

# Regulation of cytoplasmic stress granules by apoptosis-inducing factor

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## Summary

Stress granules (SG) are dynamic cytoplasmic foci in which stalled translation initiation complexes accumulate. In conditions of acute cellular redox, stress cells manipulated to lose the expression of apoptosis-inducing factor (AIF) nucleate SG signature proteins (e.g. TIA-1, PABP1) more efficiently than AIF-positive controls. AIF also inhibited SG formation induced by the RasGAP-associated endoribonuclease G3BP. Retransfection of mouse AIF into cells subjected to human AIF-specific siRNA revealed that only AIF imported into mitochondria could repress SGs and that redox-active domains of AIF, which are

dispensable for its apoptogenic action, were required for SG inhibition. In response to oxidative stress, AIF-negative cells were found to deplete non-oxidized glutathione more rapidly than AIF-expressing cells. Exogenous supplementation of glutathione inhibited SG formation elicited by arsenate or G3BP. Together, these data suggest that the oxidoreductase function of AIF is required for the maintenance of glutathione levels in stress conditions and that glutathione is a major regulator of SG.

Key words: G3BP, TIA-1, Mitochondria, Programmed cell death

## Introduction

When cells are confronted to chemical or physical stress, they can react, in principle, in two opposite ways. On the one hand they can activate defense mechanisms designed to adapt to stressful conditions, to repair damage and to resume normal cellular functions. On the other hand, they can activate the suicidal biochemical machinery leading to apoptotic demise. The choice between these two responses is dictated by the intensity of stress, as well as cell-intrinsic parameters, for instance those that set the apoptotic threshold (Garrido et al., 2003; Mosser and Morimoto, 2004; Thompson, 1995).

Stress defense mechanisms include the activation of DNA repair, as well as increased expression of inducible heat shock proteins (HSPs), a class of molecular chaperones that renature denatured proteins. Increased transcription of *hsp* genes is in part due to the activation of heat shock factor 1 (HSF-1), a transcription factor that redistributes to discrete nuclear structures, thus forming 'nuclear stress granules' or 'HSF-1 granules' (Jolly et al., 2004; Jolly et al., 1999; Sandqvist and Sistonen, 2004). In addition, cells can generate cytoplasmic 'stress granules' (SGs) in response to environmental stress (Kedersha and Anderson, 2002). Such SGs are dynamic cytoplasmic foci at which stalled translation initiation complexes accumulate. SGs appear when translation occurs in the absence of eIF2-GTP-tRNA<sub>i</sub><sup>Met</sup>, the ternary complex that normally loads tRNA<sub>i</sub><sup>Met</sup> onto the small ribosomal subunit (Kedersha et al., 2002). Stress-induced depletion of eIF2-GTP-tRNA<sub>i</sub><sup>Met</sup>, for instance as a result of inhibitor eIF-2 phosphorylation by PKR and other kinases (e.g. PERK/PEK, GCN2, HR1) or due to the lack of energy-rich

phosphates (ATP in equilibrium with GTP), allows the RNA-binding proteins TIA-1 and TIAR to promote the assembly of eIF2-eIF5-deficient pre-initiation complexes, the core constituents of SGs (Kedersha et al., 2000; Kedersha et al., 1999). As such, SGs are the morphological expression of abortive translational initiation (Kedersha and Anderson, 2002). They can be detected by following the subcellular redistribution of TIA-1 and the related protein TIAR (both normally in the nucleus), PABP-1 (the poly A-binding protein that accompanies mRNA), and small ribosomal subunits (eIF3, eIF4E, EIF4G), all of which condense into cytoplasmic foci. Another protein that redistributes into SGs (and which can induce SG formation by virtue of its RasGAP-binding activity) is G3BP, an endoribonuclease that may participate in the degradation of SG-associated RNA (Tourrière et al., 2003; Tourrière et al., 2001).

Apoptosis is morphologically defined by cellular and nuclear shrinkage (pyknosis), chromatin condensation, blebbing, nuclear fragmentation (karyorrhexis) and formation of apoptotic bodies (Kerr et al., 1972). At the biochemical level, apoptosis of mammalian cells is characterized by mitochondrial membrane permeabilization (MMP) and/or massive caspase activation (Adams, 2003; Danial and Korsmeyer, 2004; Green and Kroemer, 1998; Wang, 2002). The intrinsic (or stress) pathway leading to apoptosis involves MMP as a rate-limiting event. MMP is regulated, at least in part, by proteins of the Bcl-2 family (Zamzami and Kroemer, 2001) that are prominent apoptosis regulators. MMP causes bioenergetic failure as well as the release of potentially lethal proteins from the mitochondrial intermembrane space. Such lethal proteins include caspase

activators such as cytochrome *c*, which activates the apoptosome caspase activation complex, once in the cytosol (Wang, 2002). In addition, MMP causes the release of caspase-independent death effectors such as apoptosis-inducing factor (AIF) (Susin et al., 1999), a flavoprotein NADH oxidase (Miramar et al., 2001) that translocates to the nucleus, where it interacts with DNA (Ye et al., 2002) and forms the cyclophilin-dependent 'degradeosome', a DNA degradation complex (Cande et al., 2004; Cregan et al., 2004; Parrish and Xue, 2003).

Importantly, stress defense and apoptotic dismantling tend to occur in a mutually exclusive fashion. Thus, HSPs act as potent apoptosis inhibitors (Garrido et al., 2001; Mosser and Morimoto, 2004) whereas caspases actively destroy proteins involved in DNA repair (Creagh and Martin, 2001). Nonetheless, no information was available on the crosstalk between apoptosis and SGs. Here, we report that AIF functions as a negative regulator of stress granules. Removal of AIF by knock-out or RNA interference exacerbates SG formation. SG inhibition by AIF is mediated by the hitherto undetermined effects of AIF on GSH levels.

