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Mitochondrial release of apoptosis-inducing factor occurs downstream of cytochrome *c* release in response to several proapoptotic stimuli

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Mitochondrial outer membrane permeabilization by proapoptotic Bcl-2 family proteins, such as Bax, plays a crucial role in apoptosis induction. However, whether this only causes the intracytosolic release of inducers of caspase-dependent death, such as cytochrome *c*, or also of caspase-independent death, such as apoptosis-inducing factor (AIF) remains unknown. Here, we show that on isolated mitochondria, Bax causes the release of cytochrome *c*, but not of AIF, and the association of AIF with the mitochondrial inner membrane provides a simple explanation for its lack of release upon Bax-mediated outer membrane permeabilization. In cells overexpressing Bax or treated either with the Bax- or Bak-dependent proapoptotic drugs staurosporine or actinomycin D, or with hydrogen peroxide, caspase inhibitors did not affect the intracytosolic translocation of cytochrome *c*, but prevented that of AIF. These results provide a paradigm for mitochondria-dependent death pathways in which AIF cannot substitute for caspase executioners because its intracytosolic release occurs downstream of that of cytochrome *c*.

Introduction

Most proapoptotic stimuli require a mitochondria-dependent step, involving outer membrane permeabilization controlled by pro- and antiapoptotic members of the Bcl-2 family and leading to intracytosolic release of mitochondria intermembrane space proteins that can either trigger caspase activation, such as cytochrome *c*, or caspase-independent death pathways, such as apoptosis-inducing factor (AIF;* Martinou and Green, 2001; Zamzami and Kroemer, 2001). Bax is one of the main proapoptotic Bcl-2 family proteins; the presence of either Bax or Bak being required for most mitochondria-dependent cell death processes, including those induced by tBid, the caspase-activated form of the "BH3-domain only" proapoptotic Bcl-2 family protein Bid and by proapoptotic drugs such as staurosporine and actinomycin D (Wei et al., 2001). Bax has been reported to induce cytochrome *c* release both in Bax-expressing cells and in isolated mitochondria incubated with recombinant oligomerized Bax (Eskes et al., 1998; Jurgenmeier et al., 1998; Finucane et al., 1999; Antonsson et al., 2000). However, whether Bax-mediated mitochondria outer membrane permeabilization also induces the release of AIF (Susin et al., 1999) is so far unknown.

Here, we show that the mitochondrial outermembrane permeabilization induced either by Bax, by Bax- or Bak-dependent proapoptotic drugs, or by hydrogen peroxide (H_2O_2) results in the intracytosolic release of cytochrome *c*, but that subsequent caspase activation is required to induce the translocation of AIF into the cytosol. These findings identify the mitochondrial response to several proapoptotic stimuli as a selective process leading to a hierarchical ordering of the effectors involved in cell death induction.

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*Abbreviations used in this paper: AIF, apoptosis-inducing factor; Cox IV, cytochrome *c* oxidase subunit IV; H_2O_2, hydrogen peroxide; MIM, mitochondrial inner membrane; MOM, mitochondrial outer membrane; MP, mitoplast.

