Invitrogen), as described elsewhere (Narita et al., 1998). Irrelevant control proteins were prepared using the empty vector. Recombinant human Bid, truncated Bid (tBid), Bik, and BakΔC (lacking the COOH-terminal 21 amino acid residues) were expressed as GST fusion proteins in *Escherichia coli* strain DH5α and were purified on a glutathione-Sepharose column. Then Bid, tBid, Bik, and BakΔC were released from GST by cleavage with thrombin. The purity of Bax, Bid, tBid, Bik, and BakΔC was shown to be >80%. All purified proteins were finally dissolved in the same control buffer composed of 20 mM Hepes-K^+, pH 7.4, and 1 mM dithiothreitol. Rat liver mitochondrial VDAC was purified as described previously previously (Shimizu et al., 1999), and showed a single band on SDS-polyacrylamide gel.

Generation of Anti-VDAC Antibodies

Two oligopeptides corresponding to parts of the human VDAC1 were synthesized on a Multiple Peptide Synthesizer (model 396; Advanced Chemtech) using diisopropylcarbodiimide/1-hydroxybenzotriazole-activated fluorenylmethoxycarbonyl-protected amino acids. The purity of each peptide was determined to be >90% by matrix-assisted laser desorption ionization time of flight mass spectrometry. These peptides were used to immunize rabbits, as described elsewhere (Imamoto et al., 1992). Anti-VDAC antibodies raised against the two oligopeptides (designated as Ab#20 and Ab#25) were purified from rabbit serum on a column of Sepharose conjugated with purified rat VDAC. Antibodies that bound to VDAC-Sepharose were eluted with 0.1 M glycine-HCl (pH 2.5) and neutralized with NaOH. We used three different preparations of Ab#25 (Ab#25-1, 25-2, and 25-3) that came from different animals, but mainly used Ab#25-1 unless otherwise mentioned.

To remove antiseptic, the antibodies were dialyzed extensively against a buffer composed of 20 mM Hepes-K^+ (pH 7.4), and then were concentrated in a Micro Centricon 30 (Amicon Corp.) for use in microinjection and mitochondrial experiments.

Immunoprecipitation and Western Blot Analysis

Bax–VDAC interactions in the presence of anti-VDAC antibodies or control IgG were assessed by coimmunoprecipitation, as described elsewhere (Shimizu et al., 1999) using rat liver mitochondria. The mitochondria were lysed and immunoprecipitated with an anti-Bax antibody (N20) and the amount of coimmunoprecipitated VDAC was estimated by Western blot analysis. Interaction between rBax and VDAC on RBCs was also assessed by coimmunoprecipitation as described (Shimizu et al., 1999).

Reconstitution of VDAC and Bax in Liposomes

Plain liposomes and VDAC liposomes were prepared as described elsewhere (Shimizu et al., 1999). Liposomes in buffer consisting of 30 mM sodium sulfonate and 20 mM Tricine-NaOH (pH 5.3) were preincubated with 0.2 μg/μl antibodies for 3 min at room temperature, and with rBax (0.2 μg/μl) or the equivalent amount of irrelevant protein. VDAC activities were measured by assessing [^{14}C]sucrose uptake, as described previously (Shimizu et al., 1999). In brief, 20 μl of liposomes treated with anti-

bodies was incubated with 5 μl of [^{14}C]sucrose (97%, 200 μCi/ml) in the presence of rBax or irrelevant protein at 25°C, and filtered by centrifugation using a 30K limiting filter (Millipore) to remove free [^{14}C]sucrose. Then the [^{14}C]sucrose incorporated into the liposomes was measured with a gamma-scintillation counter (model 1414; Wallac). Bax liposomes and irrelevant protein liposomes were prepared at pH 7.4 by the same procedure as VDAC liposomes, using the same amount of rBax as VDAC protein or an equivalent amount of irrelevant protein. Bax channel activity was measured by the same radiotracer uptake procedure, except that [^{3}H]glucose (97%, 20 μCi/ml) was used instead of [^{14}C]sucrose.

Preparation of Isolated Mitochondria

Livers from male Donryu rats were homogenized with a glass Teflon Potter homogenizer. Mitochondria were isolated in 0.3 M mannitol, 10 mM potassium Hepes (pH 7.4), 0.2 mM EDTA, and 0.1% fatty acid–free BSA (MT-1 medium), as described previously (Shimizu et al., 1998). Then the mitochondria were washed twice with the same medium without EDTA (MT-2 medium).

Measurement of Mitochondrial Biochemical Parameters

For energization, isolated mitochondria (1 mg protein/ml) were incubated at 25°C in MT-2 medium plus 1 mM potassium phosphate and 4.2 mM succinate, unless otherwise indicated. Mitochondria were incubated with antibodies for 5 min, and then were treated with rBax, rBid, or Ca^{2+} at the indicated concentrations, after which Δψ and cytochrome *c* release were measured. Δψ was assessed by measuring the Δψ-dependent uptake of rhodamine 123 using a spectrophotometer (F-4500; Hitachi), as described elsewhere (Shimizu et al., 1998). For detection of cytochrome *c* release and mitochondrial association of Bax, mitochondria were spun, and the pellet and supernatant were subjected to Western blot analysis using anti-cytochrome *c* and anti-Bax antibodies. Mitochondrial respiration was measured using an O_2 electrode in the presence of succinate (state IV) or succinate plus ADP (state III). Δψ of cells was measured by incubating cells with 1 μM JC-1, followed by examination under a fluorescence microscope (IX70; Olympus).

Microinjection

HeLa cells (a human cervical cancer cell line) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. Microinjection experiments were performed using a micromanipulator (Narishige), essentially as described previously (Hieda et al., 1999). Maximum and minimum injected volume were calculated to be 1.7×10^{-14} and 1.7×10^{-15} liters (Matsuoka et al., 1994). Thus, when 12 μg/μl of antibody was injected, the injected amount was estimated to be 0.2–0.02 pg. 1 h after injection of antibodies (free from antiseptic) with or without Cy5-labeled mouse IgG into the cytoplasm of cells, rBax or rtBid with or without green fluorescent protein (GFP) was injected in the cytoplasm of the same cells. The concentrations of proteins used for microinjection are described in the fig-