## Materials and Methods

### Cell culture

HeLa and ES cells were cultured in DMEM medium supplemented with 10% (v/v) fetal calf serum, 1 mM sodium pyruvate and 10 mM Hepes buffer. ES medium was supplemented with 50  $\mu$ M  $\beta$ -mercaptoethanol and leukaemia inhibitory factor (LIF, Sigma, St Louis, MO).

### Experimental conditions

Cells were treated with sodium arsenate heptahydrate (NaHASO<sub>4</sub>, 1 mM, 3 hours), *S*-nitroso-*N*-acetyl-penicillamine (SNAP, 1 mM, 3 hours) sodium nitroprusside (SNP, 1 mM, 3 hours for HeLa cells and 0.1 mM for ES cells), paraquat (2 mM, 3 hours), *tert*-butylhydroperoxide and/or Z-VAD.fmk (100  $\mu$ M, Bachem, Torrance, CA). Cells were also pretreated for 1 hour before stimulation with glutathione ethyl ester (10 mM) or *N*-acetyl-L-cysteine (15 mM). To deplete glutathione (GSH), cells were incubated with buthionine-(*S,R*)-sulfoxamine (BSO, 100  $\mu$ M, 24 hours), an irreversible inhibitor of  $\gamma$ -glutamylcysteine synthetase. All pro- and anti-oxidants were purchased from Sigma.

### Cytofluorometric determination of mitochondrial parameters

The GSH content was determined using monochlorobimane (MCB, 50  $\mu$ M) (Macho et al., 1997). The mitochondrial membrane potential ( $\Delta\Psi_m$ ) was determined with 3,3'-dihexyl-oxacarboxyanine (DiOC<sub>(6)</sub>3, 20 nM) (Zamzami et al., 1995). Autofluorescence (424 nm emission) was measured after UV excitation at 360 nm to evaluate the NAD(P)H content (Gendron et al., 2001). These fluorochromes were purchased from Molecular Probes (Eugene, OR). Cells were also stained for the detection of phosphatidylserine exposure with an annexin-V detection kit (Bender Medsystems, Vienna, Austria).

### Transfection

HeLa cells were transfected with the pcDNA3.1 vector expressing enhanced V5/HIS (Invitrogen, Carlsbad, CA), different AIF constructs fused at their C-terminus to V5/HIS (Garrido et al., 2003; Schmitt et al., 2003), G3BP-GFP (Tourriere et al., 2003), TIA-1GFP or PABP-GFP (Kedersha et al., 2000), using Lipofectamin 2000 (Invitrogen). TIA-1GFP and PABP-GFP vectors were kindly provided by Nancy Kedersha (Harvard Medical School, Boston, MA).

### Knock-down of AIF by siRNA

HeLa cells were transfected with a small interfering RNA (siRNA) double-stranded oligonucleotide designed to interfere with the expression of human AIF (sense strain: 5'-GAUCCUCCCCG-AAUACCUCTT-3', Proligo, Boulder, CO), using an Oligofectamine procedure (Invitrogen). As a control, we used an oligonucleotide designed to downregulate the non-essential gene emerlin (Harborth et al., 2001) or an oligonucleotide specific for mouse AIF (sense strain: 5'-AUGCAGAACUCCAAGCACGTT-3') that does not affect human AIF.

### Immunofluorescence

Cells were fixed with paraformaldehyde (4% w/v) in PBS. Cells were then stained for the detection of AIF (monoclonal from Santa Cruz Biotechnology, Santa Cruz, CA) or TIA-1 (goat antiserum from Santa Cruz Biotechnology), or cytochrome *c* (monoclonal from Pharmingen, San Diego, CA) or HSF1 (rat monoclonal from Upstate Biotechnology, Charlottesville, VA) and revealed with suitable anti-IgG conjugates [donkey anti-goat Alexa568 and anti-mouse Alexa488 (or 568) and donkey anti-rat Alexa568 from Molecular Probes] and counterstained with Hoechst 33342 (Castedo et al., 2002; Perfettini et al., 2004). AIF constructs were detected with V5 antibody (monoclonal from Invitrogen). Cells were viewed with a Leika DMIRE2 microscope, and images were digitally captured using a CCD-DC 300F digital camera and compiled using Adobe Photoshop® software (v5.5).

### Immunoblot

Western blot was done with HeLa sonicated extract (15  $\mu$ g) in an isotonic buffer (250 mM sucrose, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, 10 mM Tris-HCl, pH 7.4). Protein concentration was measured by means of the Bradford protein assay (BioRad, Hercules, CA). Proteins were separated in 12% SDS-polyacrylamide gel. Immunoblot analysis was realized using specific antibodies and enhanced chemoluminescence (ECL)-based detection (Pierce, Rockford, IL). The antibodies used were the mouse monoclonal anti-human HSP27 and polyclonal anti-human HSP70, (StressGen, Victoria, Canada) and mouse antibodies raised against human AIF (Pharmingen,) and raised against human GAPDH (Chemicon, Temecula, CA).

