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COMPREHENSIVE INVITED REVIEW

Apoptosis-Inducing Factor: Structure, Function, and Redox Regulation

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Abstract

Apoptosis-inducing factor (AIF) is a flavin adenine dinucleotide-containing, NADH-dependent oxidoreductase residing in the mitochondrial intermembrane space whose specific enzymatic activity remains unknown. Upon an apoptotic insult, AIF undergoes proteolysis and translocates to the nucleus, where it triggers chromatin condensation and large-scale DNA degradation in a caspase-independent manner. Besides playing a key role in execution of caspase-independent cell death, AIF has emerged as a protein critical for cell survival. Analysis of *in vivo* phenotypes associated with AIF deficiency and defects, and identification of its mitochondrial, cytoplasmic, and nuclear partners revealed the complexity and multilevel regulation of AIF-mediated signal transduction and suggested an important role of AIF in the maintenance of mitochondrial morphology and energy metabolism. The redox activity of AIF is essential for optimal oxidative phosphorylation. Additionally, the protein is proposed to regulate the respiratory chain indirectly, through assembly and/or stabilization of complexes I and III. This review discusses accumulated data with respect to the AIF structure and outlines evidence that supports the prevalent mechanistic view on the apoptogenic actions of the flavoprotein, as well as the emerging concept of AIF as a redox sensor capable of linking NAD(H)-dependent metabolic pathways to apoptosis. *Antioxid. Redox Signal.* 14, 2545–2579.

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I. Introduction

Apoptosis is a highly regulated and energy-dependent programmed cell death (PCD) essential for early embryonic development and tissue homeostasis. Dysregulation of apoptosis may lead to various acute and chronic pathologies such as stroke, cancer, neurodegeneration, and autoimmune syndromes (217). In mammalian cells, several pro-apoptotic and cell damage pathways, which either do or do not require caspase activation, converge on mitochondria. Permeabilization of the organelle leads to release of several proteins from the intermembrane space (IMS) that participate in the organized cell demise [reviewed in (184)]. One of the soluble factors released from mouse mitochondria and capable of forcing isolated nuclei to adopt apoptotic morphology in a caspase-independent manner was discovered by Kroemer and coworkers in 1996 (214, 248). The protein was named apoptosis-inducing factor (AIF), as it could maintain the apoptogenic ability in the presence of a pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (z-vad.fmk) (212). This group demonstrated also that AIF binds favin adenine dinucleotide (FAD) and possesses various NAD(P)H-dependent redox activities (155, 212), but its specific enzymatic function is presently unknown.

In addition to the unraveling of a caspase-independent pathway of PCD, major advances in AIF research include (a) discovery of several naturally occurring and functionally distinct forms and splice variants of AIF; (b) identification of homologous proteins in lower eukaryotes, prokaryotes, and archaea and demonstration of evolutionary conservation of

the AIF-dependent cell death pathways in uni- and multicellular organisms; (c) determination of the crystal structures of different redox forms of AIF and gaining insights into its unique architecture and redox-linked conformational reorganization; (d) exploitation of natural and creation of *de novo* genetic models to pinpoint effects caused by AIF knockout, deficiency or defects; and (e) identification of a diverse group of proteins interacting with AIF in different cellular compartments that showed the complexity and multilevel regulation of the AIF-mediated signal transduction.

Numerous reviews have appeared in recent years, covering various aspects of mitochondrial and cellular physiology related to AIF (45, 86, 104, 157, 179). This article includes most recent findings on AIF and discusses the experimental data from a structural viewpoint, emphasizing the importance of folding for the structural and functional integrity of the flavoprotein. A brief overview of multiple forms, transcriptional regulation, and phylogenetic roots of AIF is followed by a detailed description of its catalytic and structural properties. Since AIF was originally discovered as an apoptosis-inducing factor and its role in PCD has been investigated more extensively and, overall, is better understood than the function in normal mitochondria, it will be discussed first. In addition to *in vitro* and *in vivo* analyses of the AIF-mediated apoptotic cell death, current views on the mechanism of AIF liberation, translocation to the nucleus and interaction with pro-life and pro-death cytoplasmic and nuclear partners are presented. Apoptogenic potential of AIF homologs from lower eukaryotes is also discussed as it helps to better understand structure/function relations and cellular roles of the flavoprotein. The following sections highlight vital functions of AIF, especially its involvement in regulation of mitochondrial respiration and cristae morphology. A hypothesis on the redox-signaling role of AIF and supporting evidence are outlined at the end of the review. Since most investigations were performed on human and murine AIF, which vary in length, the amino acid sequence numbering will be given for human AIF, with the corresponding fragments/residues for the murine protein specified when necessary.

II. Multiple Forms of AIF

A. AIF precursor

The *AIF* gene, also known as *AIFM1* and *PCDC8*, was mapped to chromosome X region A6 in mice and Xq25–26 region in humans (48). Transcription and translation of the nuclear-encoded gene gives rise to a 67 kDa precursor molecule, residues 1–613 (1–612 in mice), containing the N-terminal mitochondrial leading sequence (MLS), two nuclear leading sequences (NLS), and the NAD- and FAD-binding motifs (Fig. 1A, B) (212). The precursor can be imported to mitochondria only in a non-native form, meaning that its folding in the cytoplasm is either prevented or delayed. When refolded, full-length AIF becomes apoptogenic independently on whether FAD is bound or not (212).

B. Membrane-tethered mature AIFΔ1–54

For quite some time, the soluble 57 kDa fragment of AIF consisting of residues 103–613 (102–612 in mice; Fig. 1D) was thought to represent the mature form residing in the IMS (212). Several lines of evidence indicated, however, that mitochondrial AIF is associated with the inner membrane. In particular, it was demonstrated that (a) in response to several apoptotic stimuli, release of AIF from the IMS occurs downstream of cytochrome *c* and requires caspase activation (10); (b) during hypotonic lysis of mitochondria, AIF remains associated with the mitoplast pellet and is released only after partial solubilization of the inner membrane (222); and (c) truncated Bid (tBid), an outer membrane-permeabilizing agent, induces AIF release from isolated mitochondria only in the presence of active calpain, a calcium-dependent protease (178). Altogether, these studies suggested that AIF is somehow attached to the

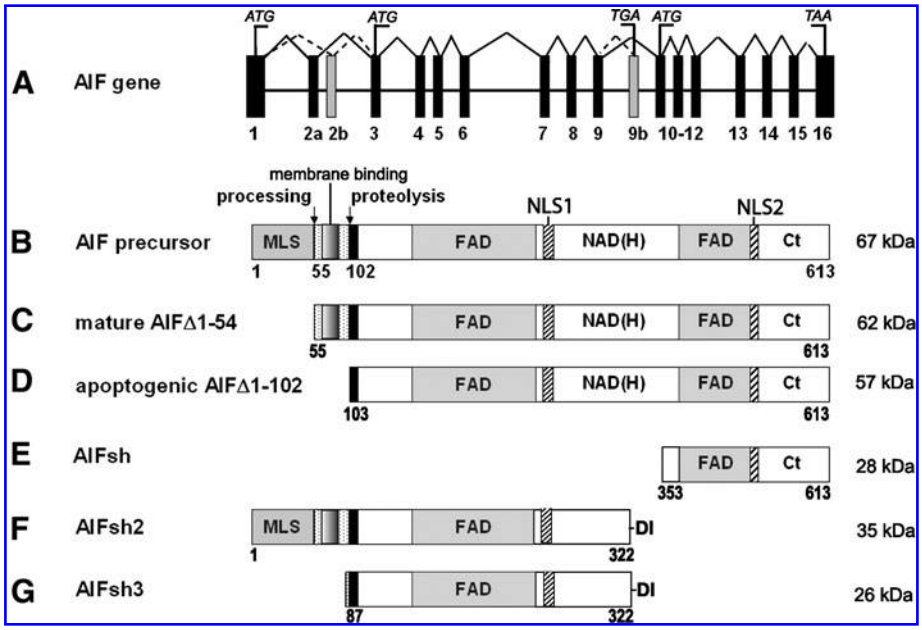

FIG. 1. Major forms and splice variants of AIF. (A) Schematic representation of the human *AIF* gene. Exons are numbered; alternative exons giving splice variants are in gray. Translation initiation (ATG) and stop codons (TGA/TAA) are indicated. **(B, E–G)** Naturally occurring transcripts corresponding to the AIF precursor, AIFsh, AIFsh2, and AIFsh3, respectively. **(C)** Mature form of AIF produced upon mitochondrial processing. Depending on the usage of exons 2a and 2b, the ubiquitously expressed AIF1 or brain-specific AIF2 isoforms can be synthesized. **(D)** Truncated apoptogenic form produced in the intermembrane space upon proteolytic processing. The FAD-binding, NAD(H)-binding, and C-terminal (Ct) domains are indicated. AIF, apoptosis-inducing factor; MLS, mitochondrial leading sequence; NLS, nuclear leading sequence.

inner membrane and may need to be cleaved to become a soluble and apoptogenic protein.

A major breakthrough was made by Otera *et al*. (168), who demonstrated that the human AIF precursor is cleaved by a mitochondrial matrix peptidase at Met54/Ala55, resulting in a mature form that is 48-residue longer (~62 kDa; Δ1–54) than originally reported (212). Residues 67–85 were identified as the IMS sorting signal and a *trans*-membrane fragment through which AIFΔ1–54 (Δ1–53 in mice), the healthy and vital form of the flavoprotein, is tethered to the inner membrane (168). However, Yu *et al.* argue against the proposed *trans*-membrane topology and suggest that AIF represents a peripheral rather than integral inner membrane protein because it can be stripped from the membranes by alkaline treatment (245). Concordantly, both endogenous and transiently overexpressed AIF1 (the ubiquitous isoform) were reported to behave as loosely membrane-associated proteins (87).

There is evidence that transport of AIF from the mitochondrial matrix into the IMS proceeds through the inner membrane channel Tim23 protein (89). Since AIF isolated from mitochondria contains FAD and the precursor does not (212), proteolytic maturation and import into the IMS seem to be required for the protein folding and flavin incorporation.

C. Soluble apoptogenic AIFΔ1–102/118

The 96–120 segment of AIFΔ1–54 has two proteolytic sites, cleavage of which can lead to formation of soluble Δ1–102 or Δ1–118 fragments (murine AIF numbering, ~57 kDa; Fig. 1D) (38, 168, 178). Proteolysis of the membrane-bound mature AIF can be mediated by mitochondrial or cytoplasmic proteases, as discussed in detail in section VII.B.1. Upon permeabilization of the outer mitochondrial membrane (OMM), the detached Δ1–102/118 fragments can translocate from the IMS to the nucleus to execute apoptosis and, therefore, represent the lethal form of the flavoprotein.

D. AIF associated with the outer mitochondrial membrane

This pool of the protein has been recently identified by Yu *et al.*, who showed that in brain mitochondria nearly 30% of total endogenous mitochondrial AIF is loosely associated with the cytoplasmic side of the OMM (245). This OMM-associated fraction, presumably an unfolded AIF precursor, can also be lethal because under certain conditions it can rapidly relocate to the nucleus and initiate PCD (see section VII.B.3 for details).

E. Splice variants AIF2, AIFsh, AIFsh2, and AIFsh3

An alternatively spliced AIF-exB (AIF2) isoform was the first splice variant discovered in mice and humans (136). AIF2 utilizes exon 2b instead of 2a (Fig. 1A) and differs from AIF1, the originally described ubiquitous form (212), only in a short N-terminal amino acid stretch that gets cleaved during apoptogenic processing. Exons 2a and 2b are phylogenetically conserved among mammals and their usage has no effect on the mitochondrial transport of AIF. AIF2 is a brain-specific isoform whose expression depends on the neuronal cell maturation status (87).

The AIFshort (AIFsh) isoform results from an alternative transcriptional start site located at intron 9 and consists of aa. 353–613 (35 kDa; Fig. 1E) (53). AIFsh lacks MLS and represents a cytoplasmic protein that upon an apoptotic insult can transport to the nucleus and promote cell death. AIFsh2 and AIFsh3, in turn, are produced *via* alternative usage of exon 9b. They are comprised of residues 1–322 and 87–322, respectively, and have additional Asp-Ile at the C-terminus (Fig. 1F, G) (54). These splice variants lack the C-terminal domain and NLS2 and cannot translocate to the nucleus. AIFsh3 is also missing the N-terminal MLS and confined exclusively to the cytoplasm. Mice have an AIFsh2 homolog but no AIFsh3. Owing to existence of multiple isoforms, the AIF-mediated function and signaling can be regulated at both the transcriptional and post-translational levels.

III. Transcriptional Regulation

A single 2.4 Kb *AIF* mRNA transcript and ubiquitous expression of *AIF1* have been detected in murine and human organs (48, 53, 212). On the contrary, *AIF2* mRNA is absent in all human tissues except brain and retina (87). Although in most areas of the human adult brain *AIF1* and *AIF2* are co-expressed, there are brain cells that solely express *AIF2* (*e.g.*, part of the anterior olfactory nucleus). The *AIF1* and *AIF2* mRNA levels are similar in the fetal brain but the *AIF1*/*AIF2* ratio decreases as brain cells differentiate (87). Expression of *AIF1* in human skeletal muscle is also age-dependent and increases by 10%–25% from the age of 10–40 (172). *AIFsh* is expressed at lower levels and regulated independently from *AIF1* (53), whereas expression of *AIFsh2* is tissue specific and, on average, lower than *AIF1* (54).

Transcriptional factors regulating AIF expression remain largely unknown. It was found though that the *AIF* gene is a transcriptional target of p53 (209). Using isogenic cell lines differing in the p53 status, Stambolsky *et al*. demonstrated that the absence of functional p53 significantly reduces *AIF* mRNA and protein levels. They also identified a p53-responsive element in the fourth intron of the *AIF* gene. Positive modulation of *AIF* expression by basal levels of p53 is cell type-specific and does not depend on DNA-damaging stress.

Conversely, downstream effectors of hepatocyte growth factor, hepatocyte growth factor receptor, and focal adhesion kinase suppress *AIF* expression (34). Bcl-2 19 kDa interacting protein, a pro-cell-death BH3-only member of the Bcl-2 family, is another transcriptional repressor that binds to a response element in the *AIF* promoter and downregulates expression (26). In highly malignant human gliomas, Bcl-2 19 kDa interacting protein 3 reduces *AIF* expression through interactions with PTB (polypyrimidine tract binding protein)-associating splicing factor and histone deacetylase 1 (26) and thus is acting as a pro-survival factor. *AIF* expression is decreased in many other tumor types, contributing to their chemoresistance (54, 96), but is upregulated in malignant colorectal epithelial cells (99).

IV. Phylogenetic Roots

Determination of multiple prokaryotic and eukaryotic genome sequences enabled analysis of phylogenetic origins of AIF and predictions on its function. Phylogenetic analysis reveals strong conservation among mammalian AIFs (~90% identity in the whole sequence) and ubiquitous presence of the

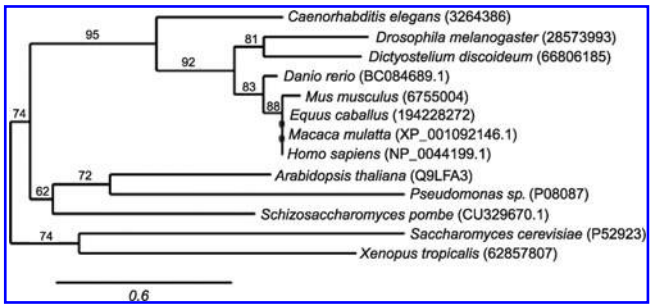

FIG. 2. Phylogenetic tree of AIF-like proteins. Analysis of the phylogenetic relationships between the full-length molecular sequences was performed using the Phylogeny.fr online server (www.phylogeny.fr), which utilizes MUSCLE for sequence alignment and PhyML for phylogeny (55). GenBank or UniProt identification numbers are indicated.

AIF-like proteins in bacteria, archaea, and eukaryotes (27, 137, 124). Clustering of eukaryotic AIFs with the archaeal orthologs suggests an existence of a universal common ancestor that was secondarily recruited for a specific mitochondrial function. Homologs from zebrafish (*Danio rerio*), fruit fly (*Drosophila melanogaster*), and slime mold (*Dictyostelium discoideum*) are closely related to mammalian AIFs (Figs. 2 and 3), whereas proteins from *Caenorhabditis elegans* and *Saccharomyces cerevisiae*, widely used as model organisms in cell biology research, are more distant on the evolutionary tree.

Most of the AIF-like proteins contain the GXGXXA and V/IXGXGXXG motifs, characteristic for the NAD(P)- and FAD-binding domains adopting the Rossmann fold (119). The N-termini of AIF from mammals and lower eukaryotes contain MLS and a stretch of hydrophobic amino acids serving as a membrane-binding fragment (Fig. 3). The plant, yeast, and bacterial homologs have a shortened N-terminus and lack both MLS and a membrane tether. The *C. elegans* protein has the longest N-terminal part that in addition to MLS contains an extended and highly charged linker region. The most important difference, however, is the presence of two insertions in mammalian proteins (191–203 and 510–560), which, as will be emphasized throughout the review, may define specific functions and signal transduction cascades. On the basis of the presence of the FAD- and NAD(H)-binding motifs and strong sequence homology to plant and bacterial ascorbate and ferredoxin reductases, AIF was suggested to possess oxidoreductase activities (212). This prediction was later confirmed *in vitro* using recombinantly expressed proteins (38, 155).

V. Redox Properties of Recombinant AIF

A. Refolded murine AIFΔ1–120

Recombinant murine AIFΔ1–120 was the first to be biochemically characterized. Cloned with the affinity tags fused to both termini, AIFΔ1–120 was expressed in *E. coli* as an apoprotein and had to be refolded to incorporate the flavin (155). FAD-bound AIFΔ1–120 can be fully reduced with sodium dithionite or NAD(P)H without accumulation of the semiquinone intermediate. However, addition of stoichiometric amounts of NAD(P)H to refolded AIFΔ1–120 is not accompanied by formation of an $FADH_2$-NAD(P) charge–transfer complex (CTC), normally produced by other flavoenzymes as a result of π-π charge–transfer interactions between the parallel-stacked isoalloxazine and nicotinamide. Nonetheless, refolded AIFΔ1–120 can oxidize NAD(P)H (20–2244 min^{-1}) and catalyze NADH-dependent reduction of small electron acceptors and cytochrome *c* directly or *via* superoxide anion, with a turnover number ranging from 0.5 to 22 min^{-1} (155). On the basis of these properties and the reductase activity of the native mitochondrial protein, AIF was proposed to function *in vivo* as a superoxide producing NADH oxidase (155).

B. Refolded human AIFsh2

Refolded human AIFsh2 is redox active and catalyses NAD(P)H oxidation and $O_2^{-\bullet}$-mediated reduction of small electron accepting molecules similar to refolded murine AIFΔ1–120 (54).