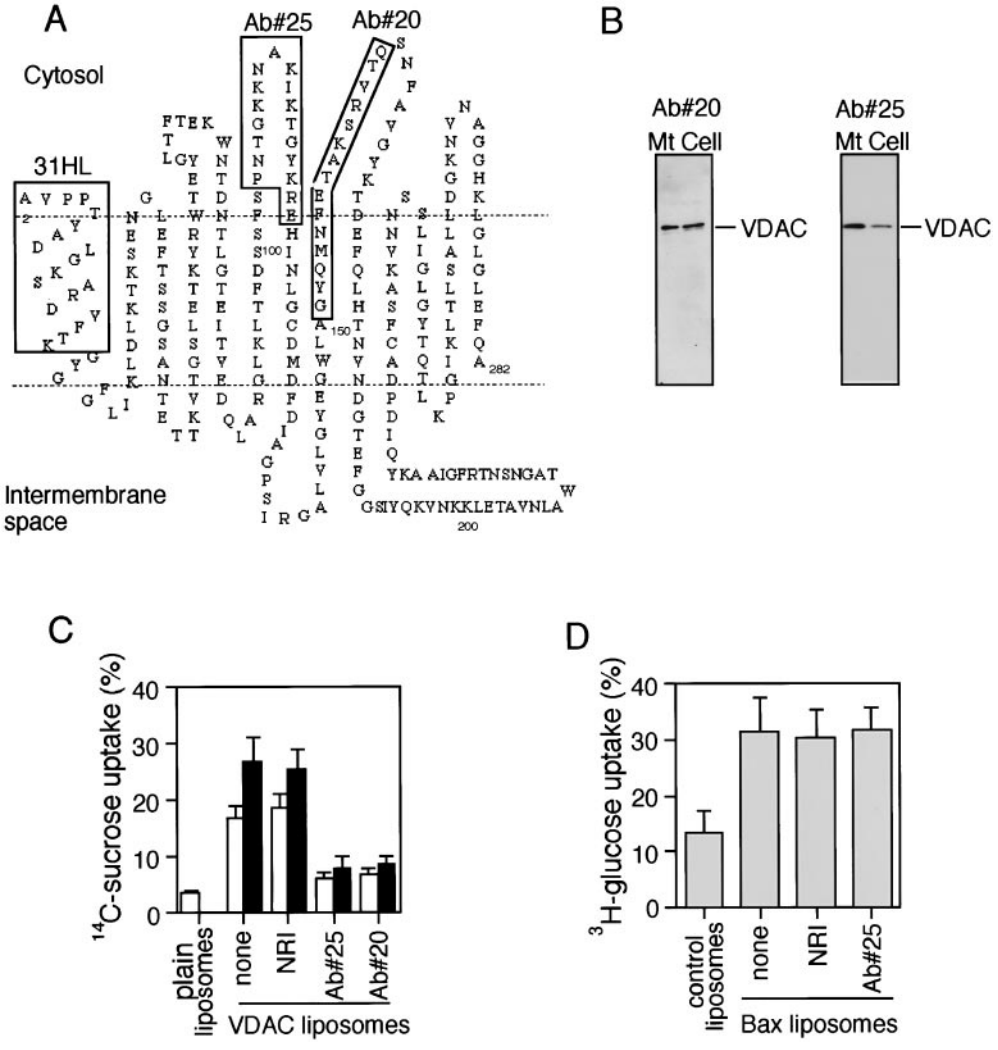

Figure 1. Inhibition of VDAC activity by anti-VDAC antibodies. (A) Putative model of human VDAC1 topology. The epitopes of the three anti-VDAC antibodies (Ab#20, Ab#25, and 31HL) are shown by boxes. (B) Specificity of Ab#20 and Ab#25. Rat liver mitochondria (Mt) lysate (15 μg) and HeLa cell lysate (10 μg) were subjected to Western blotting using Ab#20 and Ab#25. (C) Inhibition of both VDAC activity and Bax-induced enhancement of VDAC activity by Ab#20 and Ab#25. 20 μl of plain liposomes or VDAC liposomes was incubated with 0.2 μg/μl of the indicated antibodies for 3 min, and then were incubated with 5 μl of [^{14}C]sucrose (97%; 200 μCi/ml) in the presence (black bar) or absence (white bar) of rBax (0.2 μg/μl) at 25°C for the 6 min. The [^{14}C]sucrose incorporated into the liposomes was measured as described in Materials and Methods. Data are shown as the mean ± SD for three independent experiments. (D) Lack of influence of Ab#25 on Bax channel activity. Irrelevant control protein liposomes and Bax liposomes were incubated with 0.2 μg/μl of Ab#25 or NRI for 5 min, and then incubated with 5 μl of [^{3}H]glucose (97%; 20 Ci/mmol) at 25°C for 5 min. The [^{3}H]glucose incorporated into the liposomes was measured as described in Materials and Methods. Data are shown as the mean ± SD for three independent experiments.

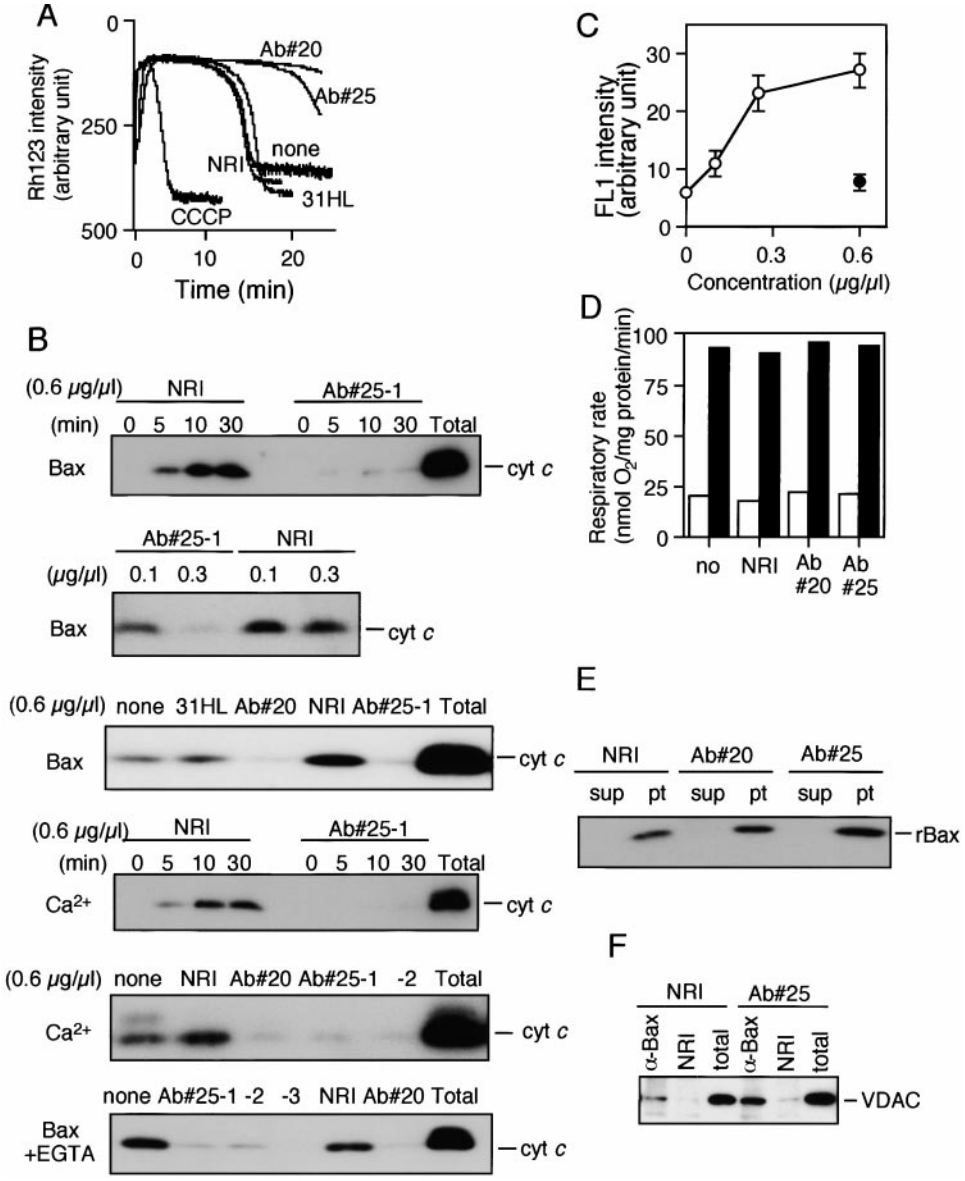

Figure 2. Inhibition of Bax-induced $\Delta\psi$ loss and cytochrome *c* release in isolated mitochondria by anti-VDAC antibodies. (A) Inhibition of Bax-induced $\Delta\psi$ loss by Ab#20 and Ab#25. Mitochondria (1 mg/ml) were preincubated with or without 0.6 μg/μl of the indicated antibodies (Ab#20, Ab#25, 31HL, or NRI) for 5 min, after which rBax (0.2 μg/μl) was added. Then $\Delta\psi$ was measured from the rhodamine 123 (Rh123) uptake over 25 min. When $\Delta\psi$ dropped, rhodamine 123 was released, resulting in an increase of rhodamine 123 intensity. Complete loss of $\Delta\psi$ was demonstrated by incubation of the mitochondria with 1 mM carbonylcyanide *m*-chlorophenylhydrazone (CCCP, protonophore). Data are representative of three independent experiments. (B) Inhibition of Bax-induced cytochrome *c* release by Ab#20 and Ab#25. Mitochondria (1 mg/ml) were preincubated with or without the indicated concentrations of antibodies (Ab#20, Ab#25-1, Ab#25-2, 31HL, or NRI) for 5 min, after which rBax (0.2 μg/μl) or Ca^{2+} (50 μM) was added (top 5 panels). Mitochondria preincubated with antibodies were also incubated with rBax in the presence of 0.2 mM EGTA (bottom panel). In the presence of EGTA, a higher concentration of rBax (1 μg/μl) was used to induce cytochrome *c* release comparable to that without EGTA. The extent of cytochrome *c* release was measured at 10 min (second, third, fifth, and bottom panels) or at the indicated times (top and fourth panels) by Western blot analysis of the supernatants. "Total" represents the total amount of cytochrome *c* in the same amount of mitochondria. Data are representative of two or three independent experiments. (C) Immunostaining of mitochondria with Ab#25. Mitochondria (1 μg/μl) were incubated with Ab#25 (open circles) or NRI (filled circle) at the indicated concentrations, and then stained with anti–rabbit IgG-Alexa488, after which the fluorescence was measured by flow cytometry as described in Materials and Methods. Data are shown as the mean ± SD for three independent experiments. (D) Lack of effect of Ab#20 and Ab#25 on mitochondrial respiration. Mitochondria (1 μg/μl) were incubated with 0.6 μg/μl of the indicated antibodies for 5 min, and then respiration was measured in the presence of 5 mM succinate (state IV; white bars) or succinate plus 0.3 mM ADP (state III; black bars). Data are representative of two independent experiments. (E) Lack of effect of Ab#20 and Ab#25 on mitochondrial association of Bax. Mitochondria were treated as described in A. At 10 min after addition of rBax, the mitochondria were spun, and the supernatants (sup) and pellets (pt) were subjected to Western blot analysis for Bax detection. (F) Lack of effect of Ab#25 on Bax–VDAC interaction. Mitochondria were treated as described in A. At 10 min after addition of rBax, mitochondria were lysed and immunoprecipitated with anti-Bax antibody (α-Bax) or NRI. Immune complexes were analyzed by Western blotting. "Total" represents 1/10 the amount of mitochondria used for the experiment.

ure legends. In some experiments, cells were stained with Hoechst 33342 (10 μM) or annexin V–Cy3 using an Annexin V–Cy3 apoptosis detection kit (MBL). Cell morphology was examined under a transmission microscope or a fluorescence microscope.

Immunostaining

Cells were washed twice with PBS and fixed with 3.7% formaldehyde in PBS at room temperature for 10 min. After permeabilization with 0.5% Triton X-100 at room temperature for 5 min, cells were incubated with 5% skim milk. Then the primary antibody in 5% skim milk was added for ~12 h at 4°C. After washing twice, cells were treated with the respective secondary antibody conjugated with Alexafluor488 or 568 and were examined under a fluorescence microscope.