## Results and Discussion

### Deficient AIF enhances the formation of cytoplasmic SGs

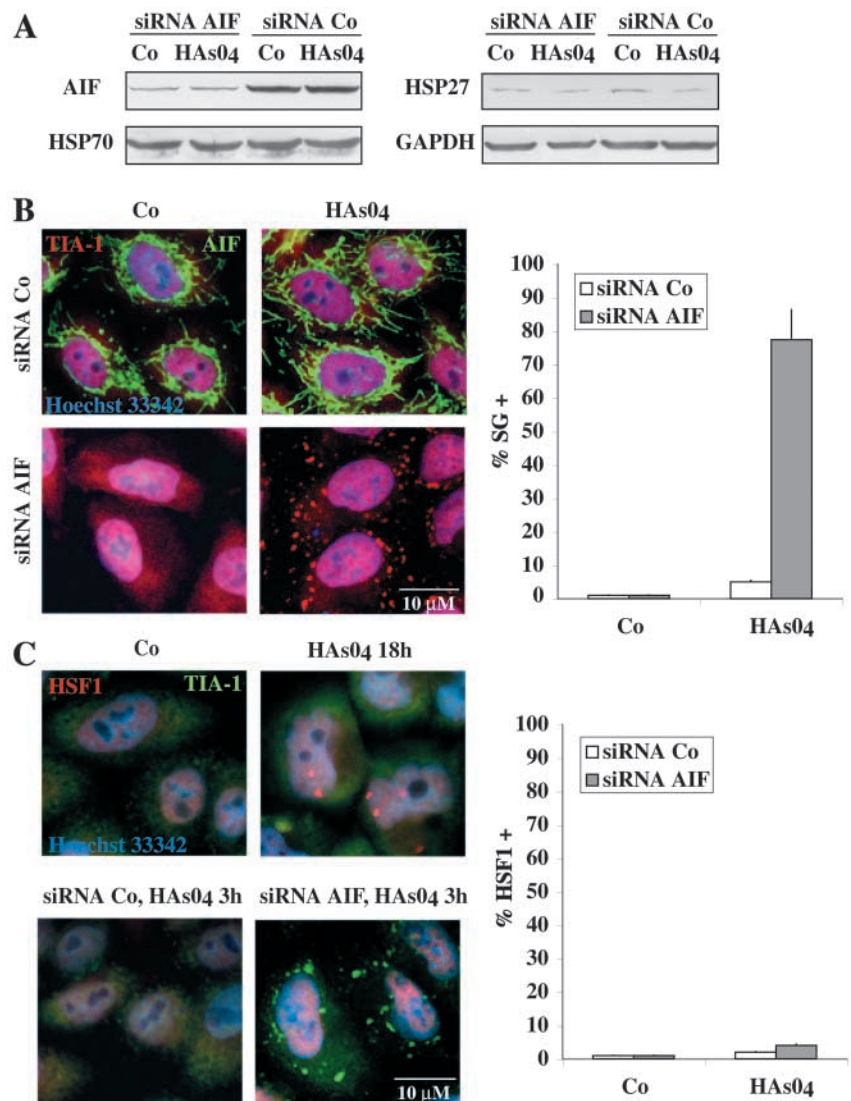
siRNA-mediated knock-down of AIF has no major effect on the abundance of the inducible HSP70 and HSP27 (Fig. 1A). By contrast, we found that AIF knock-down strongly increased the arsenate-induced SG formation, as detected by immunostaining for TIA-1, a predominantly nuclear protein that can aggregate within cytoplasmic SGs (Kedersha et al., 2000; Kedersha et al., 1999) (Fig. 1B). Co-immunoprecipitation assays (not shown) indicated that there is no interaction between AIF and cytoplasmic SG components (TIA, G3BP, not shown), and that cytoplasmic SGs did not co-localize with mitochondria (which contain AIF). Thus, the AIF effect on cytoplasmic SGs must be indirect. In the conditions in which maximum cytoplasmic SGs were induced (with 1 mM arsenate for 3 hours), no nuclear SGs (which contain HSF-1) were induced in control cells (Fig. 1C), and the absence of AIF did not stimulate the formation of nuclear SGs. Thus the effect of AIF on cytoplasmic SGs is specific. The modulation of

cytoplasmic SGs by AIF was also observed in ES cells, in which invalidation of the AIF gene (Joza et al., 2001) enhanced the formation of stress granules in response to arsenate (Fig. 2A). Similar results were obtained in HeLa cells exposed to AIF-specific siRNA (Cande et al., 2004), when the formation of arsenate-induced cytoplasmic SGs was measured in living cells, upon transfection with either a TIA-1-GFP construct (Kedersha et al., 2000) (Fig. 2B) or a PABP-GFP fusion construct (Kedersha et al., 2000) (Fig. 2C). In each case, the absence of AIF exacerbated the congregation of SG proteins in discrete cytoplasmic foci. Thus, AIF functions as an endogenous repressor of cytoplasmic SG.

### SG formation occurs independently from apoptosis

Based on the facts that AIF participates in cell death execution (Cande et al., 2002) and that arsenate can induce apoptosis (Larochette et al., 1999), we addressed the temporary and functional relationship between SG formation and apoptosis. In conditions in which arsenate (1 mM) induced massive SG formation (that is within 1-3 hours, in cells in which AIF is depleted by siRNA), mitochondria were still retaining cytochrome *c* (Fig. 3A) and maintained a near-to-normal transmembrane potential ( $\Delta\Psi_m$ ), as quantified with the  $\Delta\Psi_m$ -sensitive fluorochrome DiOC<sub>(6)3</sub> (Fig. 3B). Accordingly, such SG-positive cells lacked one of the hallmarks of apoptosis, namely phosphatidylserine exposure on the plasma membrane surface (Fig. 3C). This indicates that, when SGs are elicited by arsenate combined with siRNA of AIF, they are formed well before apoptosis is induced. In accordance with this interpretation, we found that addition of the caspase inhibitor Z-VAD.fmk did not reduce the frequency of SG-containing cells (Fig. 3D), although Z-VAD.fmk did reduce the induction of nuclear apoptosis by arsenite as an internal control of its efficacy (Fig. 3E). Similarly, no inhibitory effect of Bcl-2 overexpression on SG formation was detected (not shown). In summary, it appears that AIF functions as an endogenous repressor of SG formation independently from its capacity to modulate apoptosis.

