AMID, an Apoptosis-inducing Factor-homologous Mitochondrion-associated Protein, Induces Caspase-independent Apoptosis*

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Apoptosis-inducing factor (AIF) is a mitochondrial flavoprotein that triggers caspase-independent apoptosis. We describe here the cloning and characterization of a novel AIF-homologous molecule designated AMID (AIF-homologous mitochondrion-associated inducer of death). AMID lacks a mitochondrial localization sequence but shares significant homology with AIF and NADH oxidoreductases from bacteria to mammalian species. Immunofluorescent staining and biochemical experiments indicated that AMID was co-localized with mitochondria. Overexpression of AMID induced cell death with characteristic apoptotic morphology. Furthermore, AMID-induced apoptosis was independent of caspase activation and p53 and was not inhibited by Bcl-2. These findings suggest that AMID induces a novel caspase-independent apoptotic pathway.

Apoptosis, or programmed cell death, is one of the fundamental processes in all metazoans. It is widely believed that mitochondrion is an integrator of the cell death machinery and caspases are the central executors of apoptosis (1–6). Upon stimulation by various death signals, the outer membrane of mitochondrion is permeabilized, resulting in the release to the cytosol of molecules including cytochrome c and Smac/DIABLO (1–9). Once released into cytosol, cytochrome c binds to Apaf-1 and triggers the oligomerization of Apaf-1, which in turn recruits pro-caspase-9 to the “apoptosome” complex (10, 11). Recruitment of pro-caspase-9 into the apoptosome causes its autoactivation and further activation of downstream executioner caspases such as caspase-3 (10, 11). The activated executioner caspases cleave various cellular substrates, leading to characteristic morphological changes in apoptosis such as chromatin condensation, nucleosomal DNA fragmentation, and formation of apoptotic bodies (1–6).

Death signal-induced release of Smac from mitochondrion provides an alternative mechanism of cell death regulation. It has been proposed that cytosolic Smac released from mitochondrion interacts with inhibitors of apoptosis protein (IAPs) and competes their interaction with activated caspases, thereby promoting apoptosis (8, 9).

Although cytochrome c and caspases are critically involved in the classic apoptotic pathways, various studies indicate that cytochrome c, Apaf-1, or caspases-deficient cells can still undergo apoptosis (12–17). In addition, numerous studies have demonstrated that apoptosis induced by some stimuli cannot be inhibited by the pan-caspase inhibitor z-VAD-fmk (18–20), suggesting that caspase-independent apoptotic pathways exist.

In a search for molecules that cause caspase-independent apoptosis, Susin et al. (21) identified AIF,1 a 57-kDa flavoprotein with an ~100-amino acid (aa) mitochondrial localization sequence (MLS) at its N terminus. Like cytochrome c, AIF normally is present in the intermembrane space of mitochondria. Upon stimulation by death signals, AIF translocates from the mitochondria to the nucleus and causes chromosome condensation and large-scale DNA fragmentation (21, 22). These effects are independent of caspases and the oxidoreductase activity of AIF (21–25). Gene knock-out studies further suggest that AIF is required for apoptosis of embryonic stem cells induced by serum deprivation and is essential for apoptosis during cavitation of embryonic bodies (23). Moreover, these experiments also indicate that AIF-induced apoptosis can be uncoupled genetically from that induced by Apaf-1 and caspase-9 (23).

In this study, we have identified and cloned a novel AIF-homologous flavoprotein designated AMID. AMID is co-localized with mitochondria. Our findings suggest that AMID induces caspase and p53-independent apoptosis.

EXPERIMENTAL PROCEDURES

Reagents—The AMID EST clones (Research Genetics, Huntsville, AL), mouse monoclonal antibodies against Flag (Sigma) and HA (Covance, Berkeley, CA) epitopes, mouse monoclonal antibodies against AIF (Santa Cruz Biotechnology, Santa Cruz, CA) and caspase-3 (Transduction Laboratories, San Diego, CA), Texas Red-conjugated AffiniPure goat anti-mouse IgG (Molecular Probes, Eugene, OR), MitoTracker Green FM (Molecular Probes), and caspase inhibitor z-VAD-fmk (Medical & Biological Laboratories) were purchased from the indicated manufacturers. Human embryonic kidney 293T, monocytic U937, and T lymphoma Jurkat were kindly provided by Dr. Gary Johnson and wild type and p53-deficient colon cancer HCT116 cells and colon cancer DLD

1 The abbreviations used are: AIF, apoptosis-inducing factor; aa, amino acid(s); MLS, mitochondrial localization sequence; AMID, AIF-homologous mitochondrion-associated inducer of death; HA, hemagglutinin; CMV, cytomegalovirus; PBS, phosphate-buffered saline; GFP, green fluorescent protein; EST, expressed sequence tag; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; FADD, Fas-associated death domain protein.