Key words: mitochondria; AIF; caspases; Bax; Bid

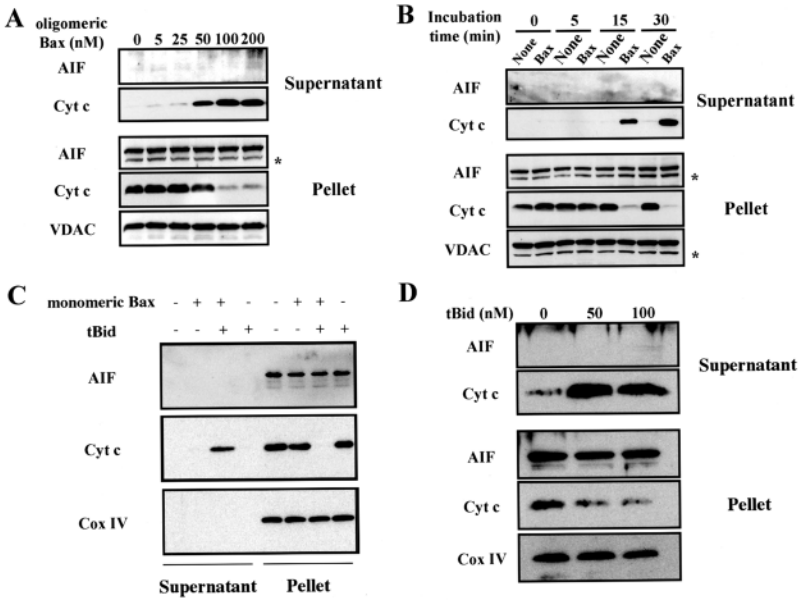

Figure 1. **Oligomeric Bax, Bax/tBid oligomers, and tBid induce the release of cytochrome *c*, but not of AIF from isolated mitochondria.** (A) Mitochondria isolated from HeLa cells were incubated for 30 min at 30°C with different concentrations (nM) of recombinant oligomeric Bax. Mitochondrial pellets and supernatant fractions were separated by SDS-PAGE, and their respective contents in AIF and cytochrome *c* (Cyt c) analyzed by Western blotting. (B) Mitochondria isolated from HeLa cells were incubated with 200 nM oligomeric recombinant Bax or with control buffer (none) at 30°C, and the mitochondria pellet and supernatant were analyzed at different time points (min), as in A. Asterisk in A and B indicates an additional band. (C) Mitochondria freshly isolated from rat liver cells were incubated for 15 min in the absence or presence of 100 nM recombinant monomeric Bax and/or 10 nM recombinant tBid, and analyzed as in A. (D) Mitochondria freshly isolated from rat liver cells were incubated for 30 min in the absence or presence of 50 or 100 nM recombinant tBid, and analyzed as in C. In all experiments, equal loading of the mitochondrial pellet was controlled using an mAb against either VDAC or cytochrome *c* oxidase subunit IV (Cox IV).

Results and discussion

To investigate whether Bax may induce the mitochondrial release of AIF, we incubated freshly purified mitochondria from human HeLa cells with recombinant oligomeric Bax, and performed Western Blot analysis of cytochrome *c* and AIF in both the mitochondria supernatants and pellets. For the detection of AIF, we used a pAb specific for human AIF (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200207071/DC1), obtained as indicated in Materials and methods. Mitochondrial release of cytochrome *c* was maximal for a Bax concentration of 100 nM (Fig. 1 A), and a kinetic analysis indicated that cytochrome *c* release was rapid; almost completed in 15 min (Fig. 1 B). In contrast, Bax did not induce any detectable mitochondrial release of AIF after incubations for 30 min with Bax concentrations up to 200 nM (Fig. 1, A and B). Because the isolated mitochondria we used were purified from HeLa cancer cells, and because cancer cells accumulate various alterations that favor cell death repression (Evan and Littlewood, 1998), we investigated the response of mitochondria from primary cells. Using another commercially available pAb specific for AIF, we explored the response of mitochondria from rat primary liver cells to either oligomeric Bax or to a form of oligomerized Bax obtained by mixing recombinant monomeric Bax with low concentrations of recombinant tBid. Although neither 100 nM monomeric Bax nor 10 nM tBid alone had any effect, together they induced the release of cytochrome *c*, but not of AIF (Fig. 1 C), as oligomeric Bax (unpublished data). At higher concentrations (50 or 100 nM), tBid by itself also induced the release of cytochrome *c*, but not of AIF (Fig. 1 D). Because tBid requires the presence of endogenous Bak on mitochondria to induce cytochrome *c* release from isolated murine liver cell mitochondria (Wei et al., 2000), tBid alone probably acted through the formation of tBid/Bak oligomers. Together, our data indicated that the lack of AIF release did not depend on the cell (primary or cancer) or species (human or rat) origin of the mitochondria.

AIF was originally described as a soluble protein localized in the mitochondrial intermembrane space (Susin et al., 1999). Therefore, a possible explanation for our findings was that Bax and tBid induce the formation of selective outer membrane pores (Martinou and Green, 2001), allowing the passage of cytochrome *c* but not of AIF. Alternately, Bax and tBid cause a nonselective process of outer membrane permeabilization (Zamzami and Kroemer, 2001), but AIF is not an intermembrane space–soluble protein. To discriminate between these two possibilities, we explored the localization of AIF and cytochrome *c* in subfractions of purified mitochondria from rat primary liver cells. When compared with its total amount in whole mitochondria, only a part of cytochrome *c* colocalized both with the mitoplasts (MP), obtained after outer membrane removal and consisting of the mitochondrial inner membranes (MIMs) and the matrix, and with the enriched, purified inner membranes (Fig. 2 A). In contrast, around the same amount of AIF colocalized with whole mitochondria, MP, and MIM, suggesting an absence of soluble AIF in the intermembrane space (Fig. 2 A). To investigate whether AIF is an integral component of the inner membrane or is rather peripherally associated with it, we subjected the MPs to sodium carbonate treatment. The alkali treatment caused a complete loss of AIF colocalization with the MPs, whereas the cytochrome *c* oxidase subunit IV (Cox IV) protein, an integral component of the inner membrane, was unaffected (Fig. 2 B). Together, these findings indicated that most AIF is peripherally associated with the mitochondrial inner membrane, presumably with its external side, and suggested that the outer membrane permeabilization induced by Bax or tBid is not sufficient to allow the detachment of AIF from the inner membrane.