To study further the role of AIF in SG assembly independently of its role in apoptosis we decided to induce SGs by transfection with G3BP (or a G3B-GFP fusion protein) (Tourriere et al., 2003), a protein that has been shown to have a phosphorylation-dependent RNase activity upon binding to myc mRNA (Tourriere et al., 2001), and actually acts as an endoribonuclease in stress granules (Tourriere et al., 2003). G3BP aggregates and leads to the accumulation of the 48S



**Fig. 1.** AIF deficiency enhances the formation of cytoplasmic SGs in response to HAsO<sub>4</sub>. (A) Failure of AIF to modulate the expression of inducible HSP70 or HSP27. HeLa cells were subjected to siRNA specific for human AIF (siRNA AIF) or mouse AIF (siRNA Co) for 48 hours, followed by culture in the absence (Co) or presence of HAsO<sub>4</sub> (3 hours, 1 mM), extraction of total cellular proteins and immunoblot detection of AIF, HSP70, HSP27 and GAPDH. (B) Abundance of cytoplasmic SGs. Cells treated as in A were stained for the detection of AIF (green fluorescence), the SG protein TIA-1 (red fluorescence) or chromatin (blue fluorescence). Note the accumulation of TIA-1<sup>+</sup> cytoplasmic granules in HAsO<sub>4</sub>-treated, AIF-negative cells. The histogram illustrates the percentage ( $\pm$ s.d.,  $n=5$ ) of cells accumulating SGs in the cytoplasm. (C) Failure of AIF to stimulate the generation of nuclear SGs. Cells treated as in A were stained with antibodies specific for constituents of nuclear SGs (HSF1, red) and cytoplasmic SGs (TIA-1, green). As a positive control for the generation of nuclear SGs, cells were treated for 18 (instead of 3) hours with HAsO<sub>4</sub>. Note that cytoplasmic SGs form in cells that lack nuclear SGs. Results are representative of five independent experiments yielding similar results.

preinitiation complex within SGs, but does not induce apoptosis. Upon G3BP-GFP transfection, HeLa cells that were depleted of AIF formed more SGs than control cells expressing AIF did (Fig. 4). This difference persisted when different deletion and phosphorylation mutants affecting the SG-inducing capacity of G3BP (as regulated by arsenate) were

assessed. In particular, mutant S149E, which is less effective at inducing SG formation, was also sensitive to AIF depletion (Fig. 4). Together these data suggest that SG formation and apoptosis are unrelated phenomena, although both are influenced by AIF.

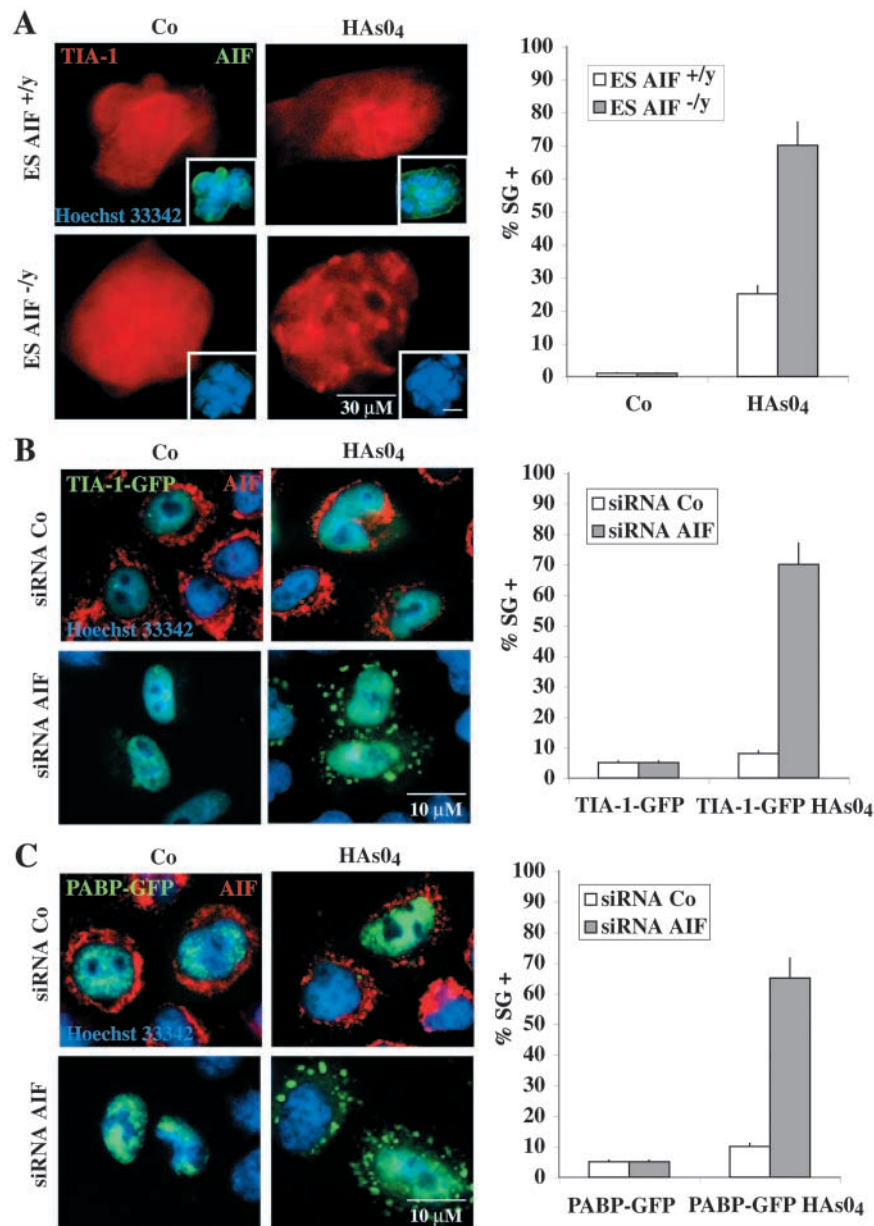
### AIF domains involved in SG regulation

