

Pro-apoptotic Apoptosis Protease-activating Factor 1 (Apaf-1) Has a Cytoplasmic Localization Distinct from Bcl-2 or Bcl-x_L

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Abstract. How Bcl-2 and its pro-survival relatives prevent activation of the caspases that mediate apoptosis is unknown, but they appear to act through the caspase activator apoptosis protease-activating factor 1 (Apaf-1). According to the apoptosome model, the Bcl-2-like proteins preclude Apaf-1 activity by sequestering the protein. To explore Apaf-1 function and to test this model, we generated monoclonal antibodies to Apaf-1 and used them to determine its localization within diverse cells by subcellular fractionation and confocal laser scanning microscopy. Whereas Bcl-2 and Bcl-x_L were prominent on organelle membranes, endogenous Apaf-1 was cytosolic and did not colocalize with them, even when these pro-survival proteins were overexpressed or after apoptosis was induced. Immunogold electron microscopy confirmed that Apaf-1 was dis-

persed in the cytoplasm and not on mitochondria or other organelles. After the death stimuli, Bcl-2 and Bcl-x_L precluded the release of the Apaf-1 cofactor cytochrome *c* from mitochondria and the formation of larger Apaf-1 complexes, which are steps that presage apoptosis. However, neither Bcl-2 nor Bcl-x_L could prevent the in vitro activation of Apaf-1 induced by the addition of exogenous cytochrome *c*. Hence, rather than sequestering Apaf-1 as proposed by the apoptosome model, Bcl-2-like proteins probably regulate Apaf-1 indirectly by controlling upstream events critical for its activation.

Key words: caspases • cell death • Bcl-2 • mitochondria • subcellular localization

Introduction

The evolutionarily conserved process of apoptosis is critical both to remove unwanted cells during development and to maintain tissue homeostasis, and its dysregulation can engender many diseases (Thompson, 1995; Strasser et al., 1997; Vaux and Korsmeyer, 1999). Apoptosis is launched when critical initiator cysteine proteases (caspases) are processed into active enzymes, which then initiate a proteolytic cascade by activating downstream caspases such as caspase-3 (Thornberry and Lazebnik, 1998). The processing of the apical caspases requires adapter molecules such as Apaf-1 (Li et al., 1997; Zou et al., 1997). In the presence of its cofactors, dATP and cytochrome *c*, Apaf-1 promotes the autocatalytic activation of caspase-9 (Li et al., 1997; Zou et al., 1997; Srinivasula et al., 1998), triggering one major pathway to apoptosis (Hakem et al., 1998; Kuida et al., 1998).

The Bcl-2 family of proteins is a critical upstream regulators of apoptosis (Adams and Cory, 1998; Gross et al., 1999). Apoptosis protease-activating factor 1 (Apaf-1)¹ probably is a crucial target for its pro-survival members because Apaf-1-deficient cells are refractory to several cytotoxic signals that are inhibitable by Bcl-2 (Ceconi et al., 1998; Yoshida et al., 1998). However, whether Bcl-2 and its close relatives control Apaf-1 activity directly remains a central unresolved issue (Adams and Cory, 1998; Green and Reed, 1998; Gross et al., 1999).

In the widely discussed apoptosome model (Hengartner, 1997), first proposed for the nematode *Caenorhabditis elegans*, the Bcl-2-like proteins directly sequester Apaf-1. Developmental cell death in the worm requires both the Apaf-1 homologue cell death abnormal (CED) 4 and the caspase CED-3, but can be prevented by the Bcl-2 homologue CED-9 (Ellis et al., 1991). Biochemical studies in

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¹Abbreviations used in this paper: Apaf, apoptosis protease-activating factor; CARD, caspase recruitment domain; CED, cell death abnormal; GFP, green fluorescent protein; HA, hemagglutinin.

heterologous overexpression systems suggested that CED-9 sequesters CED-4 and CED-3 (Chinnaiyan et al., 1997; Spector et al., 1997; Wu et al., 1997a,b). For example, CED-9, which localized to the intracellular membranes of mammalian and yeast cells, caused coexpressed CED-4 to translocate from the cytosol to those sites, suggesting that the putative CED-9/CED-4/CED-3 apoptosome complex resides on organelles (James, 1997; Wu et al., 1997b).

The conservation of the apoptotic pathway would argue for analogous mammalian apoptosomes involving Apaf-1, but coimmunoprecipitation experiments have yielded conflicting data. Overexpressed Apaf-1 was reported to associate with the pro-survival proteins Bcl-x_L (Hu et al., 1998; Pan et al., 1998), Bcl-2 (Fang et al., 1998), and Boo/Diva (Inohara et al., 1998; Song et al., 1999). A recent study from this laboratory, however, found no evidence for coimmunoprecipitation of any of the known mammalian pro-survival proteins with Apaf-1 (Moriishi et al., 1999).

As the coimmunoprecipitation data have given conflicting results, it is important to test the apoptosome model in alternative ways. The model strongly predicts that most, if not all, Apaf-1 molecules will be found at the sites where pro-survival molecules like Bcl-2 and Bcl-x_L reside, namely on the cytoplasmic aspect of the mitochondrion, the nuclear envelope, and the ER (Monaghan et al., 1992; Krajewski et al., 1993; Gonzalez-Garcia et al., 1994; Lithgow et al., 1994). Although Apaf-1 was originally isolated from the soluble fraction of HeLa cells (Zou et al., 1997) and was recently reported to behave as a soluble protein on subcellular fractionation (Zhitovitsky et al., 1999), its localization inside cells has not been definitively established. Therefore, to investigate how Apaf-1 might be regulated and to test the apoptosome model, we have generated mAbs to Apaf-1, and used them to locate the endogenous protein in diverse cell types. The subcellular fractionation and microscopic studies (confocal and electron microscopy) reported here indicate that Apaf-1 is a soluble protein with a cytoplasmic localization distinct from that of pro-survival proteins such as Bcl-2 and Bcl-x_L. Moreover, its location did not alter in response to apoptotic stimuli. These findings indicate that, contrary to the apoptosome model, Bcl-2-like proteins do not directly sequester Apaf-1 and, instead, must regulate its activity by the control of upstream events.