Lysis of RBCs

Whole blood was obtained from a healthy donor with consent. RBCs were prepared by washing the whole blood with a large amount of 0.9% NaCl, followed by centrifugation and suspending in 0.9% NaCl. The RBCs were

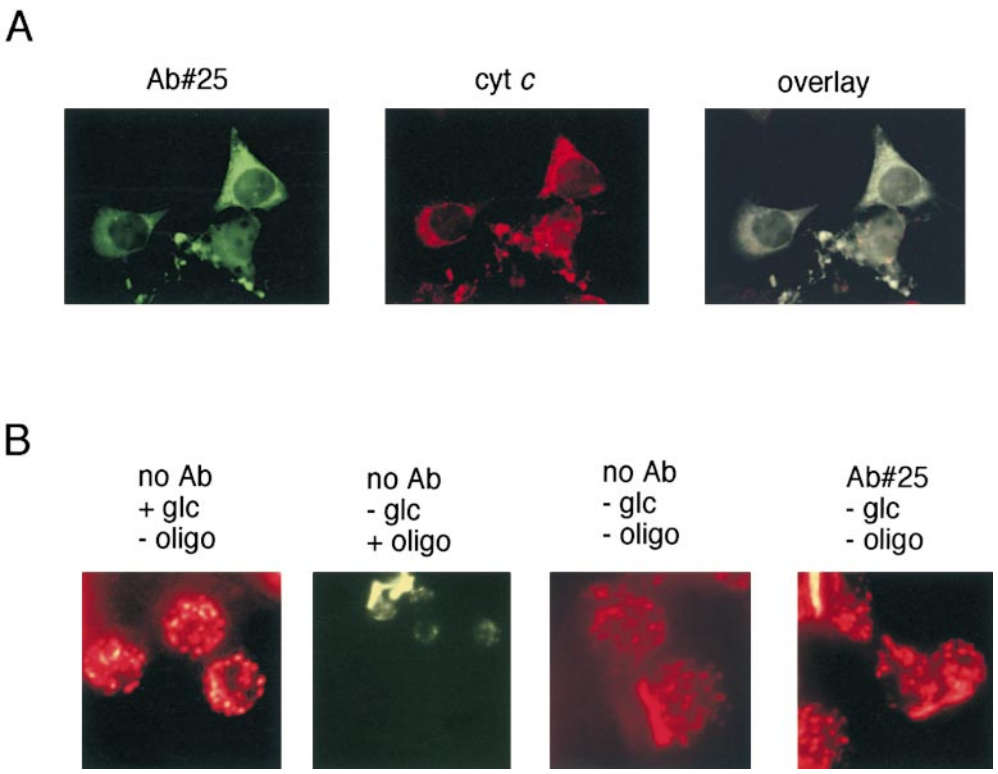

Figure 3. Effect of the microinjection of anti-VDAC antibodies. (A) Localization of injected Ab#25. HeLa cells were microinjected with 3 μg/μl of Ab#25. After fixing, the cells were incubated with anti-cytochrome *c* antibody for 12 h at 4°C, and then with anti–rabbit IgG-Alexa488 (which reacted with Ab#25) and anti–mouse IgG-Alexa568 (which reacted with anti-cytochrome *c* antibody), followed by observation under a fluorescence microscope. (B) Lack of inhibition of mitochondrial respiration by Ab#25. HeLa cells were microinjected with 12 μg/μl of Ab#25. Then noninjected and injected cells were incubated in glucose-containing (+ glc) or glucose-free (− glc) medium in the presence or absence of 10 μM oligomycin (oligo). After 24 h, cells were stained with JC-1 dye and observed under a fluorescence microscope. The dye gave an orange color to cells with a high $\Delta\psi$ and a green color to cells with a low $\Delta\psi$.

treated with antibodies or BH4 oligopeptides for 5 min, after which rBax, rBak, or Kanagawa hemolysin was added at the indicated amount. Then the RBCs were spun, and released hemoglobin was measured by the absorbance at 543 nm (the isospecific point of reduced and oxidized hemoglobin) using a spectrophotometer (UV-160A; Shimadzu).

FACS® Analysis

Mitochondria and RBCs were incubated with antibodies at the indicated concentrations for 30 min at 4°C, and then washed with MT-1 medium and 0.9% NaCl, respectively. Subsequently, the mitochondria and RBCs were incubated with 20 μg/ml of anti–rabbit IgG-Alexa488 for 1 h at 4°C. After washing twice, samples were applied to a flow cytometer (FACSCalibur™; Becton Dickinson) for analysis.

Results

Anti-VDAC Antibodies Inhibit Bax-induced $\Delta\psi$ Loss and Cytochrome c Release in Isolated Mitochondria

Recently, we showed that Bax/Bak is able to induce cytochrome *c* release by opening the VDAC through direct interaction in a proteoliposome system (Shimizu et al., 1999, 2000a). However, the essential role of the VDAC in apoptotic cytochrome *c* release and cell death in mammalian cells was still unconfirmed. For this purpose, we produced anti-VDAC antibodies that could inhibit VDAC activity. As epitopes, we selected two oligopeptides (amino acids 104–120 and 151–165) of human VDAC1 shown in Fig. 1 A, based on previous observations that VDAC in intact bovine heart mitochondria is cleaved by trypsin at lysine residues between arginine[108], which corresponds to lysine[108] in human VDAC1, and arginine[119] and by chymotrypsin at the tyrosine[117] and tyrosine[172] residues (De Pinto and Palmieri, 1992), indicating that these sites are probably exposed to the cytoplasm, and also based on the proposed three-dimensional structure (De Pinto and Palmieri, 1992; Song and Colombini, 1996) that suggests that these oligopeptides are probably localized near the entrance of the channel whereas most of the other regions form one α-helix and 12 β-strands that cross the membrane to create a β-barrel (Fig. 1 A). Furthermore, since these residues are well conserved among three isoforms of human and mouse, the antibodies were expected to react with all isoforms of various mammals. Both antibodies raised in rabbits (Ab#20 recognizing amino acids 151–165 and Ab#25 recognizing amino acids 104–120) were affinity purified and were shown to be highly specific for human and rat VDAC (Fig. 1 B). As shown in Fig. 1 C, Ab#20 and Ab#25, but not normal rabbit IgG (NRI), efficiently inhibited VDAC activity, as assessed by [^{14}C]sucrose uptake into VDAC liposomes. Ab#25 and Ab#20, but not NRI, also inhibited the Bax-induced enhancement of VDAC activity (Fig. 1 C). In contrast, both Ab#20 and Ab#25 showed no effect on Bax channel activity, as assessed by [^{3}H]glucose uptake into Bax liposomes (Fig. 1 D; data not shown).

Using these neutralizing anti-VDAC antibodies, we first examined the role of the VDAC in Bax-induced apoptotic changes of isolated mitochondria. As shown in Fig. 2, A and B (third panel from the top) NRI and a monoclonal anti-VDAC antibody (31HL: with an epitope corresponding to the NH_2-terminal region of VDAC as shown in Fig. 1 A) (Babel et al., 1991) that had no influence on VDAC activity (Benz et al., 1992; data not shown) did not inhibit, but rather slightly enhanced both Bax-induced $\Delta\psi$ loss and cytochrome *c* release. In contrast, these mitochondrial changes were efficiently inhibited by Ab#20 and Ab#25 in a