C. Naturally folded murine AIFΔ1–53 and Δ1–101

Our group showed that recombinantly expressed AIF can naturally fold and incorporate FAD if no affinity or immunetags are placed at the N-terminus, close to the flavin binding site (38). When expressed in this way, murine AIFΔ1–53 and Δ1–101 have properties distinct from those of refolded AIFΔ1–120. In particular, they have a considerably lower redox potential [−355/ −349 mV *vs.* − 308 mV at pH 7.5 (155)] and higher preference for NADH over NADPH. Both proteins react slowly with either cofactor and upon reduction form tight and long-lived $FADH_2$-NAD(P) CTC (38). Due to resistance of CTC to oxidation, the NADH oxidase activity of AIF is negligible (<0.2 min^{-1}). Nevertheless, both AIF forms directly shuttle electrons from NADH to one- and two-electron acceptors (30–60 min^{-1}) but do not reduce $O_2^{-\bullet}$, H_2O_2, ascorbate free radical, or dehydroascorbate and, hence, cannot function as reactive oxygen species (ROS) scavengers. On the basis of the kinetic parameters, such as high K_M (1–2 mM) and low K_d for NADH (150 nM), mitochondrial AIF was concluded to function as a low turnover oxidoreductase (38). Other important findings on the naturally folded AIF include its redox-linked dimerization accompanied by a conformational switch in the 509–559 insertion, and involvement of the N-terminal peptide in stabilization of the AIF-NAD(P)H complex (38).

In summary, *in vitro* studies showed that the manner of folding and FAD incorporation defines redox properties of AIF. Naturally folded AIF functions as a low-turnover NADH-dependent oxidoreductase whose reduced form is dimeric and resistant to oxidation by O_2. Although the specific enzymatic activity could not be identified, the biochemical data ruled out the possibility that AIF is an antioxidant.

VI. AIF Structure

A. X-ray structures of murine and human AIFΔ1–120

The x-ray structure of the oxidized form of refolded murine AIFΔ1–121 (150) provided first insights into the protein architecture and active site environment, defined surface properties of AIF, and enabled suggestions on how it may function in the cell. AIF has a glutathione reductase (GR) fold with the closest structural homology to BphA4, a ferredoxin reductase from *Pseudomonas* sp. strain KKS102 (200). The protein has three functional modules: the FAD- (aa. 129–170,

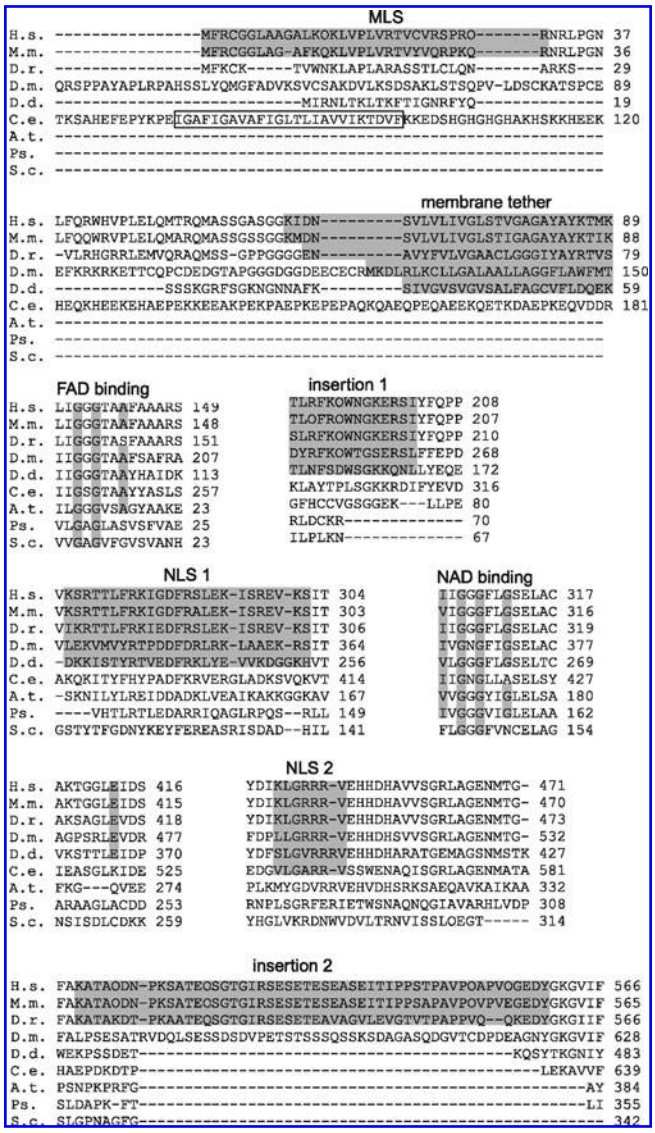

FIG. 3. Amino acid sequence alignment of AIF and AIF-like proteins from *Homo sapiens* (H. s.), *Mus musculus* (M. m.), *Danio rerio* (D. r.), *Drosophila melanogaster* (D. m.), *Dictyostelium discoideum* (D. d.), *Caenorhabditis elegans* (C. e.), *Arabidopsis thaliana* (A. t.), *Pseudomonas* sp. strain KKS102 (Ps.), and *Saccharomyces cerevisiae* (S. c.). Sequence alignment was performed with ClustalW2 (128). GenBank or UniProt accession numbers are given in Figure 2. Functionally important structural elements are highlighted in gray and indicated. The membrane-binding fragment in *C. elegans* is boxed.

203–262 and 400–479) and NAD(H)-binding domains (aa.171–202 and 263–399) with a classical Rossmann topology, and the C-terminal domain (aa. 480–610) comprised of five antiparallel β-strands and two α-helices (Fig. 4A). The FAD-binding domain is most conserved in the GR-like proteins. The Lys176-Glu313 charge–charge pair in AIF assists FAD binding and regulates catalytic efficiency (150), whereas Phe309 plays a role of a gatekeeper controlling access of the pyridine nucleotide cofactors to the active site. A unique feature of the AIF FAD-binding domain is the N-terminal insertion (aa. 190–202; Fig. 3) folded as a β-hairpin (Fig. 4A, dark cyan region). This peptide establishes multiple contacts with the main core *via* hydrophobic (Leu190, Phe192, Trp195) and polar residues (Gln194, Arg200).

The C-terminal domains are most diverse in the GR-like flavoproteins and have structurally distinct peptides located on the surface. Similar to bacterial oxidoreductases, the C-terminal part of AIF lacks two long α-helices through which the GR family enzymes dimerize and catalyze disulfide reduction. Instead, AIF contains the 509–559 insertion (Figs. 3 and 4A), which makes its structure unique. The 509–559 peptide is organized as two short α-helices (aa. 517–523) and an extended loop (a red-colored region in Fig. 4A), whose open conformation is stabilized *via* crystal contacts. Together with the β-hairpin, the α-helical part of the C-terminal insertion blocks access to the active site.

The absence of canonical DNA-binding motifs in murine AIF was quite surprising, as the protein was known to directly associate with DNA. An explanation came later, when the 1.8 Å crystal structure of refolded human AIFΔ1–120 was determined (242). The human protein has an identical fold but due to different crystal packing, its 540–559 fragment was disordered. As a result, Arg584 and Lys590 became exposed, giving rise in a positive electrostatic surface potential. Together with Lys446, Arg449, Arg450, Arg451, and Lys593, these residues form a continuous positively charged patch, proposed to guide interactions with DNA (242).

B. X-ray structure of murine AIFΔ1–77

Lacking only the membrane tether, murine AIFΔ1–77 resembles the mature Δ1–53 protein in structure and function (38). In the crystal structure of naturally folded oxidized AIFΔ1–77, there are four molecules in the asymmetric unit, which are organized in two dimers topologically similar to that of refolded AIFΔ1–120 (202). The most notable difference between the two structures is in the FAD position ($\sim$0.6 Å displacement) and conformation of the 438–453 peptide, connecting the active site with the crystallographic dimer interface (Fig. 5A). Due to conformational variations in the Arg448 side chain, the 438–450 fragment naturally folds into a 9-residue β-turn but remains as a loop upon AIF refolding. This alteration could be critical, as it may affect formation and stabilization of dimeric CTC (discussed below). The N-terminal and 538–559 peptides in all four AIFΔ1–77 molecules are structurally disordered. However, two 8- and 9-residue fragments, which could not be identified due to poor resolution and likely representing the missing N-termini, were modeled as antiparallel β-strands complementing the C-terminal β-sheets of two AIF monomers (Fig. 6).

C. X-ray structure of reduced NAD-bound murine AIFΔ1–101

The x-ray structure of reduced NAD-bound form of murine AIFΔ1–101 consists of one biological CTC dimer that resembles the crystallographic dimers of oxidized AIFΔ1–77 (Fig. 4B) (202). The CTC dimer interface has an intricate H-bonding network with two central Arg448-Glu412 intersubunit salt bridges (Fig. 5B). Since in refolded AIF Arg448 is locked in a conformation partially precluding charge–charge interactions with Glu442 and the CTC formation is perturbed (150, 155), the Arg448-Glu412 salt links are thought to promote AIF di-

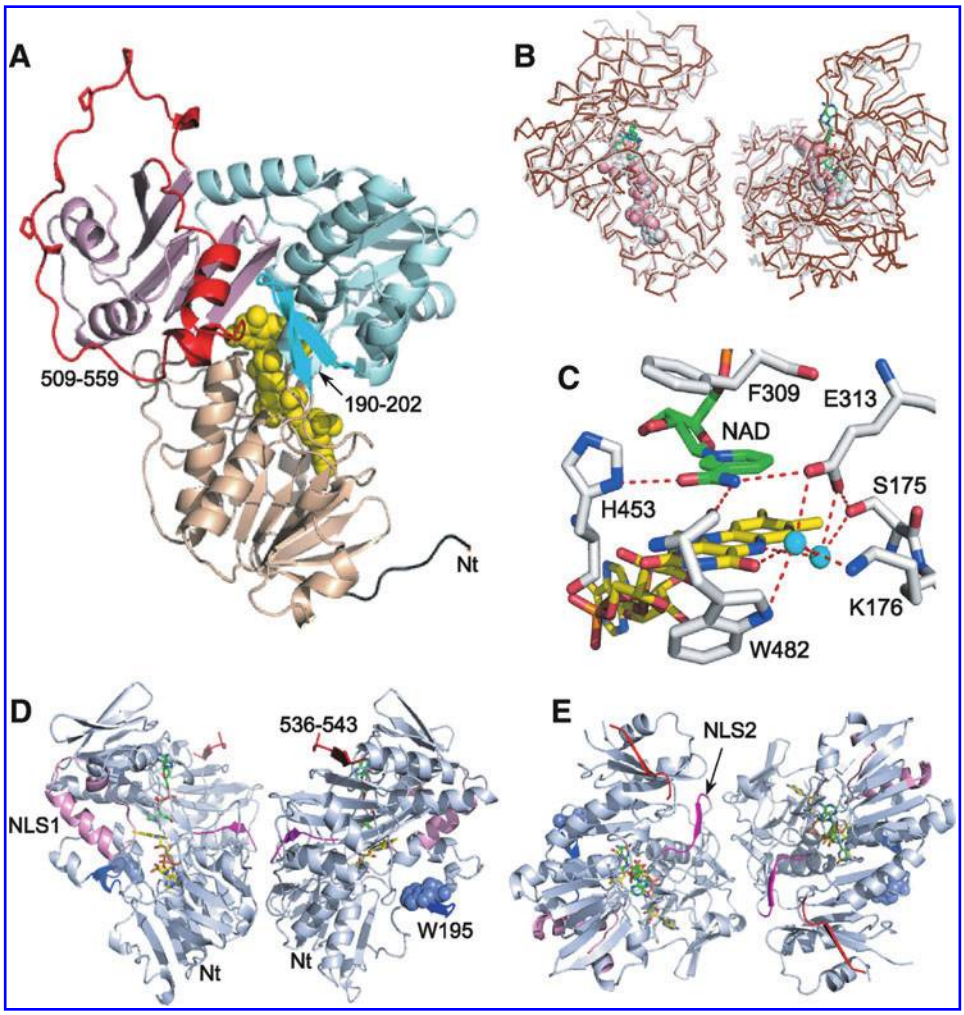

FIG. 4. Structure of AIF. (A) The x-ray model of refolded oxidized murine AIFΔ1–120 [PDB code 1GV4 (150)]. The FAD-binding, NAD(H)-binding, and C-terminal domains are depicted in beige, cyan, and purple, respectively; FAD is in CPK representation. The unique features of AIF are an extended N-terminus (shown in black), the N-terminal 190–202 insertion folded as a β-hairpin (deep cyan), and the C-terminal 509–559 insertion (red; proline residues are displayed). **(B)** Superposition of the crystallographic dimer of naturally folded oxidized murine AIFΔ1–77 [shown in gray, PDB code 3GD3 (202)] and biological dimer of the reduced NAD-bound AIFΔ1–101 [brown, PDB code 3GD4 (202)]. FAD is in CPK representation; NAD is in green. **(C)** A hydrogen-bonding network in the active site of reduced AIF. The nicotinamide group (green) is parallel stacked between Phe309 and the isoalloxazine of FAD (yellow) and establishes H-bonding network with the surrounding residues. One of these, His453, undergoes a large positional shift upon AIF reduction. **(D, E)** Views at the front side and the top of the reduced AIF dimer, respectively. The redox-sensitive 190–202 β-hairpin and Trp195 (in CPK representation) are shown in blue. NLS1 and NLS2 are in pink and magenta, respectively. NLS2, through which AIF is predominantly transported to the nucleus, comprises the dimer interface and, hence, becomes inaccessible upon AIF reduction. The α-helical portion of the regulatory peptide unwinds, and the 536–543 amino acid stretch transforms into the sixth strand of the C-terminal β-sheet (shown in red). The remaining residues of the regulatory peptide are not seen in the x-ray structure due to disorder (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

merization and stabilize CTC (202). Most importantly, Arg448 is part of NLS2 (^{445}KLGRRRV^{451}). This means that the NLS2 accessibility depends on the AIF redox/oligomeric state.

1. Redox-linked changes in the active site. Upon reduction, the flavin nucleotide portion of FAD shifts by 1.2 Å to optimize π-π charge–transfer interactions with the nicotinamide of NAD, parallel stacked between the isoalloxazine and Phe309 aromatic rings (Fig. 4C). The necessity of positional adjustments in FAD for optimization of the intercofactor charge transfer suggests that perturbations in the redox properties of refolded AIF may in part be due to an altered FAD conformation.

While the general mode of NAD(P) binding is conserved in the GR-like proteins, AIF has one distinctive feature in the active site: His453. Substituted by Leu or Ile in other flavoenzymes, His453 undergoes a large positional shift to establish an H-bond with the nicotinamide. This conformational switch is critical because it helps to orient the redox groups optimally for charge transfer and, most importantly, enables to transmit the redox signal from the active site to the surface *via* an adjacent 438–452 peptide (Fig. 5A). Thus, the redox-linked His453 movement could assist AIF dimerization and CTC stabilization. Drastic changes in the AIF redox activity caused by the H453L mutation (38) suggest that this residue also modulates the flavin redox potential and electron transfer to the acceptor molecules.

2. Reorganization in the C-terminal domain. The C-terminal domain undergoes most notable changes upon AIF reduction (202). Binding of NADH triggers rearrangement of a number of aromatic residues, whose side chains align in a tunnel spanning from the active site to the surface (Fig. 7). Tyr559 undergoes the largest movement (>9.0 Å) and shields the tunnel from the solvent. With the ring-to-ring distance of <4.0 Å, the aromatic cluster is perfectly suited for

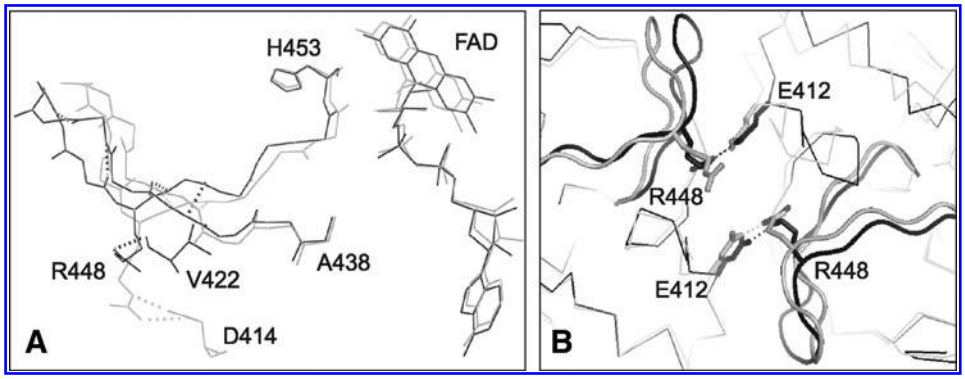

FIG. 5. Structural alterations in AIF caused by refolding. (A) Conformational differences in FAD and the 438–453 peptide, spanning from the crystallographic dimer interface to the active site. Due to conformational differences in the side chain of Arg448, forming a salt bridge with Asp414 in refolded AIF (gray) and an H-bond with the Val422 carbonyl oxygen in the naturally folded protein (black), the 438–540 fragment folds as a loop or β-turn, respectively. **(B)** By establishing an intersubunit salt bridge with Glu412, Arg448 may assist dimerization and stabilization of NADH-reduced AIF. Conformational alterations in the 438–453 peptide detected in refolded AIF (displayed in gray) could interfere with the dimerization process and be responsible for the perturbed redox properties of the protein.

transfer/delocalization of electrons, which could extend the lifetime of the reduced species. Another important consequence of the redox-linked restructuring is dislocation of the entire 509–558 peptide, the α-helical portion of which unwinds and the 536–543 stretch becomes the sixth strand of the C-terminal β-sheet. In accord with the oxidized AIFΔ1–77 structure (Fig. 6) (202), the latter observation suggests that the surface-exposed 501–508 β-strand in AIF has a high affinity for the complementary peptides and may be predisposed for the β-sheet extension.

