Identification and Characterization of AIFsh2, a Mitochondrial Apoptosis-inducing Factor (AIF) Isoform with NADH Oxidase Activity

Cécile Delettre¹, Victor J. Yuste², Rana S. Moubarak³, Marlène Bras⁴, Nadine Robert, and Santos A. Susin⁵

From the Apoptose et Système Immunitaire, CNRS-URA 1961, Institut Pasteur, 25 rue du Dr. Roux, 75015 Paris, France

Apoptosis-inducing factor (AIF) is a bifunctional NADH oxidase involved in mitochondrial respiration and caspase-independent apoptosis. Three alternatively spliced mRNA isoforms of AIF have been identified previously: AIF, AIF-exB, and AIFsh. Here, we report the cloning and the biochemical characterization of a new isoform named AIF short 2 (AIFsh2). AIFsh2 transcript includes a previously unknown exon placed between exons 9 and 10 of AIF. The resulting AIFsh2 protein, which localizes in mitochondria, corresponds to the oxidoreductase domain of AIF. In this way, AIFsh2 exhibits similar NADH oxidase activity to AIF and generates reactive oxygen species. Like AIF, AIFsh2 is released from mitochondria to cytosol after an apoptotic insult in a calpain or cathepsin-dependent manner. However, in contrast to AIF, AIFsh2 does not induce nuclear apoptosis. Thus, it seems that the reactive oxygen species produced by the oxidoreductase domain of AIF/AIFsh2 are not important for AIF-dependent nuclear apoptosis. In addition, we demonstrate that the AIFsh2 mRNA is absent in normal brain tissue, whereas it is expressed in neuroblastoma-derived cells, suggesting a different regulation in normal and transformed cells from the brain lineage. Together, our results reveal that AIF yields an original and independent genetic regulation of the two AIF functions. This is an important issue to understand the physiological role of this protein.

Apoptosis-inducing factor (AIF) is a flavoprotein, with significant homology to bacterial and plant oxidoreductases, located in the mitochondrial intermembrane space (1–3). Under physiological conditions, AIF is a NADH oxidase that plays a role in oxidative phosphorylation (4–6). Moreover, AIF plays a major role in cell death (7). Indeed, after a cellular insult, AIF is cleaved by calpains and/or cathepsins (8, 9) and translocates from mitochondria to cytosol and nucleus where it causes chromatin condensation (10) and large scale (~50 kb) DNA fragmentation in a caspase-independent fashion (1). This apoptogenic function of AIF seems essential in some types of cell death. In fact, AIF was described as a central mediator of relevant experimental models of cell death such as As2O3-induced cell death in human cervical cancer cells (11), DNA damage-mediated p53 activation (12, 13), Sulindac-induced PCD in colon cancer cells (14), geldanamycin-mediated PCD in human glioma cells (15), staurosporine-induced PCD in neuroblastoma cells (16), caspase-independent apoptosis induced by Survivin in melanoma cells (17), hexaminolevulinate-mediated photodynamic therapy in human leukemia cells (18), or poly(ADP-ribose) polymerase-mediated cell death (19, 20). In addition, blockade of the AIF signal transduction pathway seems to be implicated in the chemoresistance of non-small cell lung carcinomas (21) and other human cancers (22). On the other hand, in human colon cancer cells, AIF suppresses chemical stress-induced apoptosis and maintains the transformed state of tumor cells (22). Thus, AIF is a bifunctional protein with a vital role, via its redox activity in mitochondria, and a lethal role, via its translocation to the nucleus.

AIF comprises 16 exons and locates on chromosome X, region q25–26, and A6 in humans and mice, respectively (23). AIF is expressed as a precursor of 67 kDa. This form is addressed and compartmentalized in mitochondria by two-mitochondrial localization sequences (MLS) located in the N-terminal prodomain. Once in mitochondria, the full-length AIF is processed and the prodomain removed, yielding a mature form of ~57 kDa (1). This form comprises three structural domains: FAD-binding domain, NADH-binding domain, and the C-terminal domain (2). The first two domains compose the oxidoreductase part of AIF, which confers an electron transfer activity to the protein (4). The C-terminal part of AIF seems to be the pro-apoptotic domain (24). One splice variant of AIF, AIF-exB, was reported soon after the initial description of AIF (25). This variant contained an alternative exon 2b instead of the original exon 2. Although the function of this alternatively spliced variant is not yet deciphered, it is established that the alternative exon 2b usage does not affect AIF-exB mitochondrial import and function (25). More recently, our group identified a third AIF isoform, AIFsh (24). This isoform results from an alternate transcriptional start site located at intron 9 of AIF. As a result, AIFsh lacks the AIF N-terminal oxidoreductase domain. Intriguingly, AIFsh, which is a cytosolic protein, provokes the same effects as AIF: chromatin condensation and large-scale (50 kb) DNA fragmentation (24). Therefore, it seems that the oxidoreductase part of AIF is not necessary for the induction of cell death. However, some work suggest that, through the production of reactive oxygen species, the oxidoreduc-
tase portion of AIF could play an additional role in caspase-independent apoptosis (4, 22, 26).

In the present article, we report the identification of a novel exon in AIF, which generates two new AIF mRNA transcripts in human (AIFsh2 and AIFsh3) and one in mouse (mAIFsh2). Through biochemical, cellular, and molecular biology approaches, we localized the AIFsh2 resulting protein in mitochondria from mouse liver or human HeLa cells. In contrast, these same approaches failed to detect the AIFsh3 protein. In addition, we explored the expression pattern of AIFsh2 and its biochemical properties and showed that this novel AIF isoform, which is absent in normal brain tissue, exhibited NADH oxidase but no nuclear pro-apoptotic activity. Together with our previous results (24), our present work reveals new insights into the mechanisms regulating life and death AIF-dependent functions.

**Experimental Procedures**

**Northern Blotting**—First choice Human Blot I membrane containing total RNA from 10 tissues (Ambion) was hybridized as described previously (24) with a 330-bp 32P-labeled exon 9b fragment generated by PCR with forward 5′-TGGGACATTCTAGAAGCCTGGAGG-3′ and reverse 5′-TTCTGTGCCAAGGCTGAGGACA-3′ primers.

