

AIFsh, a Novel AIF Proapoptotic Isoform with Potential Pathological Relevance in Human Cancer

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Running Title: AIFsh triggers caspase-independent cell death

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AIF is a main mediator of caspase-independent cell death. It is encoded by a single gene located on chromosome X, region q25-26 and A6 in humans and mice, respectively. Previous studies established AIF codes for two isoforms of the protein, AIF and AIF-exB. Here, we identify a third AIF-isoform resulting from an alternate transcriptional start site located at intron 9 of AIF. The resulting mRNA encodes a cytosolic protein that corresponds to the C-terminal domain of AIF (amino acids 353-613). We named this new isoform AIFshort (AIFsh). AIFsh overexpression in HeLa cells results in nuclear translocation and caspase-independent cell death. Once in the nucleus, AIFsh provokes the same effects than AIF, namely chromatin condensation and large-scale (50 Kb) DNA fragmentation. In contrast, these apoptogenic effects are not precluded by the AIF-inhibiting protein Hsp70. These findings identify AIFsh as a new proapoptotic isoform of AIF, and also reveal that the first N-terminal 352 amino acids of AIF are not required for its apoptotic activity. In addition, we demonstrate that AIFsh is strongly down-regulated in tumor cells derived from kidney, vulva, skin, thyroid and pancreas, whereas, γ -irradiation treatment provokes AIFsh up-regulation. Overall, our results

identify a novel member of the AIF-dependent pathway and shed new light on the role of caspase-independent cell death in tumor formation/suppression.

Apoptosis or programmed cell death (PCD) is a genetically controlled process that is fundamental to the development and homeostasis of multicellular organisms (1). Aberrations in apoptosis signaling pathways result in a variety of pathological conditions and are common in cancer cells (2). In fact, cellular changes leading to inhibition of apoptosis play an essential role in tumor development (3,4). The elucidation of the apoptotic pathways is thus an important area of study that may provide insights into the causes of drug resistance and facilitate the development of novel anticancer therapies.

In the last decade, the study of PCD focused on caspases, a family of cysteine proteases specifically activated in apoptotic cells. Since the induction of apoptosis through the use of caspase activators may theoretically constitute a treatment for cancer (5), the initial pro-apoptotic anti-cancer trials have focused on caspase activity (6). Unfortunately, most of these studies are still in preclinical development because of their low efficacy. In part, this may be due to the fact that programmed cell death can proceed even when the caspase cascade is blocked (7-9).

This fact has revealed the existence of alternative pathway(s) defined as caspase-independent (8).

Apoptosis-Inducing Factor (AIF) was the first identified protein involved in caspase-independent cell death (10). AIF is expressed as a precursor of 67 kDa, which is addressed and compartmentalized into mitochondria by two mitochondrial localization sequences (MLS) located within the N-terminal prodomain of the protein. Once in mitochondria, this prodomain is removed, giving rise to a mature form of ~57 kDa (10,11). This form comprises three structural domains: FAD-binding domain, NADH-binding domain, and C-terminal domain (12). The oxidoreductase part of AIF (composed by both NADH- and FAD-binding domains) confers an electron transfer activity to the protein (13). Under physiological conditions, AIF is a mitochondrial FAD-dependent oxidoreductase that plays a role in oxidative phosphorylation (14). Thus, AIF has a protective function in some cell types (15). However, after a cellular insult, AIF is cleaved by calpains and/or cathepsins (11,16,17), then translocates from mitochondria to cytosol and nucleus where it interacts with DNA and causes a caspase-independent chromatin condensation and large-scale (~50 Kb) DNA fragmentation (10,18). Interestingly, the two AIF activities (mitochondrial oxidoreductase and nuclear pro-apoptotic) can be dissociated (13).

Several lines of evidence demonstrate that AIF plays a major role in cell death (19). In this way, AIF is a central mediator of relevant experimental models of cell death like As₂O₃-induced cell death in human cervical cancer cells (20), DNA damage-mediated p53 activation (21,22), Sulindac induced PCD in colon cancer cells (23), geldanamycin-mediated PCD in human glioma cells (24), staurosporine-induced PCD in neuroblastoma cells (25), caspase-independent apoptosis induced by Survivin in melanoma cells (26), hexaminolevulinate-mediated photodynamic therapy in human leukemia cells (27), or poly(ADP-ribose) polymerase (PARP)-mediated cell death

(28,29). In addition, blockage of the AIF signal transduction pathway seems to be implicated in the chemoresistance of non-small-cell lung carcinomas (30) and other human cancers (31). On the other hand, in human colon cancer cells, AIF suppresses chemical stress-induced apoptosis and maintains the transformed state of tumor cells (32). All these data illustrate the role of AIF and the caspase-independent death pathway in the control of PCD.

AIF, located on chromosome X, comprises 16 exons (10). Previous work revealed that *AIF* codes for two isoforms (AIF and AIF-exB) characterized by an alternative use of exon 2 or exon 2b (33). However, a detailed study allowed us to identify a novel AIF transcript present in human and mouse tissues. This novel transcript, called AIFshort (AIFsh), comprises 7 exons derived from exon 10 to 16 of *AIF*. The resulting protein corresponds to the C-terminal part of AIF, lacking its N-terminal domain. AIFsh provokes caspase-independent cell death, indicating that the proapoptotic activity of AIF resides in its C-terminal domain. In addition, we explored the potential role of this new proapoptotic AIF-isoform in tumor formation/suppression and showed that AIFsh mRNA is down-regulated in tumor cells but is transcriptionally up-regulated by γ -irradiation.

MATERIALS AND METHODS

Rapid Amplification of cDNA End (RACE) Analysis

5'- and 3'-RACE were performed using Marathon-Ready cDNA from human kidney (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's recommendation. In short, for the 5'-RACE, 2 μ l of a cDNA sample were subjected to the first round of PCR amplification using the adaptor primer 1 and the antisense primer HCR1 (Table 1). For the 3'-RACE, the first round of PCR was performed using the adaptor primer 1 and the sense primer I9F (Table 1). After amplification for 30 cycles (94°C 20 s, 66°C 30 s, and 72°C 3 min), 1 μ l of this PCR was reamplified for 30 cycles using the same reaction conditions with

nested primer adaptor primer 2 and the nested antisense primer HAIFshR for 5'-RACE. For the 3'-RACE, nested sense primer was E10F (Table 1). The PCR amplimers were synthesized with Herculase Enhanced DNA Polymerase (Stratagene, San Diego, CA) and, finally, the PCR products were cloned in the pCR[®]4-TOPO[®] vector (Invitrogen, Cergy Pontoise, France) and sequenced on an ABI Prism 310 capillary sequencer (Applied Biosystem, Foster City, CA).

