

Nuclear Apaf-1 and cytochrome *c* redistribution following stress-induced apoptosis

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Abstract Apoptotic protease activating factor-1 (Apaf-1) and cytochrome *c* are cofactors critical for inducing caspase-9 activation following stress-induced apoptosis. One consequence of caspase-9 activation is nuclear–cytoplasmic barrier disassembly, which is required for nuclear caspase-3 translocation. In the nucleus, caspase-3 triggers proteolysis of the caspase-activated DNA nuclease (CAD) inhibitor, causing CAD induction and subsequent DNA degradation. Here we demonstrate that apoptotic cells show perinuclear cytochrome *c* aggregation, which may be critical for nuclear redistribution of cytochrome *c* and Apaf-1. We thus indicate that the nuclear redistribution of these cofactors concurs with the previously reported caspase-9-induced nuclear disassembly, and may represent an early apoptotic hallmark. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Apaf-1; Caspase-9; Cytochrome *c*; Stress-induced apoptosis; Subcellular localization

1. Introduction

The execution mechanism of stress-induced apoptosis is mediated by a group of proteases known as caspases (cysteine-specific aspartate-specific proteinases) [1], which carry out the apoptotic program through a sequential activation cascade of initiator and executioner caspases [2]. Apaf-1 (apoptotic protease activating factor-1) is a cofactor that induces activation of initiator caspase-9 [3,4]. Apaf-1 binds caspase-9 via the caspase recruitment domains at their NH₂ termini, triggering the formation of a supramolecular complex called the apoptosome [3,4]. When activated, initiator caspase-9 triggers subsequent proteolytic activation of executioner caspase-3 [3].

During stress-induced apoptosis, caspase activation also requires a large number of post-translational mechanisms, including translocation to other organelles [5]; an example of these mechanisms is caspase-9 translocation to the nucleus [6,7]. In the nucleus, caspase-9 has been implicated in nuclear–cytoplasmic barrier disassembly, allowing an increase in the diffusion limit of nuclear pores [8]. This process induces caspase-3 to enter the nucleus by diffusion [8], where it triggers caspase-activated DNA nuclease by proteolysis of its inhibitor, causing DNA degradation [9,10].

Since caspase-9 redistributes from cytosolic compartments to the nucleus during stress-induced apoptosis, and caspase-9 activation requires Apaf-1 to form an active holoenzyme [11], we studied whether this cofactor also redistributes to the nucleus during apoptosis. Here we demonstrate that apoptotic cells show perinuclear cytochrome *c* aggregation as well as apparent Apaf-1 redistribution to the nucleus after stress-induced apoptosis. Taken together, these results corroborate previous reports showing caspase-9 translocation to the nucleus and indicate that, during stress-induced apoptosis, Apaf-1 appears to translocate from cytoplasmic organelles to the rest of the cell, including the nucleus. This redistribution may be required to allow the increase in the local Apaf-1 concentration necessary for caspase-9 triggering of nuclear–cytoplasmic barrier disassembly.

2. Materials and methods

2.1. Cell culture

NIH-3T3 fibroblasts and HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES and 50 µM 2-mercaptoethanol (Sigma, St. Louis, MO, USA), and maintained at 37°C in a humidified 5% CO₂ atmosphere.

2.2. Antibodies and reagents

Anti-active caspase-3 antibody was purchased from Promega (Madison, WI, USA), human anti-mitochondrial antibody was used as described [12], and monoclonal anti-cytochrome *c* antibody was from Pharmingen (San Diego, CA, USA). Mouse anti-β-tubulin (clone tub 2.1), actinomycin D and cisplatin were from Sigma. Rabbit anti-Apaf-1 antibody was used as described [13].