**Rapid Amplification of cDNA Ends (RACE) Analysis**—5′- and 3′-RACE were performed using the Marathon Ready cDNA library from human kidney (Clontech) according to the manufacturers recommendation. For the 5′-RACE, the first round of PCR amplification was done using the adaptor primer 1 and reverse primer HCR1 (5′-CCTGGTCTCAGAAGTCCATTGTGAACGC-3′). The nested PCR was done with the nested primer adaptor primer 2 and HAIshF (5′-CATAGTGGCTTTGCAACCTCTGAATAGGA-3′). For the 3′-RACE PCR the product was amplified using the adaptor primer 1 and the forward primer: 19F (5′-CGCTTACAAATGAAGTCCGGCAGGAGGA-3′).

**ARN Extraction and RT-PCR**—Total RNA from human tissues was from Stratagene. RNAs from human cancer cell lines and mouse kidney were purified using the RNeasy mini kit (Qiagen). For reverse transcription, 1 μg of total RNA was mixed with 200 units of Superscript II reverse transcriptase (Invitrogen) and 150 ng of random primers (Promega). As a control, reverse transcription was performed without reverse transcriptase. Human AIFsh and AIFsh2 isoforms were amplified simultaneously using the primers 19F (5′-CGCTTACAAATGAAGTCCGGCAGGAGGA-3′) and R4 (5′-CACCAACTGTGGGCAAAACTACT-3′). Human AIFsh3 was amplified using primers sh3F1 (5′-AGAGAACAGGCTCAGAAGCCTAA-3′) and R4 (5′-CACCAACTGTGGGCAAAACTACT-3′). The PCR products were separated on a 1% agarose gel.

**Vector Constructions**—Mammalian expression vectors for AIF, AIFsh2, and AIFsh3 were carried out by PCR amplification of the corresponding cDNA fragments and subsequently cloned into cytomegalovirus promoter-based vectors pcDNA3 (Invitrogen), pEGFP-N1 (Stratagene), or C-terminal p3xFLAG (Sigma). Final constructs are referred to as pcDNA3-AIFsh2, pcDNA3-AIFsh3, pEGFP-AIF, pEGFP-AIFsh2, or p3xFLAG-AIFsh2. Empty vectors were used as controls.

**Cell Culture, Transfections, and Cell Death Induction**—HeLa cells were cultured in complete culture medium (Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and 100 units/ml penicillin/streptomycin, Invitrogen). Cell cultures were maintained at 37°C with 5% CO2. HeLa cells were seeded at a concentration of 2 × 10^5 into 6-well plates. Transient transfections were performed using Lipofectamine 2000 in Opti-MEM (Invitrogen) according to the manufacturers protocol. 16 h after pcDNA3-AIFsh2, pcDNA3-AIFsh3, or pcDNA3-empty vector transfection, AIFsh2 and AIFsh3 protein expression was assessed by immunoblotting.

For RNAi assays, HeLa cells were transfected with specific siRNA double-stranded oligonucleotides designed against human AIF (I = 5′-GCAGAAAGGCTGGACCTTT-3′), human AIFsh (II = 5′-GAAT- GTTCTAGAAGCCTAA-3′), human AIFsh2 (III = 5′-GCAGAAGGCTGAGGACA-3′), or a common siRNA against human AIF, AIFsh, and AIFsh2 (IV = 5′-GATCTTCTACGATATGAA-3′). As a control, we used an irrelevant siRNA oligonucleotide (Co = 5′-GCAGAAGGCTGAGGACA-3′). 48 h after the indicated transfection of HeLa cells, AIF, AIFsh, or AIFsh2 mRNA expression was assessed by RT-PCR as described above. In these RNAi transfections we used Lipofectamine (Invitrogen) instead Lipofectamine 2000. Apotopsis was induced by treatment of HeLa cells for 8 h with staurosporine (STS) (1 μM, Sigma), etoposide (50 μM, Sigma), camptothecin (5 μM, Sigma), thapsigargin (10 μM, Sigma), TNF/CHX (25 ng/ml + 2 μg/ml, Sigma), and β-lapachone (20 μM, Sigma).

**Mitochondrial Purification, HeLa Cell Fractionation, and Assessment of AIF/AIFsh2 Release**—Mitochondria from HeLa cells or mouse liver were isolated as previously described (9) and resuspended in a buffer containing 10 mM Hepes/KOH, pH 7.2, 250 mM sucrose, and 5 mM EGTA. Mitochondrial protein concentration was determined by the Bio-Rad (M, Calbiochem) before addition of atractylidine (20 μM, Sigma), the cathepsin inhibitor Z-FA-fmk (10 μM, Sigma), or the calpain inhibitor calpeptin (10 μM, Calbiochem) before addition of atractylidine. The mixture was incubated for 35 min at 25°C and centrifuged (30 min, 4°C, 13,400 g) to obtain a mitochondrial pellet (P) or supernatant containing proteins released from mitochondria (SN). Supernatants were further re-clarified (90 min, 4°C, 100,000 g) to produce a soluble fraction.

In pcDNA3-AIFsh3 overexpression assays, the cytosolic purification was performed with 2 × 10^6 HeLa cells. Cells left untreated or treated with STS, etoposide, camptothecin, thapsigargin, TNF/CHX, or β-lapachone were resuspended in buffer containing 220 mM mannitol, 70 mM sucrose, 50 mM Hepes/KOH (pH 7.2), 10 mM KCl, 5 mM EGTA, 2 mM MgCl2, and 0.025% digitonin, and kept on ice for 5 min. Cells were centrifuged (16,000 × g, 5 min at 4°C) and the supernatant was retained as the cytosolic fraction. Protein content was quantified by the Bio-Rad DC Protein Assay® method.

**Immunofluorescence**—For viewing the localization of pEGFP-AIF, pEGFP-AIFsh2, and pEGFP-N1, transfected HeLa cells seeded on cov-
erslips were washed with phosphate-buffered saline three times and stained with Mitotracker Red®/H23041 (20 nM, Invitrogen) for 15 min at room temperature. Cells were mounted and red (mitochondrial) and green (GFP) fluorescence was observed in a Nikon Eclipse TE2000-U microscope and analyzed using Nikon ACT-1 software.

Recombinant Proteins—N-terminal His-tagged AIF, AIF-exB, AIFsh, AIFsh2, and AIFsh3 human recombinant proteins were produced from a Novagen pET28b expression vector, purified from *Escherichia coli* extracts on a nickel-nitrilotriacetic acid affinity matrix and stored in 50 mM Hepes, pH 7.9, 100 mM NaCl, 1 mM dithiothreitol, and 10% glycerol until use.