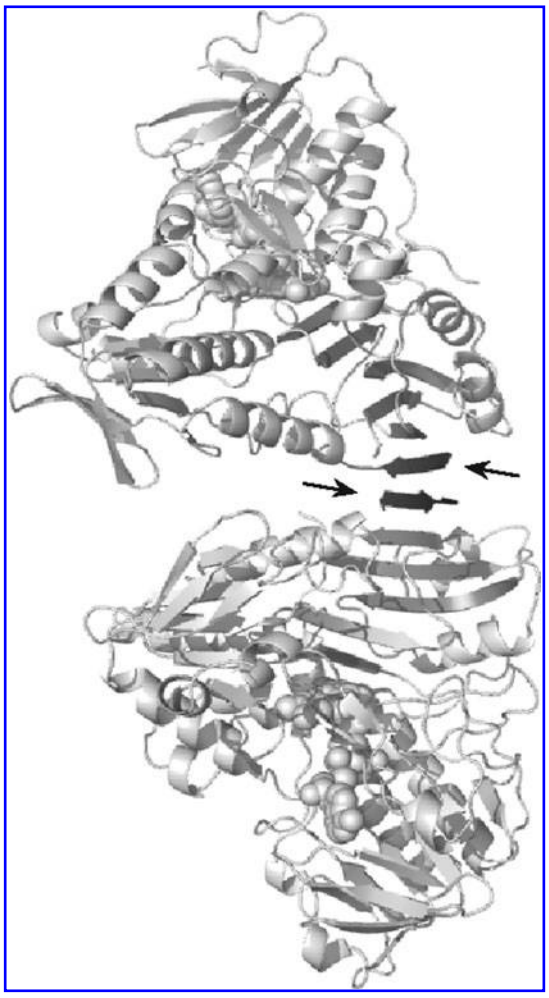

FIG. 6. Extension of the C-terminal antiparallel β-sheets in crystalline AIFΔ1–77. The N-termini of the neighboring molecules forming β-strands are shown in black and indicated by *arrows*.

3. Conformational changes in the 509–559 peptide. Conformational reorganization in the 509–559 peptide has far-reaching implications. One of the immediate effects is release of the 190–202 β-hairpin. Although the precise function(s) of this unique element are currently unknown, it has the potential to modulate redox properties and interaction of AIF with partnering proteins. Trp195, for instance, assists folding of the 509–538 peptide in oxidized AIF, modulates the FAD redox potential, and controls reactivity toward NADH and electron acceptors (202). A significantly prolonged lifetime of reduced AIF W195A suggests that Trp195 might be the site for electron exchange with oxygen and other acceptors. These features and an outward orientation make the hairpin suitable for binding to the membrane-associated or soluble partners. One protein predicted to interact with AIF through the 190–202-containing fragment is 70 kDa heat shock protein (Hsp70) (84, 196) (section VII.C.1.a). Severe and early onset mitochondrial encephalomyopathy linked to the AIF ΔR201 mutation in humans (81) (section VIII.C) also strongly argues for the functional importance of the 190–202 peptide.

Another redox-sensitive element that can modulate functioning of AIF is the 509–559 insertion. This fragment contains several regulatory elements (Fig. 8). Lysines 509 and 517 (510 and 518 in human AIF) are critical for the DNA binding and induction of apoptosis (242). It is possible that one or both of these surface lysines could undergo ubiquitination (42, 238). The proline/glutamic acid/serine/threonine-rich (PEST) sequence (aa. 528–559) is a proteolytic signal (148, 185) that, when flanked by arginine or lysine (Arg529 in human AIF; Fig. 8), correlates with the protein's short lifetime. Unstructured and easily accessible PEST-containing peptides can be cleaved by serine proteases and caspases, with the newly formed N- and C-termini acting as initiation sites promoting proteosomal degradation (19). The PEST sequence can also modulate the proteolytic action of μ-calpain (204), a protease involved in liberation of mitochondrial AIF (section VII.B.1). Further, biological activities of the PEST-containing proteins can be regulated through reversible phosphorylation, as the

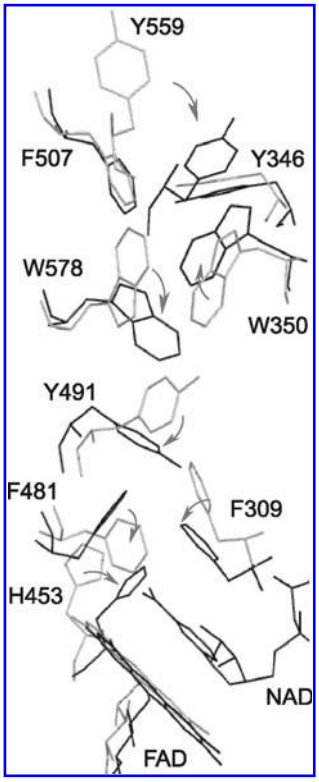

FIG. 7. Redox-induced reorganization of aromatic residues in the C-terminal domain of murine AIFΔ1–101. Displacement of Phe481 and Phe309 by the nicotinamide group initiates the rearrangement and leads to formation of an aromatic tunnel that may serve as an electron delocalization site and prolong the lifetime of the reduced species.

consensus sites of many kinases map to the PEST regions. The putative phosphorylation sites in human AIF include serines 518, 529, 531, and 538, and threonines 533 and 547 (Fig. 8). Two residues, Ser529 and Ser531, are predicted to be phosphorylated by casein kinase 2, implicated in a variety of cellular processes, including apoptosis, DNA repair, transcription, and cell cycle control (208).

The third functionally important element is a proline-rich sequence (aa. 543–554), a potential recognition and interaction site for the Src homology 3 (SH3) domain containing proteins [reviewed in (106)]. The human genome encodes ~300 SH3 domains, some of which have been implicated in intracellular signaling, cytoskeletal rearrangements, cell growth and differentiation, protein and vesicle trafficking, and immune response (82, 112). T-cell ubiquitin ligand (TULA) is one SH3-domain containing protein identified as a cytoplasmic partner of apoptogenic AIF, although the TULA-AIF association may not require the SH3 module (42) (section VII.C.2.b). Histone H2AX, on the other hand, is a nuclear partner that lacks the SH3 domain but binds to the proline-rich region of AIF (13) (section VII.D.3.d). Thus, the regulatory peptide could mediate protein–protein interactions with or without involvement of the SH3 modules. Summarizing, it can be concluded that AIF has acquired several unique structural elements that help stabilize the NADH-reduced dimer and allow redox-controlled regulation of the AIF lifetime, posttranslational modification, and interaction with partnering proteins.

VII. Role of AIF in PCD

AIF is widely accepted as a caspase-independent PCD inducer although its contribution to apoptosis may depend on species, cell type, and death stimuli [reviewed in (104, 157)]. Since the manner of folding defines structural and functional integrity of AIF and the covalently attached affinity/epitope tags not only interfere with the protein folding but can also introduce artifactual effects (63, 90, 117), the AIF constructs used in some of the cited studies will be specified.

A. Apoptogenic effects of AIF in cell free systems and live cells

AIF was originally discovered as an IMS component capable of inducing chromatin condensation and DNA loss in the nuclei isolated from healthy cells (214, 215, 248). The protein was first proposed to act as a protease or protease activator that can be inhibited by a pan-caspase inhibitor z-vad.fmk (213). Later the same group demonstrated, however, that mitochondrial AIF is a flavoprotein whose apoptogenic activity is not affected by z-vad.fmk (211).

Like native AIF, the refolded precursor and AIFΔ1–120 trigger loss of DNA and peripheral chromatin condensation in purified nuclei (155, 210, 212). This process is rapid and accompanied by large-scale (~50 Kb) DNA fragmentation which can be prevented by the divalent cation chelator EDTA. Induction of nuclear apoptosis does not require additional cytoplasmic factors but may involve nuclear protein(s), as AIF itself does not possess nuclease activity and has no effect on preheated nuclei. AIFsh induces similar effects in isolated nuclei, wherein the Δ353–613 mutant and AIFsh2 do not (53, 54). In cooperation with a heat-labile cytoplasmic factor, the precursor and AIFΔ1–120, but not the Δ1–351, Δ155–612 or Δ538–612 fragments, cause swelling of isolated mitochondria by increasing permeability of the OMM (212). Since AIF precursor becomes apoptogenic after refolding even in the absence of FAD (212) and the apoptogenic ability of polyhistidine (His)-tagged AIFΔ1–120 and Δ1–100 in cell-free systems is not affected by NAD(P)H or FAD modification (136, 155), the apoptogenic function of AIF was suggested to be defined by the protein conformation and require the C-terminal part but not the oxidoreductase activity.

The cell-death-inducing potency of recombinant AIF is abolished upon treatment with a thiol-reactive agent p-chloromercuriphenylsulfonate (pCMPS) (155). Among three

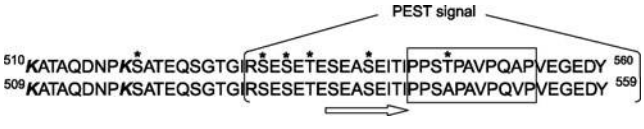

FIG. 8. Amino acid composition of the regulatory peptide in human and murine AIF (upper and lower sequences, respectively). The proline-rich motif within the PEST sequence is boxed; two lysines critical for DNA-binding are shown in bold italic. The putative phosphorylation sites were identified using the online NetPhos server (www.cbs.dtu.dk/services/NetPhos/) and are marked by asterisks. Human AIF has one additional phosphorylation site at Thr547, which is substituted by Ala in the murine protein. *An arrow* indicates the peptide transforming into the sixth strand of the C-terminal β-sheet upon AIF reduction.

cysteine residues present in AIF, only Cys441 (Cys440 in mice) is positioned close to the surface and is next to NLS2. Modification of Cys441 with pCMPS would introduce a negative charge near basic NLS2 and, hence, could interfere with the nuclear transport of AIF.

In live cells, the apoptogenic potency of AIF has been investigated by microinjection or transfection-enforced overexpression of the His- or green fluorescent protein-tagged precursor, Δ1–120, Δ1–100, and AIFsh fragments (53, 67, 136, 210, 212). The tagged Δ1–120 and Δ1–100 proteins diffusely distribute in the cytoplasm and nucleus, rapidly inducing chromatin condensation, loss of DNA, exposure of phosphatidylserine (PS) on the outer leaflet of the plasma membrane, and dissipation of the mitochondrial *trans*-membrane potential (ΔΨm). After liberation from the IMS, endogenous AIF increases mitochondrial permeability in a feedback loop (136, 212). The precursor is mainly targeted to mitochondria, but after prolonged cultivation, the cytoplasmic and nuclear distribution of AIF becomes predominant and nuclear apoptosis progresses (136). Lacking MLS, AIFsh diffusely distributes in the cytoplasm and then rapidly translocates to the nucleus, triggering large-scale DNA fragmentation in a caspase-independent manner (53).

When injected into the cytoplasm, FAD-free AIFΔ1–120 induces chromatin condensation and ΔΨm dissipation to the same degree as the FAD-bound protein, whereas the Δ1–351, Δ155–612, and Δ538–612 fragments fail to cause cell death (155, 212). To further test the role of the redox center, Loeffler *et al.* genetically modified ^{255}CLIATG^{260} and ^{303}TVIGGG^{308} motifs in the murine AIF precursor in attempt to preclude FAD and NADH binding (136). The apoptogenic potential of the ^{255}CLIAAA^{260} and ^{303}TAAAGG^{308} variants in live cells was similar to that of wild-type AIF (*i.e.*, initial mitochondrial localization followed by spontaneous translocation and apoptosis). This was another argument to support the concept that the apoptogenic effect of AIF does not rely on the oxidoreductase activity, although no proof that the mutated proteins were incapable of binding the cofactors had been presented.

In live cells, the MLS-free Δ1–120, Δ1–100, and AIFsh proteins cause cell death more rapidly than the mitochondrion-targeted precursor, with the time difference varying from several hours to several days (53, 136). The same effects are observed in the presence of z-vad.fmk, indicating that when in the cytoplasm, AIF becomes a lethal factor leading to cell demise irrespective of whether caspases are activated or not. Contrarily, the proapoptotic action of Flag-tagged human AIFΔ1–480 was found to be mediated through cytochrome *c* release in a caspase 9-dependent manner (252). Most strikingly, enforced expression of tag-free human AIF1–613, Δ1–54, or Δ1–102 is nontoxic and even cytoprotective, as all three proteins reduce the basal and stimulated ROS levels (238). Thus, the cell death-triggering ability of AIF can be modulated by the attached tags and in some cases requires caspase activation.

Whether or not AIF is essential for cell death depends on the cell type and apoptotic stimuli. *AIF*$^{-/y}$ embryonic stem (ES) cells, for instance, display normal susceptibility to death in response to a variety of apoptosis-inducing agents, including staurosporine, etoposide, azide, *tert*-butylhydroperoxide, anisomycin, UV-radiation, and growth factor withdrawal, but fail to die in response to pro-apoptotic vitamin K_3 in the presence of z-vad.fmk (105). Cells from AIF-deficient Harlequin (*Hq*) mice are more sensitive to induction of apoptosis (120), but neurons from *Hq*/*Apaf*$^{-/-}$ mice are impaired in both caspase-dependent and AIF-mediated cell death pathways (36). *AIF* gene silencing protects renal tubular epithelial cells against cisplatin-induced cell death (201), whereas AIF$^{-/y}$ human colon carcinoma cells are more sensitive to peroxide- and drug-induced apoptosis than the wild type (221).

Finally, the apoptotic machinery shares common pathways with autophagy, a self-destructive process involving lysosomes that could either link or polarize cellular responses [reviewed in (142)]. There are few studies where the functional relationship between caspase-independent cell death and autophagy has been explored. A regulatory crosstalk between AIF-mediated apoptosis and autophagy was observed in HCT116 cells treated with Lapatinib, an inhibitor of receptor tyrosine kinases (146), in malignant rhabdoid tumor cell line treated with the histone deacetylase inhibitor FK228 (235), and MCF-7 cells treated with crotoxin (241). In the presence of different inducers, cells undergo PCD that involves autophagy but not release of mitochondrial AIF (21, 80, 167). Thus, whether apoptosis and autophagy intersect depends on physiological settings.

In summary, AIF becomes a powerful lethal factor when released into the cytoplasm. Upon translocation to the nucleus, AIF triggers chromatin condensation and loss of DNA in a caspase-independent manner. The C-terminal portion of the protein but not its redox activity is responsible for the apoptogenic action. Contribution of AIF to PCD depends on the cell type and death triggers and, in some cases, may be amplified by caspases and autophagic factors, and modulated by the covalently attached tags.

B. Release of mitochondrial AIF

The mechanism regulating apoptogenic processing and translocation of mitochondrial AIF into the cytoplasm is complex and not fully understood. Here is a brief overview of the currently available data, schematically presented in Figure 9.

1. Proteolysis of mature AIF. Being tethered to the inner mitochondrial membrane, mature AIF must undergo N-terminal proteolysis to form an apoptogenic fragment that can be released into the cytoplasm (168). Studies conducted in cell-free systems demonstrated that mitochondrial μ-calpain, a Ca^{2+}-dependent neutral cysteine protease, colocalizes with AIF in the IMS and can process it locally upon activation (77, 135, 156, 169, 170). An increase in cellular Ca^{2+} (162) and association with a protein–disulfide isomerase ERp57 (170) lead to activation of mitochondrial μ-calpain and AIF proteolysis. Conversely, an endogenous calpain inhibitor, calpastatin, can prevent AIF liberation (31, 227, 233). There is controversy, however, over whether calpastatin is present in mitochondria or not (108, 109, 169). Involvement of mitochondrial μ-calpain in AIF processing has also been questioned (101).

Studies on isolated mitochondria suggest that cytoplasmic μ-calpain and lysosomal Ca^{2+}-independent cathepsins B, L, and S can gain access and process AIF after opening of the permeability transition pore (PTP) (31, 178, 246). Several lines of evidence disfavor this suggestion. First, there is no *in cellulo*

experimental evidence demonstrating that the proteases can enter mitochondria during apoptosis. Second, lysosomal permeabilization would be required for the cathepsin-dependent AIF release, which is not always observed during apoptosis [reviewed in (164)]. Finally, *in vitro* cathepsins B and L cleave recombinant AIF nonspecifically (31), and cathepsin B knockout has no effect on AIF processing (159). Thus, it is still uncertain whether external enzymes are involved in proteolysis of mitochondrial AIF.

A single Leu103/Ser104 cleavage site for μ-calpain was identified in recombinant human AIFΔ1–54 (31). Mutagenesis screening revealed that only the L101G/L103G AIF variant is completely resistant to μ-calpain activity (31). On the basis of the proteolytic pattern of endogenous murine AIF, the existence of two μ-calpain-sensitive sites within the 96–120 fragment was predicted (178). This was later confirmed *in vitro* by our group (38). We found also that one site, Glu118-Gly119, is protected in reduced AIF (38). Further, NAD(P)(H) affected the μ-calpain-mediated cleavage of endogenous AIF in mitochondria treated with atractyloside or cBid (calpain activated Bid, the proapoptotic Bcl-2 family member) (38). The protective action of the reduced cofactors was more pronounced in mitochondria respiring on malate/glutamate than in succinate/rotenone media, and the NADP/NADPH pair had a stronger effect than NAD/NADH. This led us to suggest that the externally added pyridine nucleotides exert their action indirectly *via* modulation of mitochondrial permeability, morphology, and/or protein–protein interactions.

Contrary to our study (38), Norberg *et al.* reported that NAD(P)(H) has no effect on the Ca^{2+}-stimulated AIF processing in isolated mitochondria in the presence of cyclosporin A (CsA) (163). Instead, Ca^{2+}-induced production of ROS led to AIF carbonylation and accelerated cleavage by μ-calpain. The protective effect of NAD(P)(H), therefore, is not a general phenomenon and may depend on the apoptotic insult. tBid and atractyloside, for instance, cause drastic changes in the inner mitochondrial membrane topology (144) that can be inhibited by CsA (198).

That physical interaction and subsequent degradation of AIF by μ-calpains may be redox dependent follows also from the fact that recognition and proteolysis of protein targets by μ-calpain are expedited by PEST motifs (204, 229). If AIF's PEST sequence is indeed a calpain-targeting element, then AIF processing may be regulated *via* redox-linked reorganization in the regulatory peptide (section IV.C.3).

In summary, the membrane linker in AIF has at least one proteolytic site. Mitochondrial and cytoplasmic μ-calpains, as well as lysosomal cathepsins B, L, and S, can process AIF in cell-free systems, but whether these proteases participate *in vivo* is still a matter of debate. If the involvement of μ-calpain is confirmed, processing of AIF in the IMS may be redox controlled.