2.3. Immunofluorescence and image acquisition

For immunofluorescence, cells were cultured in chamber slides, washed in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde (15 min, room temperature), pre-incubated in 2% bovine serum albumin (BSA), and incubated for 1 h with primary antibody in PBS containing 0.5% BSA and 0.1% Triton X-100. Cells were washed three times in the same buffer and incubated for 1 h with Cy2-, Cy3- or Cy5-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA). After washing, samples were incubated with TOPRO-3 (Molecular Probes, Eugene, OR, USA) in PBS for DNA staining. Serial Z-sections were obtained with an Ar-Kr laser and a TCS-NT Leica confocal imaging system equipped with a 63×1.4 oil PLAPO objective. Cy2 was analyzed at 488 nm, Cy3 at 568 nm, and Cy5/TOPRO-3 at 647 nm. Images for each channel were captured separately and assembled into a single file with TCSMERGE software (Leica Microsystems, Heidelberg, Germany) prior to analysis. All confocal images were analyzed using the Leica Vista+ (Beta Release 2) program. Images were processed digitally using Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

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Abbreviations: Apaf-1, apoptotic protease activating factor-1

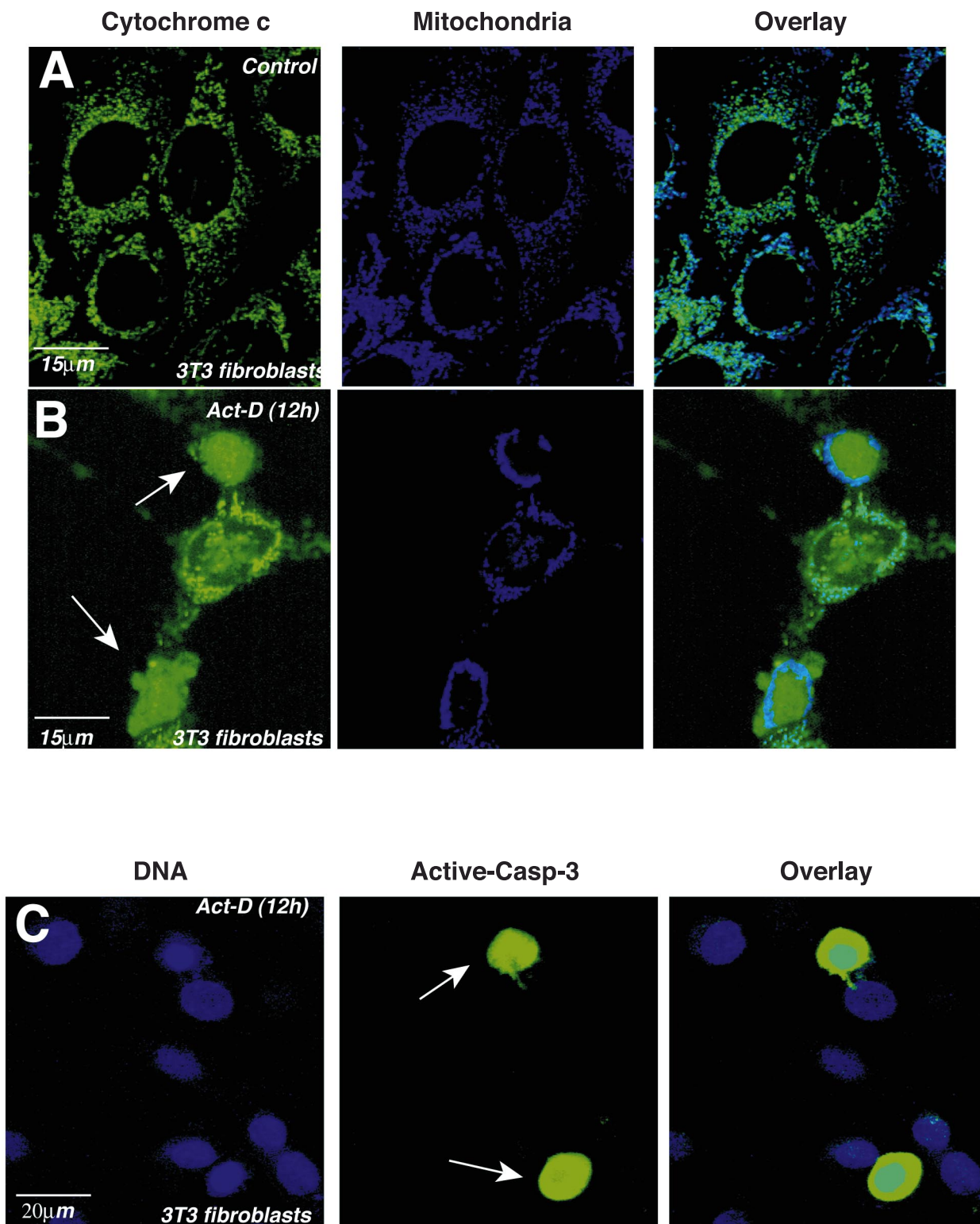


Fig. 1. Nuclear caspase-3 activation during stress-induced apoptosis. A: Untreated control NIH-3T3 fibroblasts. NIH-3T3 fibroblasts were cultured and fixed as described (Section 2), then incubated with mouse anti-cytochrome *c* and human anti-mitochondrial antibodies (Section 2); Cy2-conjugated anti-mouse (green) and Cy5 anti-human secondary antibodies (blue) were used. B: Actinomycin D-treated NIH-3T3 fibroblasts (5 $\mu\text{g}/\text{ml}$, 12 h) were fixed and stained as above. C: NIH-3T3 fibroblasts as in B were pretreated as before, then incubated with rabbit anti-active caspase-3 antibody; Cy2-conjugated anti-rabbit secondary antibody (green) was used. Samples were washed, incubated with TOPRO-3 (blue) in PBS, then analyzed by confocal microscopy (Section 2).