Next, we investigated whether the mitochondrial release of cytochrome *c* may also be dissociated from that of AIF in

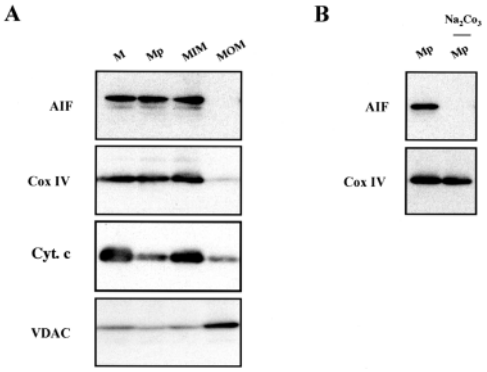

Figure 2. **AIF is associated with the mitochondrial inner membrane.** (A) Rat liver cell mitochondria (M), mitoplasts (Mp), mitochondrial inner membrane (MIM), and mitochondrial outer membrane (MOM), were resolved by SDS-PAGE, and their respective contents in AIF, cytochrome *c* (Cyt.c), Cox IV, and VDAC were analyzed by Western blotting. (B) Mp (that consists of the MIM and matrix) were further treated with 0.1 M sodium carbonate (Na_2CO_3), pH 11.5, for 30 min on ice and pelleted by centrifugation. VDAC, an integral component of MOM, is present in M and MOM, but also detectable at low levels in Mp and MIM, as VDAC is present at MIM/MOM junctions that seem unaffected by treatments used to separate MIM and MOM. Because at equal protein concentrations, MOM are enriched for VDAC, there is less VDAC in M than in MOM. Cox IV, an integral component of MIM, is present in M, Mp, and enriched in MIM, but lacking in MOM, and remains in Mp after Na_2CO_3 treatment.

cells overexpressing Bax. Bax-mediated mitochondrial release of cytochrome *c* does not require caspase activation and occurs in the presence of caspase inhibitors (Eskes et al., 1998; Finucane et al., 1999; Martinou and Green, 2001). We transiently transfected human 293T cells with an expression vector encoding Bax, in the absence or presence of the broad caspase inhibitor peptide z-VAD-fmk (100 μM), and performed a kinetic Western blot analysis. Bax expression led to a time-dependent increase in intracytosolic release of cytochrome *c* and AIF, caspase-9, and caspase-3 processing, PARP cleavage, and cell death (Fig. 3 A). z-VAD-fmk did neither prevent Bax expression nor Bax-induced intracytosolic release of cytochrome *c*, but prevented cell death induction (Fig. 3 A), nuclear DNA degradation (hypodiploidy) and mitochondrial transmembrane potential ($\Delta\Psi m$) loss (Fig. S2), caspase-9, and caspase-3 processing, PARP cleavage, and the intracytosolic release of AIF (Fig. 3 A). 100μM BAF, another broad caspase inhibitor, showed the same effect as z-VAD-fmk, preventing both cell death and the release of AIF, but not the release of cytochrome *c* (Fig. 3 B). To confirm these findings, we used immunofluorescence microscopy analysis to compare the localization of Bax, cytochrome *c*, AIF, and Hsp60 (a protein of the mitochondria matrix) in HeLa cells 18 h after transfection with a vector expressing GFP-Bax, in the absence or presence of z-VAD-fmk. Some nonspecific nuclear staining was induced by the polyclonal anti-AIF antibody (Fig. S1), and also by the control sera (unpublished data) in control HeLa cells. z-VAD-fmk prevented the induction of nuclear features of apoptosis in GFP-Bax–expressing cells (Fig. 3 C), but did neither prevent the induction of a punctuated cytosolic staining of Bax, that colocalized with Hsp 60, indicating an insertion of Bax into mitochondria, nor the induction of a diffuse cytosolic staining of cytochrome *c* (Fig. 3 C and D). In contrast, z-VAD-fmk treatment prevented the induction of a diffuse staining of AIF (Fig. 3, C and D), confirming that Bax did not induce the mitochondrial release of AIF in the presence of caspase inhibitors.

The proapoptotic drug staurosporine does not require the presence of AIF to cause cell death (Joza et al., 2001), but requires the presence of either Bax or Bak to induce mitochondrial release of cytochrome *c* and cell death (Wei et al., 2001). We treated HeLa cells with 2 μM staurosporine for 9 h in the absence or presence of 100 μM z-VAD-fmk. z-VAD-fmk prevented staurosporine-mediated apoptosis induction (Fig. 4 A), as well as caspase-9 and caspase-3 processing, PARP cleavage (Fig. 4 B), and the mitochondrial release of AIF (Fig. 4 C), but had no effect, as previously reported (Goldstein et al., 2000), on the mitochondrial release of cytochrome *c* (Fig. 4 C). Actinomycin D, as staurosporine, requires the presence of Bax or Bak to induce cytochrome *c* release and cell death (Wei et al., 2001). z-VAD-fmk also prevented apoptosis induction by 10 μM actinomycin D (16 ± 5 versus 68 ± 7), as well as mitochondrial release of AIF, but not of cytochrome *c* (Fig. 4 D).