Materials and Methods

Monoclonal Rat Anti-Apaf-1 Antibodies

Monoclonal rat anti-Apaf-1 antibodies were generated and selected according to a previous protocol (O'Reilly et al., 1998). In brief, Wistar rats were immunized with purified bacterial recombinant human Apaf-1 protein (residues 1–464: caspase recruitment domain [CARD] plus CED-4 homology regions). The hybridoma clones, resulting from a fusion of the spleen cells with Sp2/0 myeloma cells, were screened by flow cytometric analysis for immunofluorescent staining of 293T cells transfected with a plasmid encoding COOH terminally hemagglutinin (HA)-tagged full-length human Apaf-1 (Moriishi et al., 1999). The lines were cloned twice and the antibodies were purified on protein G-Sepharose columns (Amersham Pharmacia).

Cell Culture, Transfection, Induction of Cell Death and Expression Constructs

All cell lines were cultured in the high glucose version of DME medium

supplemented with 13 μ M folic acid, 250 μ M L-asparagine, 50 μ M 2-mercaptoethanol, and 10% FCS. Human T lymphoblasts were isolated and activated as described (James, 1997). 293T, COS-7, or HeLa cells were transiently transfected using Lipofectamine (Life Technologies; Huang et al., 1997b) and analyzed the next day. To generate HeLa lines that stably overexpress Bcl-2 or Bcl-x_L, cells transfected with vectors that also express the puromycin resistance gene were plated at a limiting dilution in the presence of 1 μ g ml⁻¹ puromycin (Sigma Chemical Co.). Clones expressing the relevant protein were identified by flow cytometric analysis (Huang et al., 1997a). Cell death was induced by treating cells with 1 nM–1 μ M staurosporine (Sigma Chemical Co.), 0.1–1.0 μ g ml⁻¹ etoposide (VP-16; Della West), or by UV irradiation (HeLa cells 10–100 J m⁻²; HepG2 cells at 800 J m⁻²). Cell viability was determined by counting cells stained with vital dye (0.4% eosin) in a hemacytometer or by flow cytometric analysis of propidium iodide (5 μ g ml⁻¹; Sigma Chemical Co.)-stained cells. The expression constructs for human Apaf-1, human Bcl-2, and human Bcl-x_L have been described (Huang et al., 1997a; Moriishi et al., 1999).

Subcellular Fractionation, Sucrose Gradients, Immunoprecipitation, and Immunoblotting

To prepare total cell extracts, cells were harvested in lysis buffer (20 mM Tris-HCl, pH 7.4, 135 mM NaCl, 1% Triton X-100, and 10% glycerol), supplemented with 0.5 μ g ml⁻¹ Pefabloc, 100 μ g ml⁻¹ soybean trypsin inhibitor, and 1 μ g ml⁻¹ each of leupeptin, aprotinin, and pepstatin (Sigma Chemical Co. or Roche). To separate the cytosolic fraction from other cellular components (Ramsby et al., 1994), cells were permeabilized in 0.025% digitonin (Calbiochem-Novabiochem Corp.) dissolved in HMKEE buffer (20 mM Hepes, pH 7.2, 5 mM MgCl₂, 10 mM KCl, 1 mM EDTA, and 1 mM EGTA and protease inhibitors) containing 250 mM sucrose. The organelles, cytoskeleton, and membranes were pelleted by centrifugation (13,000 rpm for 2 min at 4°C). The supernatant (cytosol; s) was carefully removed and the pellet (p) was solubilized in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris-HCl, pH 8.0, and protease inhibitors). Protein content was determined by the Bradford reaction (Bio-Rad), and equivalent portions of each fraction was used for further analysis.

For sedimentation experiments, cells were resuspended in HMKEE buffer containing 250 mM sucrose, allowed to swell on ice for 10 min, and lysed using a Dounce homogenizer (100 strokes with a type B pestle; Konte Glassware Corp.). After centrifugation at 900 g for 5 min at 4°C to remove unlysed cells, nuclei, and cell debris, the supernatant was loaded on a continuous 10–50% sucrose gradient in HMKEE buffer, centrifuged (40,000 rpm for 20 h at 4°C) in an SW40 Ti rotor (Beckman Instruments, Inc.), and the fractions were manually collected. The broad spectrum caspase inhibitor zVAD.fmk (Z-Val-Ala-DL-Asp-fluoromethylketone; Bachem Bioscience Inc.) was used at 50 μ M.

Immunoprecipitations were performed as described (Moriishi et al., 1999). Total cell lysates, immunoprecipitates, or fractionated samples were resolved by SDS-PAGE (Novex) and electroblotted onto nitrocellulose membranes (Amersham Pharmacia). Nonspecific binding was blocked by incubating the filters in PBS containing 5% skimmed milk (Diploma), 1% casein (Sigma Chemical Co.), and 0.1% Tween 20 (Sigma Chemical Co.) for \geq 1 h before incubation with the antibody. Mouse mAbs included anti-HA 16B12 (HA.11; BAbCO), anti-HSP60 and anti-HSP90 (both from Stressgen), antiporin/VDAC (Calbiochem-Novabiochem Corp.), anti-human Bcl-2 (Bcl-2-100), anti-Bcl-x (7B2.5; a gift from C. Thompson, University of Pennsylvania, Philadelphia, PA), anti-Golgi 58-kD protein (58K-9; Sigma Chemical Co.), anti-cytochrome c (7H8.2C1; PharMingen), and anti-caspase-9 (2-22; a gift from Y. Lazebnik, Cold Spring Harbor Laboratory, NY). Rabbit polyclonal antibodies were anticalnexin (Stressgen), anti-Bcl-x, and anti-caspase-9 (both from PharMingen). Bound antibodies were detected with HRP-conjugated secondary reagents (Silenus or Southern Biotechnology Associates Inc.) and enhanced chemiluminescence (Amersham Pharmacia). Blots were stripped and reprobed according to manufacturer's instructions (Amersham Pharmacia).