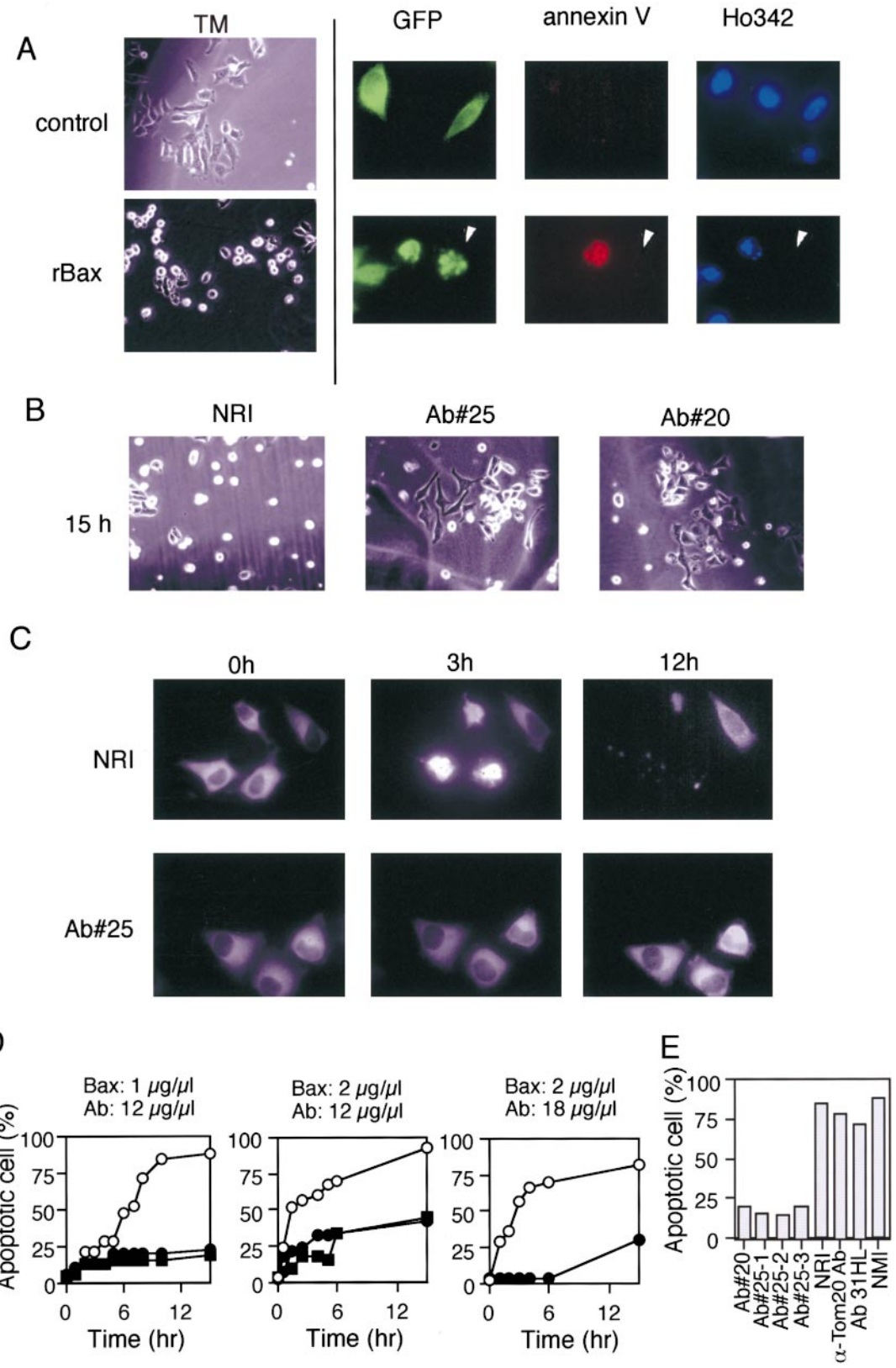

Figure 4. Inhibition of rBax-induced apoptosis by injection of anti-VDAC antibodies. (A) Induction of apoptosis by microinjection of rBax. rBax (1 μg/μl) or the equivalent amount of irrelevant control protein was microinjected into the cytoplasm of HeLa cells with GFP (3 μg/μl). After 8 h, cell morphology was assessed by transmission microscopy (TM) and fluorescence microscopy (GFP). Cells were also stained with annexin V and Hoechst 33342, and observed under a fluorescence microscope. The color photographs were taken from the same field. The arrowhead indicates an example of cells at the terminal stage of apoptosis, showing weak annexin V staining with no Hoechst 33342 staining. (B–E) Inhibition of rBax-induced apoptosis by microinjection of Ab#20 and Ab#25. (B) HeLa

concentration-dependent manner (Fig. 2, A and B, top three panels), indicating that the VDAC is required for Bax-induced cytochrome *c* release and $\Delta\psi$ loss in mammalian mitochondria under our experimental conditions, consistent with our previous findings in yeast mitochondria (Shimizu et al., 1999, 2000c). The amount of Ab#20 and Ab#25 used was near the saturation level for VDAC on the mitochondria, as judged from flow cytometric analysis of mitochondria stained with Ab#25 (Fig. 2 C) and also by comparing the amount of each antibody with the amount of VDAC in the mitochondria using PAGE (data not shown).

Since we have previously shown that Bax-induced cytochrome *c* release largely depends on mitochondrial respiration under our experimental conditions (Shimizu and Tsujimoto, 2000), and since VDAC is involved in mitochondrial respiration (for review see Colombini, 1989) by acting as a gate for respiratory substrates, the possibility was raised that the anti-VDAC antibodies inhibited respiration and thereby blocked the action of Bax on the mitochondria. However, as shown in Fig. 2 D, Ab#20 and Ab#25 had no effect on state IV and state III respiration at concentrations sufficient to inhibit cytochrome *c* release, suggesting that these antibodies inhibited cytochrome *c* release by directly inhibiting Bax-induced enhancement of VDAC activity. Since neither Bax association with the mitochondria nor Bax–VDAC interaction was inhibited by Ab#20 and Ab#25 (Fig. 2, E and F), inhibition of apoptotic mitochondrial changes by these antibodies was not due to simple blocking of Bax association with mitochondria or Bax–VDAC interaction, but was probably due to inhibition of VDAC conformational changes that are normally induced by Bax.

As can be seen in Fig. 2 B (forth and fifth panels from the top), Ca^{2+}-induced cytochrome *c* release, which was previously shown to be mediated by the PT (Narita et al., 1998), was also inhibited by Ab#20 and Ab#25 (25-1 and 25-2 were derived from different animals), indicating that the VDAC is essential for the PT.

It has previously been shown that Bax-induced cytochrome *c* release is largely dependent on the PT (i.e., blocked by PT inhibitors such as cyclosporine A and bongkrekic acid, and accompanied by $\Delta\psi$ loss) (Jürgensmeier et al., 1998; Marzo et al., 1998; Narita et al., 1998) and only partly occurs in a PT-independent manner (Narita et al., 1998; Shimizu and Tsujimoto, 2000), although other groups have reported that Bax-induced cytochrome *c* release is not dependent on the PT, particularly in the absence of Ca^{2+} (Eskes et al., 1998; Finucane et al., 1999; Kluck et al., 1999). To determine whether inhibition of Bax-induced cytochrome *c* release by Ab#20 and Ab#25 was specific to PT-associated cytochrome *c* release, we examined the effect of these antibodies on Bax-induced cytochrome *c* release in the presence of a Ca^{2+} chelator that completely inhibited the PT (Zoratti and Szabó, 1995; data not shown). As shown in Fig. 2 B (bottom), PT-independent cytochrome *c* release induced by Bax was also inhibited by both Ab#20 and Ab#25, indicating an essential role of the VDAC in Bax-induced cytochrome *c* release regardless of PT dependency. Note that a higher concentration of rBax was used to induce cytochrome *c* release in the presence of EGTA than that without EGTA.

Anti-VDAC Antibodies Inhibit Bax-induced Apoptosis

To determine whether the VDAC had an essential role in Bax-induced apoptosis in mammalian cells, we carried out an experiment in which Ab#20 and Ab#25 were microinjected into the cytoplasm of HeLa cells. Consistent with the predominant localization of VDAC in the mitochondria, Ab#25 was mainly observed in the mitochondria by immunostaining, when injected at a lower concentration (3 μg/μl) (Fig. 3 A), whereas it was observed in the mitochondria with a small amount throughout the cytoplasm when injected at a higher concentration of 12 μg/μl (data not shown), suggesting that a concentration of 3 μg/μl was less than the saturation level for mitochondrial VDAC. Therefore, microinjection experiments were mainly done with Ab#20 and Ab#25 at 12 μg/μl, which was close to the saturation level for mitochondrial VDAC. Injection of these antibodies showed little toxicity in HeLa cells (data not shown). To test the effect of Ab#20 and Ab#25 on mitochondrial respiration, cells were incubated in glucose-free medium for 24 h. Since glycolysis was halted under these conditions, inhibition of mitochondrial respiration (e.g., by the addition of oligomycin, an F_1 ATPase inhibitor) led to mitochondrial $\Delta\psi$ loss (Fig. 3 B, second panel). However, when cells were microinjected with Ab#20 or Ab#25, $\Delta\psi$ remained high during incubation in glucose-free medium (Fig. 3 B, far right panel; data not shown), indicating that these antibodies did not significantly affect mitochondrial respiration, consistent with the results obtained using isolated mitochondria (Fig. 2 D).

To examine the effect of Ab#20 and Ab#25 on Bax-induced apoptosis, these antibodies or NRI (as a control) were microinjected into the cytoplasm of HeLa cells, and rBax was microinjected into the cytoplasm of the same cells 1 h later to induce apoptosis. After injection of rBax, but not irrelevant protein, nearly all NRI-injected cells underwent apoptosis at 15 h as shown by cellular shrinkage,

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cells were microinjected with 12 μg/μl of the indicated antibodies. After 1 h, 2 μg/μl of rBax was microinjected into the same cells, and after 15 h, cells were examined under a transmission microscope. All of the cells shown were microinjected. Data are representative of seven independent experiments. (C) The same procedure shown in B was followed, except that 3 μg/μl of GFP was coinjected with rBax. Cell morphology was assessed at the indicated times under a fluorescence microscope. Data are representative of three independent experiments. (D) HeLa cells were microinjected with Ab#20 (filled squares), Ab#25 (filled circles), or NRI (open circles) at the indicated concentrations. After 1 h, rBax at the indicated concentrations was microinjected into the same cells, and apoptosis was investigated under a transmission microscope. More than 100 injected cells were analyzed. Data are representative of two or seven independent experiments. (E) HeLa cells were microinjected with 12 μg/μl of the indicated antibodies. After 1 h, the cells were injected with 1 μg/μl of rBax, and apoptosis was assessed at 12 h under a transmission microscope. NMI indicates normal mouse IgG used as a control for 31HL. Data are representative of two independent experiments.