Because staurosporine induced a rapid and marked cell shrinkage even in the presence of z-VAD-fmk, immunofluorescence studies were difficult to interpret (unpublished data). In contrast, cells did not undergo marked shrinkage when treated with actinomycin D and z-VAD-fmk. Therefore, we performed immunofluorescence analysis of cytochrome *c* and AIF in HeLa cells treated with actinomycin D, and observed that z-VAD-fmk prevented the mitochondrial release of AIF, though having no effect on the release of cytochrome *c* (Fig. 4, E and F). For further confirmation, we increased the detectability of AIF by overexpressing full-length human AIF in HeLa cells. 18 h after AIF transfection, we treated the HeLa cells for 9 h with actinomycin D in the absence or presence of z-VAD-fmk, and observed the same pattern of cytochrome *c* release and lack of AIF release in the cells treated with both actinomycin D and z-VAD-fmk (Fig. 4 G).

Cell death due to the mitochondrial release of AIF has been reported to occur in the presence of caspase inhibitors in response to certain stimuli (Joza et al., 2001; Pardo et al., 2001), including H_2O_2 (Yu et al., 2002). Using an AIF-specific mAb that did not induce background nuclear staining, we performed immunofluorescence analysis of HeLa cells treated with 400 μM H_2O_2 in the absence or presence of z-VAD-fmk. As in Bax-expressing and in actinomycin D–treated cells, z-VAD-fmk prevented both apoptosis and AIF release in H_2O_2-treated cells, while not affecting cytochrome *c* release (Fig. 5, A–C). Higher H_2O_2 concentrations caused necrotic cell death but did not induce AIF release in the presence of z-VAD-fmk (unpublished data). The very recent paper that intramitochondrial AIF acts as a free radical scavenger decreasing H_2O_2-mediated cell death (Klein et al., 2002) is consistent with the notion that AIF is not a selective effector of H_2O_2-induced death. Whether particular stimuli may allow a selective caspase-independent process of AIF release remains to be confirmed. Our observation that AIF is not a soluble mitochondrial intermembrane space protein, but rather is attached to the inner membrane, provides a