Immunofluorescence Staining and Confocal Microscopy

To stain for Apaf-1, cells were grown on glass coverslips (10-mm diam; Lomb Scientific) or in chamber slides (Becton Dickinson & Co.). Nonadherent cells or cells treated with apoptotic stimuli were attached using poly-L-lysine or Cell TAK (Becton Dickinson & Co.), fixed with 50% acetone/50% methanol for 10 min at room temperature, and permeabilized

with 0.5% Tween 20. COS cells transiently expressing Apaf-1 HA were incubated overnight at 4°C with the primary antibodies (mouse anti-HA and rat anti-Apaf-1), washed with 0.2% Tween 20 in PBS, and incubated with FITC-conjugated goat anti-mouse Ig (Southern Biotechnology Associates Inc.) and rhodamine-conjugated goat anti-rat Ig (Jackson ImmunoResearch Laboratories, Inc.). Finally, the slides were mounted in fluorescent mounting medium (DAKO). Controls included staining with primary or secondary antibody alone and staining of untransfected cells. To stain for mitochondria, cells were incubated at 37°C for 15 min with 500 nM MitoTracker red, and for lysosomal staining, with LysoTracker red for 3 h (both from Molecular Probes).

Detection of endogenous Apaf-1 required amplification of the immunofluorescence signal by tyramide signal amplification (NEN), which uses HRP to catalyze the deposition of biotin-labeled tyramide (Bobrow et al., 1992). Endogenous peroxidase activity was quenched by incubation in 3% H₂O₂, 10% methanol for 15 min, and cells were permeabilized in 0.5% Tween 20 in PBS for 15 min at room temperature. Apaf-1 was detected by incubation with mAbs 2E12 or 19G9 overnight at 4°C, followed by 30 min with HRP-conjugated goat anti-rat IgG (Southern Biotechnology Associates Inc.). After tyramide amplification for 7 min (with mAb 2E12) or 3.5 min (with 19G9), the staining was revealed with FITC- or Texas red-conjugated streptavidin (Caltag or GIBCO BRL). Between steps, the slides were washed three times in PBS containing 1% BSA and 0.1% Tween 20. Other antigens were detected by incubation with an appropriate primary antibody overnight at 4°C and an FITC-conjugated secondary reagent for 1 h (Southern Biotechnology Associates Inc.). Controls included staining with an isotype-matched antibody (anti-rat IgG2a κ R35-95; PharMingen) or staining with secondary reagents alone. Samples were analyzed with a Leica confocal laser scanning microscope using SCANware software (Leica Lasertechnik).

Immunogold Electron Microscopy

Healthy or UV-irradiated HepG2 cells were fixed for 2 h in 4% paraformaldehyde, 0.1% glutaraldehyde, 4% sucrose in HEPES-buffered saline, pH 7.4 (150 mM NaCl, 50 mM HEPES, 4 mM MgCl₂, 4 mM CaCl₂, and 2 mM KCl). After several washes with PBS, the cells were scraped off the dishes, gently pelleted, and resuspended in 0.1 M Na₃PO₄, pH 7.4, containing 10% gelatin. The pellets were cooled on ice until solid, and smaller cubes were cryoprotected in 15% polyvinyl-pyrrolidone and 1.7 M sucrose. The infused blocks were hardened in liquid nitrogen before cryosectioning at -100°C using a Diatome diamond knife on a Reichert FCS/Ultracut S. The ultra-thin sections were collected onto Formvar-coated nickel grids in 1% methylcellulose, and 1.15 M sucrose and stored at 4°C (Kleijmeer et al., 1997). The grids were floated on 2% gelatin in 0.1 M Na₃PO₄ for 30 min. Aldehyde groups and nonspecific binding were blocked by incubating the samples first in 50 mM glycine in PBS, and then in incubation buffer (10 mM Na₃PO₄, 0.1% cold fresh water fish gelatin, 0.5% ovalbumin, 150 mM NaCl, 20 mM Na₂S₂O₃) containing 5% normal goat serum. The samples were incubated with 2 μg ml⁻¹ anti-Apaf-1 2E12 or rat IgG2a κ R35-95 in IB, washed in IB, and then treated with the secondary antibody (10 nM gold-conjugated goat anti-rat IgG; British Biocell) diluted 1:40 in IB. After extensive washing, the samples were fixed for 5 min in the fixative above, washed extensively with Milli-Q water, and contrasted in 1.71% methylcellulose, 0.43% uranyl acetate (Kleijmeer et al., 1997). The sections were examined at 80 kV using a JEOL 1200EX transmission electron microscope. All chemicals were obtained from Sigma Chemical Co.

Software Applications

Immunoblots and immunogold EM images were scanned using a AGFA SNAPScan 1236 scanner and FotoLook SA 2.09.6 software. These and the confocal scanning images were edited by using Adobe Photoshop or Free-Hand software.

Results

Novel Monoclonal Anti-Apaf-1 Antibodies

To generate mAbs against human Apaf-1, myeloma cells were fused with spleen cells from rats immunized with truncated human Apaf-1 recombinant protein containing the NH₂-terminal CARD and CED-4 homologous regions (Zou et al., 1997). Clones secreting antibodies specific for