When human AIF was downregulated with specifically designed RNA oligonucleotide heteroduplexes in HeLa cells, it was possible to re-transfect the cells with murine AIF constructs (which are not affected by the human AIF-specific siRNA). Although G3BP-GFP transfection induced SGs in AIF siRNA-pretreated HeLa cells cotransfected with a  $\beta$ -galactosidase ( $\beta$ -Gal)-expressing control vector, it was much less efficient in inducing SGs in cells cotransfected with full length mouse AIF, which is imported into mitochondria (Susin et al., 1999; Loeffler et al., 2001) (Fig. 5A). The SG-

suppressive effect of re-transfected AIF disappeared upon removal of the mitochondrial localization sequence ( $\Delta$ 1-100), indicating that only mitochondrial AIF can suppress SG. To map the functional region of AIF required for SG suppression, we transfected HeLa cells lacking endogenous AIF expression (as a result of human AIF-specific siRNA) with a battery of different mouse AIF deletion constructs affecting the binding domains for flavine adenine nucleotide (FAD), nicotinic adenine dinucleotide (NAD) or the C-terminus, which is required for the apoptogenic function of AIF (Loeffler et al., 2001; Mate et al., 2002; Ye et al., 2002) (Fig. 5B). In contrast to wild-type AIF, two deletion mutants effecting the NAD-binding capacity of AIF (AIF $\Delta$ 228-347 and  $\Delta$ 322-333) (Loeffler et al., 2001; Mate et al., 2002) partially lost their SG-suppressing potential (Fig. 5C), suggesting that the redox function of AIF, which is largely determined by this region (Mate et al., 2002; Miramar et al., 2001), is important for SG inhibition. By contrast, deletion of the C-terminal region (AIF $\Delta$ 567-609), which abolishes apoptosis induction by AIF (Schmitt et al., 2003), did not affect its SG-inhibitory potential. Similar results were obtained when SGs were induced by arsenate (Fig. 5D). Thus, the SG-inhibitory effect of AIF is related to the mitochondrial, non-apoptotic function of AIF and is likely to involve the protein's redox activity.

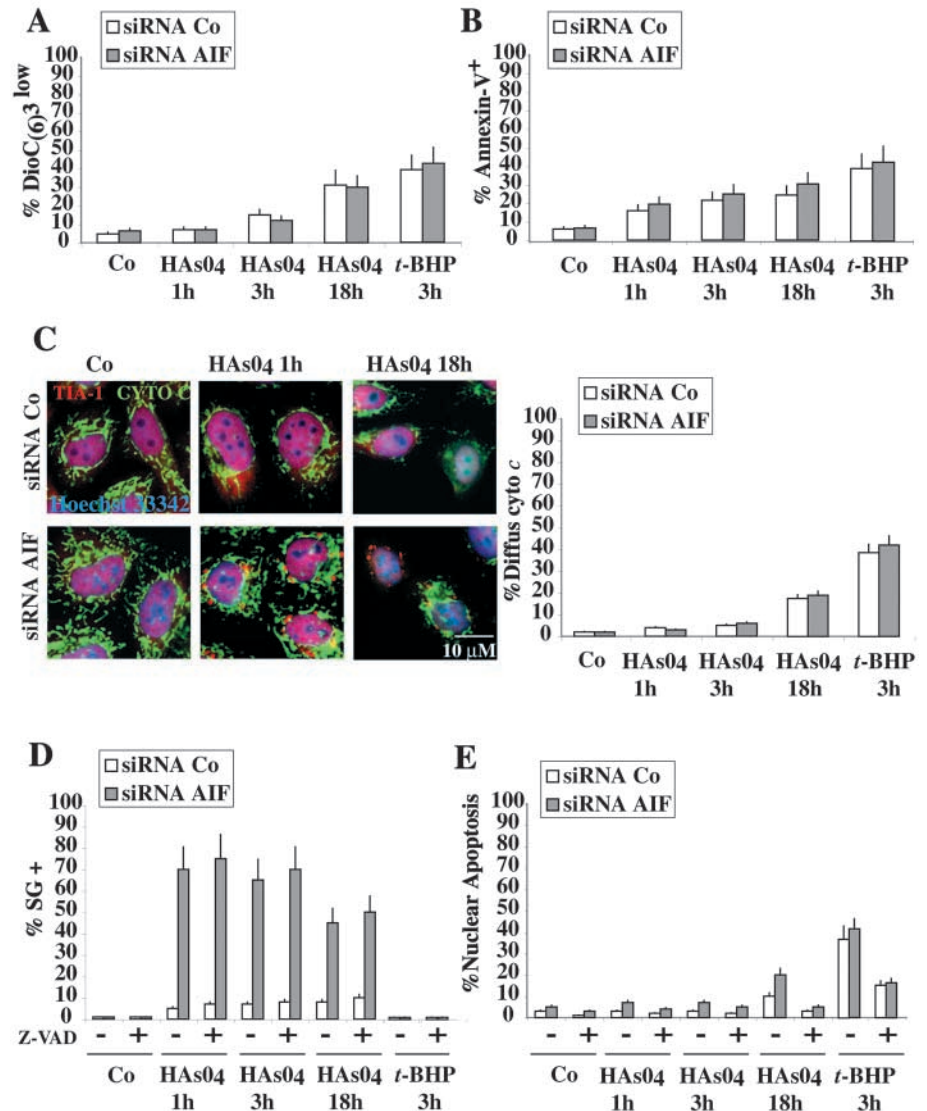
### Redox effects of AIF and their impact on SG formation

Arsenate treatment had major effects on the cellular redox metabolism. In AIF-sufficient ES cells, arsenate caused a shift in the autofluorescence, elicited at 354 nm, indicating a depletion of the pool of reduced NADH or NADPH. This arsenate-triggered NAD(P)H depletion was much attenuated in AIF-deficient ES cells (Fig. 6A). In stark



**Fig. 2.** Accumulation of cytoplasmic SGs in a variety of experimental conditions. (A) Effect of genetic invalidation of the AIF gene. Male control ES cells or cells in which the AIF gene (on the X chromosome) has been invalidated (AIF<sup>-y</sup>) were subjected to HAsO<sub>4</sub> treatment (1 mM, 3 hours), followed by fixation, permeabilization and immunostaining for the detection of TIA-1 (red) and AIF (green, shown in the insets). (B) Redistribution of TIA-1-GFP into SGs in AIF-negative cells. HeLa cells treated with siRNA for the downmodulation of AIF (or controls) for 48 hours and then transfected with TIA-1-GFP were treated with HAsO<sub>4</sub> during the last 3 hours of the experiment and then subjected to fixation and staining for AIF (red). (C) Redistribution of PABP-GFP into SGs in AIF-negative cells. The protocol was similar to Fig. 2B, with the difference that cells were transfected with PABP-GFP instead of TIA-1-GFP. Representative cells are depicted in the microphotographs and the percentage ( $\pm$ s.d.,  $n=5$ ) of SG<sup>+</sup> cells was plotted in the right panels.