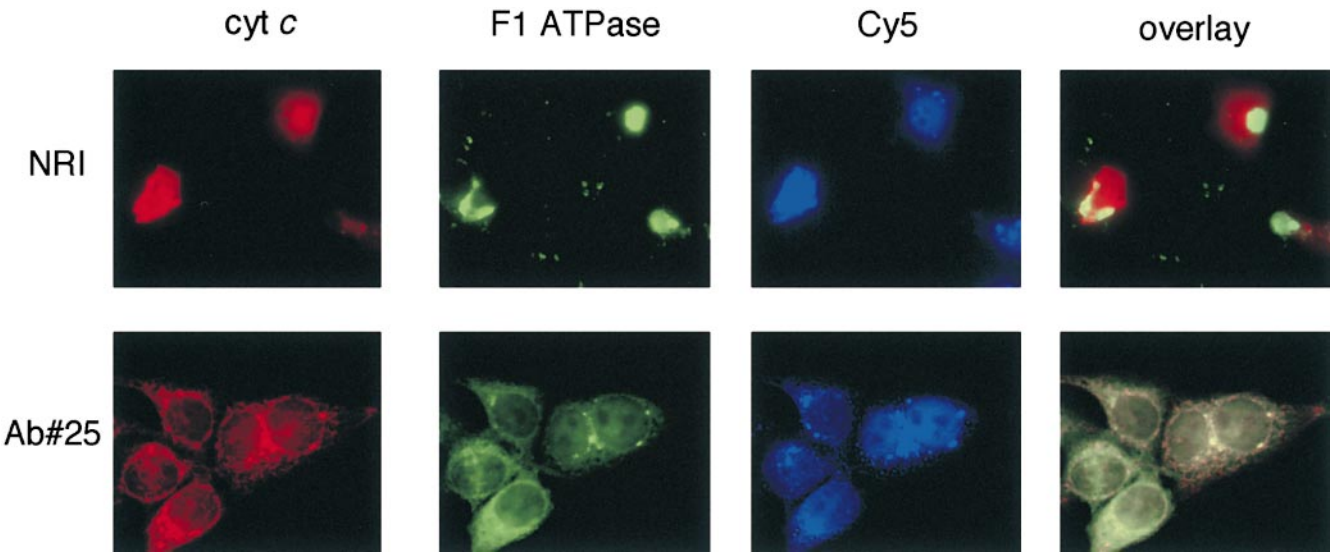

Figure 5. Inhibition of rBax-induced cytochrome *c* release by anti-VDAC antibodies. HeLa cells were microinjected with 12 μg/μl of NRI or Ab#25 plus 0.4 μg/μl of Cy5-mouse IgG (to identify the injected cells), and then 1 μg/μl of rBax was injected. After 12 h, the intracellular distribution of cytochrome *c* and that of F_1 ATPase (as a mitochondrial marker) was assessed by immunostaining. Fluorescence images of cytochrome *c* and F_1 ATPase are merged in the right panels (overlay).

rounding (Fig. 4 A), and eventual detachment from the culture dishes. Apoptosis was also confirmed by positive annexin V staining and by nuclear shrinkage and fragmentation on Hoechst 33342 staining (Fig. 4 A). Cells in the terminal stage of apoptosis (Fig. 4 A, arrowhead) only showed weak staining with annexin V and lost most of their DNA. On the other hand, depending on the amount of rBax injected (1 or 2 μg/μl), the majority and nearly half of the cells, respectively, remained alive after being preinjected with Ab#20 or Ab#25 (Fig. 4 D, left and middle), as shown by their flat morphology (Fig. 4 B) and by the division of some cells within 24 h (data not shown). Inhibition of apoptosis after injection of Ab#20 and Ab#25 was also confirmed by lack of annexin V staining as well as a normal nuclear morphology on Hoechst 33342 staining (data not shown). Fig. 4 C shows time-lapse photographs of cells injected with Ab#25 or NRI and then with rBax (1 μg/μl) and recombinant GFP (rGFP) to identify injected cells: NRI-injected cells shrank and became fragmented after 3 h, and were detached from the culture dishes by 12 h, whereas these changes were not observed when Ab#20 or Ab#25 was preinjected. Incomplete inhibition of rBax (2 μg/μl)-induced apoptosis by Ab#20 and Ab#25 at 12 μg/μl (Fig. 4 D, middle) was probably due to degradation of injected antibodies, because intracellular concentrations of Ab#20 and Ab#25, as assessed by immunostaining with anti–rabbit antibody conjugated with Alexa568, significantly decreased in a time-dependent manner (75 and 40% at 12 and 24 h after injection, respectively) and because injection of Ab#25 at a little higher concentration, 18 μg/μl, nearly completely inhibited Bax-induced apoptosis (Fig. 4 D, right). The inhibitory effect of these antibodies on Bax-induced apoptosis was not observed at lower concentrations of the antibodies, although the antibodies could still inhibit apoptosis induced by a lower concentration of rBax (data not shown). To exclude the possibility that these antibodies inhibited Bax-induced apoptosis merely by nonspecific binding to the mitochondria, we tested other antibodies, including 31HL and an antibody specific to Tom20, a protein on mitochondrial outer membrane. Both of these other antibodies did not inhibit Bax-induced apoptosis (Fig. 4 E). These results indicated that the VDAC is essential for Bax-induced apoptosis in mammalian cells.

Anti-VDAC Antibodies Inhibit Bax-induced Cytochrome c Release in Cells

To test whether Ab#20 and Ab#25 inhibited rBax-induced cytochrome *c* release from mitochondria in cells, Cy5-IgG was coinjected together with Ab#20 or Ab#25 to identify injected cells, followed by injection of rBax, and the distribution of cytochrome *c* was examined in comparison with that of mitochondrial F_1 ATPase. In cells injected with NRI and rBax, cytochrome *c* was diffusely distributed at 12 h, and in some cells, it was mainly localized in the nucleus (Fig. 5), and mitochondria tended to come together in the cells, as defined by F_1 ATPase staining. Thus the distribution of cytochrome *c* was quite different from that of F_1 ATPase as seen better in a merged photograph (Fig. 5, top right), indicating that cytochrome *c* had been released from the mitochondria. On the other hand, in cells injected with Ab#25 and rBax, the distribution of cytochrome *c* and F_1 ATPase was almost completely concordant (Fig. 5), as is the case in healthy cells (data not shown), indicating that Bax-induced cytochrome *c* release was prevented by Ab#25. Virtually identical results were obtained with Ab#20 (data not shown). These results indicated that the VDAC is essential for Bax-induced cytochrome *c* release in mammalian cells.

Effect of Anti-VDAC Antibodies on other Forms of Apoptosis

We next tested the effect of Ab#20 and Ab#25 on other forms of apoptosis. We have recently shown that BH3-only proteins, such as Bid (also tBid, an active form with an NH_2-terminal truncation) and Bik, do not bind to the VDAC, suggesting that a non-VDAC mechanism is in-