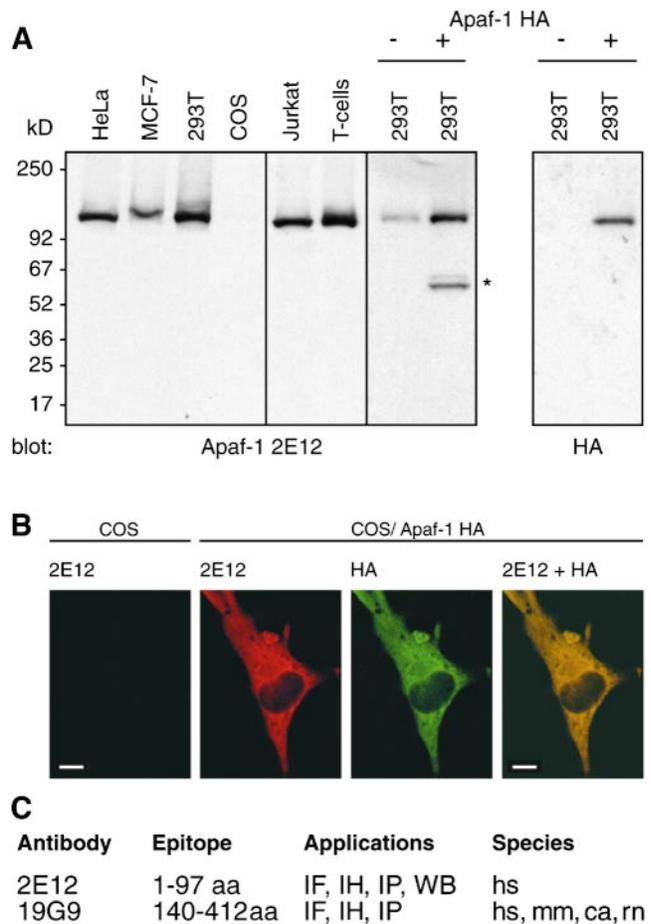


Figure 1. Characterization of anti-Apaf-1 mAbs. (A) Immunoblots with mAb 2E12 revealing endogenous Apaf-1. Lysates from 10⁶ HeLa, MCF-7, 293T, COS, Jurkat, and activated primary human T cells, and 10⁵ untransfected 293T cells or those transiently transfected with an Apaf-1 HA construct, were resolved by SDS-PAGE, and the membrane was probed with anti-Apaf-1 2E12 (left panel) or anti-HA (right panel). The asterisk indicates an NH₂-terminal cleavage product commonly seen in cells overexpressing Apaf-1. (B) Confocal microscopy showing the comparable staining of Apaf-1 HA by anti-Apaf-1 and anti-HA. Untransfected COS cells were stained with Apaf-1 mAb 2E12 and rhodamine-conjugated anti-rat IgG (first panel). COS cells transiently expressing Apaf-1 HA were similarly stained (second panel) and with anti-HA antibody plus FITC-conjugated anti-mouse IgG (third panel). In the superimposed images (fourth panel), yellow indicates regions of costaining. The images are representative of ≥20 cells examined. (C) Properties of 2E12 and 19G9, including applicability for immunofluorescence staining (IF), immunohistochemical staining (IH), immunoprecipitation (IP), and Western blotting (WB). Whereas 2E12 mAb recognizes only the human protein (hs), 19G9 mAb also recognizes monkey (ca), mouse (mm), and rat (rn) Apaf-1. Bars, 10 μm.

Apaf-1 were identified by a rapid flow cytometric screen designed to select antibodies that recognize the native protein (O'Reilly et al., 1998; see Materials and Methods). Two clones of independent origin, 2E12 and 19G9, yielded mAbs suitable for various applications (Fig. 1).

The 2E12 mAb proved to be specific for the human protein. On immunoblots, it recognized a single 130-kD protein in human epithelial cells (293T, HeLa, MCF-7, and

HepG2) and human hematopoietic cells (Jurkat, SKW6, CEM, and primary human lymphoblasts) but not in the monkey kidney epithelial COS cell line (Fig. 1 A and data not shown). The size of the endogenous protein was comparable to that of HA-tagged Apaf-1 transiently expressed in the 293T cells, though the overexpressed protein also yielded an ~60-kD NH₂-terminal proteolytic fragment (Fig. 1 A). In confirmation of the specificity of the antibodies, their staining pattern on COS cells expressing human Apaf-1 HA was identical to the staining with anti-HA antibody (Fig. 1 B). Labeling was negligible with an isotype-matched control antibody or with secondary reagents alone (data not shown) and in untransfected COS cells stained with mAb 2E12 (Fig. 1 B). The 2E12 mAb also proved useful for immunoprecipitation and immunofluorescence, whereas 19G9, which recognizes Apaf-1 from several species, worked in all applications tested except for immunoblotting (Fig. 1 C). The 2E12 epitope mapped to the NH₂-terminal CARD (residues 1–97), and the 19G9 epitope mapped to the CED-4 homology region (within residues 140–412; data not shown). Most subsequent experiments were performed using 2E12 and, where appropriate, confirmed with 19G9.

Apaf-1 Has a Cytoplasmic Localization Distinct from Bcl-2 and Bcl-x_L

Our initial confocal microscopy studies of transiently

transfected 293T, HeLa, or MDCK cells showed HA- or GFP-tagged Apaf-1 to be cytoplasmic (Fig. 1 B). We subsequently focused on the endogenous protein to preclude artifacts of overexpression, such as the proteolytic cleavage seen in Fig. 1 A or nonspecific aggregation (Johnston et al., 1998). Because of its low abundance (an estimated 5,000–8,000 molecules per epithelial cell; data not shown), detection of endogenous Apaf-1 with either antibody required tyramide signal amplification (Bobrow et al., 1992; see Materials and Methods).

In all cell types examined, endogenous Apaf-1 exhibited a granular cytoplasmic distribution (Fig. 2). In the HeLa cervical carcinoma cell line, which was not stained by a control antibody or the secondary reagents (data not shown), both the 2E12 antibody (Fig. 2 A) and 19G9 (data not shown) revealed Apaf-1 distributed throughout the cytoplasm, irrespective of the fixative used (paraformaldehyde or acetone/methanol). There was no localization to the nuclear membrane, nor any substantial overlap with the mitochondria stained with MitoTracker red (Fig. 2 A, bottom). The limited apparent costaining of Apaf-1 and MitoTracker red near the nucleus probably reflect incomplete separation of the intense signals generated, and the inadequate lateral resolution of bright signals generated by two adjacent objects, recognized limitations of confocal laser scanning microscopy (Carlsson, 1991; Brelje et al., 1993). To confirm that the Apaf-1 pattern differed from that of mitochondria, we compared its staining, in human