2.4. Measurement of caspase-9 activity

Cytosolic protein (20 μ g) was diluted five-fold in caspase buffer (25 mM HEPES pH 7.5, 0.1% CHAPS, 10% sucrose, 10 mM dithiothreitol and 0.1 mg/ml ovalbumin) and incubated (1 h, 37°C), with the fluorescent substrate Ac-LEHD-AMC (acetyl-Leu-Glu-His-Asp-7-amino-4-methylcoumarin; 10 mM); reactions were terminated by adding HPLC buffer (water/acetonitrile 75/25, 0.1% trifluoroacetic acid). Cleaved substrate fluorescence was determined by C_{18} reverse phase HPLC using fluorescence detection (338 nm excitation, 455 nm emission). Control experiments confirmed linearity of substrate release with time and protein concentration.

3. Results and discussion

Caspase-9 triggers nuclear–cytoplasmic barrier disassembly, causing caspase-3 to enter the nucleus by diffusion [8]. According to this model, active caspase-3 is localized in the nucleus during apoptosis. To validate this hypothesis, we treated NIH-3T3 cells with actinomycin D, which causes stress-induced apoptosis, including cytochrome *c* release from mitochondria and caspase-9 activation [14]. We analyzed whether cytochrome *c* redistributes during actinomycin D-induced apoptosis in NIH-3T3 cells. Co-localization analysis in healthy NIH-3T3 cells showed cytochrome *c* in mitochondria (Fig. 1A); in apoptotic NIH-3T3 cells, however, there was striking perinuclear mitochondrial aggregation, as well as cytochrome *c* redistribution to the nucleus (Fig. 1B). These data indicate that cytochrome *c* is released from mitochondria during actinomycin D-induced apoptosis. Moreover, we studied the localization of active caspase-3 during actinomycin D-induced apoptosis using an anti-caspase-3 antibody that specifically stains active caspase-3 in apoptotic cells. Analysis of actinomycin D-treated NIH-3T3 cells showed active caspase-3 localization in nuclei (Fig. 1C), confirming nuclear caspase-3 localization in apoptotic cells and thus validating the model.