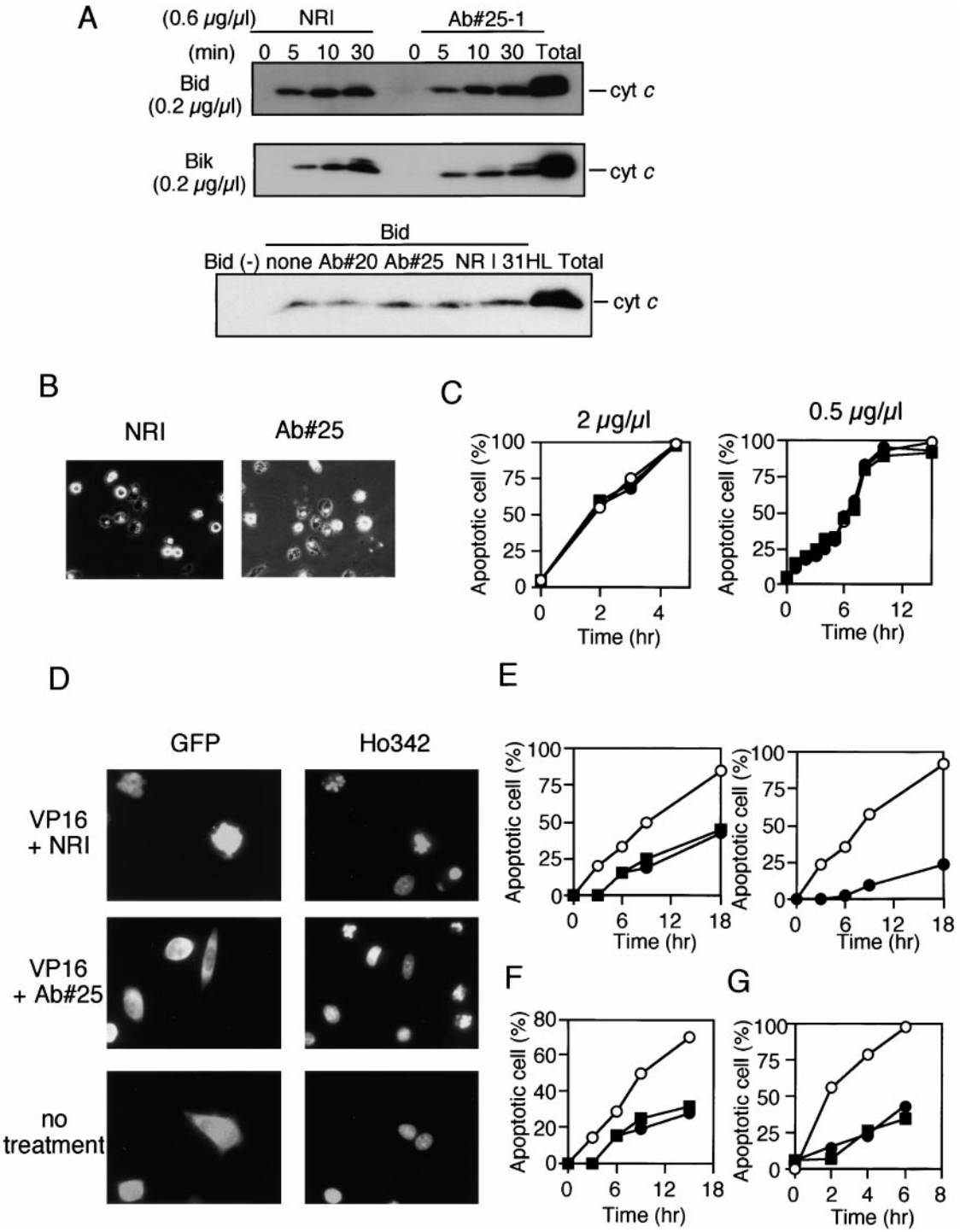

Figure 6. Inhibition of VP16-, paclitaxel- and staurosporine-induced, but not rtBid-induced, apoptosis by anti-VDAC antibodies. (A) Lack of inhibition of Bid- and Bik-induced cytochrome *c* release from isolated mitochondria by Ab#20 and Ab#25. Mitochondria (1 mg/ml) were pre-incubated with or without 0.6 μg/μl of the indicated antibodies (Ab#20, Ab#25, NRI, or 31HL) for 5 min, and then rBid (0.2 μg/μl) or rBik (0.2 μg/μl) was added. Cytochrome *c* release was measured at the indicated times (top two panels) or at 10 min (bottom panel) by Western blot analysis of supernatants obtained after centrifugation to remove the mitochondria. "Total" represents the total amount of cytochrome *c* in the same amount of mitochondria. Data are representative of two independent experiments. (B and C) Lack of effect of Ab#20 and Ab#25 on tBid-induced apoptosis. HeLa cells were microinjected with 12 μg/μl of Ab#25 (B; filled circles in C), Ab#20 (filled squares in C), or NRI (B; open circles in C). After 1 h, 2 μg/μl (C, left) or 0.5 μg/μl (B; C, right) of rtBid was microinjected into the same cells, and apoptosis was assessed from the cell morphology. More than 100 injected cells were analyzed. Data are representative of three independent experiments. Representative photographs taken at 12 h are shown in B. All cells shown in B were microinjected. (D–G) Inhibition of VP16-, paclitaxel-, and staurosporine-induced apoptosis by microinjection of Ab#25 and Ab#20. HeLa cells were microinjected with 12 μg/μl (D and E, left; F, and G) and 18 μg/μl (E, right) of antibodies together with 3 μg/μl of GFP. After 1 h, 200 μM of VP16 (D and E), 0.3 μM of paclitaxel (F), or 2 μM of staurosporine (G) was added. HeLa cells were also injected only with GFP as living cell control (no treatment in D). At 18 h, cells were stained with 1 μM Hoechst 33342 (Ho342), and photographed (D). In E–G, apoptosis was assessed from the cell morphology by fluorescence microscopy. More than 100 injected cells were analyzed. The open circles, filled circles, and filled squares correspond to NRI, Ab#25, and Ab#20, respectively. Data are representative of two or three independent experiments.

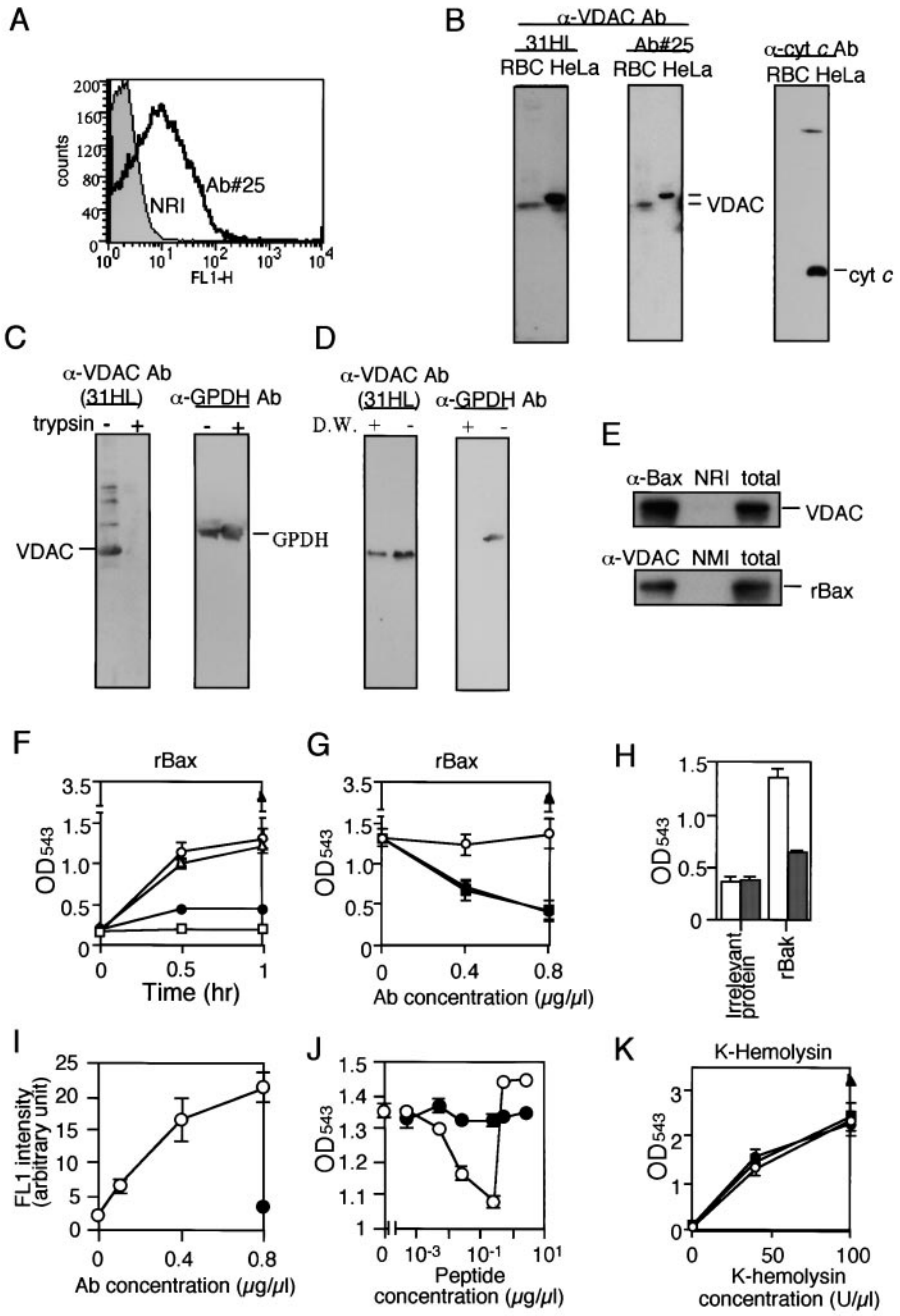

Figure 7. Inhibition of rBax-induced release of hemoglobin from RBCs by addition of anti-VDAC antibodies. (A–D) Detection of VDAC on the RBC plasma membrane. (A) RBCs, at 0.25% (vol/vol), were incubated with 0.4 μg/μl of Ab#25 or NRI for 30 min. After washing twice, anti–rabbit IgG-Alexa488 was added to the cells for 30 min and then the RBCs were analyzed using a flow cytometer. (B) Whole lysates of RBCs (40 μg) and HeLa cells (3 μg) were subjected to Western blot analysis using anti-VDAC antibody (31HL and Ab#25) and anticytochrome *c* antibody. (C and D) RBCs (40 μl), at 15% (vol/vol), were incubated with (+) or without (−) 1% trypsin for 1 h at 25°C, followed by incubation with 3% trypsin inhibitor for 30 min (C). RBCs were also incubated with an equivalent volume of distilled water (D.W.+) to produce ghost RBCs or 0.9% NaCl (D.W.−) for 30 min (D). After brief centrifugation, half of trypsin-treated RBCs and ghost RBC lysates were subjected to Western blot analysis using anti-VDAC antibody (31HL) and anti-GPDH antibody. (E) Interaction of rBax with VDAC on RBCs. RBCs (100 μl), at 15% (vol/vol), were incubated with 50 μg of rBax for 15 min at 25°C. Then, RBCs were lysed and immunoprecipitated with anti-Bax antibody (α-Bax), anti-VDAC antibody (31HL) (α-VDAC), NRI, or normal mouse IgG. Immune complexes were analyzed by Western blotting. "Total" represents 1/10 the amount of lysates used for the experiment. (F–H) Inhibition of Bax- and Bak-induced release of hemoglobin from RBCs by Ab#20 and Ab#25. All data are indicated as mean ± SD for three independent experiments. (F) RBCs, at 2.5% (vol/vol), were preincubated for 5 min with Ab#25 (filled circles), or NRI (open circles) at 0.8 μg/μl, or were preincubated without antibodies (open triangles), and then were incubated with rBax (1 μg/μl). RBCs were also incubated with an equivalent amount of irrelevant protein (open squares) for the indicated times. Then, the RBCs were spun, and free hemoglobin was detected at OD_{543} using a spectrophotometer. The total hemoglobin content was estimated after hypotonic lysis of RBCs (filled triangle). (G) A similar procedure as described in F was performed with the indicated concentrations of Ab#20 (filled squares), Ab#25 (filled circles), or NRI (open circles) for 5 min, followed by incubation with rBax (1 μg/μl) for 1 h. The total hemoglobin content was estimated after hypotonic lysis of RBCs (filled triangle). (H) A similar procedure as described in F was performed with Ab#25 (black bars), or NRI (white bars) for 5 min at 0.8 μg/μl, followed by incubation with rBak (1 μg/μl) or an equivalent amount of irrelevant protein for 1 h. Free hemoglobin was assessed. (I) Flow cytometric analysis of RBCs stained with Ab#25. RBCs, at 2.5% (vol/vol), were incubated with Ab#25 (open circles) or NRI (filled circle) at the indicated concentrations, and then stained with anti–rabbit IgG-Alexa488. Fluorescence was measured by flow cytometry as described in Materials and Methods. Data are indicated as mean ± SD for three independent experiments. (J) Inhibition of Bak-induced release of hemoglobin from RBCs by Bcl-x_L BH4 peptide. A similar procedure as described in H was performed with the BH4 peptide (open circles) or BH4 mutant ΔFL peptide (filled circles) for 5 min at the indicated concentrations, followed by incubation with rBak (1 μg/μl) for 1 h. Data are indicated as mean ± SD for three independent experiments. (K) Lack of effect of Ab#20 and Ab#25 on hemolysin-induced release of hemoglobin from RBCs. RBCs, at 2.5% (vol/vol), were preincubated with Ab#20, Ab#25, or NRI at 0.8 μg/μl for 5 min, and then were incubated with Kanagawa (K) hemolysin at the indicated concentrations for 1 h. Free hemoglobin was detected at OD_{543} using a spectrophotometer. The total hemoglobin content was estimated after hypotonic lysis (filled triangle). Data are indicated as mean ± SD for three independent experiments.