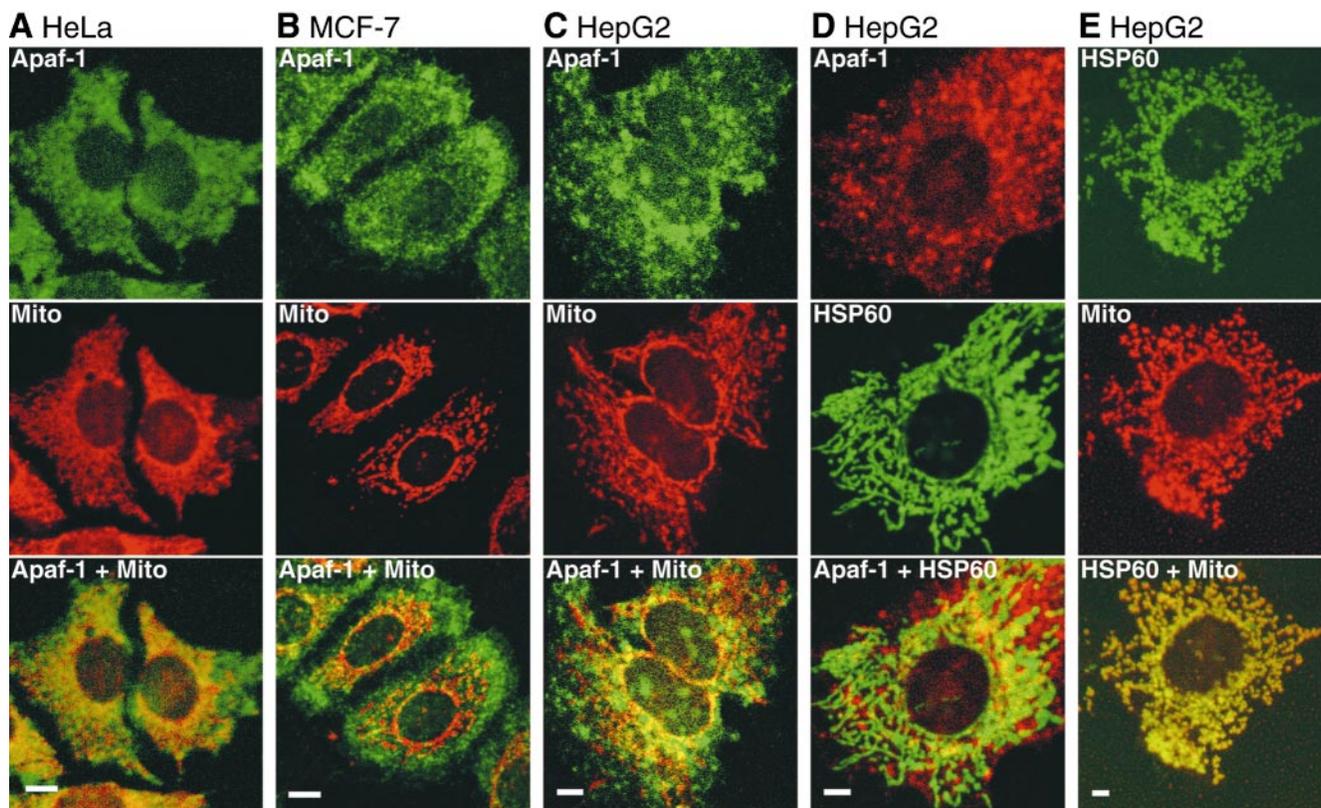


Figure 2. Confocal images showing Apaf-1 localized to the cytoplasm but not significantly to the mitochondria of epithelial cells. Staining for endogenous Apaf-1 in HeLa (A), MCF-7 (B), or HepG2 (C) cells with anti-Apaf-1 2E12 revealed by FITC-conjugated secondary reagents (top) and for mitochondria with MitoTracker red (middle). HepG2 cells were also stained with anti-HSP60 (plus FITC-conjugated anti-mouse IgG) and either Texas red-conjugated detection of anti-Apaf-1 2E12 (D) or MitoTracker red (E). In the overlaid images (bottom), yellow staining indicates potential colocalization. All data are representative of ≥ 20 cells examined. Bars, 10 μ m.

HepG2 hepatoblastoma cells, with that of the known mitochondrial protein HSP60. Whereas staining of HSP60 coincided precisely with Mitotracker red (Fig. 2 E), most of the Apaf-1 appeared distinct from mitochondria, as revealed by costaining with either MitoTracker red (Fig. 2 C) or for HSP60 (Fig. 2 D). Apaf-1 had a similar cytoplasmic distribution, distinct from that of mitochondria in the MCF-7 human breast carcinoma cell line (Fig. 2 B) as well as fibroblasts (NIH3T3, Rat-1A) and lymphocytes (Jurkat human T-lymphoma, activated primary human T cells; data not shown). Apaf-1 also failed to colocalize with markers for the Golgi apparatus (Golgi p58) or lysosomes (LysoTracker; data not shown).

The Apaf-1 confocal pattern still might conceivably reflect its association with an organelle. Alternatively, as only a few hundred Apaf-1 molecules would be expected in any optical plane, the staining might simply reflect the amplified signals from individual or small clusters of Apaf-1 molecules. To distinguish between these two possibilities and further refine its localization, we used immunogold-labeled antibodies in an electron microscopic analysis of HepG2 hepatocytes. No staining at all was observed with an isotype-matched control antibody (data not shown). Examination of over fifty fields in multiple independent preparations, however, revealed Apaf-1 molecules scattered sparsely throughout the cytoplasm but not within the nuclei (Fig. 3). The vast majority appeared as single isolated particles, and almost none were in contact with an intracytoplasmic membrane. Importantly, the numerous mitochondria present in these liver cells exhibited no significant Apaf-1 staining (Fig. 3), and identical results were obtained with HeLa cells (data not shown). Thus, in di-

verse cell types, Apaf-1 did not localize to the nuclear envelope, mitochondria, and the ER, which are the major sites where Bcl-2 resides (see also below). Instead, Apaf-1 is located diffusely throughout the cytoplasm.