ARN Extraction and RT-PCR

Total RNA from human tissues was obtained from Stratagene. RNA from *aif*^{+/-y} and *aif*^{-y} ES cells was extracted using the Rneasy kit from Qiagen (Courtaboeuf, France). For the reverse transcription, 1 µg of total RNA was mixed with 200U of Superscript II reverse transcriptase (Invitrogen) and 150 ng of random primers (Promega, Charbonnières, France). As a control, reverse transcription was performed without enzyme. AIF isoform 3 (Genbank ID AL049704) was amplified using primers: AIF is3F and R4. Human AIF was amplified using primers E2F and E10R. Human AIFsh isoform was amplified using primers I9F2 and R4. The sense and antisense primers for mouse AIF (mAIF) were mAIFex3F and E10R. The sense and antisense primers for mouse AIFsh (mAIFsh) were mI9F and R4. As a control, the cDNAs of the ribosomal protein L27 and GAPDH were amplified using primers L27F and L27R or GAPDHF, GAPDHR, respectively. The conditions used for the PCR reaction were 94°C 2 min, 30 cycles for L27 and GAPDH, 35 cycles for AIF and mAIF and 40 cycles for AIFsh and mAIFsh of 94°C 20 s, 64°C 30 s, 72°C for 2 min, and 72°C for 10 min. All PCR products were separated by electrophoresis through 2% agarose gel. All primers used were defined in Table 1.

Northern Blot, Cancer Profiling Array, and Cancer Cell Line Profiling Array

First choice Human Blot I membrane from Ambion was used for Northern blotting. Cancer Profiling Array II and Cancer Cell Line Profiling Array were purchased from

Clontech (BD Biosciences). Northern membrane or cancer arrays were hybridized with HAIF or HAIFsh specific probes amplified using E2F and E10R or I9F3 and HAIFshR primers, respectively (Table 1). The probe was radiolabeled with $\alpha^{32}\text{P}$ dCTP (ICN, Orsay, France) using the Random Priming Labeling Kit (Roche, Mannheim, Germany), and unincorporated nucleotides were removed using a nucleotide purification kit (Qiagen). After pre-hybridization in ULTRAhyb Ultrasensitive Hybridization Buffer (Ambion) for 1 h, membranes were hybridized overnight at 42°C in ULTRAhyb Ultrasensitive Hybridization Buffer. Then membranes were washed at 42°C for Northern Blot or 68°C for Cancer Arrays (x2, 15 min each) in NorthernMax Low Stringency Wash Solution (Ambion), followed by washing in NorthernMax High Stringency Wash Solution (Ambion). Finally, the membrane was exposed to X-ray film in a cassette. For cancer arrays, signal intensities were calculated for individual spots using STORM phosphoimager (Molecular Dynamics, Eugene, OR). The housekeeping gene Ubiquitin (provided by the manufacturer) was used for array normalization.

Cell Culture, Transient Transfection, RNAi assays, and Treatments

HeLa cells were cultured in complete culture medium (DMEM, supplemented with 10% FCS, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin, Invitrogen) and maintained at 37°C in a humidified 5% CO₂ atmosphere. Cells were seeded at a concentration of a 2 x 10⁵ into 6 wells plate or 1 x 10⁴ into 96 wells plate. Embryonic stem (ES) *aif*^{+/-y} and *aif*^{-y} cells were cultured as described (19). Transient transfections were performed using Lipofectamine 2000 in Optimem (Invitrogen) according to the manufacturer's protocol. For RNAi assays, HeLa cells were transfected with specific siRNA double-stranded oligonucleotides designed against human AIF (I= 5'-CCGGCTCCAGGCAACTTG-3' and II= 5'-CTTGTTCAGCGATGGCAT-3'), human AIFsh (III= 5'-

GAATGTTTCATGGCAACTA-3' and IV= 5'-GGAGGATGCTTCCTATTCA-3'), or a common siRNA against both human AIF and human AIFsh (V= 5'-GCATGCTTCTACGATATAA-3'). As a control, we used an irrelevant siRNA oligonucleotide (Co.= 5'-GCGATAAGTCGTGTCTTAC-3'). 48 h after the indicated transfection AIF or AIFsh mRNA expression was assessed by RT-PCR as described above. Apoptosis was induced by treatment of HeLa cells for 8 or 12 h with STS (1 μ M, Sigma). In a separate series of experiments, the pan-caspase inhibitors z-VAD.fmk (50 μ M, MP Biomedicals, Irvine, CA) or QVD-OPH (10 μ M, MP Biomedicals) were added 30 min before pcDNA3-AIF, pcDNA3-AIFsh, pcDNA3-empty vector transfection or STS-apoptosis induction.

Vector Construction

Mammalian expression vectors for AIF and AIFsh were carried out by PCR amplification of the corresponding human cDNA fragments and subsequently cloned into CMV promoter-based expression vectors pcDNA3 (Invitrogen), pEGFP-N1 (Statagene) or C-terminal p3xFLAG (Sigma). Final constructs are referred to as pcDNA3- Δ 5'UTRAIFsh, pcDNA3-5'UTRAIFsh (= pcDNA3-AIFsh), pcDNA3-AIF, pEGFP-AIF, pEGFP-AIFsh, p3xFLAG-AIF, or p3xFLAG-AIFsh. Empty vectors (pcDNA3, pEGFP-N1, or p3xFLAG) were used as controls.

Immunofluorescence

For viewing the localization of pEGFP-AIF, pEGFP-AIFsh, and pEGFP-N1, transfected HeLa cells seeded on coverslips were washed with PBS three times and stained by Mitotracker Red[®] (20 nM, Molecular Probes, Invitrogen) for 15 min at RT. Nuclear morphology was assessed by staining cells/nuclei with 0,5 μ g/ml Hoechst 33342. Cells were mounted and red, green, and blue fluorescence were observed in a Nikon Eclipse TE2000-U microscope and analyzed using Nikon ACT-1 software.

MTT Reduction and Propidium Iodide Cell Viability Assays

MTT is a water-soluble tetrazolium salt that is reduced by metabolically viable cells to a colored, water-insoluble formazan salt. MTT (0.5 mg/ml final concentration) was added to the culture medium of cells growing in 96-well dishes (10,000 cells/well). After incubating the dishes at 37°C for 20 min, the assay was stopped by replacement of the MTT-containing medium with 50 μ l DMSO. Formazan salts were allowed to dissolve in DMSO shaking them gently for 10 min at room temperature, and the assays were quantified by means of a Multiskan Ex ELISA plate reader (ThermoLabsystems, Cergy Pontoise, France). Final values were the result of subtracting 630 nm from 590 nm readings. For DNA loss measurements, cells were exposed for 10-15 min at RT to propidium iodide (PI, 0.5 μ g/ml) and analyzed by flow cytometry in a FACSCalibur (BD Biosciences). Data analysis was carried out in total cell population (>10,000 cells).

Protein Extraction and Immunoblot Analysis

When whole protein extracts were used, approximately 2×10^6 HeLa cells per condition were detached, gently pelleted by centrifugation and washed twice in PBS. Cells were lysed in total extraction buffer containing 125 mM Tris-HCl pH 6.8 and 2 % SDS pre-warmed at 95°C. Nuclear and cytoplasmic subfractionation extracts were performed with NE-PER[™] Extraction Reagents (Pierce, Rockford, IL) following manufacturer's instructions. In all cases, protein content was quantified by a modified Lowry assay (Bio-Rad DC protein assay, Bio-Rad). Around 25-100 μ g of protein per condition were electrophoresed in 15% SDS-polyacrylamide gels that were electrotransferred to Immobilon-PVDF membranes (Millipore, Bedford, MA). Filters were probed with the indicated primary antibodies and incubated with secondary antibodies conjugated with peroxidase (Sigma). As substrates for immunodetection, we used the ECL Western Blotting analysis system (Amersham Biosciences corp,

Piscataway, NJ). Antibodies used in this study were anti-AIF C-terminal (Sigma), anti-AIF N-terminal (Clone E-1, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Flag (Sigma), anti-Caspase-3 (Cell Signaling, Beverly, MA), anti-ICAD (Medical and Biological Laboratories, Nagoya, Japan), anti-actin (Sigma), anti-LaminA/C (Santa Cruz Biotechnology), and anti-Erk3 (BD Biosciences).