Reconstitution of AIFsh2 and Redox Activity—AIF, AIFsh, and AIFsh2 were reconstituted with FAD following the protocol described by Miramar et al. (4). *In situ* detection of nitro blue tetrazolium (NBT) reduction on native-PAGE was done using the reaction mixture described elsewhere (27). Briefly, samples were loaded onto a 8% native-PAGE. The gel was incubated 60 min in the dark with 2 mM NBT solution. Then, 1 mM NADH was added to reduce NBT and the reaction was stopped with water after the appearance of the blue band. NAD(P)H oxidase activity of AIFsh2 was performed following procedures described previously (4). Briefly, NAD(P)H oxidase activity was measured at 25 °C in a total vol-

---

**FIGURE 1.** Schematic representation of the *AIF* gene structure, and resulting protein isoforms. A, genomic organization of *AIF* and resulting AIF, AIF-exB, AIFsh, AIFsh2, and AIFsh3 mRNA transcripts (gray line). Translation start (ATG) and stop (TGA/TAA) codons are indicated, and the predicted protein product is shown at the right. Numbers in *AIF* designate exons and, in predicted proteins, amino acids. MLS, Pyr-redox, nuclear localization sequence (NLS), and C-terminal domains are indicated. I9 indicates intron 9. AIF, AIF-exB, and AIFsh isoforms are described elsewhere (1, 24, 25). The inclusion of the 203-bp exon 9b produces AIFsh2 and AIFsh3, which encodes 224- and 237-amino acid proteins, respectively. AIFsh2 contains the AIF mitochonrdial localization sequences and the oxidoreductase domain, but lacks the C-terminal amino acids of AIF. AIFsh3 has a similar structure as AIFsh2 with the splicing of exon 2, which leads to the loss of MLS. B, structure of the novel alternatively spliced exon 9b. AIFsh2 retains a 203-bp long portion of intron 9 as a novel exon, termed exon 9b (nucleotides 28309–28511 of the published sequence of *AIF*: GenBank accession number NT_011786). Conserved 5′ donor site (GT) and 3′ acceptor site (AG) that can be recognized by spliceosome are indicated by an asterisk. Y corresponds to C or T nucleotide, N corresponds to any nucleotide.
AIFsh2 Is a NADH Oxidase Located in Mitochondria

**RESULTS**

Identification of a Novel Exon in the Human AIF Gene, Which Results in Two Novel AIF Isoforms: AIFsh2 and AIFsh3—Recently, by 3′- and 5′-RACE, we have identified a new AIF transcript that results from an alternate transcriptional start site located in intron 9 of AIF (24). This new transcript encoded for a new protein called AIFsh2 (24). By a similar 3′- and 5′-RACE approach, we identified here two new AIF cDNA species in a human kidney cDNA Marathon library: AIF short 2 (AIFsh2) and AIF short 3 (AIFsh3). AIFsh2 and AIFsh3 differed from the three previously described AIF forms, AIF, AIF-exB, and AIFsh (1, 24, 25), by alternative usage of a new exon of 203 bp: exon 9b (Fig. 1, A and B). This new exon was located in intron 9 of AIF, between exons 9 and 10 (nucleotides 28309–28511 of AIF, GenBankTM accession number NT_011786, Gene ID 9131). Analysis of the flanking genomic sequences of exon 9b showed that it contained a consensus for splicing signals (29) (Fig. 1 B).

In AIFsh2, exon 9b encoded for only two amino acids (Asp and Ile), which are followed by a stop codon (Fig. 2). The resulting 324 amino acid protein did not contain the AIF C-terminal domain (Figs. 1 A and 2 A). The AIFsh3 transcript included exon 9b but lacked AIF exon 2 (Fig. 1 A). Here, the absence of exon 2 provoked a change in the open reading frame that generates a stop codon. As a result, the translation initiation site for AIFsh3 was located in exon 3 of AIF (Fig. 1 A). The resulting putative AIFsh3 protein (237 amino acids) lacked both the mitochondrial localization sequence and the C-terminal domain of AIF (Figs. 1 A and 2 A).

We next searched for a mouse ortholog of AIFsh2 and AIFsh3. An in silico analysis of the mouse AIF nucleotide sequence failed to detect an equivalent of exon 9b. Although, a RT-PCR assessment, performed between exon 1 and intron 9 (see “Experimental Procedures”), led us to amplify an mRNA transcript (mouse AIFsh2, mAIFsh2) containing the murine ortholog sequence for exon 9b. This exon encoded three amino acids (Cys, Glu, and Tyr), which were followed by a stop codon suppressing the C-terminal domain of mouse AIF (Fig. 2 B). The resulting

![Figure 2. AIF, AIFsh2, and AIFsh3 sequence comparison. A, alignment of amino acid sequences of human AIF, AIFsh2, and AIFsh3. Note that AIFsh2 mimics the N-terminal domain of AIF and lacks amino acids 323–613 at the C terminus of AIF. The AIFsh3 theoretical protein lacks both the mitochondrial localization sequence and the C-terminal domain of AIF. Black boxes indicate identical residues. Gray boxes highlight the two common AIF/AIFsh2 mitochondrial localization sequences. B, alignment of amino acid sequences of mouse AIF and AIFsh2. In this case, mAIFsh2 lacks amino acids 322–612 at the C terminus of mouse AIF. The AIFsh3 mRNA transcript was not detected in mice. Black boxes indicate identical residues.](image-url)
AIFsh2 Is a NADH Oxidase Located in Mitochondria

**FIGURE 3.** Subcellular distribution of AIFsh2 and expression pattern of AIFsh3. A, mitochondria from mouse liver or HeLa cells were purified in a Percoll® gradient as described under "Experimental Procedures" and mitochondrial fractionation quality was verified by Western blot analysis of the mitochondrial AIF protein, the lysosomal LAMP1 marker, and the ERK cytosolic protein (left panel). AIFsh2 was immunodetected in liver or HeLa mitochondrial-enriched fractions either with an anti-AIF N-terminal antibody or an anti-AIF C-terminal antibody. Note that only the anti-AIF N-terminal antibody recognizes AIFsh2. A total lysate from HeLa cells transiently transfected with the expression plasmids pcDNA3-empty vector or pcDNA3-AIFsh2 was used as a positive control (right panels). B, expression vectors encoding GFP, AIF-GFP, and AIFsh2-GFP were transfected in HeLa cells. Six hours after transfection, cells were stained with the mitochondrial specific dye Mitotracker Red (20 nM, 15 min). Mitotracker Red and GFP fusion proteins were determined by fluorescent microscopy. Note that, like AIF, AIFsh2 colocalizes with Mitotracker Red in mitochondria. C, HeLa cells were transiently transfected with expression plasmids pcDNA3-empty vector or pcDNA3-AIFsh3. AIF and AIFsh3 were immunodetected with an anti-AIF N-terminal antibody designed against an AIF peptide (amino acids 186–216). Note the absence of AIFsh3 in pcDNA3-empty vector-transfected cells. Right panels showed the expression pattern of AIFsh3 mRNA in a variety of cancer cell lines and human tissues. AIFsh3 expression was analyzed by RT-PCR with forward and reverse primers described under "Experimental Procedures." GAPDH was used as a loading control.
mAIFsh2 protein contained 324 amino acids (Fig. 2B). A similar approach failed to detect the AIFsh3 mouse ortholog. Altogether, our findings design a new map for AIF. Indeed, this gene includes a new exon, exon 9b, and codes for, at least, five different transcripts in human: AIF, AIF-exB, AIFsh, AIFsh2, and AIFsh3. In mice, the AIFsh3 mRNA is absent.