2. Release of truncated AIF into the cytoplasm. Permeabilization of mitochondria, irrespective of whether it occurs before or after AIF cleavage, is an obligatory event in the AIF-mediated apoptotic signaling. Currently, two different models have been proposed to explain how the apoptogenic IMS components are released from mitochondria. According to the first model, mitochondrial PTP, a conductance channel formed at the contact sites between the inner and outer membranes and consisting of the outer membrane voltage-dependent anion channel (VDAC), the inner membrane adenine nucleotide translocase (ANT), and associated with the matrix side of ANT cyclophilin D, is actively opened. This allows small molecules (<1.5 kDa) to enter the mitochondrial matrix, leading to swelling and rapture of the OMM. PTP opening is regulated by the mitochondrial membrane potential, $\Delta\Psi$m, dissipation of which correlates with the cytoplasmic translocation of AIF (49, 129, 171). The $\Delta\Psi$m/PTP-related liberation of AIF can be induced by Bid (127, 178), atractyloside (a competitive inhibitor of ANT), fatty and bile acids (54, 141, 159, 161, 207), α-eleostearic acid (123), and may require VDAC oligomerization (203). Conversely, bongkrekic acid, ROS scavengers, Bcl-2, and CsA inhibit translocation of mitochondrial AIF (129, 132, 163, 168, 171, 178, 212, 250). Pro-survival Bcl-2 prevents AIF liberation by sequestering proapoptotic Bax and Bak (discussed below) or *via* extramitochondrial mechanisms (20). The antibiotic bongkrekic acid acts as an ANT inhibitor, wherein CsA precludes PTP opening by forming complex with cyclophilin D on the matrix

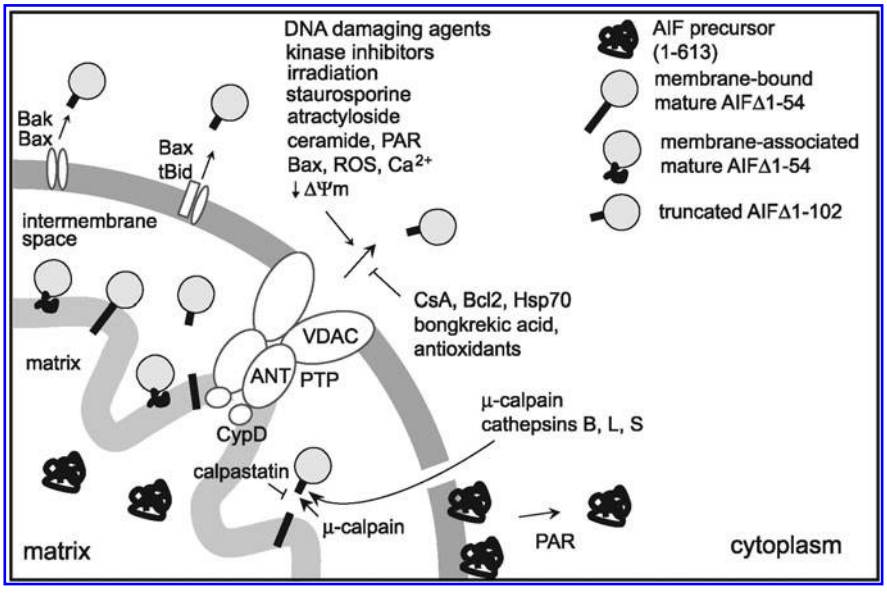

FIG. 9. Factors modulating the cleavage and release of apoptogenic AIF from mitochondria. Proteolysis of the membrane tether in mature AIF can be mediated by local or cytoplasmic proteases entering the intermembrane space upon permeabilization of the outer membrane. Detached AIF can translocate to the cytoplasm with the involvement of the PTP complex or through pores formed by proapoptotic Bcl-2 family members Bax, Bak, and Bid. AIF associated with the outer leaflet of the OMM, likely a full-length precursor, can be released and transported to the nucleus in a PAR-dependent manner. ANT, adenine nucleotide translocase; PAR, poly(-ADP-ribose); PTP, permeability transition pore; VDAC, voltage-dependent anion channel.

side of ANT (85). Intracellular Ca^{2+} dysregulation and mitochondrial Ca^{2+} overload facilitate AIF relocation by activating calpain, dissipating $\Delta\Psi m$ and inducing the OMM rupture (126, 131, 175), which could proceed in a caspase-independent manner (24, 227). In some cases, however, activation of caspases is required for the AIF release (9, 17, 201, 205).

Poly(ADP-ribose) (PAR) polymer, a byproduct of the reaction catalyzed by the activated nuclear DNA repair enzyme PAR polymerase-1 (PARP-1), triggers AIF translocation by dissipating $\Delta\Psi m$, altering mitochondrial Ca^{2+} homeostasis, and activating calpain (39, 159, 227, 244). Additionally, PARP-1 activation leads to excessive consumption of cytoplasmic NAD and generation of AMP, which facilitates mitochondrial depolarization and AIF release (3, 69). PARP-1-dependent AIF efflux may also involve Bax (159). The nuclear PARP-2 isoform was recently demonstrated to substantially contribute to nuclear translocation of AIF, in part *via* PAR accumulation (133). Finally, intramitochondrial PARP-1 can directly provoke AIF liberation *via* interactions with the mitochondrial PTP (61, 186).

The second model suggests that mitochondrial apoptogenic factors can be released through the pores in the outer membrane formed by the pro-apoptotic Bcl-2 family members such as Bax, Bak, and Bid that do not affect $\Delta\Psi m$. Bax and Bak are essential regulators of a diverse mitochondrial cell death pathways. Being in a latent state in healthy cells, they undergo conformational changes and homo- and hetero-oligomerization, forming channels in the OMM (6). While elimination of the PTP components has virtually no effect on the ability of cells to respond to apoptotic stimuli (15, 122, 194), $Bax^{-/-}/Bak^{-/-}$ double-knockout abrogates apoptosis (134, 236). Release of AIF is also blocked in $Bax^{-/-}$ human colon cancer cells (182). Bax, Bak, and full-length and proteolytically activated Bid (cBid or tBid) can initiate mitochondrial demise and facilitate AIF translocation not only through the OMM pore formation (118, 121, 125, 188), but also by increasing mitochondrial fission and fragmentation (23, 110, 127) and altering cristae formation (73, 198). In addition, a pore that permits AIF efflux can be generated by Bax, VDAC, and ceramide (191). Certain types of Bax-related mitochondrial membrane injuries and AIF release are inhibited by Hsp70 (187, 189). Thus, liberation of AIF from the IMS can be regulated by a variety of factors that may or may not involve mitochondrial PTP and $\Delta\Psi m$ dissipation.

3. Release of AIF associated with the outer mitochondrial membrane. The AIF pool loosely associated with the cytoplasmic side of the OMM can be rapidly released during parthanatos (243, 245), a unique form of PARP-1-initiated cell death triggered by DNA damage. Parthanatos is characterized by accumulation of PAR, mitochondrial depolarization, early nuclear translocation of AIF, loss of cellular NAD and ATP, and late caspase activation [reviewed in (5, 50)]. Release of the OMM-associated AIF does not involve proteases and is mediated by pathogenic PAR polymers through an unknown mechanism (234).

PAR polymers are especially toxic for neurons (5) and their cytotoxicity increases with the dose, length, and complexity of the molecule but does not depend on the negative charge (5, 243). PAR binds covalently and noncovalently to a variety of proteins, including AIF, altering their function and conformation (74, 206). PAR binding/modification can be mediated by three protein motifs: (a) an amino acid sequence rich in positively charged residues with a consensus pattern *-hxbxhhbbhhb-*, where *h*, *b,* and *x* correspond to hydrophobic, basic, and any amino acids, respectively (177); (b) a *macro* domain, capable of recognizing monomeric and polymeric ADP-ribose (113); and (c) a PAR binding zinc finger motif (PBZ) (1). AIF does not have the consensus sequence or PBZ but contains two pyrophosphate/adenine binding pockets as part of the FAD- and NAD(H)-binding sites. It is possible, hence, that folded and FAD-bound oxidized AIF could associate with the ADP-ribose monomer through an unoccupied NAD(H)-binding pocket, wherein an unfolded FAD-free precursor may have affinity for both monomeric and branched ADP-ribose. Assuming that the OMM-associated fraction represents an unfolded and non-apoptogenic AIF precursor, one can speculate that PAR directly binds to the NAD- and FAD-binding sites and refolds the protein. This, in turn, could trigger detachment and translocation of the apoptogenic PAR-AIF complex to the nucleus.

C. Cytoplasmic interactions of apoptogenic AIF

Before relocating to the nucleus, apoptogenic AIF has a capacity to exert multiple and diverse effects through interactions with the cytoplasmic proteins acting as pro-survival or pro-death effectors (summarized in Fig. 10).

1. Pro-survival partners of AIF

a. Heat shock protein Hsp70. Inducible Hsp70 (70 kDa) was the first cytoplasmic partner of apoptogenic AIF to be identified (183). Hsp's are highly conserved molecular chaperones critical for cell viability, as they assist folding, thermal tolerance, and translocation of proteins across cellular membranes, reorganization of macromolecular complexes, degradation of misfolded proteins, and regulation of signaling pathways (65, 79, 88, 93, 115). Acting as monomers, Hsp70s contain the N-terminal ATPase domain and the C-terminal module comprised of the peptide-binding domain (PBD) and an EEVD motif required for the interaction with other chaperones and regulation of protein refolding and repair (72, 153). Hydrolysis of ATP allosterically modulates affinity and kinetics of substrate binding to PBD, which recognizes hydrophobic residues or unstructured backbone regions.

Hsp70-AIF interaction: In a cell-free system, Hsp70 antagonizes the apoptogenic effect of native and recombinant AIFΔ1–100 in an energy- and caspase-independent manner (183). Likewise, downregulation of the chaperone increases the cytotoxic potency of AIF (183). Direct Hsp70-AIF association was confirmed *in vivo* by several studies showing that cytoplasmic Hsp70 specifically interacts with exogenous and endogenous AIF and inhibits its relocation to the nuclear compartment (84, 139, 151, 189). Hsp70 also binds AIF precursor (189) but whether this interaction is related to the chaperone function of Hsp70 has not been elucidated yet.

Examination of physical and functional interactions between the tagged AIF and Hsp70 under conditions of normal cell growth revealed that the chaperone's PBD but not the ATPase activity is required for association and retention of AIF in the cytoplasm (183). In contrast, investigation of dynamic protein–protein interactions under conditions of physiological stress indicated that the Hsp70 ATPase domain

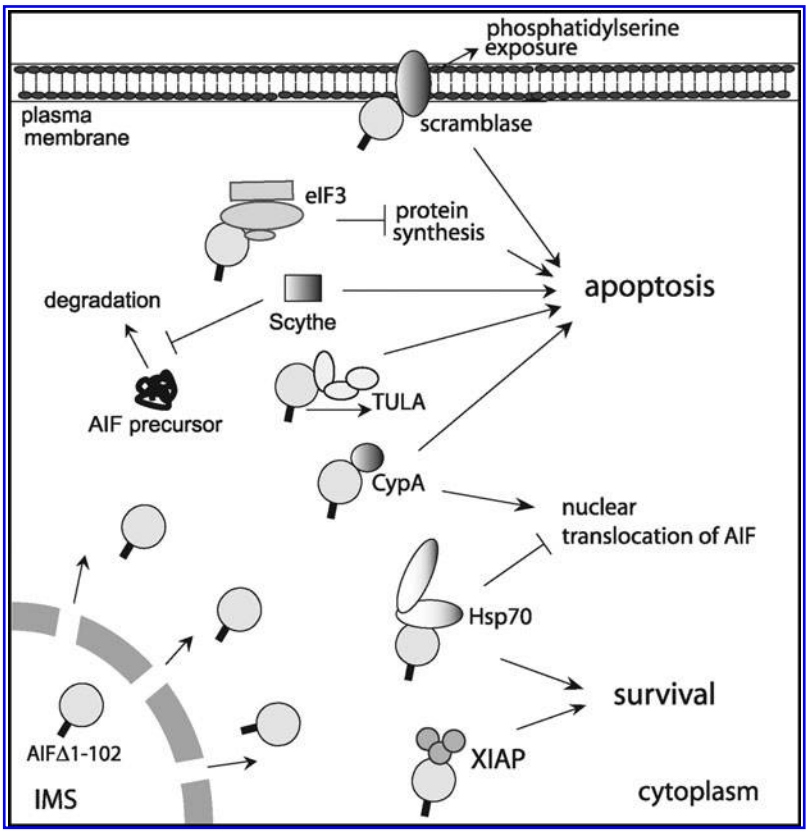

FIG. 10. Cytoplasmic partners of apoptogenic AIF. After release into the cytoplasm, AIF can promote apoptosis by interacting with TULA, eIF3g, and phospholipid scramblase, the pro-death partners. Scythe facilitates cell death by regulating stability and lifetime of the cytoplasmic AIF precursor, wherein CypA assists cytonuclear translocation of apoptogenic AIF. Contrarily, Hsp70 retains AIF in the cytoplasm and, hence, can postpone or prevent initiation of the nuclear apoptosis. XIAP is another pro-life partner, which in co-operation with AIF reduces reactive oxygen species levels and promotes cell survival. CypA, cyclophilin A; eIF3g, eukaryotic translation initiation factor 3 subunit p44; Hsp70, 70 kDa heat shock protein; TULA, T-cell ubiquitin ligand; XIAP, X-linked inhibitor of apoptosis protein.

is indispensable for interaction with native AIF (188). Again, whether these discrepancies are due to the tag attachment or/and differences in the cellular environment needs to be clarified.

Systematic deletion analysis identified the 150–228 peptide and, in particular, Arg192 and Lys194 in human AIF as critical for Hsp70 binding (Fig. 11) (84). *In silico* modeling, in turn, predicted that the protein–protein interface in the AIF-Hsp70 complex is comprised of the AIF 184–221 peptide and the 433–436, 469–473, and 534–541 fragments of the chaperone (196). The inability of Hsp70 to prevent nuclear apoptosis induced by AIFsh (53) and accelerated nuclear translocation of the R192A/K194A mutant of human AIF (242) support the notion that the N-terminal fragment containing the 190–202 β-hairpin mediates binding of the chaperone.

In general, the Hcs70/Hsp70/DnaK (bacterial Hsp70 homolog) recognition motifs represent a stretch of seven residues with two or more bulky hydrophobic/aromatic groups in alternating positions, flanked by basic amino acids and mapped to the secondary structure elements such as packed together β-strands or partially exposed α-helices (12, 70, 140). The primary sequence and the β-hairpin structure of the AIF 190–202 peptide satisfy these requirements (Figs. 3 and 11). If this element indeed serves as the Hsp70 binding site, the AIF-Hsp70 interaction could be redox controlled, as the accessibility of the hairpin changes upon AIF oxidoreduction (section VI.C.3). Another level of regulation of the AIF-Hsp70 interaction could be *via* redox-dependent Hsp70 expression (71) and S-glutathionylation of a key redox-sensitive cysteine residue in the chaperone (94).

In accord with the structural prediction, our *in vitro* experiments showed that Hsp70 does not perturb the DNA-binding ability of oxidized AIF but attenuates the inhibiting effect of NADH on the AIF-DNA interaction (38). This could result from the preferential chaperone binding to the reduced AIF dimer. Selective AIF-Hsp70 association may be physiologically beneficial because when the NADH levels are sufficiently high, cytoplasmic retention of reduced AIF could slow down the apoptotic cascade and give the damaged cell a chance to recover. Depletion of the NAD(P)(H) pools and oxidation/monomerization of AIF, in turn, would indicate drastic metabolic changes, and sequestering of apoptogenic AIF would be of little help.

As a powerful cytoprotective agent whose dysregulation either facilitates apoptosis and kills cells or contributes to carcinogenic transformation, Hsp70 represents an attractive therapeutic target. Since cytoplasmic retention of apoptogenic AIF is pro-carcinogenic, designing of noncytotoxic Hsp70-binding peptides based on the AIF structure can be one of the anticancer strategies (196). One engineered peptide, ADD70 (AIF-derived decoy for Hsp70), consists of residues 101–366, 467–566, and 610–613 of human AIF (196). ADD70 sensitizes cells to apoptosis triggered by a number of PCD inducers (DNA damaging agents, serum depletion, staurosporine, *etc*.) through binding and neutralization of Hsp70. An analogous fragment with the Hsp70 binding site omitted (Δ150–228) has no such effect (196). The chemosensitizing potential of ADD70 is observed in multiple cancer lines but not in cells lacking Hsp70, which confirms that the engineered fragment acts through Hsp70 neutralization. In syngeneic animals, expression of ADD70 delays growth and reduces metastatic potential of tumor cells and sensitizes them to the anticancer drug cisplatin (195). Another approach for anticancer therapy that is currently being developed includes a combination of Hsp70

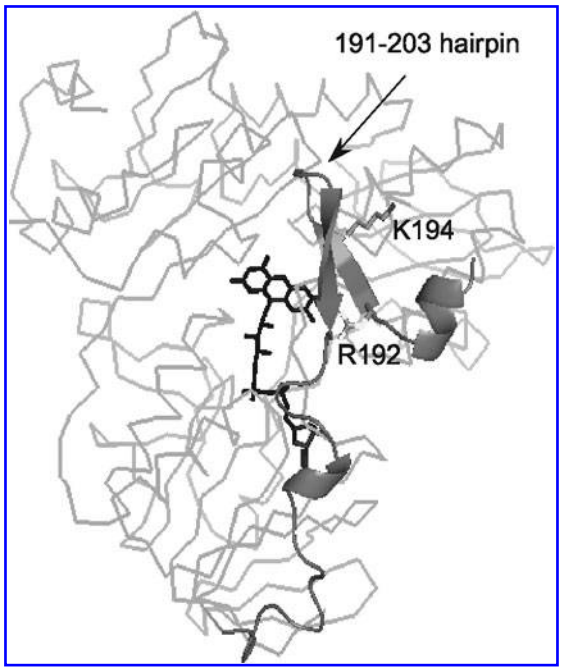

FIG. 11. An Hsp70 binding site identified by systematic deletion analysis and *in silico* modeling (84, 196). The 150–228 and, more precisely, 184–221 fragment (shown in dark gray and cartoon representation) and Arg192 and Lys194 in human AIF were identified as important for Hsp70 binding. Since the predicted site includes a redox-sensitive 191–203 β-hairpin, interaction between AIF and Hsp70 may be redox controlled.

silencing and delivery of AIFΔ1–120, which enhances chemotherapy and drug-induced cell death in tumors (254).

b. X-linked inhibitor of apoptosis protein. X-linked inhibitor of apoptosis protein (XIAP) belongs to a family of the IAPs that selectively bind and inhibit caspases 3, 7, and 9 [reviewed in (62, 192)]. Mammalian XIAP is the most potent and broad suppressor of apoptosis known to date, as it directly and with high affinity inhibits enzymatic activities of both initiator and executioner caspases (63). Human XIAP is a multifunctional cytoplasmic protein consisting of three N-terminal baculoviral IAP repeat (BIR) domains and the C-terminal zinc-binding domain, also known as a RING finger. The BIR2 and BIR3 domains inhibit caspases 3/7 and 9, respectively (59), whereas the RING finger possesses E3 ubiquitin ligase activity and enables XIAP to remove caspases and other proteins from the cell *via* ubiquitination and proteasome degradation (95). In addition to the apoptotic signaling, XIAP is involved in the NF-κB, TGF-β, and c-Jun-N-terminal kinase pathways and copper homeostasis (92, 160, 190). Downregulation of XIAP can lead to cancer, neurodegenerative disorders, and autoimmunity, which makes the protein a potential drug target (192, 193).