DNA Fragmentation Assessment

For field inverted gel electrophoresis (FIGE), DNA was prepared from agarose plugs (2 x 10⁶ cells) (25,34), followed by electrophoresis in a Bio-Rad Laboratories Fige Mapper cell (1% agarose, 0,5 X TBE). Running conditions were: 180 V (forward pulse), 120 V (reverse pulse) and 1,5 s and 3,5 s for the initial and final switch times (forward and reverse pulses, linear ramp) for 20 h. For the detection of oligonucleosomal DNA fragmentation, nuclear DNA from lysed cells (treated with protease K and RNase according to standard protocols) was subjected to conventional horizontal agarose gel electrophoresis (1%) followed by ethidium bromide staining (25).

Recombinant Proteins

N-terminal His-tagged AIF, AIFsh, and AIF Δ 353-613 human recombinant proteins were produced from a Novagen pET28b expression vector, purified from *Escherichia coli* extracts on a nickel-NTA affinity matrix and stored in 50 mM Hepes, pH 7.9, 100 mM NaCl, 1 mM DTT, and 10% Glycerol until use.

Cell Free System of Apoptosis

For the standard assessment of chromatin condensation, nuclei from HeLa cells were purified on a sucrose gradient, washed 2 x (1,000 g 10 min, 4°C) and resuspended in CFS buffer (35). In standard conditions, nuclei (10³ nuclei/ μ l) were cultured in the presence of AIF, AIFsh, or AIF Δ 353-613 for 90 min at 37°C. Nuclei were stained with Hoechst 33342 (0.5 μ g/ml, Sigma) and examined by fluorescence microscopy. Nuclei presenting chromatin condensation and/or a

translucent aspect were considered apoptotic. Alternatively, nuclei were stained with propidium iodide (PI; 0.5 μ g/ml) followed by cytofluorometric analysis. In one series of experiments, 600 nM recombinant AIF or AIFsh were preincubated 30 min with different amounts of Hsp70 human recombinant protein (Sigma) or BSA (Sigma). Furthermore, in one experiment, nuclei were pretreated with z-VAD.fmk (50 μ M, 60 min), washed 3 times (1,000 g; 10 min), and exposed to AIF and AIFsh recombinant proteins for 90 min at 37°C.

RESULTS

Identification of AIFsh, a novel AIF-isoform

In order to identify new isoforms of AIF we first analyzed, by an *in silico* approach, human *AIF* (NCBI Gene Database Accession No. 9131). This study yielded the putative existence of an alternative and uncharacterized third form of AIF (NCBI Nucleotide Database, Accession No. NM_145813) corresponding to a short splice variant lacking exons 2 to 9 of *AIF*. This predicted isoform of AIF has a mitochondrial targeting sequence, but lacks a part of the AIF oxidoreductase domain. Using the total cDNA obtained from both HeLa cells and different human tissues as templates, we performed a PCR amplification to find this putative mRNA. However, using two specific oligonucleotides as primers (AIFis3F and R4), we failed in the amplification of this isoform. In fact, a more careful assessment of the nucleotide sequence observed in the junction between exons 1 and 10 of *AIF* revealed that the same six nucleotides sequence (TCCCAG) is present at the end of exon 1 and intron 9 (nucleotides 279-284 and 28643-28648 of *AIF*, respectively). Thus, it seems possible that this predicted *in silico* variant is only derived from a bioinformatic comparative approach between these TCCCAG overlapping sequences.

However, a general study performed in the GenBank database allowed us to find three expressed sequence tags (EST) derived from different human cell types: skin (BG 675614), colon (BG985116), and head neck

(AI834231). These ESTs include exon 10 of *AIF* and downstream cDNA sequences, but differ at the 5' start site, which corresponds to a part of intron 9. Interestingly, the three alternative 5' UTR transcripts generate a potential open reading frame that starts in exon 10, creating a novel *AIF*-isoform. This potentially new *AIF*-isoform is also founded in *Sus scrofa* EST database (BF079256). These initial findings led us to further investigate the existence of this putative new *AIF*-isoform in human and mouse cells.

By using a 3'- and 5'-Rapid Amplification of cDNA End (RACE) on a Marathon Ready human kidney cDNA library, we reconstituted the full-length cDNA of this novel variant, which we named *AIF* short (*AIFsh*). This cDNA contains an entire open reading frame (ORF) of 822 bp. For the 3'-RACE, the first round of PCR amplification using I9F primer showed a distinct amplification product which corresponded to the 3'-end of *AIF*. For the 5'-RACE, the first round of PCR amplification, using HCR1 primer, did not show a specific amplification product. However, the second nested PCR carried out with HAI*Fsh*R showed several bands. After sequencing, we observed that these bands belonged to the same transcript. In fact, all sequences obtained differed at the 5'-end and retained a part of the 3'-end of intron 9 (Fig. 1A). The most 5'-end obtained by 5'-RACE, corresponding to nucleotides 27794 of the sequence of *AIF*, was considered as the transcription start site of *AIFsh*. In a similar manner, using a mouse brain Marathon cDNA library, we also reconstituted the mouse *AIFsh* full-length cDNA (data not shown). The mouse ORF displays 91 % nucleotide sequence identity with human *AIFsh* coding sequence.

AIFsh mRNA expression in normal tissues and cancer cell lines

Through Northern and RT-PCR approaches, we next sought to determine *AIFsh* mRNA tissue expression. Northern blot analysis was performed using specific *AIF* or *AIFsh* cDNA fragments as a probe (Table 1). This approach

revealed one mRNA transcript, which is different for *AIF* and *AIFsh* (Fig. 1B). Interestingly, *AIF* and *AIFsh* presented different tissue expression, suggesting an independent regulation of the *AIF* and *AIFsh* mRNA transcripts (Fig. 1B). A RT-PCR approach, which provided an improved signal, allowed us to further evaluate *AIF* and *AIFsh* mRNA tissue expression. In this assessment, the ribosomal L27 mRNA amplification was used as a loading and semi-quantitative control. Primers I9F2, in 5'UTR of *AIFsh* (intron 9 of *AIF*), and R4 in exon 5 (exon 14 of *AIF*) were used to detect *AIFsh*, whereas E2F (exon 2 of *AIF*) and E10R (exon 10 of *AIF*) were used to amplify *AIF* (Fig. 1C). Standard primers were used for L27 (Table 1). This approach led us to observe that *AIFsh* was less expressed in brain and thyroid compared with other tissues (Figure 1D). As assessed by Northern blot, a different expression pattern was observed between *AIF* and *AIFsh* (e.g. kidney, liver, lung, and colon tissues). Indeed, a multiplex PCR approach used to amplify *AIF* and *AIFsh* simultaneously in liver allowed us to calculate that, in this tissue, the expression level of *AIFsh* was about 7 times lower than the expression of *AIF* (data not shown).