AIFsh2 Localizes in Mouse and Human Mitochondria—We next focused on the detection of the AIFsh2 and AIFsh3 mRNA resulting proteins. In this way, given that AIF is confined in mitochondria (1), we checked whether AIFsh2 and/or AIFsh3 were also localized in this organelle. Thus, we first separated mitochondria from other organelles and debris on a discontinuous density gradient. The use of specific antibodies against AIF, LAMP1, and ERK, three markers of the mitochondrial, lysosomal, or cytoplasmic compartments, confirmed the mitochondrial enrichment of our preparations (Fig. 3A, left panel). For AIFsh2 protein detection, we used two complementary approaches:

(i) An immunoblotting assessment performed with an N-terminal anti-AIF antibody in the mitochondrial-enriched fractions obtained from mouse liver or HeLa cells. This approach led us to recognize AIF and a second band of ~35 kDa (Fig. 3A). The molecular mass of this band was compatible with the theoretical molecular mass of AIFsh2, suggesting that AIFsh2 existed and was localized in mitochondria. In this way, immunoblotting of mitochondrial extracts using an antibody against a C-terminal epitope of AIF did not reveal the ~35-kDa band, indicating the specificity of AIFsh2 detection. Indeed, as the C-terminal domain of AIF was absent in AIFsh2, with this antibody we detected AIF but failed to detect AIFsh2 (Fig. 3A). The apparent molecular mass of mitochondrial AIFsh2 was consistent with the molecular mass of the main product resulting from AIFsh2 overexpression assays (Fig. 3A, right panels), further establishing the specificity of the mitochondrial AIFsh2 detection.

(ii) In a second immunofluorescent approach, we generated expression plasmids encoding AIF-GFP and AIFsh2-GFP fusion proteins. Upon transfection into HeLa cells AIF-GFP and AIFsh2-GFP distribution patterns were assessed. As depicted in Fig. 3B, in contrast to the cytosolic GFP protein, AIF-GFP and AIFsh2-GFP exhibited a filamentous pattern 6 h after transfection, which colocalized with the specific mitochondrial marker Mitotracker Red. These data corroborated that, like AIF, AIFsh2 localized on mitochondria. It is important to mention that the location of AIFsh2 in mitochondria is in line with the presence of the two MLS previously described for AIF in the AIFsh2 mRNA (Fig. 1A) (1).

Similar cellular subfractionation and immunoblotting approaches failed to identify the AIFsh3 protein in whole cell, cytosolic or mitochondrial extracts from human HeLa cells (data not shown). This negative result was also confirmed in AIFsh3 overexpression assays. Indeed, this approach helped to visualize a ~27-kDa protein in HeLa-transfected cells, which was absent in whole extracts from control cells (Fig. 3C). Thus, it seems that, in this cell line, the human AIFsh3 mRNA is absent or not translated.

To examine whether AIFsh3 mRNA is expressed in HeLa and other commonly used cancer cell lines, a specific RT-PCR approach was developed (Fig. 3C). In this assessment, the GAPDH mRNA amplification was used as a control. Primers sh3F1 and HCR1 were used to detect the AIFsh3 mRNA as a 920-bp product. We amplified AIFsh3 mRNA only in OV10 and 293T cell lines (Fig. 3C). A similar RT-PCR method was used to determine the AIFsh3 mRNA expression pattern in a panel of tissues. In this case, AIFsh3 mRNA was detected as a faint band only in kidney, brain, and colon (Fig. 3C). These results indicate that the AIFsh3 transcript displays different tissue expression both in normal and transformed cells. In HeLa cells, the AIFsh3 mRNA is absent.

Expression of AIFsh2 mRNA in Human Normal Tissues and Cancer Cells—The tissue distribution and function of AIFsh2 were analyzed further in detail. First, through a Northern blot assessment performed with a specific probe located on exon 9b, we sought to determine its expression pattern in tissues. This approach revealed a single ~2.5-kb
mRNA transcript in kidney, skeletal muscle, heart, liver, and colon. Surprisingly, this transcript seemed to be absent in brain (Fig. 4A). To further confirm AIFsh2 distribution, we complemented our Northern blot by a RT-PCR approach. We used total RNA from a panel of tissues (Fig. 4B) and I9F and R4 as primers (described under "Experimental Procedures"). These two primers are designed to simultaneously amplify the AIFsh and AIFsh2 transcripts (as 736- and 599-bp products, respectively), allowing us to compare the expression of these two mRNAs at the same time. GAPDH amplification was used as an internal control. We observed that AIFsh2 mRNA was expressed in all tested tissues except brain (Fig. 4B), thereby confirming the Northern blot result. Interestingly, a different expression pattern was detected between AIFsh and AIFsh2 (Fig. 4B). In fact, quantification of the RT-PCR result allowed us to calculate that, in thyroid, the expression level of AIFsh2 was about 4 times higher than that of AIFsh. A similar expression of the two AIF isoforms was measured in lung and, finally, a more abundant AIFsh transcript was detected in kidney, ovary, muscle, liver, heart, and colon tissues (Fig. 4B).

Transcription of AIFsh2 was also investigated, by a similar RT-PCR approach, in 12 human cancer cell lines (Fig. 4C). AIFsh2 transcript was amplified in all cancer cell lines tested with a lower expression in SH-SYSY, IMR5, U937, IMR32, and HeLa cells (Fig. 4C). In contrast, AIFsh2 was highly expressed (8 times more than AIFsh) in Ramos. Note that even if the AIFsh2 mRNA is absent from brain tissue (Fig. 4, A and B), it is detected in neuroblastoma-derived cell lines (e.g. SH-SYSY, IMR5, and IMR32) (Fig. 4C). Thus there seems to be a different regulation in normal and tumor tissues from the brain lineage. In any case, as it happens in normal tissues, AIFsh and AIFsh2 are differentially expressed in tumor cells.