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RNA Isolation and Northern Blot Hybridization—Human multiple tissue mRNA blots were purchased from CLONTECH (Palo Alto, CA). To detect AMID expression in cancer cell lines, total RNA was isolated by the Trizol (Invitrogen) method following the manufacturer’s protocol. The purified RNA (20 \mu g) was fractionated in a 1.5% agarose gel and transferred to nitrocellulose membrane as described (27). The blots were hybridized with the indicated probes in Rapid Hybridization Buffer (CLONTECH, Palo Alto, CA) under high stringency condition.

Vector Construction and Transfection—Mammalian expression vectors for HA- or Flag-tagged AIF, AMID, and their deletion mutants were constructed by PCR amplification of the corresponding cDNA fragments and subsequently cloned into CMV promoter-based vectors containing an N- or C-terminal HA or Flag tag. Transfection of 293T and COS cells was performed with a standard calcium phosphate precipitation method (27). Transfection of HCT116-p53(+/-) and (-/-) cells was performed with LipofectAMINE (Invitrogen) following procedures suggested by the manufacturer.

Cell Fractionation and Western Blot—293T cells were transfected with 20 \mu g of expression plasmid for C-terminal HA-tagged AMID for 16 h. The transfected cells were harvested and centrifuged at

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**Fig. 1. Sequence and expression of AMID.** A, alignment of amino acid sequences of human AMID, human AIF, and *Escherichia coli* NADH oxidoreductases. The three possible starting methionines are underlined. The GenBank™ accession number for the AMID sequence is AF506757. B, Northern blot analysis of AMID expression. RNA blots of the indicated cell lines were hybridized with a cDNA probe corresponding to the AMID coding sequence or an ubiquitin probe. C, protein expression of AMID. 293T cells were transfected with expression plasmids for N-terminal Flag-tagged or C-terminal HA-tagged full-length AMID, and protein expression was analyzed by Western blot with anti-Flag and anti-HA antibodies.

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cells (26) by Dr. Bert Vogelstein. The B lymphoma RPMI8226 cell line was purchased from ATCC (Manassas, VA).

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Cell Fractionation and Western Blot—293T cells were transfected with 20 \mu g of expression plasmid for C-terminal HA-tagged AMID for 16 h. The transfected cells were harvested and centrifuged at
RESULTS

Identification and Cloning of AMID—To identify a novel AIF-homologous gene, we searched the GenBank™ EST data base. This search identified more than 10 EST clones that encode a potential AIF-homologous protein, which we designated AMID (for AIF-homologous mitochondrion-associated inducer of death). Sequence analysis of the longest EST clones suggests that AMID encodes a 373-aa protein (Fig. 1A). The EST clone (GenBank™ accession number BG285370) that we obtained encodes for full-length AMID because there is an in-frame stop codon at the 5′ end upstream of the putative ATG start codon and a poly(A) sequence at the 3′ end (data not shown).

BLAST searches of the GenBank™ data bases suggested that AMID, like AIF, has significant homology with NADH oxidoreductases/flavoproteins from bacteria to mammalian species (Fig. 1A). AMID, however, does not contain a recognizable MLS that is found in AIF.

Expression of AMID—We examined the expression of AMID in various human tissues by Northern blot analysis. These experiments indicated that AMID mRNA was undetectable in all tissues tested, including heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood leukocyte (data not shown). However, AMID expression could be detected easily in colon cancer cell lines DLD and HCT116 and could be detected weakly in B lymphoma cell line RPMI8226 (Fig. 1B).

To examine the possible functions of AMID, we made mammalian expression constructs for N-terminal Flag-tagged, C-terminal HA-tagged, and untagged AMID. As expected, the N-terminal Flag-tagged AMID was expressed as an ~43-kDa protein. Surprisingly, the C-terminal HA-tagged AMID was expressed as an ~39-kDa protein, which was ~4 kDa smaller than expected. Moreover, all C-terminal tagged C-terminal deletion mutants were also ~4 kDa smaller than expected (see below and Fig. 4). It is possible that a 4-kDa N-terminal fragment in AMID is cleaved after translation. Alternatively, it is possible that AMID translation does not start from the putative start codon but from the second or third methionine located at aa 37 and 41, respectively, downstream of the first methionine (Fig. 1A).