volved in Bid/Bik-induced cytochrome *c* release and cell death (Shimizu and Tsujimoto, 2000). Consistently, addition of Ab#20 or Ab#25 did not inhibit rBid- and rBik-induced cytochrome *c* release from isolated mitochondria (Fig. 6 A, compare with Fig. 2 B). Similar results were obtained with rtBid (data not shown). Furthermore, rtBid-induced apoptosis was not inhibited by preinjection of Ab#20 or Ab#25 into HeLa cells, irrespective of the injected amount of tBid (Fig. 6, B and C). The fact that the onset of apoptosis induced by 0.5 μg/μl of rtBid (Fig. 6 C) was slower than that induced by 2 μg/μl of rBax (Fig. 4 D) excluded the possibility that the amount of rtBid used supplied a death signal that overwhelmed the inhibitory capacity of Ab#25. These findings, taken together with our previous results (Shimizu and Tsujimoto, 2000), indicated that the VDAC is not required for tBid-induced cytochrome *c* release and cell death. Failure of Ab#20 and Ab#25 to inhibit tBid-induced cytochrome *c* release and apoptosis also indicated that inhibition of Bax-induced apoptosis and cytochrome *c* release by the anti-VDAC antibodies was not due to a nonspecific effect.

When incubated with isolated mitochondria, lysates of VP16 (etoposide)-induced apoptotic HeLa cells induce cytochrome *c* release that is largely dependent on Bax (Nomura et al., 1999), suggesting that Bax plays an important role in VP16-induced cytochrome *c* release. As expected, VP16-induced apoptosis was significantly inhibited by microinjection of Ab#20 or Ab#25, but not by NRI (Fig. 6, D and E). Note that in the Ab#25-injection experiment shown in Fig. 6 D, most of the cells that were not injected with the antibody died of apoptosis. Although VP16 might have activated not only Bax but also some of the BH3-only proteins that do not target the VDAC, incomplete inhibition of VP16-induced apoptosis by Ab#20 or Ab#25 (Fig. 6 E, left) was largely due to degradation of antibodies, because injection of Ab#25 at 18 μg/μl more efficiently inhibited VP16-induced apoptosis (Fig. 6 E, right). We also found that paclitaxel-induced and staurosporine-induced apoptosis were significantly inhibited by microinjection of Ab#20 or Ab#25 (12 μg/μl), but not by NRI (Fig. 6, F and G). Since paclitaxel-induced apoptosis was reported to depend on Bim, one of the BH3-only proteins (Bouillet et al., 1999), this suggested that Bim might target the VDAC or that Bax/Bak may contribute to this form of apoptosis in HeLa cells.

Anti-VDAC Antibodies Inhibit Bax-induced Lysis of RBCs

It has previously been reported that addition of rBax induces lysis of RBCs, and this hemolysis has been considered to be due to formation of the Bax channel on the RBC membrane (Antonsson et al., 1997). Since VDAC is also known to be located on the plasma membrane (Cole et al., 1992; Buettner et al., 2000), the possibility was raised that Bax-mediated RBC lysis was also mediated by the VDAC. The presence of the VDAC on RBC plasma membrane was confirmed by flow cytometry (Fig. 7 A), which also showed that it was oriented with the epitope for Ab#25 facing outside, consistent with previous observations (Cole et al., 1992). Since RBCs do not possess mitochondria, which was confirmed by the absence of cytochrome *c* (Fig. 7 B), and since they have no ER and Golgi apparatus, the VDAC shown by Western blotting (Fig. 7 B) probably largely represented VDAC on the plasma membrane. The VDAC on RBCs was ~1 kD smaller than that in HeLa cells (Fig. 7 B), but reacted with all the anti-VDAC antibodies (31HL, Ab#25 [Fig. 7 B], Ab#20, and an antibody whose epitope was amino acids 177–192 [data not shown]), and, therefore, the VDAC on RBCs probably represents a splicing variant or is subjected to RBC-specific modifications. Several erythrocyte-specific alterations of proteins have been reported, for example, hexokinase and pyruvate kinases (Lacrinique et al., 1992; Murakami and Piomelli, 1997). The possibility was not excluded that the VDAC on RBCs represents a protein highly related to VDAC or another member of the VDAC family. The presence of the VDAC on RBC plasma membrane was also confirmed by tryptic digestion of VDAC but not glyceraldehyde 3-phosphate dehydrogenase (GPDH), a cytosolic glycolytic enzyme (Fig. 7 C), and by the presence of VDAC in ghost RBCs (Fig. 7 D). Like mitochondrial VDAC, VDAC in RBCs bound to rBax (Fig. 7 E).

When RBCs were treated with rBax, hemolysis took place as shown by the release of hemoglobin (Fig. 7 F), whereas addition of Ab#20 or Ab#25 (but not NRI) efficiently inhibited Bax-induced hemolysis in a dose-dependent manner (Fig. 7, F and G). Consistent results were also observed when rBak was used instead of Bax (Fig. 7 H). These data, together with our previous observation that Bax/Bak directly and functionally interact with VDAC, indicated that Bax/Bak targeted the VDAC on the RBC plasma membrane to induce hemolysis, although the possibility was not formally excluded that Bax/Bak acted on RBCs independent of VDAC to trigger hemolysis that was somehow regulated by VDAC. The amount of Ab#20 and Ab#25 used was less than would have saturated the VDAC sites on RBCs, as judged by flow cytometric analysis (Fig. 7 I). Since we have previously shown that VDAC activity is inhibited by the BH4 peptide of Bcl-2 and Bcl-x_L (Shimizu et al., 2000b), we also tested the effect of the BH4 peptide on Bak-induced hemolysis. As shown in Fig. 7 J, the BH4 peptide, but not a mutant BH4 peptide (ΔFL) lacking the ability to close VDAC (Shimizu et al., 2000b), inhibited hemolysis in a dose-dependent manner, although to a lesser extent comparing with Ab#20 and 25. This partial inhibition of Bax-induced hemolysis by the BH4 peptide was partly due to toxicity of the BH4 peptide to induce extensive aggregation that was prominent at higher concentrations (Fig. 7 J).

As shown in Fig. 7 K, neither Ab#20 nor Ab#25 inhibited hemolysis induced by Kanagawa hemolysin, which is known to occur via formation of pores by the hemolysin itself. These results indicated that, as in the mitochondria, Bax increased plasma membrane permeability by acting on the VDAC.

Discussion

We have previously shown that Bax/Bak and Bcl-x_L bind to the VDAC and modulate its activity: Bax and Bak enhance/open the VDAC so that cytochrome *c* can pass through the channel and Bcl-x_L closes it (Shimizu et al., 1999, 2000a). Mutational studies have indicated that the ability of Bax/Bak and Bcl-x_L to regulate apoptosis paral-

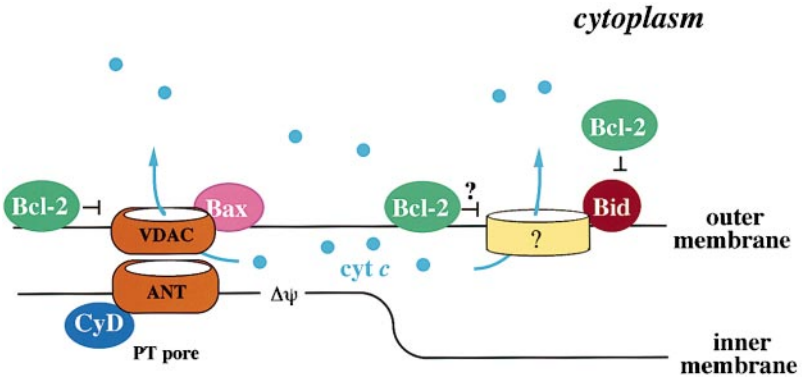

Figure 8. Functional interaction of Bcl-2/Bcl-x_L and Bax/Bak, but not Bid/Bik, with the VDAC. Bax/Bak directly opens the VDAC to induce cytochrome *c* release, while Bcl-2/Bcl-x_L closes this channel. Bid/Bik does not interact with the VDAC, but probably has the ability to open an unidentified channel(s) that is involved in cytochrome *c* release. The VDAC is a component of the PT pore, which contains ANT and cyclophilin D (CyD) and would explain the concomitant induction of Δψ loss by Bax/Bak and inhibition of Bax/Bak-induced cytochrome *c* and Δψ loss by PT inhibitors. Bcl-2/Bcl-x_L inhibits Bid/Bik-induced cytochrome *c* release, probably through heterodimerization with Bid/Bik or by closing an unidentified channel(s).