Our above RT-PCR data strongly suggested that AIFsh2 and AIFsh expression are regulated separately. To confirm this possibility, we used an RNAi approach (Fig. 5A). Indeed, if AIFsh2 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. In this way, using the RNAi approach depicted in Fig. 5A, we fully confirmed this outcome (Fig. 5B). Interestingly, a similar result was found in RNAi assays designed to independently down-regulate AIF, AIFsh, or AIFsh2 (Fig. 5B). Thus, there seems to be three individual AIF, AIFsh, and AIFsh2 mRNAs, which could be autonomously regulated.

**AIFsh2 Redox Activities**—Under physiological conditions, AIF is a mitochondrial NADH oxidase that plays a role in oxidative phosphorylation (4–5). In fact, the oxidoreductase part of AIF, which is conserved in AIFsh2, confers an electron transfer activity to the protein (4–6). Thus, we generated the human AIFsh2 recombinant protein to investigate whether AIFsh2 presents the same NADH oxidase activity as AIF. AIFsh, which lacks the AIF oxidoreductase domain, was used as a negative control. AIF, AIFsh, and AIFsh2 recombinant proteins were synthesized in *E. coli* and the FAD moiety was reconstituted by external addition. As depicted in Fig. 6A, the absorption spectrum of reconstituted AIFsh2 showed the typical features of an oxidized flavoprotein, with visible maxima at 381 and 453 nm and a shoulder at 478 nm. These absorbance characteristics are similar to the spectrum obtained with AIF. In contrast, reconstituted AIFsh showed no visible maxima (Fig. 6A). Thus, AIF and AIFsh2 proteins associated FAD, whereas, in contrast, AIFsh did not incorporate this flavin. Consequently, AIF- and AIFsh2-reconstituted holoproteins showed NADH oxidase activity measured by in situ NBT detection (Fig. 6A, inset), whereas the reconstituted AIFsh lacked any detectable NADH oxidase-NBT reductase activity (Fig. 6A). This fact suggests that, like AIF, AIFsh2 exhibits an oxidoreductase activity.

We next investigated the characteristics of the AIFsh2 redox function and showed that AIFsh2 features NADH and NADPH oxidase activities (Fig. 6B). NAD(P)H oxidation in the presence of AIFsh2 was followed by measuring the initial rates of A340 nm. The apparent *K*$_{_{m}}$ for NADH was calculated as 102.6 ± 7 μM and the turnover number 2.7 min$^{-1}$. When NADPH was used as electron donor, the apparent *K*$_{_{m}}$ was 45.3 ± 9 and the turnover number 3.5 min$^{-1}$). These kinetic parameters are very similar to previous values described for AIF (4), indicating that AIFsh2 has
FIGURE 6. Redox activity of AIFsh2. A, absorption spectrum of FAD and reconstituted recombinant AIF, AIFsh, and AIFsh2. The inset shows the recovery of the NADH oxidase activity of recombinant proteins after FAD reconstitution measured by in situ NBT detection. B, NAD(P)H oxidation by AIFsh2. NADH (●) and NADPH (○) oxidation were measured following absorbance variation at 340 nm, after addition of different amounts of NAD(P)H. AIFsh2 was added at a concentration of 3 mM. C, AIFsh2 induced NBT reduction with NADH (●) and NADPH (○) as electron donors. AIFsh2 was added at a concentration of 100 nM. D, NADH (●) and NADPH (○) oxidase activities of AIFsh2 result in generation of superoxide anion. AIFsh2 concentration was 100 nM.
AIFsh2 Is a NADH Oxidase Located in Mitochondria

In Pro-apoptotic Conditions, AIFsh2 Is Released from Mitochondria to Cytosol in a Calpain/Cathepsin-dependent Manner—After a cellular insult, AIF is released from mitochondria and translocated to cytosol and nucleus, where it achieves a caspase-independent pro-apoptotic function (1, 12, 19, 28, 30, 31). It is well established that, to be released from mitochondria AIF is cleaved into tAIF by proteolytic processing at position Gly102-Leu103 of the mouse AIF amino acid sequence (9). This processing is accomplished by μ-calpain or cathepsins B, L, or S (8, 9).

AIFsh2 mimics the N-terminal sequence of AIF, which includes the calpain/cathepsin cleavage site. Thus, accepting the complexity of cell death regulation and execution (32), we could assume that AIFsh2 can be cleaved into tAIFsh2 in a μ-calpain-dependent or cathepsin-dependent fashion. To verify this possibility, we used two independent tools: (i) purified mitochondria. If AIF and AIFsh2 are similarly released from mitochondria, it should be possible to detect tAIF and tAIFsh2 in supernatants from mitochondria treated with Atractyloside, an agent highly effective in inducing mitochondrial AIF liberation (9); and (ii) AIFsh2 overexpression in HeLa cells. With this approach, we searched whether AIFsh2 was released from mitochondria to cytosol after classical pro-apoptotic insults, such as the tyrosine kinase inhibitor STS, the topoisomerase I inhibitor camptothecin (Ctp, 20 μM), thapsigargin (Thap, 20 μM), and subjected to subcellular fractionation. Cytoplasmic fractions were blotted for immunodetection of the flagged protein (right panel). Note the presence of tAIFsh2-FLAG in the cytosol of STS, etoposide, cathepsin, thapsigargin, TNF/CHX, or β-lapachone-treated cells. It is important to underline that each proapoptotic treatment produces the loss of 75% in cell viability measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (data not shown). Cytosolic fractionation quality and protein loading were verified by the distribution of the specific subcellular marker ERK. The mitochondrial COX IV protein was used to confirm the purity of the cytosolic fractions.