To assess the distribution of *AIFsh* in cancer cells, we used the above-described RT-PCR approach in 12 different cancer cell lines. This method showed that *AIFsh* is present in all cell lines tested with a lower expression level in Cos7, IMR5, U937, 293T, and IMR32 (Fig. 1E). As in normal tissues, *AIF* and *AIFsh* display a tissue specific expression pattern in tumor cells.

Overall, our Northern blot and RT-PCR data strongly suggested that the expression of *AIFsh* is regulated independently from the expression of *AIF*. To confirm this assertion, we used two independent tools: 1) RNAi double-stranded oligonucleotides designed specifically against human *AIF* or human *AIFsh*. If *AIF* and *AIFsh* transcripts are regulated independently, it should be possible to interfere with the expression of one mRNA without disturbing the transcription of the other mRNA; and 2)

AIF-deficient ES cells (19). In these AIF KO cells, we searched whether the AIFsh mRNA is transcribed even when the AIF mRNA was suppressed by genetic recombination. Using the first approach (Fig. 2A), we confirmed that it is possible to regulate AIF or AIFsh mRNA transcripts independently (Fig. 2B). Interestingly, the design of a common siRNA double-stranded oligonucleotide designed against AIF and AIFsh (RNAi V), led us to eliminate of both mRNAs. Our second approach, the detection of AIF and AIFsh mRNA in AIF-deficient ES cells (*aif*^γ) (19) definitely confirmed that two independent AIF and AIFsh mRNAs exist. Indeed, in contrast to AIF, AIFsh was normally transcribed in AIF-deficient ES cells (Fig 2C).

Overall, these results demonstrated the widespread distribution of AIFsh transcript. Our findings also revealed the existence of individual AIF and AIFsh mRNAs, which could be independently regulated.

AIFsh a ~35 kDa protein corresponding to the C-terminal part of AIF

We next focused on the characterization of the AIFsh mRNA resulting protein. Sequence analysis of AIFsh mRNA revealed three potentially different ATG start sites in exon 10 that can produce in-frame translational products starting from M340 (M1), M353 (M2) or M363 (M3) of AIF protein sequence (Fig. 3A). To identify the real start codon of AIFsh, we produced two expression constructs: Δ 5'UTR AIFsh, containing the AIFsh sequence without the 5'UTR, and 5'UTR AIFsh, containing the complete AIFsh sequence identified by RACE. As shown in Fig. 3B, the expression of the Δ 5'UTR AIFsh plasmid in HeLa cells generates three products corresponding to the three start codons M1, M2, and M3, with a major expression for the longest product. Surprisingly, the expression of 5'UTR AIFsh plasmid generated only two products, corresponding to the start codons M2 and M3. Between these two proteins, the longest product is mainly expressed (Fig. 3B). This finding indicates that the 5'UTR is necessary to control AIFsh translation. Indeed, the start

codon M2 flanked with a Kozac sequence (36) (ACC ATG G), conserved in both human and mouse, is the most probable translational initiation site.

In order to confirm the initial aminoacid of AIFsh, we first performed an immunoblot analysis. Our working hypothesis was that a C-terminal anti-AIF antibody must recognize AIF and AIFsh in a whole cellular extract. Indeed, we detected the presence of AIFsh in HeLa, Jurkat and 3T3 cell lysates. In contrast, using an antibody against a N-terminal AIF epitope absent in AIFsh (aminoacids 1 to 300 of AIF), we failed to detect AIFsh (Fig. 3C). Together, these data confirm the existence of endogenous AIFsh. Interestingly, as shown in Fig. 3C, the apparent molecular mass of AIFsh is consistent with the apparent molecular mass of the main product observed in 5'UTR AIFsh overexpression assays. The Edman microsequencing of this overexpressed product confirmed that AIFsh presents an N-terminal sequence starting at the Methionine 353 (M2) of the AIF precursor. Thus, AIFsh is a protein of 261 amino acids with a relative molecular weight of ~35 kDa. AIFsh mimics the C-terminal region of AIF (aminoacids 353 to 613) including a nuclear localization sequence (10).

AIFsh is a cytosolic protein that induces caspase-independent cell death

AIF was first reported to be located in the intermembrane space of the mitochondria (10). In fact, the protein is addressed and compartmentalized into mitochondria by two localization sequences located within the N-terminal prodomain of AIF (10). As this domain is absent in AIFsh, it was therefore necessary to determine the AIFsh subcellular localization. To address this issue, expression plasmids encoding AIF-EGFP or AIFsh-EGFP fusion proteins were generated. Upon transfection into HeLa cells, AIF-EGFP or AIFsh-EGFP display distinct distribution patterns as shown in Fig. 4. AIFsh presents a diffuse cytosolic distribution, similar to that observed for GFP alone, whereas AIF-GFP exhibits a filamentous distribution that

colocalizes with the mitochondrial marker Mitotracker Red[®].

To examine if AIFsh can induce PCD, AIFsh was overexpressed in HeLa cells and cell death was quantified using two independent methods: MTT reduction, which assesses the mitochondrial functionality, and propidium iodide (PI) staining, which measures cellular viability (Fig. 5A). These two approaches showed a progressive disappearance of viable cells expressing AIF or AIFsh, thereby confirming the previously described lethality of AIF (10) and, most importantly, revealing a cytotoxic role for AIFsh. AIFsh-mediated cytotoxicity is a rapid and time-dependent process with significant alterations observed already 6 h after transfection. By contrast, AIF-induced PCD is a slower process, starting only 12 h after transfection (Fig. 5A). As AIF and AIFsh were efficiently overexpressed in HeLa cells (see upper panel in Fig. 5A), we attributed the different cytotoxic kinetics between AIF and AIFsh to their above-described cellular localization (Fig. 4). Indeed, in contrast to the cytosolic protein AIFsh, AIF needs to overcome its mitochondrial distribution to induce PCD (10). In this context, we found that overexpressed AIF initially localizes into mitochondria, and is redistributed to cytoplasm and nucleus only 12 h after transfection (Fig. 4 and data not shown). In contrast, overexpressed AIFsh redistributes from cytosol to the nucleus 6 h post-transfection (Fig. 4 and 5B). Thus, the redistribution of AIF or AIFsh to the nucleus seems to determine the cytotoxic kinetics of these two proteins. In fact, like AIF (7,10), AIFsh seems to provoke the loss of viability in transfected HeLa cells through the induction of nuclear apoptosis. In this way, as shown in Fig. 5C, when translocated from cytosol to nucleus, AIFsh provokes one of the main hallmarks of caspase-independent cell death: large-scale (50 Kb) DNA fragmentation. Overall, our findings show that overexpression of AIFsh induces loss of cell viability and nuclear apoptosis in HeLa cells.