Apaf-1 Is a Soluble Protein, Not Associated with Bcl-2, Bcl-x_L, or Procaspase-9

It is important to establish the Apaf-1 localization using different fractionation techniques, because some can generate artifacts (Hsu and Youle, 1997). To confirm that Apaf-1 was cytosolic, HeLa cells were lysed with 0.025% digitonin, which permeabilizes only the plasma membrane, and the lysates were centrifuged to generate a cytosolic fraction (s) and a pellet (p) containing the organelles, membranes, and cytoskeleton (Ramsby et al., 1994). Immunoblotting (Fig. 4 A) revealed Apaf-1 almost exclusively in the cytosolic fraction, whereas markers for the mitochondria (VDAC/porin) or the ER (calnexin) were confined to the pellet. The outer mitochondrial membrane clearly remained intact because the intermembrane proteins cytochrome *c* (see Fig. 6) and HSP60 (data not shown) were confined to the pellet fraction. As expected (Lithgow et al., 1994), Bcl-2 appeared only in the insoluble fraction. As in another fractionation technique (Hsu et al., 1997), Bcl-x_L resided in both fractions, possibly reflecting a weaker interaction with intracytoplasmic membranes. Apaf-1 was also exclusively cytosolic in MCF-7 (Fig. 4 B), Jurkat (Fig. 4 C), HepG2 (Fig. 4 D), 293T, and activated primary human T cells (data not shown).

In case detergents had altered Apaf-1 behavior, we also prepared HeLa cell lysates by Dounce homogenization,

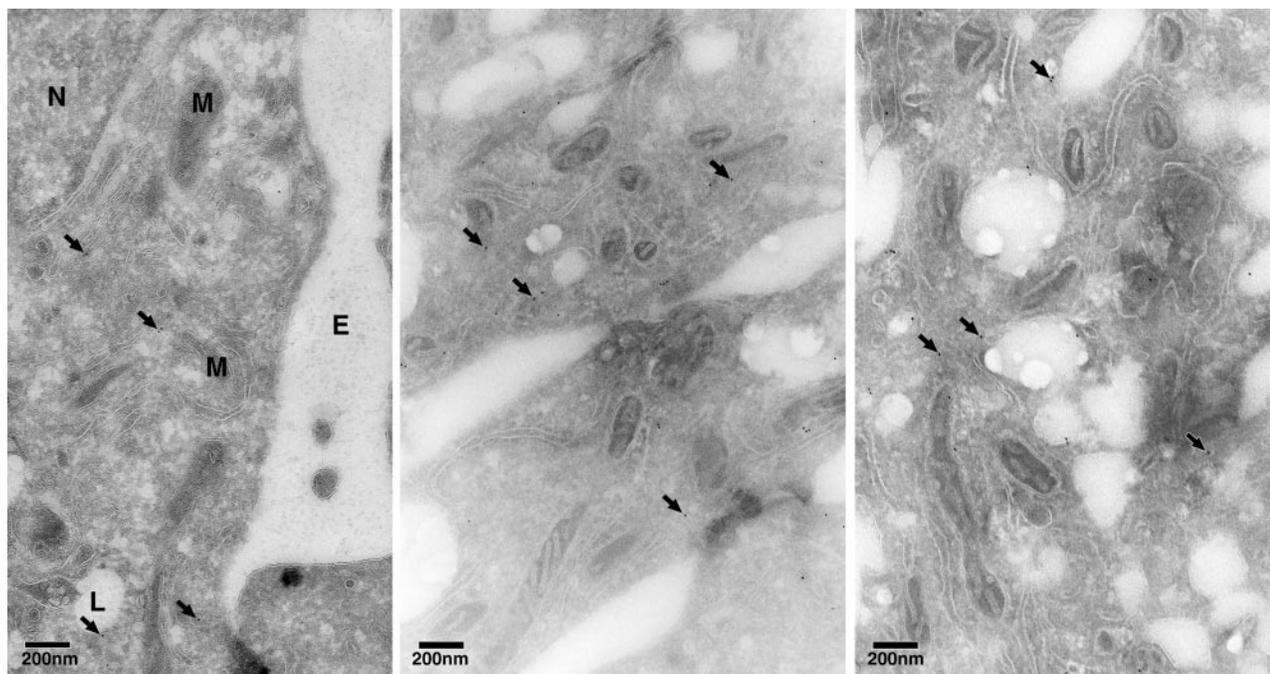


Figure 3. Diffuse cytoplasmic localization of endogenous Apaf-1 revealed by immunogold electron microscopy. HepG2 cells prepared by cryosectioning were immunolabeled using anti-Apaf-1 2E12 and 10 nM gold-conjugated goat anti-rat IgG antibody. The extracellular matrix (E), a representative nuclear section (N), lipid-containing vacuoles (L), mitochondria (M), and some of the gold particles (arrows) are indicated.