XIAP-AIF interaction: Using a tandem affinity purification technique and a D148A/W310A variant of XIAP as a bait, Wilkinson *et al.* identified AIF as one of the XIAP-associated proteins in HEK 293 cell lysates (238). The AIF binding site was mapped to the XIAP BIR2 module. Further, it was demonstrated that AIF could be ubiquitinated by XIAP or alternative E3 ligases. This modification plays a regulatory role, as transient XIAP-AIF complexes formed in the cytoplasm decay quickly upon AIF ubiquitination but become more long-lived if the E3 ubiquitine ligase activity of XIAP is eliminated (238). Thus, although ubiquitination does not target AIF to the proteosome, it may modulate the apoptogenic potency of the flavoprotein.

Two lines of evidence suggest that the AIF-XIAP association plays a pro-survival role. First, AIF inhibits interaction of XIAP with Smac/DIABLO, another death-signaling protein released from mitochondria that prevents XIAP from binding to caspases (238). Second, coexpression of XIAP with either full-length, Δ1–54, or Δ1–102 AIF progressively decreases ROS levels in control and dying cells. Although the underlying mechanism is currently unknown, cooperation between AIF and XIAP is thought to affect caspase-independent functions of XIAP and promote cell survival by reducing cellular oxidative stress (238).

Using the docking program GRAMM (220), which performs a computational search of all possible configurations to find protein complexes with the highest surface complementarity, we compared preferable binding sites for the human XIAP BIR2 domain in the oxidized monomer and reduced dimer of murine AIF. In both types of computer-generated complexes (Fig. 12), the top ranking solutions for the BIR2 domain are clustered at two different sites that have similar binding energy (*i.e.*, surface complementarity). This suggests that XIAP may bind the two redox forms of AIF with equal affinity. Independence of the XIAP-AIF association on the AIF redox state would be physiologically beneficial because it could increase the probability of the interprotein complex formation and promote cell survival.

2. Pro-death partners of AIF

a. Eukaryotic translation initiation factor 3 subunit p44. Eukaryotic translation initiation factor 3 (eIF3) is a 10–13 subunit complex that promotes binding of the 40S ribosomal subunit to mRNA (33). Although evolutionary conserved, the eIF3 subunit p44 (eIF3g) is not essential for the formation of an active mammalian eIF3 (149). eIF3g was reported to associate with the eIF3a subunit, stabilize the eIF3i subunit, and bind to rRNA through the C-terminal RNA recognition domain (22, 149).

eIF3g-AIF interaction: The AIF-binding ability of eIF3g was first detected by the yeast two-hybrid screening method where the N-terminally LexA-fused human AIFΔ1–101 was used as a bait (116). Direct interaction between eIF3g and AIF was confirmed both *in vitro* and *in vivo* and required the N-terminal part of eIF3g and the C-terminal portion of AIF (116). By specifically interacting with eIF3g, AIFΔ1–101 and Δ1–439 inhibit *de novo* protein synthesis in live cells and cell-free extracts, whereas the full-length precursor and 102–439 fragments have no such effect. Thus, the inhibitory properties of AIF could in part be defined by its folding. In MCF-7 cells undergoing cisplatin-induced apoptosis, AIFΔ1–101 not only inhibits the eIF3 machinery and protein synthesis but also amplifies apoptosis *via* caspase 7 activation and proteolytic cleavage of eIF3g, and colocalizes with eIF3g in apoptotic nuclei (116). This suggests that eIF3g and AIF may act synergistically in promoting cell death.

b. T-cell ubiquitin ligand. TULA is a cytoplasmic protein primarily expressed in T and B lymphocytes and other lym-

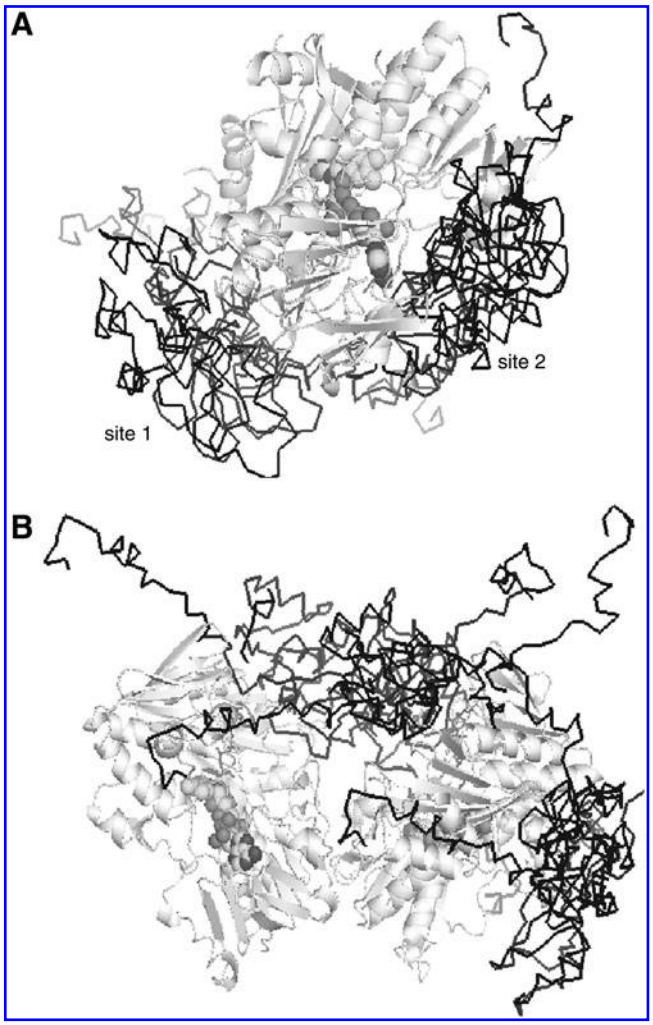

FIG. 12. Complexes between the BIR2 domain of human XIAP (PDB code 1I30) and the oxidized monomer and reduced dimer of AIF (A and B, respectively) generated with the program GRAMM (220). AIF molecules are in light gray and cartoon representation; BIR2 is in black and ribbon representation. In both types of complexes, the top ranking solutions for BIR2 are clustered at two docking sites that have similar binding energy. This suggests that association of AIF with XIAP may be redox independent.

phoid cells where it acts as a negative regulator of c-Cbl-mediated inhibition of protein tyrosine kinases and epidermal growth factor receptor, induces c-Cbl degradation, and promotes cellular transformation (68). The N-terminal part of TULA represents a ubiquitin-associated domain (UBA), through which binding of ubiquitin and ubiquitinated proteins takes place. In addition, TULA has the central SH3 domain, the c-Cbl association site, and a region homologous to phosphoglyceromutases with unknown function (68).

TULA-AIF interaction: TULA was identified as a cytoplasmic partner of AIF by Collingwood *et al.*, who showed that in HEK293T cell lysates AIF coimmunoprecipitates with the full-length and UBA/SH3-domain-containing 1–299 fragment of TULA (42). The UBA/SH3 module interacts with endogenous AIF stronger than the intact TULA, wherein the C-terminal portion (residues 300–623) is incapable of binding AIF. Despite a strict requirement of the N-terminal part of TULA for association with AIF, deletion of the SH3 or UBA domains or introduction of the W279L point mutation to inactivate the SH3 module have no effect on the protein–protein interaction. At the same time, both UBA and SH3 domains are indispensable for the apoptogenic action of TULA on T-cells (42). Further, in Jurkat T cells TULA plays a critical role in apoptosis induced by growth factor withdrawal that does not involve caspases or T-cell receptor/CD3 ligation but requires physical association with AIF liberated from mitochondria. On the basis of these findings, the TULA-AIF pair was proposed to amplify the cytoplasmic apoptotic cascade through cooperation with other factors. Whether this regulatory mechanism is specific for the malignant Jurkat cells (T-cell leukemia) or is more widespread remains to be established.

c. Cyclophilin A. Cyclophilins possess a peptidyl prolyl isomerase (PPI) activity and catalyze *cis-trans* isomerization of peptidylprolyl bonds. Cyclophilins have high affinity for CsA, an immunosuppressive drug and inhibitor of the PPI activity, and are implicated in protein folding, assembly and trafficking, immune response, and cell signaling [reviewed in (14, 154, 230)]. The proteins share a common fold but differ in surrounding structural elements that define subcellular compartmentalization and functional specialization of the individual family members (60). By recognizing and binding to a specific β-turn motif, cyclophilins can modulate functions of the protein targets and stabilize multiprotein complexes (98). Cyclophilin A (CypA) is the most abundant and ubiquitously expressed cyclophilin that, although not essential for cell viability (40), plays an important role in cellular biochemistry and pathogenesis of viral infection, cardiovascular disease, and cancer (165). A type VIb β-turn is one of the elements through which CypA selectively binds to its targets (98).

CypA-AIF interaction: CypA interacts with apoptogenic AIF in the cytoplasm (29) and cotranslocates to the nucleus (253). Formation of the CypA-AIF complex does not require any other factors or the PPI activity of CypA but may involve the AIF 367–399 fragment (Fig. 13A) and one of the helices (aa. 136–146) and part of the β-barrel of CypA (29). Binding kinetics between recombinant AIF (unspecified construct) and human CypA was investigated by surface plasmon resonance in the presence of NADPH (253). Assuming that under studied conditions the flavoprotein was predominantly reduced, the derived dissociation constant (1 μM) suggests that CypA has considerable affinity for the CTC dimer. Since no experimental data on the association of CypA with oxidized AIF had been reported, we utilized *in silico* modeling to test whether the AIF redox state affects CypA binding. CypA was found to preferably dock to the oxidized monomer at two low affinity sites, distinct from that predicted by Cande *et al.* (29), and clustered in the groove above the monomer–monomer interface in the CTC dimer (Fig. 13). While biological relevance of the predicted docking sites remains to be tested, the manner of the AIF-CypA interaction corroborates the kinetic data (253) and suggests that the cyclophilin has a higher affinity for the dimeric form of the flavoprotein. Since oxidized AIF has a tendency to oligomerize when present at high concentrations (38), it would be of interest to elucidate whether the binding preference of CypA for the reduced dimers is specific or the protein can associate as tightly with the oxidized AIF oligomers. In the latter case, cytoplasmic or nuclear

accumulation/clustering of any form of AIF would facilitate CypA association and promote cell demise (see sections VII.D.3.b/c for additional information on the AIF-CypA interaction).

d. Phospholipid scramblase. Scramblases are located in the cell membrane and belong to the family of *trans*-membrane lipid transporters, also known as flippases, that transport (scramble) negatively charged phospholipids from the inner- to outer-leaflet and *vice versa*. By translocating phospholipids against the concentration gradient, scramblases can establish or diminish asymmetrical distribution of *trans*-membrane lipids. Exposure of new lipid-head groups on the membrane leaflet, in turn, can serve as a specific signal for physiological modifications [reviewed in (58)]. Externalization of PS, normally distributed in the inner monolayer of the plasma membrane, is a hallmark event in mammalian apoptosis that serves as a signal for induction of phagocytosis (147). This process is promoted by AIF *via* an unknown mechanism (136, 212).

Transport of PS to the outer-leaflet of the plasma membrane also takes place during apoptosis in *C. elegans* and involves two proteins: scramblase SCRM-1, homologous to human scramblases, and the AIF homolog WAH-1 (231). WAH-1 specifically interacts and activates SCRM-1 in a Ca^{2+}-independent manner. Further, genetic inactivation of WAH-1 diminishes PS exposure on the surface of apoptotic germ cells and compromises cell-corps engulfment. On the basis of these results, WAH-1 was proposed to mediate a mitochondrial-to-plasma-membrane signaling pathway that promotes changes on the surface of apoptotic cells (231). The 380–550 region in WAH-1 was identified as sufficient and Lys446 as critical for SCRM-1 binding (corresponding to the 270–440 fragment and Lys336 in human AIF; Fig. 14).

The results obtained with *C. elegans* should be extrapolated to humans with caution. Although mammalian AIF promotes PS externalization (136, 212), direct interaction of AIF with the plasma membrane scramblase has never been reported. Besides, there are significant structural differences between human and worm proteins. The *C. elegans* SCRM-1, for instance, is lacking part of the N-terminal proline-rich domain and may have fewer, if any, SH3-domain containing partners than the human enzyme. WAH-1, on the other hand, is missing functionally important residues corresponding to Trp195, a Glu412-Arg448 salt bridge, the active site His453, and the regulatory 509–559 insertion (murine AIF numbering; see section VI.C for details). Instead, WAH-1 contains an extended and highly charged membrane linker whose function is currently unknown (Fig. 3). Owing to these dissimilarities, the human mechanism of PS exposure and AIF-mediated signal transduction could be distinct and more complex.

e. Scythe. Scythe, also known as BAT3 (HLA-*B*-associated transcript 3), is a 120 kDa protein that contains the N-terminal ubiquitin-like domain, a central proline-rich region, the C-terminal zinc finger motif, NLS, and the BAG-1(Bcl-2-associated gene *1*)-like domain (16, 143, 219). Originally, Scythe had been identified as a regulator of apoptosis in *Drosophila* (218) and later was shown to play an important role during mammalian development. Inactivation of Scythe in mice leads to embryonic or perinatal lethality due to multiple developmental defects (56). *Scythe*$^{-/-}$ cells are defective in

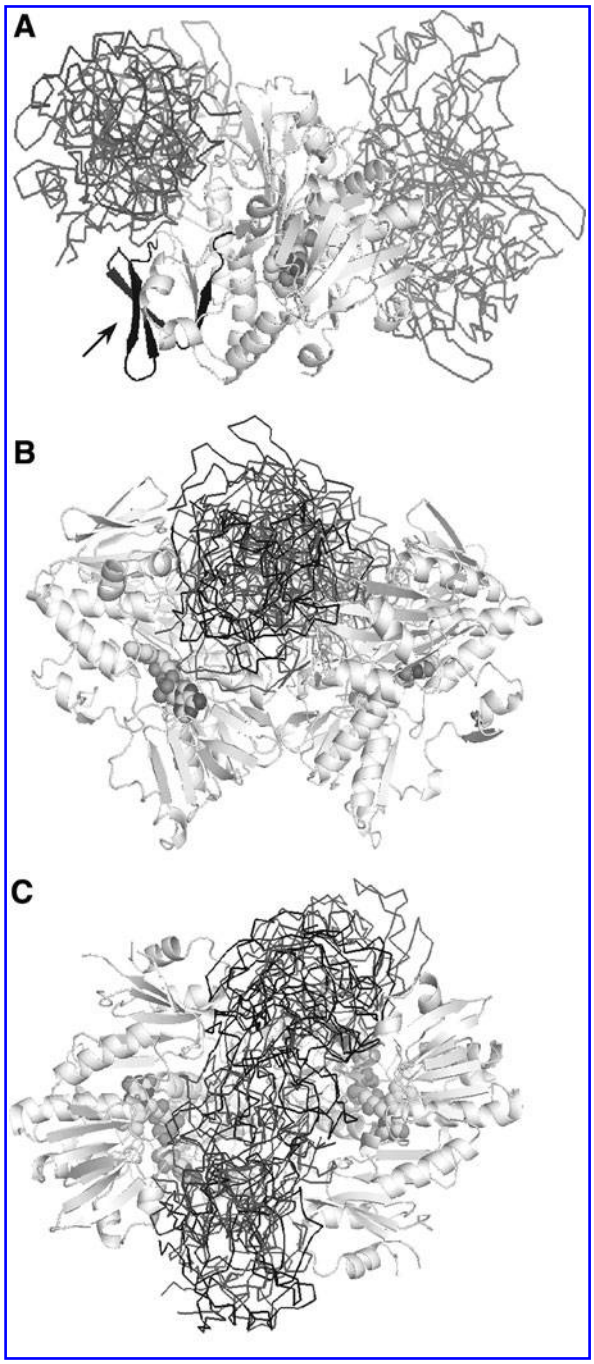

FIG. 13. Complexes between CypA (PDB code 3KOM) and the oxidized monomer (A) and reduced dimer of AIF (B, C) generated with the program GRAMM (220). AIF molecules are depicted in light gray and carton representation. Computer modeling suggests that CypA (in ribbon representation) has a higher affinity for the dimeric form of AIF and preferably binds to the grove above the monomer–monomer interface. In the oxidized monomer, two favorable CypA docking positions are predicted, distinct from the site identified by Cande *et al.* [residues 367–399 (29); shown in black and indicated by *an arrow*]. Physiological relevance of the predicted CypA binding sites and whether the AIF-CypA interaction is redox dependent or simply requires clustering and oligomerization of AIF remain to be established.

specific apoptotic signaling pathways and are resistant to apoptosis caused by thapsigargin (TG) or menadione, the endoplasmic reticulum stress inducers (56). An investigation of TG-related apoptosis in mouse ES cells revealed that (a) Scythe regulates expression and stability of the AIF precursor, (b) *Scythe*$^{-/-}$ cells become resistant to apoptosis because of decreased levels of AIF, and (c) expression of AIF can be regulated on a post-translational level and restored by reintroduction of Scythe into *Scythe*$^{-/-}$ cells (57). The Scythe-dependent regulation of AIF stability was suggested to take place in the cytoplasm *via* a mechanism that involves the proteosome and requires direct physical interaction of AIF with the N-terminal domain but not ubiquitin-like module of Scythe (57).

The cytoplasmic site for the functional association between AIF and Scythe proposed by Desmots *et al.* (57) contradicts the notion that Scythe is a nuclear protein that does not relocate during apoptosis (143). Another drawback of their study is utilization of the N-terminally green fluorescent protein-fused AIF precursor for investigating Scythe-mediated regulation of the AIF stability (54). The N-terminal tag could interfere with the mitochondrial sorting and artificially retain the AIF precursor in the cytoplasm, thus promoting interactions with the apoptotic machinery. Further studies are also necessary to clarify how the Scythe-mediated protection of a nonapoptogenic AIF precursor facilitates cell death.

In summary, upon translocation into the cytoplasm, apoptogenic AIF interacts with a group of structurally and functionally diverse proteins. These interactions are critical because they could promote, postpone, or even prevent cell death. To fully understand how the AIF-mediated signal transduction is regulated, more detailed investigations on the aforementioned partners and identification of other AIF-binding proteins are required.