AIFsh2 treatment provokes both AIF cleavage into tAIF and mitochondrial release of the AIF soluble form. Like AIF, AIFsh2 is cleaved and released from mitochondria after Atractyloside treatment. Leupeptin, Z-FA-fmk, and calpeptin significantly alleviated AIF/AIFsh2 cleavage and release. To verify this possibility, we used two independent tools: (i) pretreated with the broad cysteine protease inhibitor leupeptin (Leup, 10 μM), the cathepsin inhibitor Z-FA-fmk (10 μM), or the calpain inhibitor calpeptin (Calpept, 10 μM) before addition of Atractyloside. After incubation, the mixture was centrifuged to separate mitochondrial pellet (P) or supernatants (SN) containing proteins released from mitochondria. Atractyloside treatment provokes both AIF cleavage into tAIF and mitochondrial release of the AIF soluble form. Like AIF, AIFsh2 is cleaved and released from mitochondria after Atractyloside treatment. Leupeptin, Z-FA-fmk, and calpeptin significantly alleviated AIF/AIFsh2 cleavage and release. B, STS, etoposide, camptothecin, thapsigargin, TNF/CHX, or β-lapachone proapoptotic treatment provokes AIFsh2 redistribution from mitochondria to cytosol. HeLa cells were transfected with p3xFLAG-AIFsh2. 16 h after transfection total cell lysates were subjected to a double immunoblot analysis performed with a N-terminal anti-AIF antibody or an anti-FLAG antibody. As shown in the left panels, the anti-FLAG antibody specifically recognizes AIFsh2-FLAG. In a similar set of experiments, 16 h after transfection HeLa cells were left untreated (Co.) or treated 8 h with STS (1 μM), etoposide (Etop, 50 μM), camptothecin (Ctp, 20 μM), thapsigargin (Thap, 20 μM), or β-lapachone (β-lap, 20 μM) and subjected to subcellular fractionation. Cytoplasmic fractions were blotted for immunodetection of the flagged protein (right panel). Note the presence of tAIFsh2-FLAG in the cytosol of STS, etoposide, cathepsin, thapsigargin, TNF/CHX, or β-lapachone-treated cells. It is important to underline that each proapoptotic treatment produces the loss of 75% in cell viability measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (data not shown). Cytosolic fractionation quality and protein loading were verified by the distribution of the specific subcellular marker ERK. The mitochondrial COX IV protein was used to confirm the purity of the cytosolic fractions.

In the right panel, purified mitochondria were left untreated (Mit.) or pretreated with 5 mM Atractyloside (A). After incubation, the mixture was centrifuged to separate mitochondrial pellet (P) or supernatants (SN) containing proteins released from mitochondria. Atractyloside treatment provokes both AIF cleavage into tAIF and mitochondrial release of the AIF soluble form. As shown in the left panels, the anti-FLAG antibody specifically recognizes AIFsh2-FLAG. In a similar set of experiments, 16 h after transfection HeLa cells were left untreated (Co.) or treated 8 h with STS (1 μM), etoposide (Etop, 50 μM), camptothecin (Ctp, 20 μM), thapsigargin (Thap, 20 μM), or β-lapachone (β-lap, 20 μM) and subjected to subcellular fractionation. Cytoplasmic fractions were blotted for immunodetection of the flagged protein (right panel). Note the presence of tAIFsh2-FLAG in the cytosol of STS, etoposide, cathepsin, thapsigargin, TNF/CHX, or β-lapachone-treated cells. It is important to underline that each proapoptotic treatment produces the loss of 75% in cell viability measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (data not shown). Cytosolic fractionation quality and protein loading were verified by the distribution of the specific subcellular marker ERK. The mitochondrial COX IV protein was used to confirm the purity of the cytosolic fractions.
AIFsh2 in cytosol purified from STS, etoposide, camptothecin, thapsigargin, TNF/CHX, or H9252-lapachone-treated HeLa cells, fully confirmed that, like AIF, AIFsh2 is released from the mitochondria to cytosol after an apoptotic insult (Fig. 7B).

Effect of AIFsh2 on Isolated Nuclei—We previously demonstrated that the C-terminal domain of AIF contains the information to induce nuclear apoptosis (24). However, is there an additional role of the oxidoreductase part of AIF, which is mimicked by AIFsh2, in nuclear cell death? Using a cell-free in vitro system in which the recombinant AIFsh2 protein was confronted to purified HeLa nuclei, we answered this question (30). AIF and AIFsh were used as positive controls. After a 90-min incubation, nuclear morphology was assessed by fluorescent and cytofluorometric approaches (33). As expected, recombinant AIF and AIFsh caused peripheral chromatin condensation and loss of DNA content. In contrast, recombinant AIFsh2 yielded negative results (Fig. 8, A–C). Addition of NADH or NADPH failed to disclose apoptogenic activity on AIFsh2. Moreover, AIFsh2 was unable to provoke large scale DNA fragmentation or the “ladder type” of chromatin digestion in purified HeLa nuclei (data not shown), confirming that AIFsh2 was not able to reproduce the nuclear features characterizing AIF/AIFsh action on nuclei (1, 24). Altogether these results indicate that the reactive oxygen species produced by AIF/AIFsh2 do not induce nuclear apoptosis.

DISCUSSION

The identification of the new exon 9b illustrates a continuously increasing complexity of the human AIF gene. There are now five variants described for AIF: AIF, AIF-exB, AIFsh, AIFsh2, and AIFsh3. The first two isoforms differ in the alternative use of exon 2 or exon 2b (25) and are targeted to the mitochondrial intermembrane space. We recently described a third variant of AIF, AIFsh, which results from an alternate transcriptional start site located at intron 9 of AIF (24). In the present study we identified exon 9b, an additional exon located between exons 9 and 10 of AIF. This novel exon leads to two new AIF cDNA species: AIFsh2 and AIFsh3. We failed to detect the AIFsh3 protein in
human HeLa cells. However, AIFsh2 mRNA transcript yields a novel mitochondrial form of AIF composed by 324 amino acids, which include the two MLS and the pyridine nucleotide-disulfide oxidoreductase (Pyr-redox) domain of AIF. In this context, AIFsh2 is able to incorporate FAD and exhibit NADH oxidase activity. In fact, like AIF, AIFsh2 reacts rapidly to O₂⁻, forming O₂•-. AIFsh2 Is a NADH Oxidase Located in Mitochondria

AIFsh2 differs in a main feature: AIFsh2 lacks the C-terminal domain of AIF and has no apoptogenic activity on purified nuclei. In contrast, AIFsh lacks the N-terminal domain of AIF and has no oxidoreductase activity (Figs. 1A and 6A and Ref. 24). These complementary results confirm that AIF is a bifunctional protein with dissociable redox and apoptogenic moieties. Indeed, contrary to other bifunctional proapoptotic proteins (e.g. cytochrome c) (34, 35), AIF yields an independent genetic regulation of its two different functions. This is an important issue to understand the physiological role of AIF.