AMID Is Co-localized with Mitochondria—AIF has an MLS and is localized to the intermembrane space of mitochondria under physiological condition (21, 22). AMID, however, lacks a recognizable MLS (Fig. 1A). To determine the cellular localization of AMID, we performed immunofluorescent staining and

600 × g for 10 min. The pellet was washed with 1 ml of PBS and resuspended in 300 µl of Buffer A (20 mM Hepes, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 250 mM sucrose, pH 7.5), homogenized by a syringe with an 18-gauge needle for 15–20 times. The lysate was centrifuged at 10,000 × g for 10 min, and the supernatant was further centrifuged at 10,000 × g for 1 h. The mitochondrion-enriched pellet was resuspended in 50 µl of Buffer A. The cytosolic supernatant (10 µl) and the mitochondrial fraction (10 µl) were fractionated on SDS-PAGE, and subsequent Western blot analyses were performed as described (28, 29).

Apoptosis Assays—β-Galactosidase co-transfection assays for determination of cell death were performed as described previously (30, 31). Briefly, 293T cells (~2 × 105) were seeded on 6-well (35-mm) dishes and transfected the following day by calcium phosphate precipitation with 1 µg of pCMV-β-galactosidase plasmid and the indicated testing plasmids. Within the same experiment, each transfection was performed in triplicate, and where necessary, a sufficient amount of empty control plasmid was added so that each transfection received the same amount of total DNA. Approximately 36–48 h after transfection, the cells were stained with X-gal as described previously (30). The numbers of surviving blue cells from five representative viewing fields were determined microscopically. Data shown are the averages and standard deviations of one representative experiments in which each transfection was performed in triplicate.

Immunofluorescent Staining—293T cells cultured on glass coverslips were plunged sequentially into methanol and acetone at −20 °C, each for 10 min. The cells were rehydrated in PBS, blocked with 1% bovine serum albumin in PBS for 15 min, and stained with primary antibodies (2 µg/ml) in blocking buffer for 1 h at room temperature. The cells were rinsed with PBS, stained with Texas Red-conjugated Affinipure goat anti-mouse IgG (1:200 dilution) and MitoTracker Green FM (10 µM) for 45 min at room temperature. The cells were then rinsed with PBS and mounted in Prolong Antifade (Molecular Probes, Eugene, OR). The cells were observed with a Leica DMR/XA immunofluorescent microscope using a 100 × plan objective.

Electron Microscopy—Sorted GFP-positive cells were fixed in 3% glutaraldehyde, stained with 1% osmium tetroxide, enroled in SeaPlaque agarose, dehydrated with ethanol, and embedded in Epon/Araldite resin. Thin sections were cut, placed on Butvar-coated 200 mesh copper grids, post-stained with 3% aqueous uranyl acetate and Reynolds lead citrate, and observed in a Philips 400 transmission electron microscope.

AMID Induces Caspase-independent Apoptosis

293T cells were transfected with expression plasmid for c-terminal HA-tagged AMID for 16 h. Transfected cells were stained with MitoTracker Green FM for mitochondria (A-a, in green) and anti-HA antibody for AMID (A-b, in red). The merged image of A-a and A-b, as well as DNA staining (in blue), is shown in A-c. The magnified image of the boxed area in A-c is shown in A-d. Arrows point to AMID (red)-surrounded mitochondria (green). B, cellular fractionation study of AMID. Lysate of 293T cells transfected with C-terminal HA-tagged AMID was fractionated, analyzed by Western blots with anti-HA antibody (top panel), anti-caspase-3 antibody (middle panel), and anti-AIF antibody (bottom panel).
biochemical experiments. To visualize AMID localization in mammalian cells, we transfected 293T cells with expression plasmid for C-terminal HA-tagged AMID and performed double immunofluorescent staining with an anti-HA antibody and a fluorescent dye for mitochondria. These experiments indicated that a major fraction of AMID was co-localized with mitochondria (Fig. 2A). It seems that AMID is adhered to the outer membrane of mitochondria and forms a ring-like structure (Fig. 2A). Another fraction of AMID was localized in the cytosol (Fig. 2A). We also examined the localization of N-terminal tagged full-length AMID, and similar results were obtained (data not shown). To further confirm this observation, we isolated the mitochondrial and cytosolic fractions from 293T cells transfected with C-terminal HA-tagged AMID and then performed Western blot analysis with anti-HA antibody. As shown in Fig. 2B, we found that AMID was detected in both the mitochondrial and the cytosolic fractions. As controls for these cell fractionation experiments, AIF was detected only in the mitochondrial fraction, whereas caspase-3, a cytosolic protein, was detected only in the cytosolic fraction (Fig. 2B). Taken together, these data suggest that AMID is associated with the outer membrane of mitochondria and localized in the cytosol.