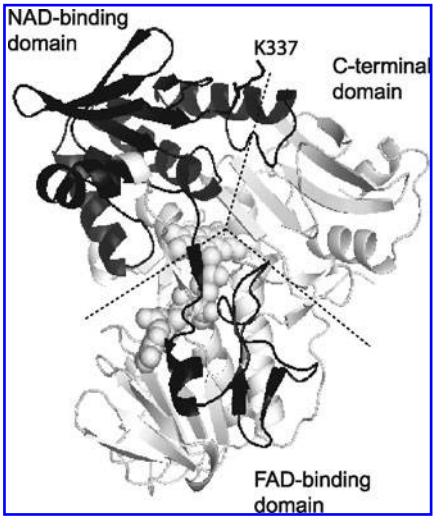

FIG. 14. A potential binding site for phospholipid scramblase derived based on the sequence homology between human AIF and *C. elegans* WAH-1 (231). The 270–440 fragment and Lys337 in human AIF (highlighted in black) correspond to the WAH-1 380–550 peptide and Lys446, which are critical for interaction with SCRM-1, a worm homolog of human scramblase. The predicted scramblase-binding site includes residues comprising the NAD(H)- and FAD-binding domains.

D. Nuclear effects of apoptogenic AIF

Whether the release of mitochondrial AIF is a primary execution step or occurs downstream of caspase activation, AIF must enter the nucleus to promote cell death (35, 46, 210, 212, 242, 244). Relocation of apoptogenic AIF to the nuclear compartment is observed in different species during various but not all types of cell death.

1. Transport of AIF to the nucleus. Cytoplasmic proteins are imported to the nucleus by nuclear transport receptors that recognize an NLS, a surface peptide that generally contains a cluster of positively charged residues. On the basis of the sequence analysis, two NLS motifs were identified in murine AIF: NLS1 and NLS2, corresponding to the 277–301 and 445–451 fragments, respectively (Fig. 1) (212). Among the two, the C-terminal ^{445}KLGRRRV^{451} peptide was proven to be the predominant NLS through which AIF is transported to the nucleus (84). The minor role of NLS1 is also evidenced by the inability of AIFsh2 and AIFsh3 to relocate from the cytoplasm to the nuclear compartment (54).

NLS2 is fully accessible in the oxidized monomer but becomes buried at the dimer interface upon AIF reduction (Fig. 4D, E; section VI.C). This means that the NLS2 accessibility to the nuclear transporters and the apoptogenic potency of AIF, in general, will depend on the cellular redox status. Besides NLS2, several positively charged surface residues collectively assist nuclear import of AIF (242). Cytoplasmic partners (Fig. 10) could also affect AIF trafficking. Accelerated relocation of human AIF R192A/K194A (242), for example, is likely caused by the perturbed association with Hsp70 (section VII.C.1.a) rather than nuclear transporters. Proteases degrading Hsp70 (32) or other AIF-binding proteins could be another potent regulatory factor. The importance of cytoplasmic interactions for the apoptogenic function of AIF is further evidenced by the ability of CypA to comigrate to the nucleus (216, 253) (Fig. 15) and mediate binding of AIF to DNA and nuclear components that promote chromatinolysis (13, 232) (sections VII.D.b/c).

2. Interaction of AIF with DNA. Genomic DNA degradation, a key feature of PCD, is a two-step process during which chromatin first condenses along the nuclear membrane and undergoes large-scale fragmentation (>50 kb; stage I), then DNA gets cleaved at the inter- and intranucleosomal sites, and apoptotic bodies are formed (stage II) (251). Both endogenous and recombinant AIF can trigger peripheral chromatin condensation and large-scale DNA fragmentation (36, 46, 75, 107, 173). There is also evidence suggesting that AIF is responsible only for stage I nuclear morphology but not large-scale DNA degradation (199, 247).

Lacking the nuclease activity, AIF is proposed to directly interact with DNA and disrupt/collapse chromatin structure by displacing chromatin-associated proteins and/or by recruiting proteases and nucleases to form DNA degrading complexes, degradosomes (210, 212). Colocalization of AIF with DNA during stage I chromatin condensation, as well as the inability of the K510A/K518A and K255A/R265A variants of human AIF to bind DNA *in vitro* and induce apoptosis in live cells are consistent with this hypothesis and indicate that direct AIF-DNA association is critical for execution of PCD (242). Concordantly, several RNA-binding proteins associating

with AIF were identified in rat brain lysates and nuclear HeLa extracts (224). One of these, heterogeneous nuclear ribonucleoprotein, interacts with AIF *via* the RNA moiety.

Refolded His-tagged AIF binds to all kinds of nucleic acids in a sequence-independent manner but preferentially associates with single-stranded DNA (224). According to another *in vitro* study, recombinant AIF binds to double-stranded DNA but fails to interact with single-stranded oligonucleotides (242). Mg^{2+} ions promote AIF-DNA association, leading to thickening and shortening of DNA fragments and formation of intermolecular complexes *via* AIF-AIF contacts (224). NAD(P)H was reported to increase the mean size of the AIF-DNA complexes, NAD had no effect, and NADP promoted generation of high-order AIF-DNA and AIF-RNA agglomerates (224). These *in vitro* results led to the proposal that AIF invades chromatin at pre-existing DNA breakage sites and cooperatively propagates through the adjacent double-stranded DNA, causing chromatin condensation and aggregation into higher order structures.

In our study (38), naturally folded and tag-free AIF interacted with linearized double-stranded DNA quite differently. Formation of the AIF-DNA complexes was observed at considerably higher AIF concentrations [10–100 μg *vs.* 1 μg (224)] and higher AIF:DNA ratios [>40:1 *vs.* 20–30:1 (224)]. The DNA-binding ability of AIF was unaffected by Mg^{2+} and NADP but markedly inhibited by NAD(P)H. The latter finding is another supporting argument for the redox dependency of the apoptogenic action of AIF. The discrepancies in the manner of AIF-DNA interaction could result from structural aberrations in the refolded flavoprotein or/and attachment of the His-tag, which increases the AIF affinity for DNA by 10-fold (38).

In conclusion, AIF is transported from the cytoplasm to the nuclear compartment predominantly *via* NLS2 and with the assistance of CypA. In the nucleus AIF initiates chromatin condensation likely *via* direct interaction with DNA. Both nuclear transport and interaction with DNA may be redox controlled because the NLS2 accessibility and DNA-binding ability depend on the AIF redox state. Further clarification is needed on whether AIF participates in large-scale chromatin degradation or not.

3. Nuclear partners of AIF

a. Endonuclease G. Endonuclease G (EndoG) is an evolutionary conserved, nonspecific nuclease that cleaves single- and double-stranded DNA and RNA in an Mg^{2+}/Mn^{2+}-dependent fashion [reviewed in (237)]. Mammalian EndoG is a nuclear-encoded protein targeted to mitochondria, the 29 kDa mature form of which is compartmentalized in the IMS and implicated in mitochondrial DNA replication and apoptosis. Upon an apoptotic insult and release from the IMS, EndoG is activated and imported to the nucleus. In isolated nonapoptotic nuclei, EndoG first generates large fragments of DNA (>50kb), then cleaves at inter- and intranucleosomal sites.

In *C. elegans*, the EndoG homolog Csp-6 cooperates with WAH-1, the worm's homolog of AIF (Fig. 3; sections III and VII.C.2.d), in DNA degradation during apoptosis (232). The 196–700 fragment of WAH-1 (corresponding to human AIFΔ1–101) was shown to directly interact with Csp-6 and promote chromatinolysis *in vitro* and cell demise *in vivo*. On the basis of the similarity in cellular localization and apoptotic effects caused by AIF and EndoG and their worm homologs, the AIF/EndoG pair was proposed to define an evolutionary conserved DNA degradation pathway (232).

However, there is evidence that does not support this hypothesis. First, the WAH-1 deficient worms are vital although they grow slower and are smaller in size. Unlike mammalian AIFs, crucial for cell survival (section VIII), downregulation of *WAH-1* expression by RNAi has little effect on the fate of the cells undergoing apoptosis but enhances the cell survival phenotypes of mutants that are partially or strongly defective in cell death. This indicates that WAH-1 normally promotes cell demise. Second, release of WAH-1 from mitochondria strongly depends on caspase activity and, thus, may be part of the caspase-dependent apoptotic cascade. The lack of a regulatory insertion in WAH-1 is another indication that signal transduction in the nematode may proceed *via* different pathways. Third, although WAH-1 is predicted to be a flavoprotein (Fig. 3, section III), the investigated 196–700 fragment does not contain FAD (232) and could be misfolded. Next, there is no direct evidence for cooperation between AIF

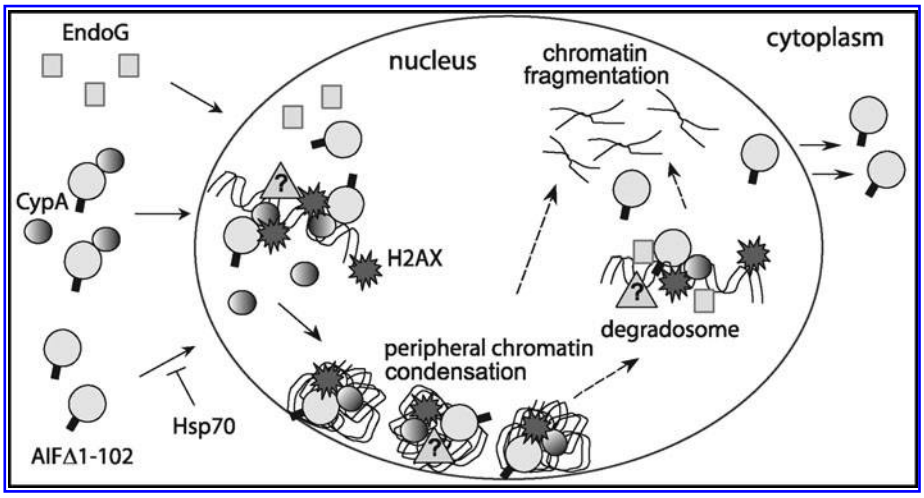

FIG. 15. Nuclear effects of apoptogenic AIF. AIF is transported from the cytoplasm to the nucleus by nuclear transport receptors recognizing NLS1 and NLS2. CypA assists and Hsp70 prevents cytonuclear translocation of AIF. In the nucleus, AIF directly binds to DNA and in cooperation with other proteins causes peripheral chromatin condensation and, possibly, large-scale chromatin fragmentation. Histone H2AX and CypA cooperatively promote AIF-mediated nuclear apoptosis. Co-operation between AIF and endonuclease G was suggested but has not been proven thus far. Other unidentified proteins (indicated in the figure as triangles with a question mark) may interact with AIF as well, forming a DNA degrading complex, "degradosome." Upon chromatinolysis, AIF might dissociate from oligonucleosomal DNA and translocate back to the cytoplasm.

and EndoG in DNA degradation during apoptosis in mammalian cells. Contrarily, one study showed that during caspase-independent noise-induced cell death in the inner ear, only EndoG translocates to the nucleus, whereas AIF is retained in the cytoplasm (240). Finally, EndoG null mice have no obvious defects in embryonic development and regulation of apoptosis (51), which also argues against the essential role of EndoG in PCD. Thus, the evolutionary conservation of the EndoG/AIF-mediated DNA degradation pathway in higher eukaryotes remains an open question.

b. Cyclophilin A. Presently, it is unclear whether cyclophilins possess or lack nucleolytic activities (145, 158). Nonetheless, recombinant CypA was demonstrated to cooperate with AIF *in vitro* in chromatinolysis and plasmid DNA degradation (29). That two proteins act synergistically is also evidenced by the facts that (a) CypA colocalizes with endogenous AIF in isolated nuclei during the initial stage of chromatin condensation, (b) transfection of $CypA^{+/+}$ but not $CypA^{-/-}$ Jurkat cells with AIF increases frequency of staurosporine-induced apoptosis, and (c) the difference between CypA-sufficient and -deficient cells disappears when *AIF* expression is downregulated (29). The CypA- or DNA-binding domains of AIF (Δ269–442 and Δ263–399 or Δ473–552 and Δ567–609, respectively) but not the PPI activity of CypA are necessary for induction of apoptosis in $CypA^{+/+}$ cells (29).

In our *in vitro* study, CypA had no effect on the DNA-binding ability of AIF, and the two proteins did not display any nuclease activity individually or in cooperation (38). It is unclear at the moment whether these functional dissimilarities are caused by aberrations in the AIF folding, covalent tag attachment, or contamination of CypA preparations with nucleases. Importance of the AIF-CypA interaction in triggering and promoting apoptosis is undermined also by the finding that CypA-deficient mice have no defects in cell death (41).

c. Histone H2AX. One possible role of CypA in the nucleus could be facilitation of AIF association with the proteins promoting chromatinolysis, in particular histone H2AX (13) (Fig. 15). As an integral component of the chromatin packaging, H2AX contributes to genome stability by participating in the assembly of the DNA damage repair foci [reviewed in (176)]. This H2AX function is regulated through post-translational modification. In response to treatments causing double-strand DNA breaks, the histone is rapidly phosphorylated at Ser139, which modifies chromatin and makes it accessible for repair. Phosphorylated H2AX (γH2AX) is known to regulate caspase-dependent apoptosis *via* the caspase-activated DNase pathway (138).

Artus *et al.* demonstrated that H2AX is also involved in caspase-independent necrosis *via* association with AIF (13). Interaction between H2AX and AIF is observed only in cells treated with the DNA alkylating agents that cause double-strand DNA breaks, such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) (13). The proline-rich peptide of AIF (aa. 543–559) and phosphorylation of Ser139 in H2AX are required for their association. The AIF-H2AX interaction is essential for AIF-mediated chromatin condensation and degradation, as deletion of the proline-rich fragment in AIF and knockout of *H2AX* abolish chromatinolysis and nuclear apoptosis (13). The MNNG toxicity and all hallmarks of nuclear apoptosis are also eliminated when the *CypA* gene is silenced. This suggests that a synchronized action of AIF, H2AX, and CypA is necessary for initiation of nuclear DNA degradation. Downregulation of *EndoG*, on the other hand, has no effect on the AIF/H2AX-dependent chromatinolysis (13), which undermines once again the importance of the AIF/EndoG-mediated PCD pathway in mammalian cells.

It would be of interest to investigate whether the AIF redox state affects the AIF-H2AX interaction. If the proline-rich fragment is indeed a key element through which AIF binds to H2AX, association between the proteins should be redox controlled. Elucidation of the phosphorylation status of H2AX Tyr142 during AIF-mediated necrosis is also important because after DNA damage, the fate of the cell is determined by the Tyr142 but not Ser139 modification (43).

4. Relocation of AIF in late apoptosis. While association of apoptogenic AIF with the chromatin in cells undergoing early apoptosis is well documented (36, 46, 75, 107, 173, 247), little is known about what happens to the protein during the later stages of PCD. One of the first reports on AIF and a recent immunofluorescent study indicate that during stage II of nuclear apoptosis, when DNA is degraded to small oligonucleosomal fragments, colocalization of AIF with the chromatin is diminished (199, 242). It was observed also that during the late stages of etoposide- and actinomycin-induced apoptosis in HeLa cells, AIF relocates from the nuclei of karyorrhexic cells back to the cytoplasm and mitochondria (199). If confirmed, physiological relevance of this relocation should be investigated.

To summarize, AIF does not possess nuclease activities and can trigger DNA degradation only *via* co-operation with other proteins such as histone H2AX and CypA. EndoG is another potential partner but its involvement in AIF-mediated chromatinolysis in mammals has not been demonstrated yet.

E. Apoptogenic properties of the AIF homologs

As emphasized in sections VII.A–D, the mechanism of AIF-mediated apoptosis in mammalian cells is complex and, despite extensive research, is not fully understood. Comparative analysis of effects caused by downregulation of AIF in higher and lower eukaryotes helps better understand structure/function relations and cellular roles of the flavoprotein. In addition to the *C. elegans* WAH-1 (section VII.D.3.a), apoptogenic properties of four other AIF homologs have been investigated. The *D. melanogaster* and *D. discoideum* proteins are closely related to mammalian AIFs, whereas the *Tetrahymena* and *S. cerevisiae* counterparts are more distant on the evolutionary tree.

1. *D. melanogaster*. As in mammals, the PCD in *D. melanogaster* involves mitochondrial remodeling (83) that may lead to caspase activation or proceed in a caspase-independent manner (8, 152). The fly's homolog of AIF (DmAIF, 50% identity and 68% similarity) contains all structural elements characteristic for mammalian AIFs, including the N- and C-terminal insertions (Fig. 3). When transfected into live cells, the full-length DmAIF precursor is targeted to mitochondria, wherein the 178–674 fragment (corresponding to human AIFΔ1–120) is diffusely distributed in the cytoplasm (102). However, in the cells undergoing apoptosis triggered by UV/γ-irradiation or overproduction of the lethal protein

GRIM, DmAIF remains compartmentalized in mitochondria (102). This raises doubts over the DmAIF ability to mediate PCD similarly to mammalian counterparts. One reason for the inability to transport from mitochondria could be the lack of a canonical PEST motif in DmAIF (Fig. 3). As discussed in section VII.B.1, PEST motifs regulate interaction of calpain proteases with protein targets (204, 229). Having a distinct C-terminal insertion, flies might be resistant to calpains.

The apoptogenic potential of extramitochondrial DmAIF was evaluated on transgenic flies that were expressing in the eye either full-length or Δ1–177 DmAIF fragments (102). Enforced expression of truncated DmAIF leads to a marked reduction in eye size and disruption in ommatidial architecture caused by ectopic cell death. This phenotype is also observed in the mutant fly lines deficient in caspases or caspase activators, and could be partially ameliorated by expression of caspase inhibitors Diap1 and Diap2 (*Drosophila* IAPs; homologous to human XIAP). On the contrary, coexpression of the baculoviral caspase inhibitor p35 enhanced the phenotypic changes induced by DmAIFΔ1–177 both in the eye and dorsal and thoracic bristles. Thus, extramitochondrial DmAIF can promote cell demise and alter tissue morphology in a manner that, in part, involves caspase proteases.

Drosophila thioredoxin-2 (DmTrx-2) was shown to be a natural mediator of DmAIFΔ1–177 cytotoxicity (102). Homologous to human thioredoxin, cytoplasmic DmTrx-2 participates in peroxide metabolism and redox signaling (18). Downregulation of *DmTrx-2* abolishes the eye-damaging effects caused by DmAIFΔ1–177 overexpression and reverses to normal eye architecture. It was concluded therefore that the two proteins cooperate *in vivo* to facilitate PCD (102). That DmTrx-2 coimmunoprecipitates with endogenous murine AIF in ES cell lysates, as well as direct interaction between murine thioredoxin and AIF detected by yeast two-hybrid screening (102) support the interpretation that the thioredoxin-AIF partnership may be evolutionary conserved.

2. *D. discoideum*. *Dictyostelium*, also known as slime mold, undergoes a complex developmental process within its lifetime, transitioning from a collection of unicellular amoebae into a multicellular slug and a fruiting body. The cell death phenotype in *D. discoideum* is highly similar to that of multicellular animals and plants, and includes ΔΨm dissipation, plasma membrane PS exposure, intense vacuolization, large-scale DNA fragmentation, autophagy, and engulfment of apoptotic corpses by neighboring cells (11, 44). The slime mold homolog of AIF (DdAIF, 30% identity and 60% similarity) translocates from mitochondria to the nucleus during initiated or developmental cell death and triggers chromatin condensation and fragmentation in a caspase-independent fashion (11). DdAIF-containing cytosolic extracts induce condensation and partial degradation of DNA not only in healthy nuclei isolated from the slime mold but also in nuclei obtained from mammalian cells.