Although AIFsh2 mitochondrial function remains unknown, the presence of the Pyr-redox domain and oxidoreductase activity suggests that, like other Pyr-redox proteins (36, 37), AIFsh2 may be involved in a variety of redox-dependent processes such as antioxidant defense and regulation of the cellular redox state. Moreover, because AIFsh2 shares similar redox functions to AIF, we may speculate that these two proteins have similar functions in mitochondria. In this sense, the different expression levels of AIF and AIFsh2 tested in a variety of tissues and cancer cell lines indicated that the two variants are differentially regulated. This was confirmed by an RNAi approach. It is important to remark that our RNAi approach provides new instruments to evaluate the specific role of AIF, AIFsh, and AIFsh2 in cell death and in oxidative phosphorylation. Indeed, from now on it should be possible to avoid generation of AIF and AIFsh, two caspase-independent death effectors. Moreover, it would be possible to obtain, for the first time, a cellular system in which AIF, AIFsh, and AIFsh2 isoforms are down-regulated. In any case, it is possible that AIFsh2 substitutes the redox function of AIF in some particular conditions (e.g. normal versus tumor cells). The different AIFsh2 expression observed in brain normal/tumor tissue suggests this possibility.

AIF redox activity was described as having a protective function in some cell types on the Harlequin mutant mouse, which has an 80% reduction in AIF. This mutant mouse appeared normal except for premature neurodegeneration and increased peroxide sensitivity in specific subsets of neurons in adult animals (38). The precise mechanism of action of AIF in free radical scavenging and counteracting oxidative stress in this mouse model remains to be established. Therefore, a particular study of AIFsh2 in these animals could greatly help to understand AIF/AIFsh2 function. More recently, it has also been shown that a redox-active domain of AIF and reduced glutathione are required for the inhibition of cytoplasmic stress granule formation under conditions of chemical stress, suggesting that AIF is involved in an adaptive response (39). Moreover, AIF deficiency compromises oxidative phosphorylation by inhibiting respiratory chain complex I in vitro and in vivo, revealing a "life" function for AIF (5, 6). Finally, AIF maintains the transformed state of colon cancer cells through its NADH oxidase activity, by mechanisms that involve complex I function (22). Unfortunately, the mechanistic relations between the redox activity of AIF, complex I, oxidative phosphorylation, and cell survival remain unclear. Moreover, in these systems, the genetic suppression of the AIF transcript will lead to AIFsh2 ablation. Thus, it is difficult to evaluate whether, like AIF, AIFsh2 may play a role in electron transfer and have a function in the defense against oxidative damage.

We demonstrated here that the cysteine protease mechanism implicated in mitochondrial AIF cleavage and release provokes a similar outcome in AIFsh2. Thus, it is possible that, in apoptotic conditions, deficiencies in oxidative phosphorylation may not only be related to AIF mitochondrial loss. The AIFsh2 ectopic presence could contribute to the defects in oxidative phosphorylation detected in apoptotic cells. Obviously, a mutant mouse specifically suppressing AIF or AIFsh2 could greatly help in the understanding of the mitochondrial function of these two proteins.

In summary, and despite the functional similarities between AIFsh2 and AIF (e.g. electron transfer activity), AIFsh2 has three different features. First, AIFsh2 mRNA is not expressed in brain. Second, AIFsh2 lacks the C-terminal domain of AIF. Third, AIFsh2 lacks the proapoptotic activity of AIF. These interesting features definitely confirm that the C-terminal domain of AIF is necessary and sufficient to induce AIF-dependent nuclear apoptosis. Indeed, by using the recombinant protein AIFsh2, we have demonstrated that the first 323 amino acids of the N-terminal domain in AIF are not required for its nuclear proapoptotic activity. Thus, AIF does not induce chromatin condensation and 50-kb DNA fragmentation through the production of the O₂•- reactive oxygen radical associated with this part of the protein.

In conclusion, our present study completes the knowledge of AIF. The identification and characterization of a novel AIF variant, lacking its apoptotic domain, provides a "natural mutant" that would greatly help in the understanding of the functional role of AIF in mitochondria and, by extension, in the understanding of the bifunctional role of AIF in life and death.

Acknowledgments—We thank Martine Cohen-Salmon and Marcela Segade for critical comments and review of the manuscript and Sophie Laine for p3xFLAG-AIFsh2 plasmid construction.

REFERENCES
1. Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Snow, B. E., Brothers, G. M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D. R., Aebersold, R., Siderovski, D. P., Penninger, J. M., and Kroemer, G. (1999) Nature 397, 441–446
2. Lorenzo, H. K., Susin, S. A., Penninger, J., and Kroemer, G. (1999) Cell Death Differ. 6, 516–524
3. Lorenzo, H. K., and Susin, S. A. (2004) FEBS Lett. 557, 14–20
4. Miramar, M. D., Costantini, P., Ravagnan, L., Saravia, L. M., Haouzi, D., Brothers, G., Penninger, J. M., Peleato, M. L., Kroemer, G., and Susin, S. A. (2001) J. Biol. Chem. 276, 16391–16398
5. Vales, N., Canoe, C., Briere, J. J., Benit, P., Joza, N., Larochette, N., Masterbroersdoro, P. G., Pequignot, M. O., Casares, N., Lazar, V., Feraud, O., Debili, N., Wissing, S., Engelhardt, S., Madero, F., Pacientini, P., Penninger, J. M., Schagger, H., Rustin, P., and Kroemer, G. (2004) EMBO J. 23, 4679–4689
6. Joza, N., Oudit, G. Y., Brown, D., Benit, P., Kassiri, Z., Vahsen, N., Benoit, L., Patel, M. M., Nowikovsky, K., Vassault, A., Backx, P. H., Wada, T., Kroemer, G., Rustin, P., and Penninger, J. M. (2005) Mol. Cell. Biol. 25, 10261–10272
7. Joza, N., Susin, S. A., Dasugas, E., Stanford, W. L., Cho, S. K., Li, C. Y., Sasaki, T., Elia, A. J., Cheng, H. Y., Ravagnan, L., Ferri, K. F., Zamzami, N., Wakeham, A., Hukrem, R., Yoshida, H., Kong, Y. Y., Mak, T. W., Zuniga-Pflucker, J. C., Kroemer, G., and Penninger, J. M. (2001) Nature 410, 549–554
8. Polster, B. M., Basanez, G., Etexbarria, A., Hardwick, J. M., and Nicholls, D. G. (2005) J. Biol. Chem. 280, 6447–6454
9. Yuste, V., Moubarak, R. S., Deleit, C., Bras, M., Sancho, P., Robert, N., d’Aleyzer, J., and Susin, S. A. (2005) Cell Death Differ. 12, 1445–1448
10. Yuste, V. J., Sanchez-Lopez, I., Sole, C., Moubarak, R. S., Bayascas, J. R., Dolcet, X., Encinas, M., Susin, S. A., and Comella, J. X. (2005) J. Biol. Chem. 280, 35670–35683
11. Kang, Y. H., Yi, M. J., Kim, M. J., Park, M. T., Bae, S., Kang, C. M., Cho, C. K., Park, I. C., Park, M. J., Rhee, C. H., Hong, S. I., Chung, H. Y., Lee, Y. S., and Lee, S. J. (2004) Cancer Res. 64, 8960–8967
12. Cregan, S. P., Fortin, A., MacLaurin, I. G., Callaghan, S. M., Ciecconii, F., Yu, S. W., Dawson, T. M., Dawson, V. L., Park, D. S., Kroemer, G., and Slack, R. S. (2002) J. Biol. Chem. 277, 507–517
13. Corbierre, C., Liagre, B., Terro, F., and Beneytout, J. L. (2004) Cell Res. 14, 188–196
14. Park, Y. C., Jeong, J. H., Park, K. J., Choi, H. J., Park, Y. M., Jeong, B. K., Higuchi, Y., and Yoo, Y. H. (2005) Life Sci. 77, 2059–2070
AIFsh2 Is a NADH Oxidase Located in Mitochondria