We next analyzed further in detail whether AIFsh-provoked PCD is caspase-

dependent or caspase-independent. We first assessed the activation of a key executioner of the caspase-dependent type of cell death: caspase-3. This protease is synthesized as an inactive proenzyme of 32 kDa. However, after a caspase-dependent apoptotic insult, such as STS, caspase-3 is cleaved to yield an active subunit of p17 kDa (37). Thus, the presence of active caspase-3 was analyzed by Western blot, demonstrating that in contrast to the caspase-dependent staurosporine (STS) treatment, caspase-3 did not become activated at 8, 12 or 24 h after AIFsh transfection (Fig. 5D). This result indicates that AIFsh induced cytotoxicity in the absence of caspase-3 activation. This was further confirmed by measuring the cleavage of ICAD, one of the main caspase-3 substrates (38). Again, and in contrast to STS, our immunoblot assessment revealed the absence of caspase-3 dependent ICAD cleavage in AIFsh transfected cells (Fig. 5D). More generally, a pharmacological approach further confirmed that AIFsh induced a caspase-independent cytotoxic process. In fact, AIFsh-mediated PCD was not precluded by the broad-spectrum caspase-inhibitor QVD-OPH or by inhibitors of individual caspases, including those against caspase-2, -3, -6, -7, -8, -9 and -10 (Fig. 5E and data not shown).

Recombinant AIFsh provokes chromatin condensation and DNA fragmentation on isolated nuclei.

Next, we generated the human AIFsh recombinant protein to investigate whether the features observed in the nucleus of AIFsh HeLa transfected cells are directly due to the protein itself. AIFsh was tested in a cell-free *in vitro* system in which this recombinant protein was confronted to highly purified HeLa nuclei (35). After 90 minutes, nuclear apoptosis was quantified by two independent methods: immunofluorescence, to assess nuclear morphology, and cytofluorometry, to measure DNA alterations (Fig. 6). Using both systems, we observed that recombinant AIFsh induces peripheral chromatin condensation (Fig. 6A) and DNA loss (Fig. 6B) in the nuclei. In fact, and as shown in Fig. 6A, 6B,

and 6C, recombinant AIFsh causes *in vitro* the same nuclear apoptogenic effects than those observed in AIFsh-transfected cells. These results corroborate that AIFsh induces caspase-independent PCD through a direct effect of the protein on the nucleus.

Intriguingly, these nuclear modifications are similar to those observed after addition of the entire AIF recombinant protein to the purified nuclei, but they are not evident after addition of the deletion mutant AIF Δ 353-613, an AIFsh complementary protein which corresponds to the N-terminal region of AIF (aminoacids 1-352) (Fig. 6A). Thus, the first 352 amino acids of AIF are not required for its apoptotic activity. Therefore our results confirm that AIFsh presents similar proapoptotic effects to AIF because they share the apoptogenic part of the protein: the C-terminal domain.

The nuclear apoptotic effects of AIFsh are not inhibited by the chaperone Hsp70

Heat-shock protein 70 (Hsp70) has been reported to block AIF-mediated apoptosis through a physical interaction with AIF (31,39). Thus, to gain insight into the possible cellular regulation of AIFsh, we investigated if Hsp70 also neutralizes AIFsh. Using the cell free-system of apoptosis described above, we found that Hsp70 antagonized the apoptogenic effects of recombinant AIF, but failed to prevent the AIFsh-induced nuclear PCD (Fig. 7A and 7B). These results confirm previous studies indicating that Hsp70 blocks the AIF proapoptotic function by interaction with the N-terminal part of the protein (31). As this domain is absent in AIFsh, the AIF-cytoprotective properties of the chaperone Hsp70 are overcome.

AIFsh is down-regulated in human cancer

It has been shown that a shift in the relative ratio of a specific isoform of the same gene may lead to an impaired cell death program and consequently to tumor formation (40,41). This prompted us to investigate AIFsh potential pathological relevance, and we thus studied if AIFsh could be specifically

modulated in human cancer tissues. To this end, we analyzed on a Cancer Profiling Array the expression of AIFsh mRNA transcript in paired normal/tumor tissues (Fig. 8). This array is spotted with a complex cDNA representing the entire mRNA message expressed in a given normal or tumor tissue from an individual patient (154 cDNAs pair samples derived from 19 different tissues and 3 to 11 patients by tissue). Hybridization results revealed disease-related as well as patient-specific AIFsh gene expression patterns. We analyzed this array by quantification of the autoradiography depicted in Figure 8A. Data obtained from the normal tissue of each patient is considered as one unit, and we judged AIFsh to be down-regulated when we assessed less than 0.85 units in the paired tumor tissue (42). After quantification, we expressed the results obtained by percentage of patients showing AIFsh down-regulation in each tumor category (Fig. 8B). According to this, AIFsh was found to be frequently suppressed (over 80% of patients) in tumors derived from kidney, vulva, thyroid, skin, and pancreas but also in tumor samples derived from stomach, trachea, prostate, cervix, rectum and small intestine (around 60% of patients). In contrast, in ovary and colon carcinomas of some patients, AIF is up-regulated compared to normal tissues. In any case, we found that AIFsh was suppressed in 56% (86/154) of all tumors examined. Overall, these results indicated that AIFsh is frequently down-regulated in human tumor cells.

Expression of AIFsh is up-regulated by γ -irradiation

Finally, we focused on the relationship between AIFsh expression levels and sensitivity to cancer drugs, asking if AIFsh could confer resistance or sensitivity to these anti-tumor agents. In fact, and because AIFsh is down-regulated in tumor cells, it was necessary to further examine the possible role of AIFsh in tumor formation/suppression. To this end, we profiled AIFsh expression in cDNA samples generated from 26 human cancer cell lines, each individually treated

with 1 of 26 chemotherapeutic agents, oxidative stress inducers, or radiation (Fig. 9A). Each proapoptotic treatment produces, after 48 h, the loss of 50% in cell viability (IC_{50}) measured by the MTT assay. As shown in Fig. 9B, most of these treatments have moderate effects on AIFsh expression with the important exception of the γ -irradiation. Indeed, irradiation of cells, using a ^{60}Co source to produce an overall dose of 2 Gy, provokes an enhancement of the AIFsh expression in about 77% of the cancer cell lines tested (Fig. 9C). AIFsh up-regulation was more notable in cells derived from colorectal adenocarcinoma (HT-29), breast adenocarcinoma (MDA-MB-435S), melanoma (SK-MEL-28), and hepatocellular carcinoma (HepG2) (Figure 9C). Strikingly, in the two prostate cancer cell lines (DU-145 and PC-3), γ -irradiation is the only proapoptotic treatment up-regulating AIFsh (Fig. 9D). In a similar experiment, we assessed whether AIF was up-regulated by gamma-irradiation. Indeed, AIF expression was enhanced in 38% of the cancer cell lines tested but down-regulated in 50% (Fig. 10A). Interestingly, when AIF and AIFsh expression were compared in prostate DU-145, skin SK-MEL-5, and kidney 786-O cancer cell lines, γ -irradiation up-regulated AIFsh but not AIF (Fig. 10B). Interestingly enough, in the prostate DU-145 cancer cell line, γ -irradiation specifically up-regulated AIFsh (Fig. 10B). These results allow us to suggest that the expression of AIFsh is a good marker for γ -irradiation response in some types of cancer, and to support a potential role for AIFsh in the apoptotic program associated with DNA-damage.