**Induction of Apoptosis by AMID**

Because AMID is an AIF-homologous protein that is associated with the outer membrane of mitochondria, we determined whether AMID could induce apoptosis. When overexpressed, AMID induced cell death of 293T cells in a dose-dependent manner (Fig. 3A and B). AMID-induced cell death occurred ~30 h after transfection, as compared with 14 h for apoptosis induced by death receptors or caspase-8 (data not shown). To determine whether AMID induces apoptosis or necrosis, we performed electron microscopy analysis after co-transfecting 293T cells with expression plasmid for AMID and an expression plasmid for GFP. Most cells (90 of 113) transfected with GFP alone are morphologically normal (a), whereas a high proportion of cells (45 of 80) transfected with AMID display apoptotic morphology with peripheral, condensed chromatin and apoptotic bodies (b).
plasmids for AMID and green fluorescent protein (GFP). Forty hours after transfection, cells were sorted for GFP expression by flow cytometry and then analyzed by electron microscopy. As shown in Fig. 3C, overexpression of AMID in 293T cells caused chromatin condensation and formation of apoptotic bodies. The condensed chromatin induced by AMID was accumulated at the periphery of the nucleus, a phenotype similar to that induced by AIF but distinguishable from most caspase-dependent apoptosis (21, 22). Interestingly, overexpression of AMID also caused a loss of structurally preserved mitochondria (Fig. 3C).

Domain Mapping of AMID-induced Apoptosis—To determine the domains that are required for AMID-induced apoptosis, we made a series of N- and C-terminal deletion mutants and determined their abilities to induce apoptosis. These experiments indicated that the C-terminal fragments (aa 77–373 and 186–373) were sufficient to induce apoptosis, whereas the two N-terminal fragments (aa 1–185 and 1–300) did not induce apoptosis (Fig. 4A). These data suggest that an intact flavoprotein domain spanning approximately aa 80 to 300 is not required for AMID-induced apoptosis, which is consistent with the observation that the intact flavoprotein domain of AIF is not required for AIF-induced apoptosis (25). Interestingly, immunofluorescent staining indicated that aa 77–373 and 186–373 were associated with the outer membrane of mitochondria or localized in the cytosol, whereas aa 1–185 and 1–300 were localized in the nucleus (Fig. 4B). These data imply that the association of AMID to the outer membrane of mitochondria is critical for AMID-induced apoptosis.

AMID-induced Apoptosis Is Independent of Caspases and p53 and Is Not Inhibited by Bcl-2—Although caspases are the central executioners of most classical apoptotic pathways, AIF-induced apoptosis is caspase-independent. We investigated whether AMID-induced apoptosis is caspase-dependent. We co-transfected 293T cells with expression plasmids for AMID and crmA, a specific caspase inhibitor. The results indicated that crmA could not inhibit AMID-induced apoptosis (Fig. 5A). In contrast, crmA inhibited apoptosis induced by FADD, a death domain-containing protein involved in death receptor-mediated apoptosis (30) (Fig. 5A). In addition, a pan-caspase inhibitor, z-VAD-fmk, also did not inhibit AMID-induced apoptosis but inhibited FADD-induced apoptosis (Fig. 5A). These data suggest that AMID-induced apoptosis is caspase-independent.
ANTIBODIES. Constructs were measured by Western blot with anti-Flag and anti-HA performed as described for Fig. 3. The relative expression levels of the constructs were measured by Western blot with anti-Flag and anti-HA antibodies (lower panel).

Bel-2, a prototypic member of the Bcl-2 family, can inhibit certain types of mitochondrion-mediated apoptosis (1–5). We examined whether Bel-2 could inhibit AMID-induced apoptosis. Our results indicated that Bel-2 could not inhibit AMID-induced apoptosis (Fig. 5A), pointing to the possibility that AMID functions either downstream of mitochondrion-mediated release of death-triggering molecules or in a distinct apoptotic pathway.