This similarity of action could arise from the common structural fold and functional elements shared by mammalian and *Dictyostelium* AIFs (Fig. 3). However, DdAIF does not contain a regulatory peptide but has a canonical DNA-binding motif (aa. 501–523; Fig. 16A) (11). In mammalian AIFs, the corresponding helix-turn-helix is surface-exposed and has the Asp-Gly-Glu cluster instead of basic Lys-Arg-Arg (Fig. 16B). This makes the surface more acidic and less suitable for DNA binding. An identical substitution is present in *D. rerio*, whereas in other species the turn between helices contains at least one basic residue (Fig. 16A). This suggests that the AIF ancestors were binding to DNA stronger. The lesser DNA-binding ability of mammalian AIFs may be beneficial as it enables to slow down and finely tune the apoptotic cascade.

3. *Tetrahymena thermophila*. *T. thermophila* are ciliates that have two functionally and morphologically distinct nuclei: a small diploid micronucleus and a large polyploid macronucleus responsible for reproduction and general cell regulation, respectively. Sexual reproduction in ciliates involves conjugation and programmed nuclear death, during which the parental macronucleus is eliminated (52). Degradation of the macronucleus proceeds in three stages that include large-scale DNA fragmentation (stage I), chromatin condensation and oligonucleosomal DNA fragmentation (stage II), and lysosomal autophagy (stage III).

The *Tetrahymena* homolog of AIF (TtAIF, 24% identity and 45% similarity) plays an important role in DNA degradation during early stages of programmed nuclear death (2). TtAIF contains MLS and NLS but lacks two insertions characteristic for mammalian AIFs. TtAIF is expressed continuously during conjugation and translocates to the parental macronucleus before nuclear differentiation. Δ*AIF Tetrahymena* strains have reduced growth rates and can initiate normal nuclear events during mating, although condensation and large-scale DNA fragmentation in the parental nuclei are delayed (2). Further, mitochondria isolated from Δ*AIF* cells display a considerably lower DNase activity relative to controls. These findings indicated that TtAIF is involved but not crucial for the initial stages of nuclear degradation, during which it may cooperate or activate a yet-unidentified mitochondrial DNase similar to EndoG. Unfortunately, it has not been investigated whether TtAIF mediates cell demise. Presently, *Tetrahymena* appear to employ TtAIF for a very specific task: elimination of a parental macronucleus, which requires precise temporal regulation of TtAIF expression, proteolysis, and translocation. It would be worthwhile to investigate how the TtAIF-mediated cascade is put into action. The mechanism by which two nuclei are distinguished is also intriguing.

4. *S. cerevisiae*. Aif1p, a yeast homolog of AIF (22% identity and 41% similarity), does not contain MLS and a membrane-binding fragment but associates with mitochondria and transports to the nucleus of yeast cells during H_2O_2-induced or physiologically induced apoptosis (*i.e.*, chronological aging) (239). Aif1p is required for efficient apoptotic cell death in budding yeast. In synergy with mild oxidative stress, overexpression of Aif1p stimulates cell demise and leads to chromatin condensation and DNA fragmentation mostly in a caspase-dependent fashion. Further, recombinant Aif1p can degrade DNA in isolated nuclei. This activity does not require binding of FAD but may depend on other cofactors *in vivo*. The yeast homolog of human CypA is one of the proteins that promotes Aif1p-induced apoptosis (239). Similar to the human AIF-CypA pair (29), the cooperative apoptogenic action of yeast Aif1p and CypA does not require the cyclophilin's PPI activity.

In summary, a strong ability to promote PCD and high similarity of the apoptogenic effects exerted by the AIF-like proteins suggest that the mitochondrion-initiated apoptotic

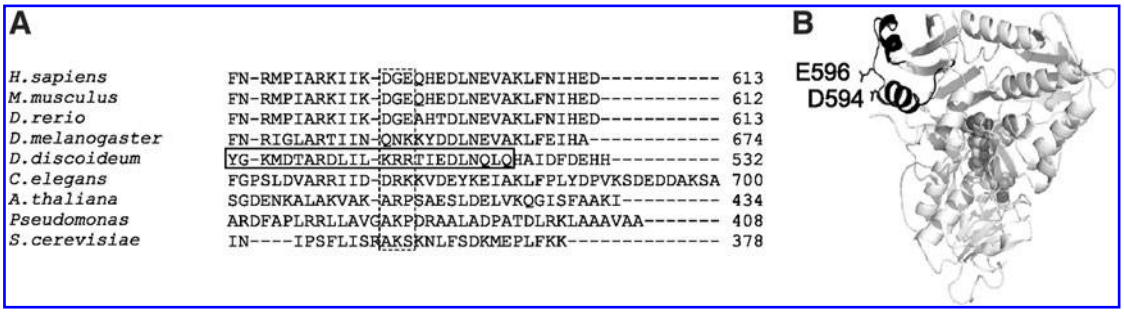

FIG. 16. (A) The C-terminal sequence alignment of the AIF-like proteins and (B) the x-ray structure of human AIF. (A) A DNA-binding helix-turn-helix motif predicted in *D. discoideum* is boxed. Residues corresponding to the ^{512}Lys-Arg-Arg^{514} cluster in *D. discoideum* are also indicated. **(B)** The 595–605 peptide corresponding to the DNA-binding motif in *D. discoideum* is displayed in black. Instead of the basic cluster predicted to comprise the turn between helices in *D. discoideum,* human AIF has ^{594}Asp-Gly-Glu^{596}, which makes the surface more acidic and less suitable for DNA binding.

DNA degradation pathway involving AIF is phylogenetically old and conserved among eukaryotes (28). Similar to mammalian counterparts, the AIF-like proteins act in cooperation with the cytoplasmic and nuclear partners analogous to thioredoxin, CypA, EndoG, and possibly others. However, owing to structural and functional differences in AIFs, the PCD execution mechanisms may vary among species. During evolution, mammalian AIFs acquired the regulatory peptide and lost the DNA-binding motif. These features may be beneficial because they allow to slow down the apoptotic cascade and introduce additional regulatory steps through which the cell can more precisely regulate its demise.

VIII. Vital Functions of Mitochondrial AIF

Over the past years, compelling evidence has emerged to indicate that in addition to the apoptogenic function mitochondrial AIF has an essential pro-survival role. The precise role of the flavoprotein in the IMS, however, remains a puzzle. One obstacle to solving this problem is the difficulty in separating vital and lethal actions of AIF, as some of the cell-death-inducing effects may result from removal of the protein from the mitochondrial compartment. Analyses of phenotypes associated with the AIF deficiency and defects in cells, animal models and human patients strongly suggest that the flavoprotein plays an important role in regulation of mitochondrial morphology and energy metabolism.

A. Role of AIF in mitochondrial respiration

The most straightforward way to analyze function(s) of a protein would be its genetic inactivation through gene knockout. Homozygous *AIF* knockout in mice is embryonically lethal. The death of embryos was first reported to be the result of abolishment of the first wave of developmental cell death during early embryogenesis (105). This conclusion was later revised, as it was demonstrated that AIF is not required for cavitation and apoptotic cell death in early mouse embryos (25, 103). Moreover, the cavitation defects in $AIF^{-/y}$ ES cells were found to be secondary and caused by abnormalities in the function of respiratory complex I (66).

1. *Hq* mouse phenotype. An array of metabolic changes caused by AIF deficiency *in vivo* was first identified in *Hq* mice, a rodent strain that has 80% decreased AIF expression due to a retroviral insertion into the first intron of the *AIF* gene. Both *Hq*/Y males and *Hq*/*Hq* females are viable and do not exhibit obvious phenotypic alterations at early age. However, adult animals exhibit lack of hair and develop progressive neurodegeneration, ataxia, and blindness caused by retinal degeneration (120), but are resistant to a progressive weight gain and lipid accumulation during high-fat feeding (180). AIF-deficient neurons and cardiomyocytes are sensitized to peroxides and ROS-induced damage (120, 225). Further, AIF ablation leads terminally differentiated neurons to abortive cell cycle re-entry (120) and reduces oxidative phosphorylation (OXPHOS) in the brain and retina, but not in the liver and the heart, by diminishing expression of complex I subunits (223).

On the basis of strong correlation between AIF deficiency and oxidative stress, mitochondrial AIF was proposed to act as an antioxidant that plays a role in OXPHOS and assists free radical scavenging (120, 223, 225, 226). This conclusion contradicts *in vitro* data showing that AIF does not possess antioxidant activities (38) (section V.C). Concordantly, experiments on isolated brain mitochondria from wild-type and *Hq* mice demonstrated that AIF does not directly modulate ROS release, and the organelles can tolerate a 90% decrease in AIF and >40% in complex I without apparent changes in the mitochondrial ROS production (37). These findings indicated that oxidative stress in the AIF-deficient *Hq* brain is a consequence rather than a cause of mitochondrial dysfunction.

2. Tissue-specific AIF defects. Results obtained from genetic and RNAi knockdown models of AIF deficiency in various cells are inconsistent and suggest that downregulation of mitochondrial AIF elevates (7), decreases (180, 221), or has no effect on the cellular ROS levels (223). A general feature of cells lacking AIF is high lactate production and increased dependence on glycolytic ATP generation due to an altered oxidative metabolism (223). A 50% decrease in complex I activity is detected in both $AIF^{-/y}$ ES and AIF siRNA HeLa cells, whereas a proportional decrease in complex III activity is observed only when AIF expression is eliminated but not downregulated (223). Defects in the OXPHOS activity are caused in part by decreased expression of complex I subunits NDUFB7, NDUFS7, NDUFA9, and Grim19 and, to a lower extent, complex III subunits UQCRC1, UQCRC2, and UQCRFS1 (7, 223). Ablation of AIF also affects the NADH

oxidase activity of complex I (223), indicating that there are defects both in the assembly and function of the enzyme. However, AIF-negative cells have no perturbations in expression of mitochondrial DNA-encoded respiratory chain subunits and functioning of complexes II, IV and V, PTP, or translocator of the outer membrane (223). Further, the AIF null cells have normal levels of oxidized cardiolipin, reduced glutathione, NAD(P)H, quinone-dependent, and superoxide dismutase activities (223). These findings are consistent with the interpretation that *AIF* knockout does not lead to oxidative stress.

Mice with cardiac- and skeletal-muscle-specific *AIF* knockout develop severe dilated cardiomyopathy and skeletal atrophy (103). A comprehensive metabolic analysis showed that the animals have reduced complex I-derived electron transfer and decreased ROS generation through increased coupling in the respiratory chain (180). Consequently, these functional changes improve insulin sensitivity and upregulate glucose uptake machinery, prevent diet-induced obesity and diabetes, and cause metabolic shifts in ATP, NAD, and AMP. Mice lacking AIF only in cortical neurons have defective cortical development and reduced neuronal survival due to defects in mitochondrial respiration (35). Depletion of AIF in the insulin-producing β-cells, on the other hand, results in an age-dependent mass decrease caused by misregulation of the cell cycle control and arrest in the G2 phase (197).

Despite in-depth *in vivo* analyses of metabolic changes caused by AIF ablation, the precise role of the mitochondrial flavoprotein remains elusive. A crucial role of the AIF redox activity for normal mitochondrial functioning is evidenced by the fact that only expression of full-length AIF can restore defects in complex I and the cell growth supportive function in $AIF^{-/y}$ cells (221, 223). Additionally, the flavoprotein was proposed to regulate the OXPHOS function and energy homeostasis indirectly, by acting as an assembly factor that assists biogenesis and/or stabilizes multisubunit complexes I and III (223). This hypothesis is mainly based on the findings that AIF does not tightly associate with any of the respiratory complexes and has no effect on their transcription at the mRNA level (223).

3. Role of AIF in neurodegeneration, neurogenesis, and neuroprotection. *In vivo* studies consistently demonstrate that AIF targeting has the largest effect on neurons. Having a high demand for energy and being unable to switch to anaerobic glycolysis when OXPHOS is limited, neurons are more dependent on mitochondrial metabolism than other types of cells. AIF deficiency in neurons leads to oxidative damage and, consequently, to neurodegeneration and blindness (35, 97, 103, 120, 225). What ROS molecules exert harmful effects is currently unknown.

Analysis of mouse embryos that had AIF deleted in the prospective midbrain and cerebellum at an early embryonic stage showed that AIF is critical for both postmitotic neuron survival and cerebellar development (97). Histological examination of the brains of these animals, which died soon after birth of unknown causes, revealed severe cerebellar hypoplasia with a marked reduction in the Purkinje and granule cell precursors. Deletion of AIF in the Purkinje cell precursors causes premature entry into S-phase and cell death *via* apoptosis, wherein the G1-S phase transition is disrupted in the precursors of granule cell. Thus, AIF is essential for neurogenesis and its loss causes cell cycle abnormalities in a neuron-specific manner. The mechanism underlying the abortive cell cycle re-entry in AIF-deficient neurons (97, 120) has not been established yet.

Predominant expression of AIF2 in the developing and adult central nervous system and retina correlates with decreased AIF-dependent neurotoxicity and, hence, may be neuroprotective (87). The *trans*-membrane fragment in brain-specific AIF2 is one residue longer and more hydrophobic than in AIF1, due to which AIF2 binds to the inner membrane tighter and is more difficult to extract. Upon MNNG treatment, AIF2 displays less relocation into the cytoplasm and, most importantly, retains AIF1 in the IMS, promoting neuronal survival (87).

In some cases, a decrease in the AIF levels can be neuroprotective as well. This effect is observed in neurons after acute injury, where AIF is the major factor mediating neuronal death [reviewed in (76)]. Further, compared to wild-type littermates, *Hq* mice display smaller infarct volumes and show dramatically reduced neuronal death in the ischemic zone after transient cerebral artery occlusion (47). The *Hq* mutation provides significant protection against photoreceptor apoptosis (91), whereas neurons carrying *Hq* and $Apaf1^{-/-}$ (apoptosis proteases-activating factor) mutations exhibit extended neuroprotection against DNA damage and glutamate-induced excitotoxicity (36). Downregulation of *AIF* by siRNA significantly reduces neuronal apoptosis induced by glutamate- and oxygen-glucose deprivation (47), and a neutralizing antibody to AIF prevents excitotoxic neuronal death (228). Thus, AIF can be beneficial or harmful for neurons, depending on physiological settings.

4. AIF deficiency in lower eukaryotes. Loss of zygotic expression of *Drosophila* DmAIF results in arrested larval growth, decreased viability, and OXPHOS defects mainly due to reduced enzymatic activities of complexes I and IV (102). Knockdown of *C. elegans* WAH-1 and *Tetrahymena* TtAIF, on the other hand, leads to a slower growth rate but has no affect on the viability of the mutants (2, 232). Compared to wild type, the Δ*Aif1p S. cerevisiae* strain exhibits a significantly longer doubling time when grown on nonfermentable carbon sources, but not in glucose-rich media (223). These findings suggest that the AIF-like proteins may also be required for optimal OXPHOS.

Summarizing, AIF targeting mostly affects cells with high demand for energy, especially neurons. Analyses of metabolic changes caused by AIF deficiency showed that a yet unidentified redox activity of AIF is crucial for functioning of the respiratory chain and energy homeostasis. Additionally, the flavoprotein is thought to act as an assembly factor that regulates OXPHOS indirectly by assisting biogenesis/maintenance of complexes I and III.

B. AIF and mitochondrial morphology

Mitochondrial respiration and metabolism are spatially and temporarily regulated by the organelle's architecture, aberrations in which may lead to metabolic and energy failures. There is experimental evidence strongly suggesting that, solely or in cooperation with other proteins, AIF is involved in regulation of mitochondrial structure.

1. Mitochondrial abnormalities in telencephalon-specific AIF$^{\Delta}$ mice. In an elegant study on the telencephalon-specific AIF$^{\Delta}$ mice (*tel* AIF$^{\Delta}$), lacking the *AIF* gene only in cortical neurons, Cheung *et al.* were able to dissociate the physiological and apoptogenic roles of AIF (35). This group showed that translocation of AIF to the nucleus rather than depletion of the protein in mitochondria is the major event in apoptotic signaling. Most importantly, they found that *tel* AIF$^{\Delta}$ neurons exhibit short and fragmented mitochondria with hyperpolarized membrane potential and abnormally dilated cristae. The altered cristae morphology and respiratory defects could be restored to normal by expression of AIF targeted to the outer leaflet of the inner mitochondrial membrane. It was suggested, therefore, that AIF regulates mitochondrial morphology and cristae formation, and that mitochondrial fragmentation could be the main cause of bioenergetic failure and reduced viability of the *tel* AIF$^{\Delta}$ neurons (35).

2. Association of AIF with the optic atrophy 1 protein. Mitochondrial cristae are connected to the periphery of the inner membrane by narrow tubular junctions, which together with mitochondrial fusion and fission define the morphology of the organelle [reviewed in (144)]. The principal regulators of mitochondrial fusion and fission are the evolutionary conserved dynamin family GTPases (181). One of these, optic atrophy 1 (OPA1), is located on the outer leaflet of the inner membrane and directly involved in mitochondrial fusion and cristae remodeling (73, 130). In humans, mutations in *OPA1* have been associated with familial autosomal dominant optic atrophy, characterized by progressive blindness due to loss of retinal ganglion cells (166).

Cells derived from the dominant optic atrophy patients display impaired complex I-dependent ATP synthesis, fragmented and balloon-like mitochondria, and 50% inhibition in mitochondrial fusion when grown in galactose medium (*i.e.*, under forced oxidative metabolism), although expression and assembly of the respiratory complexes are normal (249). The same study found that AIF forms complexes with OPA1, even when complex I is partially assembled. This led to the hypothesis that AIF and OPA1 cooperatively regulate and stabilize the respiratory chain (249). The lack of difference in the OPA1-AIF interaction in the mutant and control cell lines was explained by the fact that all investigated mutations of OPA1 were located in the regions outside its coiled-coil domain, predicted to serve as a protein–protein interaction site (249).

Besides the dynamin family of enzymes, there are other inner membrane proteins shaping mitochondria. Mitofilin (100), SH3 domain containing endophilins (111), ATP synthase (174), and proteases (64) are some of the known cristae remodelers whose connection to AIF should be explored as well.