Caspase-9 activation requires the cofactor Apaf-1 to form an active holoenzyme [11]. We analyzed whether, during apoptosis, Apaf-1 undergoes subcellular redistribution, which may be required for caspase-9-induced nuclear–cytoplasmic barrier disassembly. Caspase activation induces cytoskeletal collapse during apoptosis; this process is due to the degradation of multiple structural proteins associated with the cytoskeletal network [15]. To study whether Apaf-1 redistributes to the nucleus during apoptosis, we used cytoskeletal collapse as an indicator of actinomycin D-induced apoptosis. Cytoskeletal collapse is detected during apoptosis using the β -tubulin microtubule marker. Confocal microscopy analysis of Apaf-1 and β -tubulin in untreated cells indicated that Apaf-1 did not co-localize with the microtubule network (Fig. 2A). Apaf-1 and β -tubulin localization was analyzed by immunostaining after 12 h of actinomycin D treatment. In actinomycin D-treated cells, both a marked collapse in the microtubule network and cell shrinkage were observed (Fig. 2B). We also detected Apaf-1 in the nucleus of apoptotic cells, indicating apparent Apaf-1 redistribution during actinomycin D-induced apoptosis (Fig. 2B). These data suggest that, during stress-induced apoptosis, Apaf-1 translocates from cytoplasmic organelles to the rest of the cell, including the nucleus.

To test whether Apaf-1 and cytochrome *c* accumulated within the nucleus or were adsorbed to the nuclear envelope early in apoptosis, we studied Apaf-1 and cytochrome *c* redistribution in HeLa cells at different times after cisplatin treatment. We found that cytochrome *c* redistributes 4 h after cisplatin treatment, forming a ring structure (Fig. 3A,B). In