DISCUSSION

Concomitant with the evolution of higher eukaryotes, cells were required to raise their protein diversity in order to cope with the increasing broad spectrum of functional and behavioral complexity. One of the major approaches to accomplish this task is the generation of multiple transcripts from a common gene. As a matter of fact, this mode of regulation of gene expression plays a major

role in the control of programmed cell death (41,43). Concerning AIF, two isoforms have been previously described (33). These two isoforms, which differ in the alternative use of exon 2 vs. exon 2b (see scheme in Fig 1A), are both targeted to the mitochondrial intermembrane space, suggesting a similar function for these two proteins. In the present work, we identified a third form of AIF: AIFsh. AIFsh mRNA and protein are ubiquitously expressed in human tissues and in a variety of cancer cell lines. Interestingly, the different AIF and AIFsh mRNA expression, the specific AIF and AIFsh RNAi regulation, and the presence of the AIFsh transcript in AIF KO cells, reveal that the generation of the AIFsh mRNA was independent from the generation of the AIF transcript. In this context, a careful analysis of the sequence upstream of the 5'UTR of the AIFsh transcript demonstrated the existence of two Alu repeated elements. These sequences are candidates to regulate the AIFsh expression. In this sense, previous studies reported that interspersed Alu repeat sequences are involved in the regulation of human genes transcriptional activity (44). In addition, we showed that the 5'UTR of AIFsh contributes to regulating the initiation of the mRNA translation. Thus, our data suggested that the 5'UTR of AIFsh modulates the expression of the transcript, probably through the repeated Alu sequences that are present in intron 9 of *AIF*.

An Edman microsequencing approach allowed us to identify the aminoacid sequence of AIFsh, which corresponds to the AIF C-terminal domain (amino acids 353-613). Contrary to the oxidoreductase domain of AIF, which shares strong homology to ferredoxin oxidoreductases (12), the C-terminal domain is the most intriguing part of the protein, given the absence of homology with any other protein. A structural analysis of the AIF C-terminal domain showed that a flexible loop resides in this part of the molecule (45). The extended structure of this loop suggests a potential binding region for DNA (18) and some chaperones, such as Hsp70. In fact, this protein has been shown to

interact with AIF, impairing its apoptotic function (39). However, our data demonstrate that Hsp70 fails to block the cytotoxic properties provoked by AIFsh. This indicates that Hsp70 does not interact with the C-terminal domain of AIF, or that AIFsh has not the same structure as AIF. In any case, and taking into account that, through its binding-inhibitory properties, Hsp70 has been implicated in tumor formation (46), the induction of AIFsh overexpression can serve as a new tool to overcome the Hsp70-cytoprotective properties.

AIF is initially compartmentalized in mitochondria (10) showing an NADH oxidase-dependent complex I function that is important for oxidative phosphorylation and tumorigenicity (14,32). Upon an apoptotic insult, AIF is cleaved and released from mitochondria (11,16,17), translocates to nucleus and triggers caspase-independent PCD. Because AIF itself does not have DNase activity (35), how AIF exerts its nuclear DNA fragmentation function remains unclear. Regarding this point, a recent paper demonstrated that AIF interacts with cyclophilin A to form an active DNase (47). Also, in the nematode *C. elegans*, AIF was thought to be working together with endonuclease G to promote DNA degradation (48). However, whether these two mechanisms apply to other apoptotic systems is yet unknown. In fact, we showed in a recent manuscript that AIF is not involved in the large-scale DNA fragmentation induced by STS in SH-SY5Y neuroblastoma cells (25). These results indicate that AIF function could be rather specific for cell and tissue type.

In contrast to AIF, our immunofluorescent and biochemical approaches suggested that AIFsh directly localizes in cytosol. Interestingly, AIFsh overexpression results in a rapid translocation to the nucleus where the protein induces cell death in a time-dependent manner. The comparison of cell death kinetics between AIF and AIFsh indicates that AIFsh induces cell death more rapidly than AIF. This could be explained by the fact that accumulation of AIFsh in the cytosol fulfills an apoptogenic

function directly on the nucleus. In contrast, AIF needs to translocate from mitochondria to cytosol before targeting the nucleus. Obviously, the presence of AIFsh in cytosol (and not in the nucleus) of non-apoptotic cells implies the presence of a yet-unidentified AIFsh-regulatory protein that is able to sequester AIFsh. When overexpressed, AIFsh would be capable of overcoming this regulatory protein by translocating to the nucleus.

Like AIF, AIFsh-induced PCD is not precluded by inhibitors of caspases, confirming that AIFsh-induced PCD is caspase-independent. Interestingly, AIFsh induces peripheral chromatin condensation and 50 Kb DNA fragmentation, both in transient transfected cells and in purified nuclei. These two biochemical hallmarks are similar to that induced by AIF. In contrast, AIFsh-mediated PCD is not inhibited by Bcl-2 or Bcl-X_L overexpression (unpublished observations), suggesting the possibility that AIFsh-mediated PCD might not be controlled by members of the Bcl-2 family.

Summing up, despite the functional similarities between AIFsh and AIF (e.g. nuclear translocation, induction of chromatin condensation, and 50 Kb DNA fragmentation), AIFsh has five different features: 1) AIFsh mRNA is expressed independently from the AIF mRNA; 2) AIFsh lacks the oxidoreductase domain of AIF, meaning that AIFsh does not present electron transfer activity (C. Delettre and S. A. Susin, manuscript in preparation); 3) In non-apoptotic cells, AIFsh localizes in the cytosol; 4) AIFsh apoptotic activity is not controlled by Hsp70; and finally 5) AIFsh PCD action does not seem to be regulated by the Bcl-2 family of proteins.

Our data strongly support that the C-terminal domain of AIF, which is mimicked by AIFsh, is necessary and sufficient to induce AIF-dependent caspase-independent apoptosis. Indeed, by using the recombinant protein AIF Δ 353-613, we demonstrate that the first 352 amino acids of the N-terminal domain in AIF are not required for its nuclear apoptotic activity. In fact, through the

identification and characterization of AIFsh, we further demonstrate that: (i) the oxidoreductase domain of AIF is not necessary to induce nuclear apoptosis. Thus, AIF does not induce chromatin condensation and 50 Kb DNA fragmentation through the production of the reactive oxygen species associated with this part of the protein (13); (ii) the C-terminal domain of AIF contains all the information to interact with nuclear proteins (or DNA) and to induce chromatin condensation and large-scale DNA fragmentation; (iii) because the effect of AIF and AIFsh are analogous, the putative target of AIF/AIFsh in the nucleus (e.g. cyclophilin A) might be the same. In this sense, the region of AIF interacting with cyclophilin A (47) is present in AIFsh.