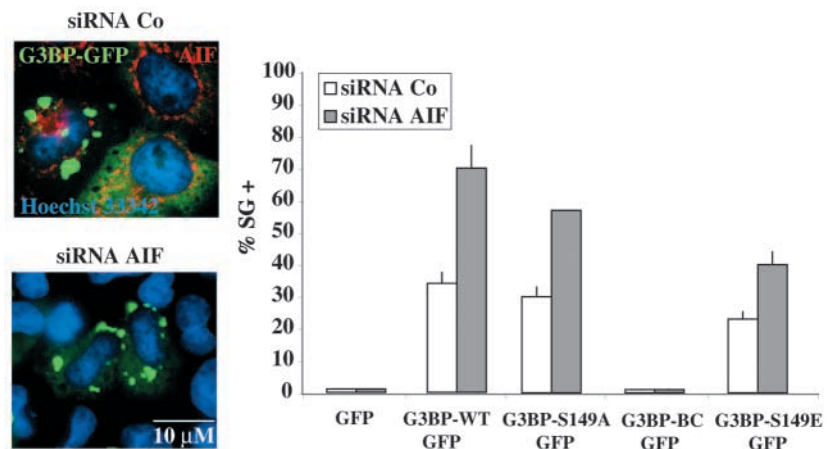
**Fig. 3.** Formation of cytoplasmic SGs occurs independently from apoptosis. (A,B) SGs form before the  $\Delta\Psi_m$  is lost and phosphatidylserine residues are exposed on the cell surface. Cells treated to obtain maximal SG formation (1-3 hours HAsO<sub>4</sub>, Figs 1, 2) do not manifest a loss of the  $\Delta\Psi_m$ , as detectable with the  $\Delta\Psi_m$ -sensitive dye DiOC<sub>6</sub>(3) (A), nor do they stain with Annexin V (B). (C) SGs form before cytochrome *c* (Cyt *c*) is released from mitochondria. After the indicated treatment, HeLa cells were stained for the simultaneous detection of Cyt *c* (green) and TIA-1 (red). Note that an 18 hour incubation period with HAsO<sub>4</sub> is required to detect diffuse or absent Cyt *c* staining in a fraction of cells. (D,E) Effect of the broad spectrum caspase inhibitor Z-VAD.fmk on SG formation. Cells were treated for the indicated period with HAsO<sub>4</sub>, in the presence or absence of Z-VAD.fmk and the frequency ( $\pm$ s.d.,  $n=3$ ), of SG+ cells (D) or cells exhibiting nuclear apoptosis (E) was determined by staining with anti-TIA-1 or Hoechst 33342, respectively.



contrast, the absence of AIF sensitized cells to the depletion of non-oxidized glutathione (GSH) (Fig. 6B). Thus, the absence of AIF shifts the cellular response to oxidative stress from NAD(P)H oxidation to GSH depletion, in line with the fact that the protein has an NADH oxidase activity (Mate et al., 2002; Miramar et al., 2001). This AIF effect was also observed in response to other inducers of oxidative stress, namely the two NO donors SNAP (*S*-nitroso-*N*-acetyl-penicillamine) and sodium nitroprusside, as well as paraquat, which induces mitochondrial oxidative stress (Costantini et al., 1995). These AIF effects on the redox balance were found both in HeLa cells subjected to AIF knock-down and in ES cells subjected to AIF knock-out (Fig. 6C-F). Next, we determined whether the maintenance of elevated

GSH levels by addition of a cell-permeable GSH ester or *N*-acetylcysteine (NAC) (Droge et al., 1994) would suppress the induction of SGs. The stimulation of SG formation by AIF was blunted by either GSH ester or NAC. This was found both

**Fig. 4.** Increased formation of G3BP-elicited SGs in the absence of AIF. Cells were treated for knock-down of AIF and then transfected with G3BP-GFP fusion constructs for 24 hours. The micrographs exemplify representative staining patterns obtained after transfection with the non-mutated G3BP-GFP construct. The histograms illustrate the quantitative difference in the formation of SG<sup>+</sup> cells as a function of AIF expression and as a result of transfections with the mutant G3BP-GFP construct. Results are expressed as the percentage of SG<sup>+</sup> cells exhibiting a GFP-dependent fluorescence ( $\pm$ s.d.,  $n=3$ ).