lels their ability to modulate the VDAC (Shimizu et al., 1999, 2000a,b; Shimizu and Tsujimoto, 2000). These results, taken together with our observation that Bax/Bak is able to induce cytochrome *c* release and Δψ loss in mitochondria isolated from wild-type yeast cells but not from VDAC1-deficient yeast cells (Shimizu et al., 1999, 2000c), led us to propose that cytochrome *c* is released through the VDAC during apoptosis and the Bcl-2 family of proteins regulate apoptosis by modulating VDAC activity in mammalian cells. Several other models have been proposed to explain how Bax induces cytochrome *c* release: (a) by rupture of the outer mitochondrial membrane due to swelling (Vander Heiden et al., 1997, 1999) that could result from Bax–adenine nucleotide translator (ANT) interaction (Marzo et al., 1998) or due to Bax-mediated destabilization of the lipid membrane (Basanez et al., 1999); or (b) via the multimeric channels of Bax (Eskes et al., 2000; Saito et al., 2000). In this study, using neutralizing anti-VDAC antibodies, we obtained direct evidence that the VDAC is essential for Bax-induced apoptotic mitochondrial changes (including cytochrome *c* release and Δψ loss) and for apoptotic death of mammalian cells. We also showed that anti-VDAC antibodies could significantly inhibit etoposide-, paclitaxel-, and staurosporine-induced apoptosis. These results support our model that the VDAC is involved in apoptogenic cytochrome *c* release and apoptosis that are dependent on members of the Bax subfamily. Although the results in this study confirmed that VDAC is essential for Bax-induced apoptosis, they did not formally confirm that cytochrome *c* indeed passes through VDAC in mammalian cells during apoptosis. However, our previous demonstration that cytochrome *c* can pass through the VDAC in the presence of Bax/Bak in the proteoliposome system (Shimizu et al., 1999, 2000a) strongly suggests that VDAC is a channel for apoptotic cytochrome *c* release in mammalian cells. To confirm this notion requires further investigation. Since our neutralizing anti-VDAC antibodies inhibited VDAC activity, leading to the inhibition of apoptotic mitochondrial changes and cell death, our results excluded the possibility that the VDAC needs to be inhibited for the induction of apoptosis, leading to hyperpolarization and subsequent mitochondrial swelling (Vander Heiden et al., 1997, 1999) that is blocked by the action of Bcl-2 to maintain VDAC function (Vander Heiden et al., 2000). We also showed that the VDAC is essential for Bax-induced rupture of RBCs, although this phenomenon was previously considered to be mediated by large pores formed by Bax alone (Antonsson et al., 1997). Thus, the VDAC is essential for Bax to modulate membrane permeability at two independent sites, the mitochondrial membrane and the plasma membrane, further supporting the idea that the VDAC is the functional target of Bax. Given that none of the Bcl-2 family proteins is secreted, a Bax-mediated increase of plasma membrane permeability does not seem to be physiologically relevant. However, the VDAC on the plasma membrane is involved in cell volume regulation (Thinnes et al., 2000), so there might be secreted protein(s) that modulate this VDAC in a similar fashion to Bax/Bak.

It has previously been suggested that Bax induces cytochrome *c* release via two different mechanisms in isolated mitochondria: a mechanism that is accompanied by Δψ loss and is blocked by PT inhibitors such as cyclosporine A (Jürgensmeier et al., 1998; Marzo et al., 1998; Narita et al., 1998) or a mechanism that is not accompanied by Δψ loss and is resistant to PT inhibitors (Eskes et al., 1998; Finucane et al., 1999; Kluck et al., 1999), although it seems likely that the former mechanism is more physiologically relevant because Bax induces cytochrome *c* release together with Δψ loss in mammalian cells (Xiang et al., 1996; Marzo et al., 1998; Pastorino et al., 1998; Shimizu and Tsujimoto, 2000; our unpublished observations). In the present study, our neutralizing anti-VDAC antibodies inhibited Bax-induced cytochrome *c* release that was largely PT inhibitor-sensitive. Importantly, the same antibodies inhibited Bax-induced cytochrome *c* release in the absence of Ca^{2+}, that was PT inhibitor resistant. Thus, irrespective of PT dependence, Bax-induced cytochrome *c* release always requires the VDAC (Fig. 8). Accordingly, the likely scenario is that Bax triggers VDAC opening and cytochrome *c* release, leading to opening of inner membrane channel(s) such as ANTs via a mutual interaction that results in Δψ loss and the PT, which might subsequently induce another phase of cytochrome *c* release.

The VDAC has been considered to be a component of the PT pore (for reviews see Bernardi et al., 1994; Zoratti and Szabó, 1995), although conclusive evidence for direct involvement of the VDAC in the PT has not been obtained. Our result that anti-VDAC antibodies inhibited the Ca^{2+}-induced PT is the first direct evidence that the VDAC is essential for the PT. Possible mutual interaction among different components of the PT pore complex could explain how the ANT inhibitor bongkrekic acid (which is assumed to close the ANT channel) and cy-

closporine A (which targets mitochondrial cyclophilin D) can prevent Bax/Bak-induced VDAC opening and thereby inhibit cytochrome *c* release (Fig. 8).

We have previously shown that Bax-induced apoptotic mitochondrial changes are largely dependent on mitochondrial respiration (Shimizu and Tsujimoto, 2000). Various respiratory substrates and products, including succinate and adenine nucleotides, pass through the VDAC (for review see Colombini, 1989). Our finding that the neutralizing anti-VDAC antibodies blocked cytochrome *c* release, but not respiration, suggests that only a small fraction of VDAC activity is sufficient for respiration although a larger fraction is required for apoptogenic cytochrome *c* release, or that the antibodies may not close the mitochondrial VDAC completely so that substances involved in respiration still pass through the channel.

We have previously shown that BH3-only proteins such as Bid (also tBid) and Bik do not bind to VDAC (Shimizu and Tsujimoto, 2000). We have also shown that Bid/Bik-induced cytochrome *c* release is substantially different from Bax/Bak-induced cytochrome *c* release: (a) Bax/Bak-induced, but not Bid/Bik-induced, cytochrome *c* release is accompanied by $\Delta\psi$ loss; and (b) PT inhibitors and respiratory chain inhibitors block Bax/Bak-induced, but not Bid/Bik-induced, cytochrome *c* release (Shimizu and Tsujimoto, 2000). Based on these observations, we have proposed that Bax/Bak target VDAC to induce cytochrome *c* release, whereas Bid/Bik induces cytochrome *c* release by a mechanism not involving the VDAC (Shimizu and Tsujimoto, 2000; Tsujimoto and Shimizu, 2000b). Consistently, we also showed here that VDAC is required for Bax-induced, but not for Bid-induced, cytochrome *c* release and cell death. These results, together with our observation that Bax/Bak but not Bid/Bik induce cytochrome *c* release in mitochondria isolated from yeast cells (unpublished observation), suggest that Bid/Bik probably targets a distinct molecule on the mitochondrial outer membrane to induce cytochrome *c* release (Fig. 8). It has been suggested that Bid (tBid) exerts its activity by heterodimerizing with antiapoptotic members of the Bcl-2 family (Wang et al., 1996), or by facilitating oligomerization of Bax and Bak to form pores permeable for cytochrome *c* (Eskes et al., 2000; Wei et al., 2000). Bid (tBid) has also been suggested to have an ability to form a pore by itself (Schendel et al., 1999) or destabilizing lipid membrane (Kudla et al., 2000). The substantial difference between Bax/Bak-induced and Bid/Bik-induced cytochrome *c* release and apoptosis (Shimizu and Tsujimoto, 2000), however, makes the possibility unlikely that Bid functions only through heterodimerizing with Bcl-2/Bcl-x_L or facilitating Bax/Bak oligomerization, because these two models suggest no difference between Bid-induced and Bax/Bak-induced cytochrome *c* release and cell death. The possibility is also unlikely that Bid/Bik forms a channel by itself or destabilizes mitochondrial membrane for cytochrome *c* release, because, unlike Bax/Bak, Bid is unable to induce cytochrome *c* release in yeast mitochondria (our unpublished results). Since Bid/Bik-induced cytochrome *c* release is prevented by Bcl-2/Bcl-x_L, these antiapoptotic Bcl-2 family members might form heterodimers with Bid/Bik to inhibit their activity or directly close the Bid/Bik target channel in a similar manner as occurs with the VDAC (Fig. 8).

In conclusion, we obtained evidence that the VDAC is essential for Bax-induced (but not Bid-induced) apoptotic cytochrome *c* release and apoptotic death and apoptosis induced by etoposide, paclitaxel, and staurosporine in mammalian cells. These results, taken together with our previous observation that Bax opens the VDAC and releases cytochrome *c* in a proteoliposome system (Shimizu et al., 1999, 2000a), suggest that the VDAC is a channel for apoptotic cytochrome *c* release that is directly regulated by the Bcl-2 family of proteins.

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