Because alterations in the expression and function of apoptosis-regulating genes play a prominent role in the resistance to chemotherapy and radiotherapy, the regulation of AIFsh expression in cancer cells deserves a particular analysis. In fact, the development of cancer is a multistep process involving mutations in proto-oncogenes and tumor suppressor genes. Therefore, acquisition of apoptotic resistance might be an important and even necessary step during progression of tumors to a fully malignant "metastatic" phenotype. Further supporting this theory, numerous reports depict dysfunction of some apoptosis regulatory genes like *bcl-2* gene family, *p53*, and *Rb* or expression of certain oncogenes like *ras*, which are apparently associated to enhanced metastatic ability of cancer cells (2,49). Hence, defining the mechanisms of regulation of apoptotic effectors is a major priority in cancer research. In our manuscript, we showed that AIFsh mRNA is down-regulated in a large variety of tumors. Special mention should be made of the reduced tumor-specific expression observed in renal, thyroid and pancreas tissues compared with their normal counterparts (detected in more than 80 % of the patients tested). The down-regulation of AIFsh expression that may be caused, for example, by point mutation in the ORF (region of regulatory elements) or by

hypermethylation at the CpG islands in the AIFsh promoter region, requires further investigation. In any case, the apoptotic properties of AIFsh and the reduction of expression of AIFsh mRNA in tumors, suggest a potential tumor-suppressing function for this new identified AIF-isoform.

Additionally, our analysis indicates that the proportion observed in AIFsh isoform can serve as a prognostic factor for radiotherapy outcome. By studying the effects of multiple chemotherapeutic agents, oxidative stress inducers and radiation in human cancer cell lines, we have found that AIFsh was transcriptionally up-regulated by γ -irradiation. Therefore, our findings may provide novel opportunities to understand the cellular pathways that lead to death in response to DNA damage. In fact, we can hypothesize that AIFsh may be one of the mediators of radiation-induced apoptosis. The AIFsh up-regulation in response to DNA damage, which is independent from AIF up-regulation, is more relevant when analyzing the array by single cell line. This approach has allowed us to observe that γ -irradiation is the only pro-apoptotic treatment up-regulating AIFsh in the two tested prostate cancer cell lines (Fig. 9D). One of the major problems in the management of prostate cancer is the lack of reliable genetic markers predicting the outcome of an anticancer therapy (50). Widely used biochemical, histopathological, and clinical criteria such as prostate-specific antigen (PSA) level, Gleason score, the clinical tumor stage, and molecular genetic approaches assaying loss of tumor suppressors or gain of oncogenes only had limited success and demonstrated a significant variability in predictive value. Thus, our study on AIFsh may provide the basis for novel therapeutic approaches in the management of prostate cancer.

In conclusion, the present report provides substantial progress in three major areas: (i) the understanding of *AIF*, (ii) the comprehension of the mechanisms regulating AIF proapoptotic function, and (iii) the development of new tools for the treatment of some types of cancer. In this sense, the

identification of AIFsh might pave the way for novel diagnostic, therapeutic and pharmacological tools for designing new strategies targeting the caspase-independent cell death pathway.

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FOOTNOTES

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The abbreviations used are: CMV, Cytomegalovirus; DMEM, Dulbecco's Modified Eagle's Medium; GFP, Green Fluorescent Protein; ICAD, Inhibitor of Caspase-Activated DNase; PBS, Phosphate-Buffered Saline; QVD-OPH, Gln-Val-Asp(non-Omethylated)-Oph; STS, Staurosporine; z-VAD.fmk, N-benzyloxycarbonyl-Val-Ala-Asp.fluoromethylketone.

The GenBank Accession numbers for the nucleotide sequences of human and mouse AIFsh are DQ016496 and DQ016497, respectively.

FIGURE LEGENDS

FIGURE 1. AIFsh mRNA identification and expression. A, Genomic organization of *AIF* and resulting AIF and AIFsh mRNA transcripts (grey line). Translation start (ATG) and stop (TAA) codons are indicated, and the predicted AIF and AIFsh protein products are shown to the right. The identification of an alternative transcriptional start site in intron 9 (I9) of *AIF* yields the AIFsh isoform. AIFsh contains the C-terminal of AIF, from exon 10 to 16, which includes a nuclear localization sequence (NLS), and lacks the mitochondrial leader sequence (MLS) and a part of the oxidoreductase domain (Pyr-Redox). B, Expression profile of AIF and AIFsh in human tissues. Specific AIF or AIFsh cDNA probes were hybridized to a Northern blot membrane of polyadenylated RNA from the indicated human tissue. Note the different expression pattern between AIF and AIFsh. C, Schematic representation of the two primer pairs used in the identification of AIF and AIFsh transcripts by RT-PCR. D, Expression pattern of AIFsh in a variety of human tissues. AIFsh expression was analysed by RT-PCR with a forward primer located in the 5'UTR of AIFsh and a reverse primer located in exon 14 of AIF. AIF mRNA was analyzed with a forward primer located in exon 2 and a reverse primer located in exon 10. The ribosomal protein L27 was used as a semi-quantitative control. E, Expression pattern of AIFsh in a variety of cancer cells lines. AIFsh mRNA on the indicated cell line was analysed by RT-PCR as in D.

FIGURE 2. Different modulation of AIFsh and AIF transcripts. A, Schematic representation of the five siRNA double-stranded oligonucleotides used in RNAi assays. siRNA I and II were AIF specific, siRNA III and IV were AIFsh specific, and siRNA V interferes with the expression of both AIF and AIFsh. B, HeLa cells were transfected with a scramble siRNA double-stranded oligonucleotide (Co.), two siRNAs against human AIF (I and II), two siRNAs against human AIFsh (III and IV), or a siRNA designed against AIF and AIFsh (V). 48 h after the indicated transfection, the expression of AIF or AIFsh was analyzed by RT-PCR as in Figure 1D. The ribosomal protein L27 was used as a semi-quantitative control. C, RT-PCR of mouse AIF (mAIF) and mouse AIFsh (mAIFsh) in *aif*^{+/-y} and *aif*^{-/-y} ES

cells. GAPDH expression was used as control for RNA quality. Note the presence of the AIFsh transcript in *aif*^{-ly} ES cells.

FIGURE 3. AIFsh protein expression. A, Alignment of aminoacid sequences of human AIF, human AIFsh and mouse AIFsh. Identical residues are shaded and the three possible starting methionines of AIFsh/mAIFsh are marked with an asterisk. B, HeLa cells were transiently transfected with expression plasmids pcDNA3-Δ5'UTRAIFsh (upper) or pcDNA3-5'UTRAIFsh (lower), and protein expression was analyzed by Western Blot with an anti-AIF C-terminal antibody (epitope being aa 593-613). The scheme explains the three ATG codons (M1, M2, and M3) encoding for a protein after pcDNA-Δ5'UTRAIFsh transfection and the two ATG codons (M2, and M3) encoding for a product after pcDNA-5'UTRAIFsh transfection, respectively. Note that the pcDNA-5'UTRAIFsh transfection results in a main product of 261 aminoacids beginning at the Methionine M2. C, Total cell lysates from 3T3, Jurkat, and HeLa cells were prepared, and the expression level of AIFsh was analyzed by Western Blot either with an anti-AIF C-terminal antibody (epitope being aa 593-613) or an anti-AIF N-terminal antibody (epitope being aa 1-300). Actin was used to control protein loading. Note that only the anti-AIF C-terminal antibody recognizes AIFsh. A total lysate from HeLa cells transiently transfected with the expression plasmid pcDNA3-5'UTRAIFsh was used as a positive control.