We also examined whether AMID-induced apoptosis is p53-dependent. To do this, we determined the effects of AMID overexpression on wild type and p53-deficient colon cancer HCT116 cells. As shown in Fig. 5B, overexpression of AMID induced comparable cell death in both wild type and p53-deficient HCT116 cells, suggesting that AMID-induced apoptosis is independent of p53.

Overexpression of AIF Is Insufficient to Induce Apoptosis—We attempted to determine whether AMID is involved in AIF-induced apoptosis. However, to our surprise and in contrast with previous reports (21–24), we found that overexpression of AIF in 293T cells (Fig. 6) and all other cell lines tested (data not shown), including COS cells and embryonic fibroblasts used in previous reports, did not induce cell death. Similar data were obtained using a C-terminal tagged AIF and a AIF mutant lacking the MLS, expressed for various times ranging from 24 to 96 h. In these experiments, AMID, which was expressed at an even lower level, induced apoptosis (Fig. 6).

**DISCUSSION**

Mitochondrion plays a central role in apoptosis induced by various stimuli. Upon stimulation by death signals, mitochondrion releases to the cytosol of molecules including cytochrome c, Smac/DIABLO, and AIF, among others (1–6). Once released into cytosol, cytochrome c and Smac trigger well defined caspase-dependent apoptotic pathways. In contrast, AIF-induced apoptosis is caspase-independent. In this report, we provide evidence that a novel AIF-homologous molecule, AMID, can also induce caspase-independent apoptosis.

AIF is confined to the intermembrane space of mitochondria under physiological condition (21, 22). Upon certain apoptotic stimulation, AIF translocates into the nucleus and induces nuclear apoptosis by a unresolved mechanism (21, 22). Although AMID shares significant homology with AIF, it lacks an N-terminal MLS. Our immunofluorescent staining and biochemical experiments suggest that AMID is co-localized with mitochondria, most likely adhering to the outer membrane of mitochondria.

Overexpression of AMID induces cell death in a dose-dependent manner. Like AIF, AMID-induced apoptosis is not inhibited by the pan-caspase inhibitors VAD-fmk or crmA, suggesting that AMID-induced apoptosis is caspase-independent. Interestingly, electronic microscopy studies suggest that AMID induces peripheral-type chromatin condensation, a phenotype that is very similar to that induced by AIF but is in contrast to that induced by caspase-mediated apoptosis (22, 23).

AMID-induced apoptosis is not inhibited by Bel-2, pointing to the possibility that AMID-induced apoptosis is not mediated by Bel-2 family member-induced mitochondrial permeability. Previously, it has been suggested that apoptosis induced by overexpression of mitochondrion-targeting AIF precursor can be inhibited by Bel-2. However, apoptosis induced by overexpression of cytosolic AIF lacking its MLS cannot be inhibited by Bel-2 (24). In this context, AMID, which is associated with the outer membrane of mitochondria or localized in the cytosol, behaves similarly to the cytosolic AIF.

The domain mapping experiments indicate that the C-terminal 187 aa of AMID is sufficient to induce apoptosis. The two N-terminal deletion mutants (aa 77–373 or 186–373), which are co-localized with mitochondria, induce apoptosis. Although no nuclear localization signal sequence is found in AMID, the two C-terminal deletion mutants (aa 1–185 and 1–300) are localized to the nucleus and cannot induce apoptosis. The simplest explanation for these observations is that association with mitochondria is required for AMID-induced apoptosis.

The exact mechanism by which AMID induces apoptosis is unknown. It is possible that the association of AMID with mitochondria causes disruption of the mitochondrial structure and a release of mitochondrial content, thereby inducing apoptosis. One piece of evidence to support this hypothesis is our electronic microscopy observation that the AMID-induced apoptotic cells lack structurally preserved mitochondria. However, it is equally possible that AMID-induced apoptosis is independent of mitochondria and the loss of mitochondria in cells over-expressing AMID is a result of AMID-induced apoptosis.

Because AMID is homologous to AIF and also induces caspase-independent apoptosis, we attempted to determine whether AMID and AIF are connected functionally. However, in contrast to previous reports (21–24), our extensive efforts failed to demonstrate any apoptotic effects induced by overexpression of AIF or its cytosolic mutant lacking the MLS. Currently, we have no explanations for this discrepancy.

In conclusion, we have identified an AIF-homologous protein, the flavoprotein AMID. AMID is associated with the outer membrane of mitochondria and induces caspase-independent apoptosis. The mechanism of AMID-induced apoptosis, like that of AIF, requires further investigation.

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