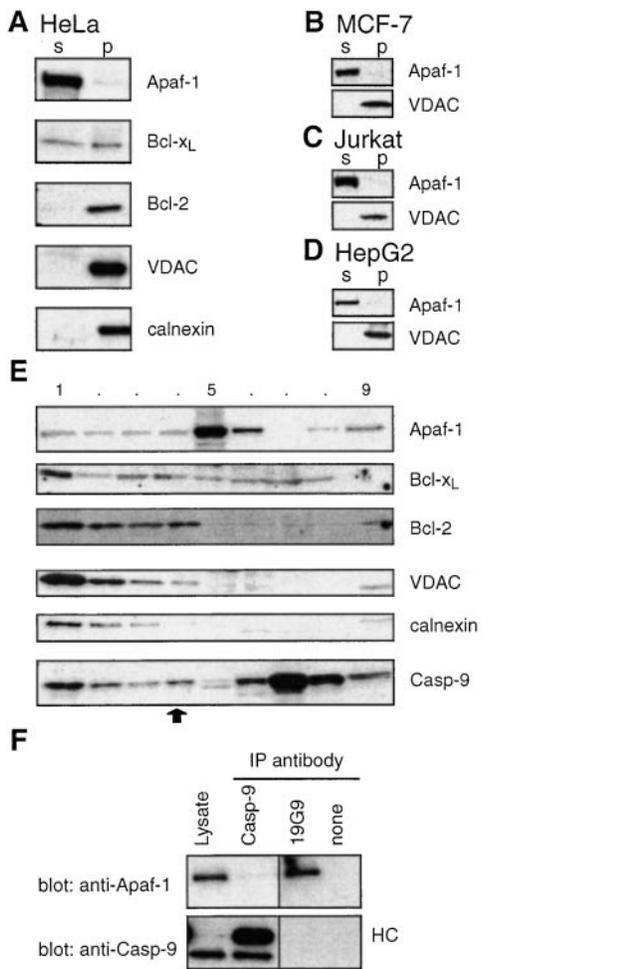


Figure 4. Biochemical evidence that Apaf-1 is a cytosolic monomeric protein that is not associated with Bcl-2, Bcl-x_L, or procaspase-9. (A) HeLa cells lysed in 0.025% digitonin were fractionated into soluble (s) and insoluble (p) fractions, equivalent aliquots of each were resolved by SDS-PAGE, and the filter was probed with antibodies to Apaf-1, Bcl-x, Bcl-2, VDAC/porin (marker for mitochondrial membranes) and calnexin (marker for ER). Lysates from MCF-7 (B), Jurkat (C), or HepG2 (D) cells were analyzed similarly. (E) Apaf-1 sedimented separately from most of the Bcl-x_L, Bcl-2, and procaspase-9 molecules. HeLa cell lysates prepared by Dounce homogenization were centrifuged through a 50–10% sucrose gradient, and nine equivalent fractions (collected and numbered from the bottom) were resolved electrophoretically and immunoblotted with antibodies to the indicated proteins. The arrow marks the mobility of the 232-kD catalase marker. (F) Endogenous Apaf-1 and procaspase-9 do not coprecipitate. Lysate from 10⁶ HeLa cells (lane 1) and anti-procaspase-9 and anti-Apaf-1 immunoprecipitates from 5 × 10⁶ HeLa cells were blotted with anti-Apaf-1 2E12 or anti-caspase-9 antibodies. HC marks immunoglobulin heavy chains from the immunoprecipitate. Data shown are representative of three or more independent experiments.

centrifuged them on sucrose gradients, and analyzed the fractions by immunoblotting (Fig. 4 E). Whereas organelle markers such as VDAC/porin and calnexin appeared in the denser fractions, Apaf-1 behaved like a free monomeric protein of 130 kD (Zou et al., 1999). In contrast, Bcl-2 tracked with the organelle markers. Consistent with

the results above, Bcl-x_L was more dispersed. Importantly, however, the fraction containing the vast majority of Apaf-1 (fraction 5) had very little Bcl-x_L (<1% by densitometric measurements) and no detectable Bcl-2. Thus, even in the absence of detergent, most Apaf-1 was not associated with these pro-survival molecules. Nor was Apaf-1 detectable in either nuclei or mitochondria purified from HeLa or Jurkat cells by Dounce homogenization in the absence of detergent (data not shown).

Interestingly, the vast majority of procaspase-9 also sedimented distinctly from Apaf-1 (Fig. 4 E). The coimmunoprecipitation of these two proteins from lysates of cells in which either or both are overexpressed (Hu et al., 1998; Pan et al., 1998; Moriishi et al., 1999) must be produced by overexpression because we detected no association of the endogenous proteins (Fig. 4 F), even though the antibodies used can precipitate the complexes of the overexpressed proteins (Moriishi et al., 1999). Hence, as also concluded by others (Rodriguez and Lazebnik, 1999; Zou et al., 1999), Apaf-1, and procaspase-9 do not associate inside healthy cells.

Apaf-1 Localization Was Unaltered by Elevated Bcl-2

Although we found no evidence that endogenous Bcl-2-like molecules sequester Apaf-1 to cytoplasmic membranes (Figs. 1–3 and data not shown), it seemed possible that raising the Bcl-2 concentration might cause Apaf-1 to translocate, as overexpressed CED-9 did with CED-4 (James et al., 1997; Wu et al., 1997b). Therefore, we analyzed HeLa cells that stably overexpress Bcl-2 or Bcl-x_L and were thereby rendered refractory to death induced by UV irradiation, staurosporine, or etoposide (data not shown). The Apaf-1 staining pattern in these cells was indistinguishable from that of the parental HeLa cells, and was almost entirely distinct from that of Bcl-2 (Fig. 5 A) and largely distinct from that of Bcl-x_L (Fig. 5 B). In contrast to the pan-cytoplasmic distribution of Apaf-1, Bcl-2 was predominantly perinuclear, with smaller amounts on the mitochondria (Fig. 5 A), and Bcl-x_L was mainly mitochondrial (Fig. 5, B and C). Moreover, in fractionated lysates of HeLa cells overexpressing either Bcl-2 or Bcl-x_L, Apaf-1 remained cytosolic (Fig. 5, right panels). Furthermore, even an overexpression of Apaf-1 in HeLa cells that overexpress Bcl-2 or Bcl-x_L did not lead to its colocalization with the pro-survival proteins (data not shown).

Apaf-1 Localization Was Unchanged by Apoptotic Stimuli

The recruitment of the cytosolic adapter FADD/MORT1 to the plasma membrane upon ligation of a death receptor leads to the formation of a multimeric complex that promotes the activation of caspase-8 (Kischkel et al., 1995; Ashkenazi and Dixit, 1998), most likely by the induced proximity of zymogen molecules (Salvesen and Dixit, 1999). By analogy, Apaf-1 might also shift location as apoptosis is initiated. Hence, we examined Apaf-1 in HeLa cells exposed to cell death stimuli that require its function, namely UV irradiation and etoposide (VP-16; Cecconi et al., 1998; Yoshida et al., 1998). These stimuli induced cytochrome *c* release into the cytosol and procaspase-9 cleavage (Fig. 6, compare A with B and C), accompanied by some