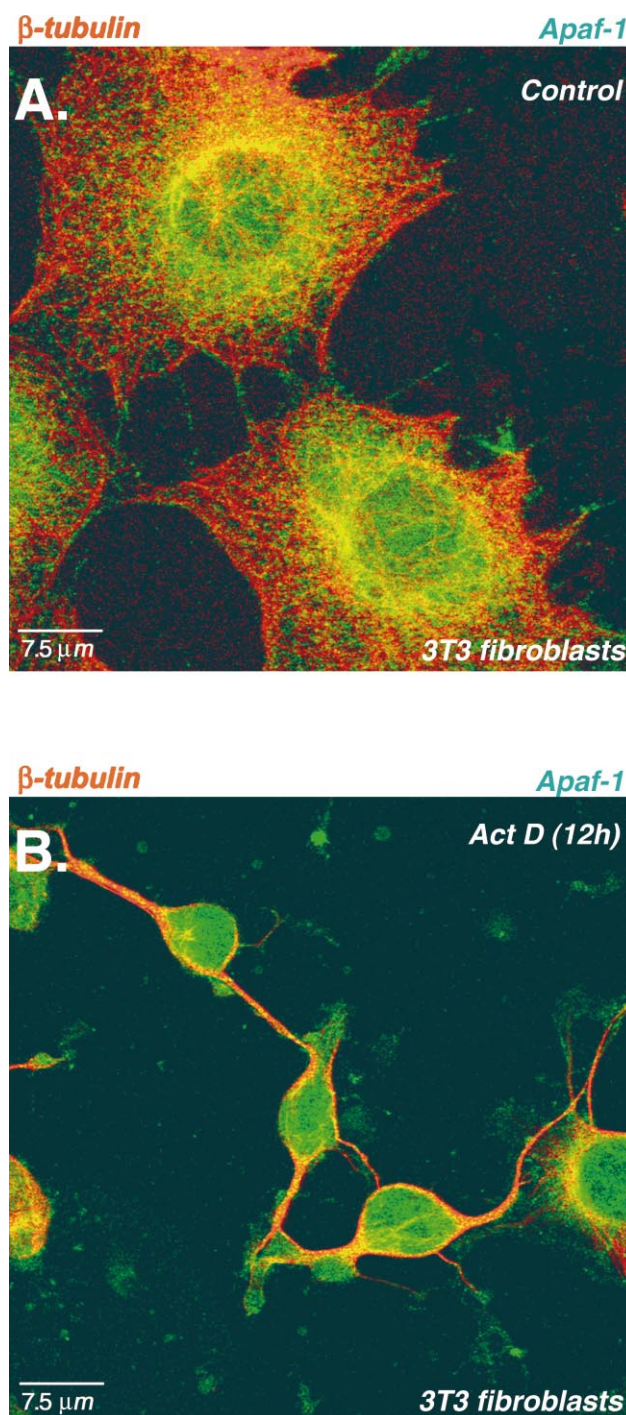


Fig. 2. Cytoskeletal collapse and nuclear redistribution of Apaf-1 during stress-induced apoptosis. A: Untreated NIH-3T3 fibroblasts were pretreated as in Fig. 1, then incubated (1 h) with rabbit anti-Apaf-1 (green) and mouse anti- β -tubulin (red). After incubation, samples were washed, then incubated (1 h) in Cy2 anti-rabbit and Cy3 anti-mouse antibodies. After washing, samples were analyzed and serial Z-sections obtained as above. B: Cells were actinomycin D-treated for 12 h, then processed as above. The data are representative of the total cell population.

addition, we detected a diffuse cytochrome *c* pattern in the nucleus at 8 h (Fig. 3C), a time point at which more than 60% of cells are clearly apoptotic (not shown). These data indicate that the cytochrome *c* release process may have two stages; in