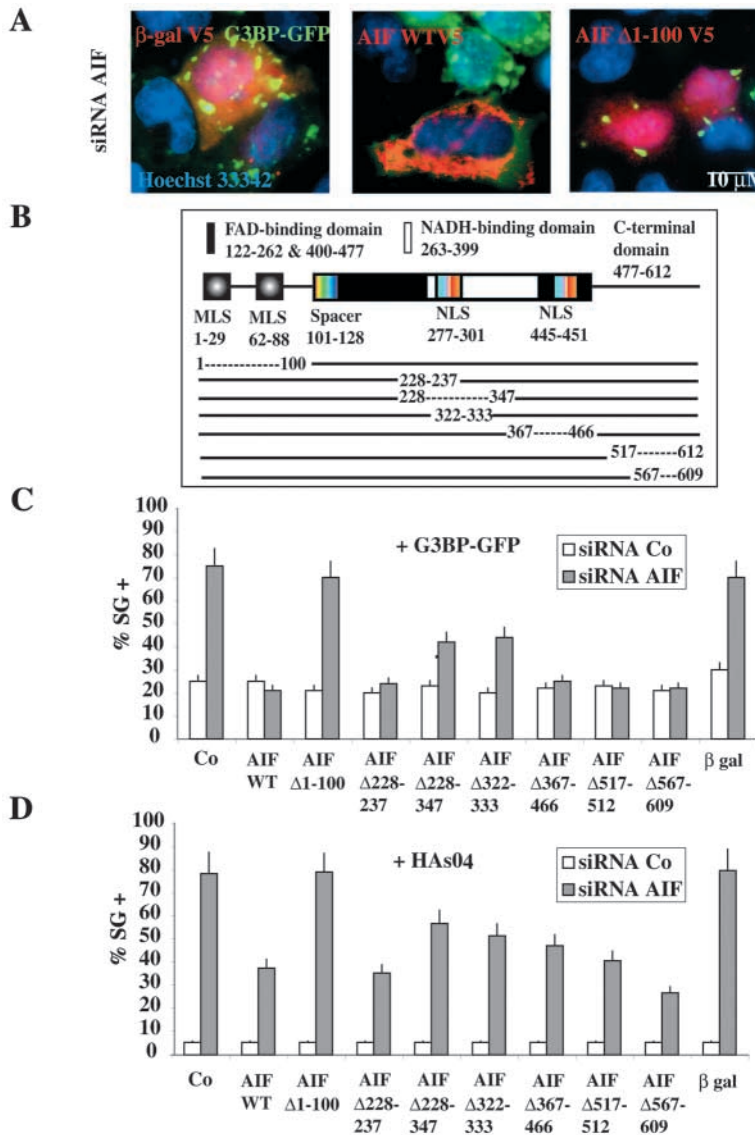


when SGs were induced by arsenate (Fig. 7A) and when SGs were stimulated by G3BP-GFP (Fig. 7B). Thus, it is likely that GSH is (one of) the endogenous repressor(s) of SG formation and that AIF acts indirectly to repress SG aggregation, by maintaining normal GSH levels.

### Concluding remarks

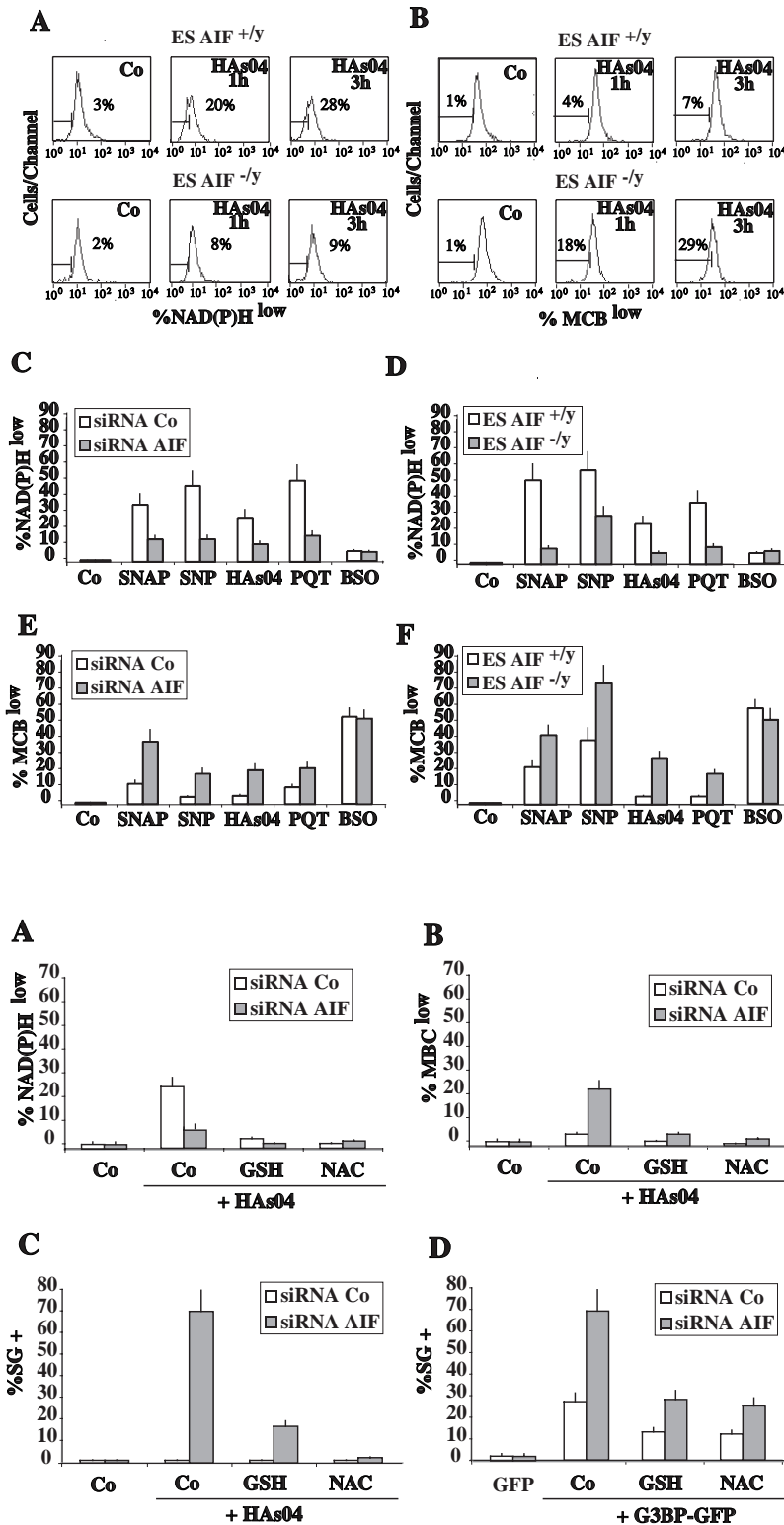
Depending on the intensity of physical or chemical stress, cells can activate defense mechanisms or rather activate the apoptotic default program. One of the possible defense mechanisms induced by oxidative stress is the formation of cytoplasmic SGs, a multimolecular aggregate that manifests an arrest of mRNA translation and might avoid the generation of misfolded proteins. The data contained in this paper reveal that AIF functions as an endogenous repressor of cytoplasmic SG formation (Figs 1, 2, 4), but has no effects on other stress responses (such as the formation of nuclear SGs linked to the cytoplasm-to-nuclear translocation of HSF-1 with subsequent induction of heat shock proteins, Fig. 1). AIF, which normally is present in the mitochondrial intermembrane space, possesses an NADH oxidoreductase activity (Cande et al., 2002). Upon outer mitochondrial membrane permeabilization, AIF translocates to the nucleus and mediates caspase-independent alterations in chromatin structure. Importantly, the pro-apoptotic and the anti-SG activities of AIF can be completely dissociated at several levels. First, SG formation occurs in conditions in which mitochondria retain cytochrome *c* as well as the  $\Delta\Psi_m$ , that is well before signs of apoptosis such as caspase activation and phosphatidylserine exposure become apparent (Fig. 3). Second, to suppress SG formation, AIF has to be localized within mitochondria, as indicated by the re-introduction of mouse AIF (92% identical to human AIF) (Fig. 5). Full-length AIF, which is imported into mitochondria and which does not induce apoptosis, represses SG formation. By contrast, AIF manipulated to be withheld in the cytoplasm (by deletion of the MLS) is highly apoptogenic [(Cande et al., 2002; Cande et al., 2004; Ye et al., 2002) and data not shown] and does not repress SGs (Fig. 5). Third, AIF mutants that lose their apoptogenic function (for instance the deletion mutant  $\Delta 567-609$ ) (Schmitt et al., 2003) can maintain their anti-SG activity, while mutations that selectively affect the redox function of AIF (and have no effect on AIF-mediated apoptosis) (Gurbuxani et al., 2003; Schmitt et al., 2003) invalidate SG modulation by AIF (Fig. 5). In accordance with this latter interpretation, AIF had dramatic effects on the stress-induced depletion of cellular redox equivalents, and removal of AIF from the cell accelerated the oxidation/depletion of GSH (Fig. 6). Correction of this GSH deficiency fully reverted the phenotype of the AIF knock-out or knock-down as far as SG formation was concerned (Fig. 7).