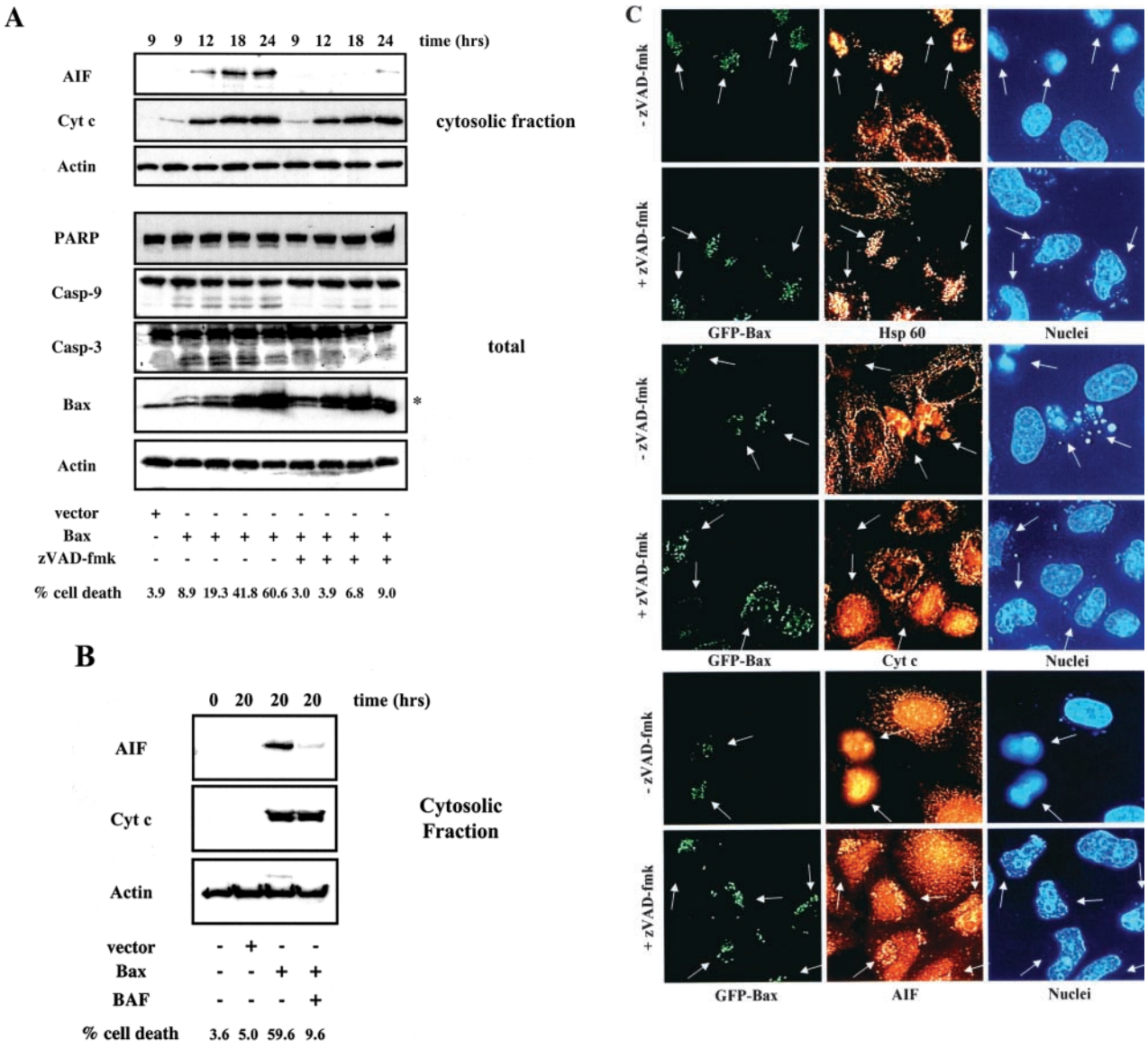

Figure 3. **Caspase inhibitors prevent mitochondrial release of AIF in Bax-overexpressing cells.** (A) Western blot analysis of AIF and cytochrome *c* (Cyt c) release in the cytosolic fraction, and of Bax expression, caspase-9 (Casp-9), and caspase-3 (Casp-3) processing and PARP cleavage in total cell extracts (total) at various time points after transient transfection of 293T cells with either a vector encoding HA-Bax (Bax) or the empty control vector (vector), in the absence (−) or presence (+) of the caspase inhibitor z-VAD-fmk (100 μM). Actin was used as loading control. Asterisk indicates the HA-Bax, the NH_2-terminal HA-tag providing an additional molecular mass of ~1.5 kD. (B) The cytosolic fraction of Bax transfected 293 T cells in the absence or presence of the caspase inhibitor BAF (100 μM) was analyzed by Western blotting for cytochrome *c* (Cyt c) and AIF release, as in A. (C) GFP-Bax expression, and immunostaining of Hsp60, cytochrome *c* (Cyt c), and AIF together with nuclear Hoechst staining in HeLa cells 18 h after transient transfection with a vector encoding GFP-Bax in the absence (−zVAD-fmk) or presence (+zVAD-fmk) of the caspase inhibitor z-VAD-fmk (100 μM). Nuclear staining by the polyclonal anti-AIF antibody is nonspecific, and also induced by control sera (not depicted). (D) Quantitative analysis of the numbers of GFP-Bax–transfected cells with intracytosolic release of cytochrome *c* and/or AIF in the absence or presence of 100 μM z-VAD-fmk. Each histogram indicates mean ± SD of three fields of at least 100 cells within a representative experiment.

simple potential explanation for our findings, and for recent findings by others, indicating that AIF is not among the proteins released by isolated mitochondria on incubation with tBid (Van Loo et al., 2002). Our observation that caspase inhibitors prevent AIF release in cells exposed to several proapoptotic stimuli, including Bax, Bax- or Bak-dependent proapoptotic drugs, and H_2O_2 strongly suggests that activation of given caspases after mitochondrial outer membrane permeabilization may be the limiting step for AIF detachment from the inner membrane and intracytosolic release.