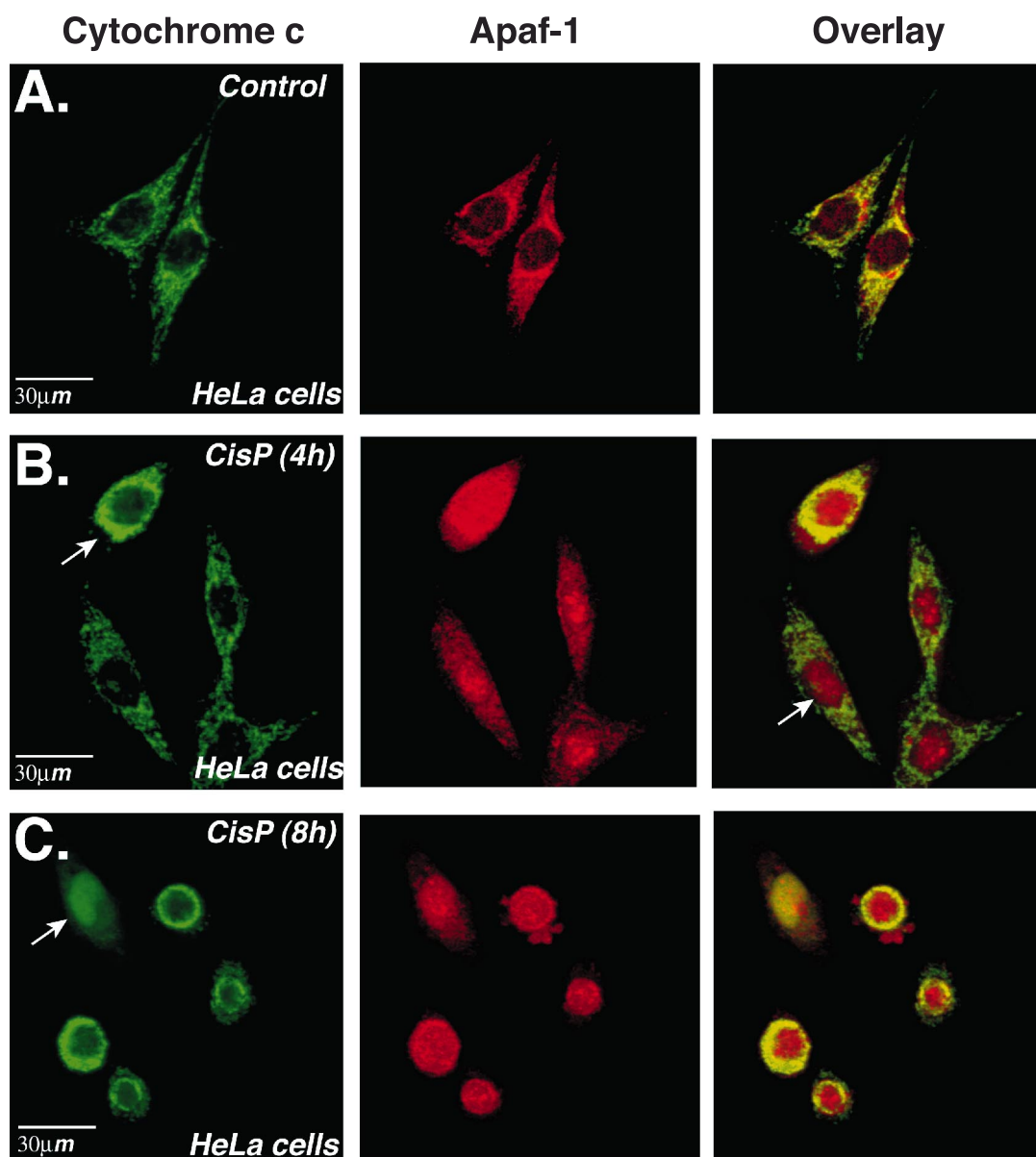


Fig. 3. Apaf-1 redistributes earlier than cytochrome *c*. A: Untreated HeLa cells. B: Cisplatin-treated HeLa cells (10 $\mu\text{g/ml}$, 4 h). C: Cisplatin-treated HeLa cells (10 $\mu\text{g/ml}$, 8 h). After treatment, cells were fixed and pretreated as in Fig. 1, then incubated (1 h) with rabbit anti-Apaf-1 (red) and mouse anti-cytochrome *c* antibodies (green). After washing, samples were incubated (1 h) in Cy3 anti-rabbit and Cy2 anti-mouse antibodies, and analyzed as before.

the first, cytochrome *c* would move to the nuclear envelope, in the second, it would be redistributed within the nucleus. Apaf-1 is nonetheless detected inside the nucleus as early as 4 h after cisplatin treatment, a time at which cytochrome *c* does not show nuclear localization.

We next analyzed whether Apaf-1 and cytochrome *c* redistribution is associated with caspase-9 activation. NIH-3T3 cells were cisplatin-treated at several time points, after which Apaf-1 and cytochrome *c* localization were analyzed by immunostaining; caspase-9 activity was measured in parallel. We found caspase-9 activity as well as Apaf-1 redistribution at a time at which cytochrome *c* did not show nuclear localization, suggesting that caspase-9 activation and Apaf-1 translocation occur earlier than this characteristic pattern of cytochrome *c* release (Fig. 4A,B).

A growing number of studies suggest that relocation and aggregation of apoptotic signaling proteins are important steps in caspase activation. Apaf-1 triggers its apoptotic effect by oligomerization and subsequent activation of caspase-9 [16]. The fact that Apaf-1 is a transcriptional target of p53 also indicates that the cellular concentration of Apaf-1 is an important molecular determinant of the stress-induced apoptosis threshold [17–19]. In this study, we show apparent sub-cellular redistribution of Apaf-1, which may be required to allow the increase of the local Apaf-1 concentration necessary for caspase-9 triggering of nuclear–cytoplasmic barrier disassembly. Further studies are needed, however, to determine whether the relationship described here between Apaf-1 sub-cellular redistribution and stress-induced apoptosis has a role in *in vivo* caspase-9 activation.