FIGURE 4. AIFsh localizes in cytosol. HeLa cells were transfected with vectors encoding GFP or the fusion proteins AIF-GFP or AIFsh-GFP. Six hours after transfection cells were stained with the mitochondrial specific dye Mitotracker Red[®] (red fluorescence). Representative cells show that AIFsh has a diffuse cytosolic distribution, whereas AIF displays a filamentous pattern that colocalizes with mitochondria.

FIGURE 5. AIFsh translocates to nucleus and induces large-scale DNA fragmentation and caspase-independent cell death. A, Kinetic analysis of AIFsh-mediated cytotoxicity. HeLa cells were transfected with pcDNA3-empty vector (Empty vector), pcDNA3-AIF (AIF), or pcDNA3-AIFsh (AIFsh). After the indicated transfection time, cell viability was measured by MTT assay (upper panel) or PI labeling (lower panel). In a similar set of experiments, HeLa cells were transfected with p3xFLAG-AIF (AIF) or p3xFLAG-AIFsh (AIFsh). At the time indicated after transfection, total cell lysates were prepared and the expression levels of AIF or AIFsh were analyzed by immunoblotting with an anti-Flag antibody. PVDF membrane was stained with Naphtol Blue (NB) to control protein loading. B, AIFsh overexpression provokes its redistribution from cytosol to nucleus. HeLa cells were transfected with pcDNA3-AIFsh and subjected to subcellular fractionation. Cytoplasmic and nuclear fractions were blotted for immunodetection of AIFsh. Note the presence of AIFsh in the nucleus of AIFsh-transfected cells. Fractionation quality and protein loading were verified by the distribution of the specific subcellular markers: Erk3 for cytoplasm and Lamin A/C for nucleus. C, Assessment of large-scale (upper panel) and oligonucleosomal (lower panel) DNA fragmentation of HeLa cells transfected with pcDNA3-empty vector (Empty vector), pcDNA3-AIF (AIF), or pcDNA3-AIFsh (AIFsh). DNA analysis was performed 12 h after transfection. The tyrosine kinase inhibitor staurosporine (STS) was used as a positive control. D, AIFsh induces death in a caspase-3 independent manner. Total cell lysates from HeLa cells treated with STS for 8 h or transfected with pcDNA3-AIF (AIF) or pcDNA3-AIFsh (AIFsh) were obtained at different times and probed for the detection of AIF, caspase3, or ICAD. Equal loading was confirmed by actin detection. The precursor forms of caspase-3 and ICAD were cleaved only in HeLa cells treated by STS but not in cells transfected with AIF or AIFsh. E, AIFsh induced death is caspase-independent. HeLa cells were transfected or treated

with STS as in B in presence or absence of 10 μ M QVD-OPH. Cell viability was determined by MTT test at 12 h post-transfection.

FIGURE 6. Effects of AIFsh recombinant protein on isolated nuclei. A, B, Effects of AIF and AIFsh on nuclear morphology and DNA content. Purified HeLa nuclei were left untreated (Control) or incubated with the human recombinant proteins AIF, AIFsh or AIF Δ 353-613 at the indicated concentrations (5 μ g/ml in A), stained by Hoescht 33342 or PI, and then subjected to determination of chromatin condensation or nuclear DNA loss by fluorescent microscopy (A) and flow cytometry (B), respectively. Fluorescent microscopy showed representative results of each treatment. C, Agarose or pulse-field electrophoresis of purified HeLa nuclei that were left untreated (Control), or incubated with 5 μ g/ml of AIF or AIFsh in the absence or presence of the pan caspase-inhibitor z-VAD.fmk. Recombinant Caspase-Activated DNase (CAD) was used as positive control. AIF and AIFsh cause large-scale (50 Kb) DNA fragmentation in purified HeLa nuclei even in the presence of z-VAD.fmk. However, in contrast to CAD, these two proteins do not induce oligonucleosomal DNA fragmentation.

FIGURE 7. Hsp70 effects on AIF and AIFsh activities determined in a cell-free system. A, B, Purified HeLa nuclei were incubated with recombinant AIF (5 μ g/ml), AIFsh (5 μ g/ml), BSA (used as a control) or Hsp70 at the indicated molar ratio (1:10 in A), stained by Hoescht 33342 or PI, and then subjected to determination of chromatin condensation or nuclear DNA loss by fluorescent microscopy (A) or flow cytometry (B) as in Fig. 6. Hsp70 blocks AIF nuclear proapoptotic action, but fails to inhibit the apoptogenic effects of AIFsh.

FIGURE 8. AIFsh expression in paired normal/tumor tissues by cDNA array. The Cancer profiling array II (Clontech) was hybridized with a 333 bp 32 P-dCTP-labeled specific probe from the 5'UTR region of AIFsh. Data of hybridization were normalized to ubiquitin expression and converted into numbers by densitometric evaluation in a STORM phosphorimager. AIFsh expression in paired tumor/normal tissues from the same patient was quantified considering expression in normal tissue as one unit. Upper panel represents the phosphorimage autoradiography of the array. N = Normal, T = Tumor, cc = cancer cell line cDNA control. Lower panel represents the quantification of the autoradiography by type of tumor expressed as percentage of patients showing AIFsh down-regulation (less than 0.85 than in normal tissue) (42).

FIGURE 9. AIFsh modulation in response to chemotherapy and radiotherapy proapoptotic treatments. A, AIFsh mRNA expression was profiled in 26 diverse cancer cell lines treated with a large panel of chemotherapeutic agents, oxidative stress inducers or radiation. Left panel represents the phosphorimage autoradiography of the array hybridized with an AIFsh specific probe as in Fig. 8. Hybridization with the Ubiquitin probe furnished by the supplier was used as a control (right panel). Columns: Treatments. Rows: Cell lines. Ubi: Ubiquitin cDNA. B, Changes in AIFsh expression level in response to the treatments described in A. Quantification was done in a STORM phosphorimager by cell line (●) or by the average effect of each treatment in all cell lines (○). Fold change = (AIFsh expression / Ubiquitin expression - 1) x 100 as described by Li et al. (51). C, Variations in AIFsh expression observed after γ -irradiation treatment quantified as in B in the entire panel of cancer cell lines. D, Detail of AIFsh expression observed in DU-145 and PC-3 prostate cells after the apoptogenic treatments described in A. AIFsh expression level was quantified as in B and C. Note that γ -irradiation is the sole treatment which provokes AIFsh up-regulation.

FIGURE 10. Independent AIF/AIFsh mRNA regulation in response to γ -irradiation. A, AIF mRNA expression was profiled as in Fig. 9. Variations observed in the panel of cancer cell lines after γ -irradiation treatment were quantified and compared to the AIFsh mRNA changes measured in Fig. 9C. B, Detail of AIF and AIFsh expression observed in DU-145, 786-O, and SK-MEL-5 cancer cell lines after the proapoptotic treatments described in Fig. 9A. AIF and AIFsh expression levels were quantified as in A. Note that AIF and AIFsh are independently regulated and that γ -irradiation specifically up-regulated AIFsh in DU-145 cells.