3. AIF isoform-specific cristae morphology. What AIF isoform predominates in the inner membrane may also define mitochondrial cristae morphology. Overexpression of AIF2 in human U2OS cells increases the curvature of cristae and forces them to adopt an onion-like shape, whereas overproduction of AIF1 leads to cristae rarefaction (87). Since AIF2 is expressed only in the central nervous system and the AIF1/AIF2 ratio depends on the brain cell maturation status (87), mitochondrial architecture in neurons is likely to be dynamic and affected by both the total and relative AIF1 and AIF2 contents.

In conclusion, several studies suggest that AIF may act as a regulator of mitochondrial cristae formation, and that mitochondrial fragmentation and abnormal cristae structure observed in AIF-deficient cells could be the primary cause of bioenergetic failure. Although the underlying mechanism is presently unknown, the AIF isoform content and AIF-OPA1 interaction are proposed to be the factors that could define mitochondrial morphology.

C. Human mitochondrial encephalomyopathy linked to the AIF*Δ*201 mutation

Only one mitochondrial disorder in humans directly linked to AIF has been described thus far (81). A 1601–1603 nucleotide deletion in exon 5 of the *AIF* gene was found in two related male infants. This mutation results in expression of AIF with the Arg201 deletion (AIFΔR210) leading to progressive mitochondrial encephalomyopathy characterized by axonal sensory and motor peripheral neuropathy, severe muscular atrophy, reduced OXPHOS activities, tissue-specific mitochondrial DNA depletion, and increased levels of plasma lactate. In contrast to the *Hq* mutation in mice, where residual levels of wild-type AIF are sufficient to provide normal embryonic development and support mitochondrial respiration in young offspring (120), the Arg201 deletion causes severe and early onset mitochondrial deficiency without affecting AIF expression levels.

Patient-derived fibroblasts have fragmented mitochondria and decreased complex I, III, and IV activities when grown under forced oxidative metabolism (81). Supplementation of *AIF*$^{\Delta R201}$ cells with wild-type AIF recovers ~30%–50% of complex III and IV activity, confirming a direct link between the AIF mutation and impairment of mitochondrial respiration. Deletion of Arg201 also increases the apoptogenic potency of AIF, as evidenced by increased frequency of staurosporine-induced apoptosis in *AIF*$^{\Delta R201}$ fibroblasts relative to control, irrespective of whether z-vad.fmk was present or not.

Computer modeling provided some insights into why the ΔR201 mutation has such deleterious effects (81). Being part of the 191–203 β-hairpin (Figs. 3 and 4A), Arg201 (Arg200 in mice) establishes a salt link with Glu531 in oxidized AIF, thus, assisting folding of the regulatory peptide and limiting solvent access to the active site (Fig. 17A). By analogy with the murine protein (202), redox-induced restructuring of the regulatory peptide in human AIF is expected to release the hairpin, allowing Arg201 to form a hydrogen bond with the carbonyl oxygen of Phe205 (Fig. 17B). *In silico* modeling suggests that deletion of Arg201 shortens the hairpin and disrupts the β-turn structure. Since Arg201 defines the active site environment, as well as the length and positioning of the functionally important hairpin, the ΔR201 mutation could perturb both the folding and oxidoreductase function of AIF and, possibly, interaction with the partnering proteins.

Indeed, *in vitro* characterization showed that recombinant AIFΔR201 is structurally unstable and tends to lose FAD (81). Further, compared to wild type, the mutant reacts with NADH two-orders of magnitude faster, produces shorter-lived CTC, and has a higher capacity to bind DNA. The latter property and conformational distortions in the β-hairpin, predicted to serve as a Hsp70 binding site (84, 196) (section VII.C.1.a), explain the higher potency of the mutant to induce nuclear apoptosis. Thus, the available data suggest that the

ΔR201 mutation alters structural and functional properties of AIF, ultimately leading to destabilization of the inner membrane, defects in the OXPHOS assembly/functioning, and reduction of mitochondrial DNA content. Conformational instability of the mutant, on the other hand, could facilitate the N-terminal proteolysis and promote caspase-independent cell death. The fact that $AIF^{\Delta R201}$ and control cells have similar OPA1 content (81) does not exclude participation of AIF in mitochondrial morphogenesis.

It would certainly be worthwhile to investigate effects of Arg201 deletion in AIF in model animals and tissue-specific cells because this mutation creates unique physiological settings where expression levels and redox activity of AIF are preserved, but, nonetheless, mitochondrial morphology and OXPHOS are altered. Since one of the potential damaging effects of the ΔR201 mutation is disruption/perturbation of protein–protein interactions, the studies on the model systems might help to identify mitochondrial partners of AIF and clarify whether AIF is indeed an assembly factor or plays other roles in the IMS.

D. Involvement of AIF in regulation of cytoplasmic stress granules

Besides regulation of mitochondrial respiration and morphology, AIF controls cytoplasmic stress granule (SG) formation. SGs appear in the cytoplasm of mammalian cells under environmental stress and represent aggregates of stalled translation preinitiation complexes due to failed attempts to make proteins from mRNA [reviewed in (4, 114)]. SGs associate with the endoplasmic reticulum and are formed either to store/protect RNA from harmful conditions or serve as sites of mRNA triage by determining whether individual mRNAs are degraded or reinitiated. That AIF could act as an endogenous repressor of cytoplasmic SGs was suggested based on enhanced formation of SGs in AIF-deficient cells in response to arsenate (29, 30, 105). Since AIF does not coprecipitate with the cytoplasmic SG components, its inhibitory action on SG formation is thought to be indirect. AIF-mediated SG regulation requires the mitochondrial redox activity but not apoptogenic capacity of the flavoprotein. The Δ228–347 and Δ322–333 mutations, postulated to interfere with the FAD and NAD(H) binding (136), partially abolish the SG suppressing activity of AIF, whereas deletion of the C-terminal 567–609 fragment, indispensable for induction of apoptosis (196), has no effect (30). Therefore, it was suggested that mitochondrial AIF maintains a balance between the NAD(P)H and oxidized/reduced glutathione levels, which, in turn, directly control stress-induced SG formation (30).

IX. Possible Redox Sensing Role of AIF

As outlined throughout the review, a number of studies indicated that the apoptogenic activity of AIF is independent on its oxidoreductase function (53, 136, 155, 212, 242, 252). Indeed, the death-inducing potency of AIF is defined by the structural elements positioned on the surface remote from the redox center [*e.g.*, NLS2, the N- and C-terminal insertions, lysines 510 and 518 (84, 195, 242)], and naturally occurring flavin-free AIFsh can be apoptogenic as well (53). However, there is sufficient experimental and structural evidence allowing to suggest that both vital and lethal functions of the major FAD-containing forms, AIF1, AIF2, and AIFΔ1–102/118, may be redox controlled (38, 202).

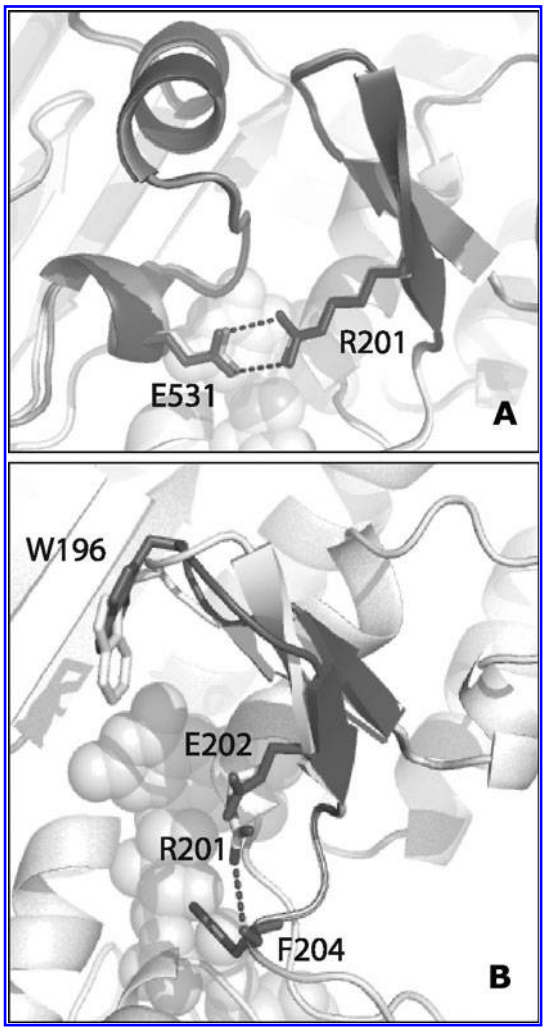

FIG. 17. Functional importance of Arg201. (A) Superposition of the x-ray structures of oxidized human and murine AIF. By forming a salt bridge with Glu531, Arg201 assists folding of the β-hairpin and the helical part of the regulatory peptide (highlighted in black), which stabilizes FAD binding. **(B)** Superposition of the reduced forms of wild-type (light gray) and ΔR201 mutant (black) of human AIF, modeled based on the structure of the murine protein. In the wild-type protein, Arg201 forms an H-bond with the carbonyl oxygen of Phe204 and limits the flexibility of the β-hairpin. Deletion of Arg201 shortens the hairpin and disrupts the β-turn structure. In oxidized AIF this may lead to weaker FAD association, whereas in the reduced form, where binding of FAD is stabilized through charge–transfer and π-π stacking interactions with NAD, the Arg201 deletion and elimination of the H-bond with Phe204 may increase flexibility of the partially unstructured 191–203 peptide and, possibly, perturb protein–protein interactions. Through these changes, the ΔR201 mutation can affect both vital and apoptogenic functions of AIF.

Ligand-induced monomer–oligomer transition is a key process in cell signaling. Reduction of AIF with NADH not only promotes protein dimerization but also leads to significant structural reorganization. As a result, AIF can exist in two functionally distinct forms (Fig. 4A, D): monomeric, where the

active site is blocked by the β-hairpin and regulatory peptides, and the NLS2 and N-terminal proteolytic sites are accessible, and dimeric, where the active site and the β-hairpin are accessible but NLS2 and one of the N-terminal proteolytic sites are not. In normal mitochondria, where reduced cofactors are abundant, the reduced AIF dimer would be the predominant form. Although it is still unknown what exactly AIF does in the IMS and whether other proteins are needed to support its function(s), severe abnormalities in mitochondrial morphology caused by defects in AIF expression or structure/function (35, 81) favor the hypothesis that the redox active dimer is part of a multiprotein complex and its enzymatic activity is critical for biogenesis/restructuring of mitochondrial membranes and assembly/functioning of the OXPHOS system (38, 202). If interaction of AIF with the membrane-associated partners is mediated by the redox-sensitive β-hairpin and regulatory peptides, the IMS redox status could control the enzymatic function through both the oxidoreduction of AIF and AIF-mediated protein–protein interactions.

Depletion of NAD(P)(H) occurs early in apoptosis and together with other metabolic changes eventually leads to mitochondrial $\Delta\Psi m$ dissipation and PTP opening (78). Presently, little is known about pyridine nucleotide homeostasis in the IMS and its relevance to the AIF function. It is evident though that mitochondrial NAD(H) can directly regulate AIF monomer–dimer equilibrium. Small fluctuations in the NAD(H) levels could transiently affect the oligomeric state and association of AIF with the IMS partners. Depletion of NAD(H), in turn, would shift equilibrium toward the inactive monomeric form, more susceptible to the N-terminal proteolysis (38). It still needs to be determined whether dimeric AIF can undergo proteolysis and pass through the OMM openings. Even if it does, it would interact with the cytoplasmic partners and mediate signal transduction differently than the oxidized monomer. The lifetime, posttranslational modifications, and interactions of AIF with the cytoplasmic partners, for instance, could be differently regulated *via* the distinctly folded regulatory peptide and β-hairpin (Figs. 4A, D and 8). Transport to the nucleus, on the other hand, could be modulated by NADH through changes in NLS2 accessibility (202) (Fig. 4D, E). Reduced dimers may possess a lesser capacity, if any, for cyto-nuclear translocation owing to unreachable NLS2. This and the lower DNA-binding ability (38, 202) make reduced AIF less apoptogenic than the oxidized monomeric form.

Via the outlined mechanism (Fig. 18), both redox and apoptogenic functions of the flavoprotein, as well as

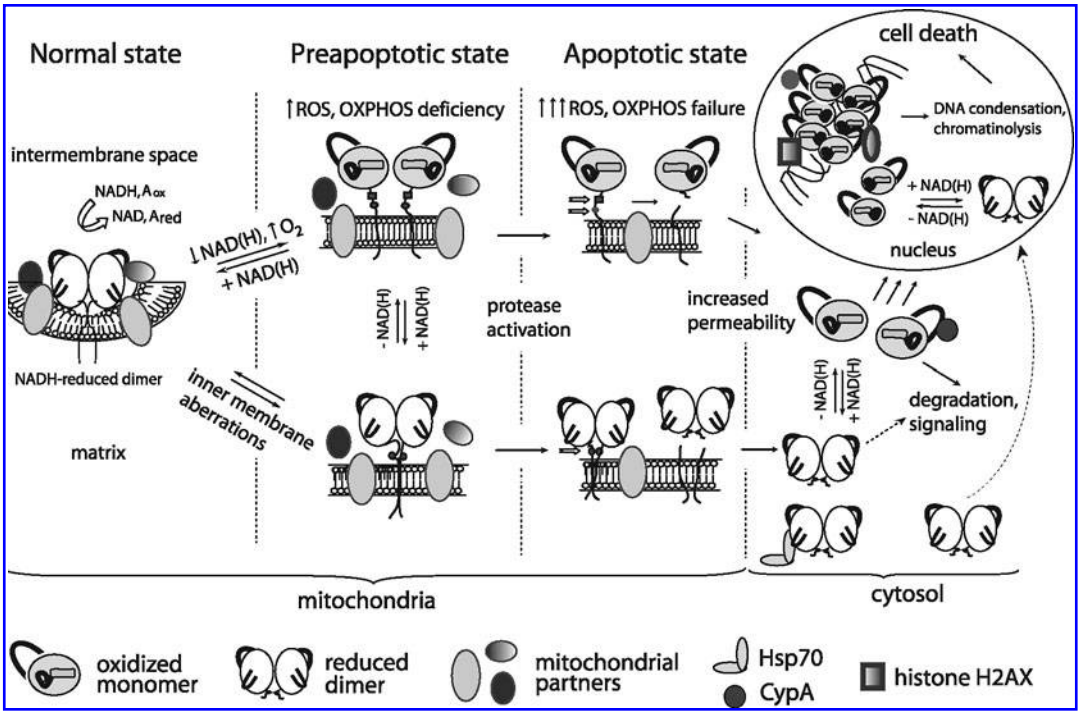

FIG. 18. Possible redox-sensing mechanism of AIF. In normal mitochondria, the reduced dimer is likely to be the predominant form of AIF and could assist cristae formation and OXPHOS functioning through a specific redox activity or/and protein–protein interactions. Fluctuations in the NAD(H) levels and aberrations in the inner membrane could lead to transient changes in monomer–dimer equilibrium and dissociation of the AIF-mediated protein complexes (preapoptotic state). Depletion of pyridine nucleotides and severe defects in the inner membrane coupled with the protease activation and OMM permeabilization would lead to AIF monomerization, proteolysis, and translocation into the cytoplasm. Owing to the distinctly folded β-hairpin and regulatory peptides (shown as bold lines) and differences in the accessibility of NLS2, the oxidized and reduced forms of AIF could recruit different cytoplasmic partners and, consequently, initiate different signaling pathways. The loss of redox active mitochondrial AIF, leading to OXPHOS failure and increased production of reactive oxygen species, may be another factor promoting cell death. If the reduced dimer does relocate to the nucleus, it will be less apoptogenic than the oxidized monomer due to the lower affinity for DNA. Existence of separate mitochondrial, cytoplasmic, and nuclear NAD(H) pools allows compartmental regulation of the AIF-mediated signal transduction and creates multilevel check points through which the cell can reverse, postpone, or promote its demise. OXPHOS, oxidative phosphorylation.

AIF-mediated signal transduction could be controlled by NAD(H) at the mitochondrial, cytoplasmic, and nuclear levels. By undergoing conformational and oligomeric state changes, AIF could respond to fluctuations in the compartmental redox status and transmit the signal to the interacting proteins, thus linking the NAD(H)-dependent metabolic pathways to apoptosis. Existence of multilevel checkpoints in the AIF-mediated apoptotic cascade is highly beneficial, as it enables the cell to reverse, postpone, or promote its demise depending on the severity of metabolic changes.

X. Concluding Remarks

Despite the tremendous progress that has been made over the past decade in AIF research, there are still some controversies, highlighted in this review, that need to be resolved. The major remaining challenge is elucidation of the vital activity of the flavoprotein and how it relates to OXPHOS functioning, ROS detoxification, mitochondrial morphology, and cell cycle regulation. Being structurally distinct from other NAD(P)H-dependent oxidoreductases, mammalian AIFs appear to operate *via* a unique redox mechanism that should be further explored. Biochemical characterization of the AIF homologs from lower eukaryotes might also be helpful in solving the AIF-related puzzles as it could provide valuable insights into the enzymatic mechanism, assist the identification of a specific redox activity, and relate structure to function. Development of new genetic animal models, on the other hand, particularly those with the $AIF^{\Delta R201}$ mutation, may help to unravel the pathophysiological mechanism associated with AIF defects and pinpoint mitochondrial proteins dependent on the AIF activity. Identification of novel cytoplasmic and nuclear partners of AIF and detailed investigations on those already identified are also critical, as they could test the proposed redox-sensing mechanism and provide deeper insights into relations between the cellular redox status and signaling networks. Considering the wide range and importance of cellular processes regulated by AIF and its partners, there is little doubt that future studies will make a significant contribution to better understanding of normal development, degenerative diseases, and carcinogenesis.

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Abbreviations Used

ΔΨm = mitochondrial *trans*-membrane potential
AIF = apoptosis-inducing factor
ANT = adenine nucleotide translocase
CsA = cyclosporin A
CTC = charge–transfer complex
CypA = cyclophilin A
eIF3g = eukaryotic translation initiation factor 3 subunit p44
EndoG = endonuclease G
ES = embryonic stem
GR = glutathione reductase
Hq = Harlequin
Hsp70 = 70kDa heat shock protein
IMS = intermembrane space
MLS = mitochondrial leading sequence
MNNG = *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine
NLS = nuclear leading sequence
OMM = outer mitochondrial membrane
OPA1 = optic atrophy 1
OXPHOS = oxidative phosphorylation
PAR = poly(ADP-ribose)
PARP = PAR polymerase
PCD = programmed cell death
PPI = peptidyl prolyl isomerase
PS = phosphatidylserine
PTP = permeability transition pore
ROS = reactive oxygen species
SG = stress granule
SH3 = Src homology 3
TULA = T-cell ubiquitin ligand
VDAC = voltage-dependent anion channel
XIAP = X-linked inhibitor of apoptosis protein
z-vad.fmk = benzyloxycarbonyl-Val-Ala-Asp (Ome) fluoromethylketone

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