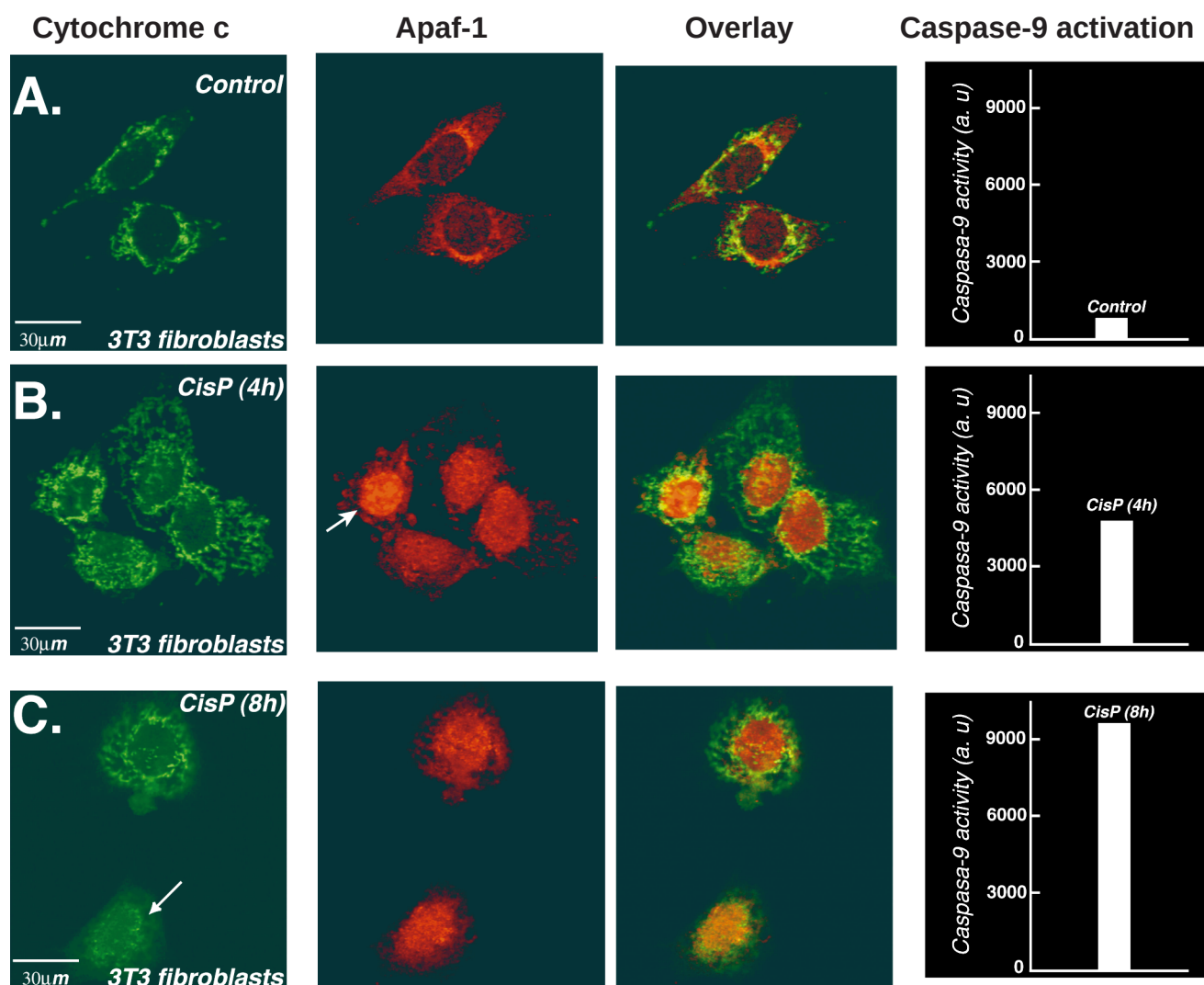


Fig. 4. Apaf-1 redistribution is associated with caspase-9 activation. A: Untreated NIH-3T3 fibroblasts. B: Cisplatin-treated NIH-3T3 fibroblasts (10 μ g/ml, 4 h). C: Cisplatin-treated NIH-3T3 fibroblasts (10 μ g/ml, 8 h). After treatment, cells were fixed, pretreated, stained and analyzed as in Fig. 3C. Untreated and cisplatin-treated cells were lysed in parallel to measure caspase-9 activity.

In the nematode *Caenorhabditis elegans*, the CED-4 protein (the Apaf-1 homologue) triggers activation of CED-3 (caspase homologue) following CED-4 nuclear redistribution [20]. This phenomenon is observed here in mammalian cells, suggesting the conservation of this Apaf-1 function throughout evolution.

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