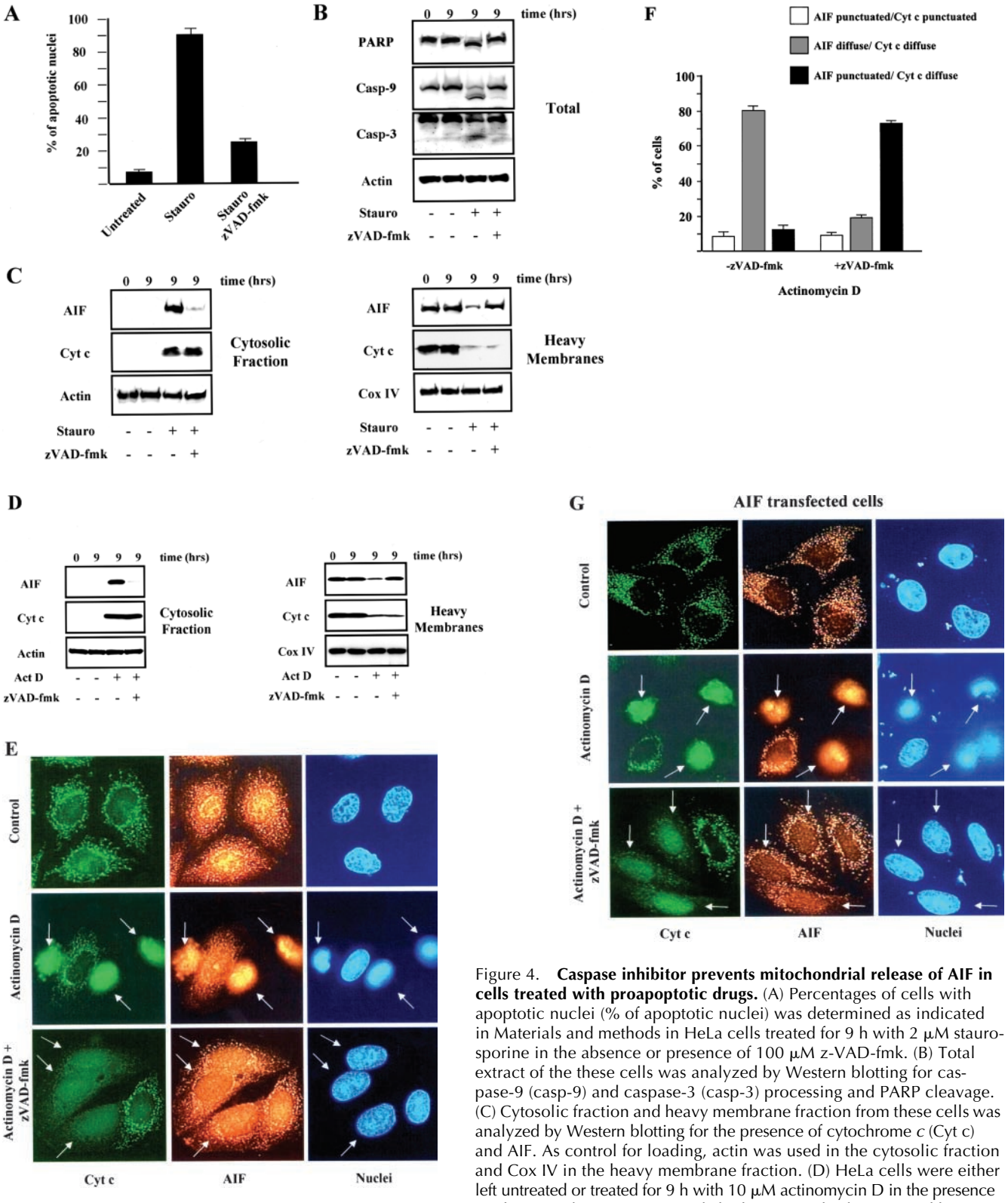

Figure 4. **Caspase inhibitor prevents mitochondrial release of AIF in cells treated with proapoptotic drugs.** (A) Percentages of cells with apoptotic nuclei (% of apoptotic nuclei) was determined as indicated in Materials and methods in HeLa cells treated for 9 h with 2 μM staurosporine in the absence or presence of 100 μM z-VAD-fmk. (B) Total extract of the these cells was analyzed by Western blotting for caspase-9 (casp-9) and caspase-3 (casp-3) processing and PARP cleavage. (C) Cytosolic fraction and heavy membrane fraction from these cells was analyzed by Western blotting for the presence of cytochrome *c* (Cyt c) and AIF. As control for loading, actin was used in the cytosolic fraction and Cox IV in the heavy membrane fraction. (D) HeLa cells were either left untreated or treated for 9 h with 10 μM actinomycin D in the presence or absence of 100 μM z-VAD-fmk, then cytosolic fraction and heavy membrane fraction were analyzed as in C. (E) The cells were also immunostained with the anti–cytochrome *c* (Cyt c) and anti-AIF antibodies together with Hoechst nuclear staining. (F) Quantitative analysis of the numbers of actinomycin D–treated cells with intracytosolic release of cytochrome *c* and/or AIF in the absence or presence of 100 μM z-VAD-fmk. Each histogram indicates mean ± SD of three fields of at least 100 cells within a representative experiment. (G) HeLa cells were transiently transfected for 18 h with a vector encoding full length AIF, and then either left untreated (Control) or treated for 9 h with 10 μM actinomycin D in the presence or absence of 100 μM z-VAD-fmk, and then immunostained with the anti–cytochrome *c* (Cyt c) and anti-AIF antibodies together with Hoechst nuclear staining. Arrows in E and G indicate cells showing intracytosolic release of cytochrome *c*.