15. Nomura, M., Nomura, N., Newcomb, E. W., Lukyanov, Y., Tamasdan, C., and Zagzag, D. (2004) J. Cell. Physiol. 201, 374–384
16. Liu, T., Brouha, B., and Grossman, D. (2004) Oncogene 23, 39–48
17. Furre, I. E., Shahzidi, S., Luksiene, Z., Moller, M. T., Borgen, E., Morgan, J., Tamasdan, C., and Zagzag, D. (2004) J. Cell. Physiol. 201, 374–384
18. Yu, S. W., Wang, H., Potiras, M. F., Coombs, C., Bowers, W. J., Federoff, H. J., Poirier, G. G., Dawson, T. M., and Dawson, V. L. (2002) Science 297, 259–263
19. Wang, H., Yu, S. W., Koh, D. W., Le, J., Coombs, C., Bowers, W., Federoff, H. J., Poirier, G. G., Dawson, T. M., and Dawson, V. L. (2004) J. Neurosci. 24, 10963–10973
20. Gallego, M. A., Joseph, B., Hemstrom, T. H., Tamiji, S., Mortier, L., Kroemer, G., Formstecher, P., Zhivotovsky, B., and Marchetti, P. (2004) Oncogene 23, 6282–6291
21. Schmitt, E., Parcellier, A., Gurbuxani, S., Cande, C., Hammann, A., Morales, M. C., Hunt, C. R., Diz, D. J., Kroemer, R. T., Giordanetto, F., Jazdzela, M., Penninger, J. M., Pance, A., Kroemer, G., and Garrido, C. (2003) Cancer Res. 63, 8233–8240
22. Urbano, A., Lakshmiman, U., Choo, P. H., Kwan, J. C., Ng, P. Y., Guo, K., Dhakshina, S., and Porter, A. (2005) EMBO J. 24, 2815–2826
23. Daugas, E., Nochy, D., Ravagnan, L., Loeffler, M., Susin, S. A., Zamzami, N., and Kroemer, G. (2000) FEBS Lett. 476, 118–123
24. Delettre, C., Yuste, V. J., Moubarak, R. S., Brao, M., Lesbordes-Brion, J. C., Petres, S., Bellalou, J., and Susin, S. A. (2006) J. Biol. Chem. 281, 6413–6427
25. Loeffler, M., Daugas, E., Susin, S. A., Zamzami, N., Metivier, D., Nieminen, A. L., Brothers, G., Penninger, J. M., and Kroemer, G. (2001) FASEB J. 15, 758–767
26. Wissing, S., Ludovico, P., Herker, E., Buttner, S., Engelhardt, S. M., Decker, T., Link, A., Prokch, A., Rodrigues, F., Corte-Real, M., Freibich, K. U., Manns, J., Cande, C., Sigrist, S. J., Kroemer, G., and Madeo, F. (2004) J. Cell. Biol. 166, 969–974
27. Lopez-Huertas, E., Corpas, F. J., Sandalio, L. M., and Del Rio, L. A. (1999) Biochem. J. 337, 531–536
28. Susin, S. A., Zamzami, N., Castedo, M., Hirsch, T., Marchetti, P., Macho, A., Daugas, E., Geuskens, M., and Kroemer, G. (1996) J. Exp. Med. 184, 1331–1341
29. Breathnach, R., and Chambon, P. (1981) Annu. Rev. Biochem. 50, 349–383
30. Susin, S. A., Zamzami, N., Castedo, M., Daugas, E., Wang, H. G., Geley, S., Fass, F., Reed, J. C., and Kroemer, G. (1997) J. Exp. Med. 186, 25–37
31. Susin, S. A., Daugas, E., Ravagnan, L., Samejima, K., Zamzami, N., Loeffler, M., Costantini, P., Ferri, K., Farnoudou, T., Prevost, M. C., Brothers, G., Mak, T. W., Penninger, J., Earnshaw, W. C., and Kroemer, G. (2000) J. Exp. Med. 192, 571–580
32. Bras, M., Queeman, B., and Susin, S. A. (2005) Biochemistry (Mosc.) 70, 231–239
33. Susin, S. A., Zamzami, N., Larochette, N., Dellaporta, B., Marzo, I., Brenner, C., Hirsch, T., Petit, P. X., Geuskens, M., and Kroemer, G. (1997) Exp. Cell Res. 236, 397–403
34. Skulachev, V. P. (1998) FEBS Lett. 423, 275–280
35. Abdullaev, Z., Bodrova, M. E., Chernyak, B. V., Dolgikh, D. A., Kuk, R. M., Pereverzev, M. O., Arseniev, A. S., Efremov, R. G., Kneipchikin, M. P., Mokhova, E. N., Nemeyer, D. D., Roder, H., and Skulachev, V. P. (2002) Biochem. J. 362, 749–754
36. Halliwell, B. (1999) Free Radic. Res. 31, 261–272
37. Sies, H. (1999) Free Radic. Biol. Med. 27, 916–921
38. Klein, J. A., Longo-Guess, C. M., Rossman, M. P., Seburn, L. H., Hurd, R. E., Frankel, W. N., Bronson, R. T., and Ackerman, S. L. (2002) Nature 419, 367–374
39. Cande, C., Vahsen, N., Metivier, D., Tourriere, H., Chebli, K., Garrido, C., Tazi, J., and Kroemer, G. (2004) J. Cell Sci. 117, 4461–4468