Together, these data point to a hitherto unexpected crosstalk between apoptosis and the stress response. Although the execution of apoptotic cell death and SG formation are mechanistically unrelated, AIF can be placed in the intersection of the two phenomena. When present in the nucleus, AIF actively participates in the formation of the 'degradosome' to digest DNA (Cande et al., 2004; Parrish and Xue, 2003) and to seal the cell's irreversible fate. When present in



**Fig. 5.** Functional mapping of the AIF domains regulating SG formation.

(A) Repression of SG formation by retransfection with mouse AIF. HeLa cells manipulated to downregulate endogenous (human) AIF (siRNA-AIF) were re-transfected with V5-tagged mouse AIF, either full length (AIF WT) or deleted in its N-terminus (AIF $\Delta 1-100$ ) which contains the mitochondrial localization sequence, together with the SG inducer G3BP-GFP. Twenty-four hours after transfection, the cells were stained with an antibody recognizing the V5 epitope. Note that no SGs are formed upon transfection with wild-type AIF, which accumulates in mitochondria (middle panel), whereas AIF $\Delta 1-100$  (which distributes in a diffuse fashion) does not suppress SG formation. (B) Deletion mutations that affect different regions of the AIF molecule. (C,D) Effect of different mouse AIF deletion constructs on SG formation elicited with G3BP (C, done as in A) or HAsO<sub>4</sub> (D, incubation for 3 hours with 1 mM HAsO<sub>4</sub>, 24 hours after re-transfection of AIF into cells). Note that the control for siRNA-AIF (targeting human AIF) used in C and D was a construct designed to downmodulate emerin.



**Fig. 7.** GSH represses SG formation. HeLa cells expressing AIF or not were pretreated with glutathione ethyl ester (GSH) or *N*-acetyl-L-cysteine (NAC) for 1 hour, before addition of HAsO<sub>4</sub>, followed by detection of NAD(P)H (by autofluorescence) (A), intracellular GSH levels (with monochlorobimane, B), or the abundance of SGs (with anti-TIA-1, C). Alternatively, cells were treated with GSH or NAC immediately after liposome-mediated transfection of the SG inducer G3BP-GFP. After 24 hours, SG formation was quantified by assessing the cytoplasmic nucleation of G3BP-GFP.

**Fig. 6.** Effect of AIF on redox metabolism. ES cells positive or negative for AIF (A,B,D,F) or HeLa subjected to AIF knock-down (C,E) were treated with HAsO<sub>4</sub> (A,B) or a panel of different pro-oxidants (and as an internal control for GSH depletion with BSO) for 1-3 hours (A,B) or 3 hours (C-F). Then, the level of NAD(P)H was determined by cytofluorometry, based on its autofluorescence (A,C-F). Alternatively, the cells were stained with the GSH-reactive fluorochrome monochlorobimane (MCB, B,C-F). Results are expressed as the specific variation obtained in triplicate determinations (+s.d.) in one experiment representative of three. PQT, paraquat.

mitochondria, AIF catalyzes redox reactions whose optimal and physiologically relevant electron donors and acceptors remain elusive. Nonetheless, AIF determines the balance between NAD(P)H and GSH under stress conditions and (directly and indirectly) maintains the levels of non-oxidized GSH, which in turn determines the level of SG formation. Both functions of AIF, the pro-apoptotic function and the redox-active, SG-modulatory one, can be separated because they rely on distinct molecular domains. Nonetheless, it remains intriguing that the same molecule can regulate apoptosis as well as the defense against stress, depending on its subcellular localization. Future studies will have to address the possibility that other pro-apoptotic proteins, similarly to AIF, have a second function that blunts adaptive stress responses such as SG formation. If so, it could be postulated that apoptosis regulators not only determine the probability of fulminant self-execution but also modulate the cell's capacity to respond to environmental challenges and to mount a slow, adaptive response against stress.

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