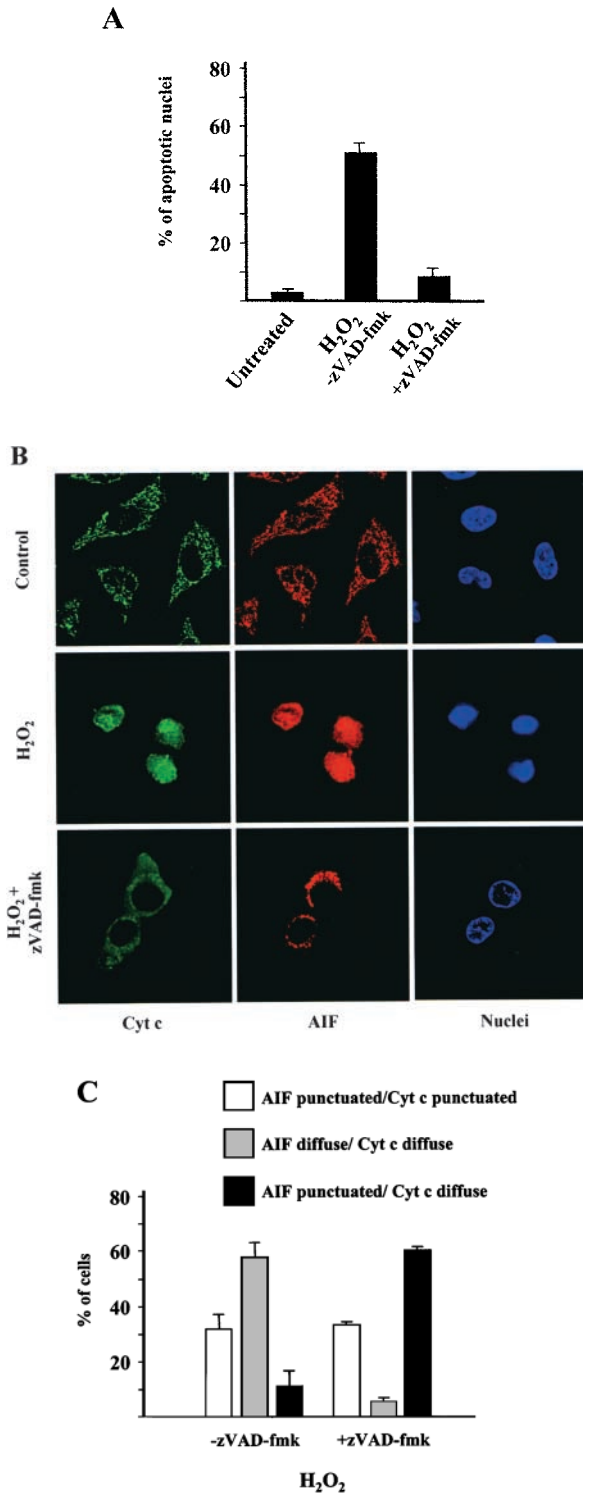

Figure 5. **AIF release is caspase dependent in H_2O_2-mediated cell death.** (A) Percentages of apoptotic nuclei were determined in HeLa cells treated for 6 h with 400 μM H_2O_2 in the absence or presence of 100 μM z-VAD-fmk. (B) HeLa cells were treated as in A and immunostained with a sheep anti–cytochrome *c* (Cyt c) and a mouse anti-AIF mAb together with Hoechst nuclear staining. (C) Quantitative analysis of the numbers of H_2O_2-treated cells with intracytosolic release of cytochrome *c* and/or AIF in the absence or presence of 100 μM z-VAD-fmk. Each histogram indicates mean ± SD of three fields of at least 100 cells within a representative experiment.

However, because caspase inhibitors may also prevent the activity of other proteases (Wolf et al., 1999), the precise pathway leading to AIF release downstream of cytochrome *c* release remains to be investigated.

Here, we have identified the mitochondrial response to Bax and other proapoptotic stimuli as a very selective process leading to a hierarchical ordering of the effectors involved in cell death induction. Our findings provide a paradigm for mitochondria-dependent cell death pathways involving a postmitochondrial level of pharmacological and possibly endogenous regulation that precedes the intracytosolic release of the caspase-independent death effector AIF.

Materials and methods

Mitochondria isolation and in vitro cytochrome *c* and AIF release

Mitochondria were isolated from HeLa cells and rat liver by sucrose density gradient centrifugation, and in vitro cytochrome *c* and AIF release was assessed as described previously (Eskes et al., 1998; Desagher et al., 1999). Production of full-length recombinant oligomeric, monomeric Bax and monomeric tBid (Antonsson et al., 2000; Kudla et al., 2000), and preparation of mitochondrial membrane vesicles from rat liver mitochondria (Mayer et al., 1993) were performed as described previously.

Cell culture and transfection

HEK 293T and HeLa cells were cultured in DME (GIBCO BRL) supplemented with 10% FCS, 2 mM L-glutamine, 50 IU penicillin, and 50 $\mu g \cdot ml^{-1}$ streptomycin under standard conditions. Transient transfections of 293T and of HeLa cells were performed using calcium phosphate and LipofectAMINE™ (GIBCO BRL), respectively. pcDNA-AIF (Susin et al., 1999) was provided by G. Kroemer and S. Susin (CNRS-UMR 8125, Institut Gustave Roussy, Villejuif, France).

Cell death measurement

At different time points after transfection or drug treatment, cells were recovered after incubation in PBS containing 1 mM EDTA, washed in PBS, and fixed with 4% PFA. Nuclei were then colored with Hoechst 33342 (Sigma-Aldrich). Green cells with blebbing, shrinkage, and apoptotic (condensed and fragmented) nuclei were considered as dying.

Subcellular fractionation

Cells were harvested in isotonic mitochondrial buffer (210 mM mannitol, 70 mM sucrose, 1 mM EDTA, and 10 mM Hepes, pH 7.5) supplemented with protease inhibitor cocktail Complete (Boehringer), and homogenized for 30–40 strokes with a Dounce homogenizer. Samples were transferred to Eppendorf centrifuge tubes and centrifuged at 500 *g* for 5 min at 4°C to eliminate nuclei and unbroken cells. Supernatant was then centrifuged at 10,000 *g* for 30 min at 4°C to obtain the heavy membrane pellet enriched for mitochondria, and the resulting supernatant was stored as the cytosolic fraction.

Protein studies

Preparation of cellular lysates, immunoblotting, and immunofluorescence were performed as described previously (Desagher et al., 1999; Finucane et al., 1999). Antibodies used in immunoblotting were as follows: mouse mAb against cytochrome *c* (clone 7H8.2C12; PharMingen), PARP (BD Biosciences), Bax (N20; Santa Cruz Biotechnology, Inc.), VDAC (Calbiochem), Cox IV (Molecular Probes, Inc.), or actin (Sigma-Aldrich), and rabbit pAbs specific for caspase-9 (Cayman Chemical). For human AIF detection, we used a rabbit polyclonal anti-AIF antibody that we obtained from rabbits immunized against a mixture of three different human AIF peptides (amino acids 106–120, 512–526, and 588–602). Each peptide was detected by the anti-AIF antibodies up to the dilution of 1/10,000 by ELISA. The anti-AIF antibody also detected recombinant human AIF protein (Susin et al., 1999), provided by G. Kroemer and S. Susin. For rat AIF detection, we used a goat polyclonal anti-AIF antibody (D-20; Santa Cruz Biotechnology, Inc.). All antibodies were used at dilution 1/1,000, then visualized using HRP-conjugated secondary antibodies (Amersham Biosciences), followed by enhanced chemiluminescence (Amersham Biosciences). Antibodies used in immunofluorescence were as follows: our rabbit polyclonal antibody against human AIF (1/200) or in Fig. 5, a commercially available mouse IgG2b mAb against human AIF (AIF [E-1]; Santa Cruz Biotechnology, Inc.)

(1/50), that was raised against a recombinant protein corresponding to amino acids 1–300 of human AIF; an anti–cytochrome *c* mAb (6H2.B4, 1/200; BD Biosciences) or a sheep polyclonal anti–cytochrome *c* (1/600; Sigma-Aldrich), and an anti-Hsp60 mAb (1/200; StressGen Biotechnologies). Antibodies were then detected using TRITC-labeled or FITC-labeled secondary antibody (1/200), and cells examined under a UV Fluorescence Microscope (3CCD; Leica) or a Confocal Microscope (LSM 510; Carl Zeiss MicroImaging, Inc.).

Online supplemental material

The specificity of our rabbit polyclonal antibody against human AIF was assessed by Western blotting and immunofluorescence assays (Fig. S1). The preventive effect of the caspase inhibitor z-VAD-fmk on Bax-induced cell death was investigated by flow cytometry analysis of DNA degradation and mitochondrial permeability ($\Delta\Psi m$) loss in Bax-transfected 293 T cells (Fig. S2). Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200207071/DC1.

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Note added in proof. While our paper was in proof, it was reported that the mitochondrial release of the AIF homologue WAH-1 is also caspase (CED-3) dependent in the nematode *Caenorhabditis elegans* (Wang, X., C. Yang, J. Chai, Y. Shi, D. Xue. 2002. *Science*. 298:1587–1592). Thus, the process that we describe here, in mammalian cells, may be evolutionarily conserved.

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