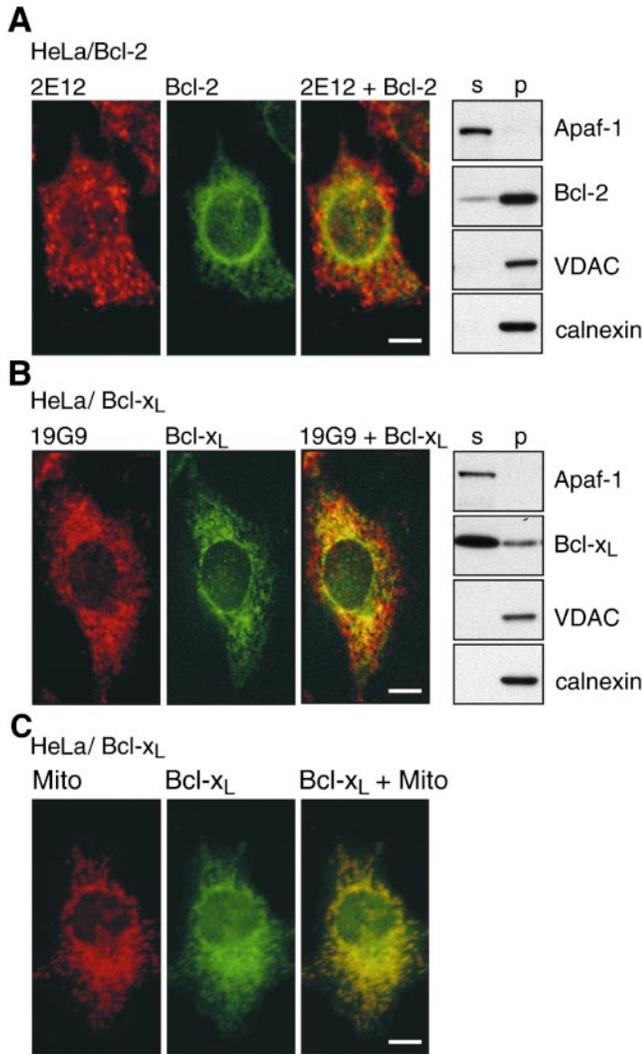


Figure 5. Endogenous Apaf-1 does not colocalize with overexpressed Bcl-2 or Bcl-x_L. Apaf-1 localization in HeLa cells that stably overexpress Bcl-2 (A) or Bcl-x_L (B) was analyzed by confocal microscopy and subcellular fractionation. Apaf-1 was revealed with Texas red-conjugated detection of anti-Apaf-1 2E12 or 19G9 staining (first panels), for Bcl-2 (with anti-human Bcl-2-100) or Bcl-x_L (anti-Bcl-x 7B2.5) revealed with FITC-conjugated. In the overlays (third panels), yellow staining indicates the extent of colocalization. Data shown are representative of ≥ 20 cells examined. The blots (right-hand panels) show equivalent aliquots of soluble (s) and pellet (p) fractions from digitonin lysates of these cells, which were probed for the indicated proteins. (C) Colocalization of Bcl-x_L with mitochondria. HeLa cells that stably overexpress Bcl-x_L were analyzed by confocal microscopy after staining with MitoTracker red (first panel) and anti-Bcl-x plus FITC-conjugated anti-mouse IgG (second panel); the images were overlaid in the third panel. Data shown are representative of at least three experiments. Bars, 10 μm .

clustering of mitochondria near the nucleus, as has been previously noted (De Vos et al., 1998; Li et al., 1998; Degli Esposti et al., 1999). Nevertheless, Apaf-1 remained entirely cytosolic, and its staining pattern was unchanged. When overexpressed, Bcl-x_L efficiently inhibited cytochrome *c* release, procaspase-9 activation, and cell death,

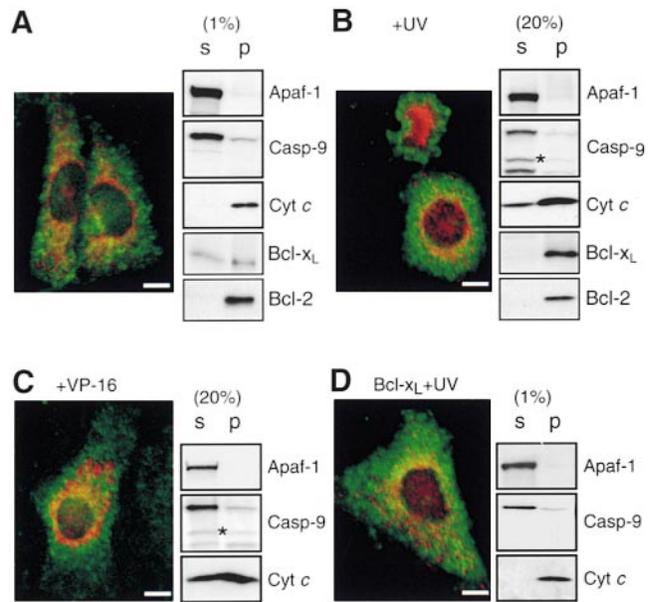


Figure 6. The location of Apaf-1 is unchanged after apoptotic stimuli, even when Bcl-x_L is overexpressed. Apaf-1 in HeLa (A–C) or a subline overexpressing Bcl-x_L (D) was examined by confocal microscopy (left) after staining with both anti-Apaf-1 2E12 (green) and MitoTracker red, or by subcellular fractionation and blotting with antibodies to the indicated proteins (right). HeLa cells were left untreated (A), or incubated for 6 h after exposure to 50 J m⁻² UV irradiation (B), or incubated for 24 h with 100 ng ml⁻¹ etoposide (VP-16) continuously (C). The HeLa/Bcl-x_L cells were incubated for 6 h after exposure to 50 J m⁻² UV irradiation (D). The proportion of dead cells at harvest is indicated in parentheses, but all the HeLa cells die by 48 h with these treatments. The asterisk marks the 35-kD-processed caspase-9 fragment. The immunofluorescence images are representative of ≥ 20 cells examined. Bars, 10 μm .

but it did not affect the location of Apaf-1 (Fig. 6 D). Immunogold EM on HepG2 cells that were induced to die by UV irradiation also failed to reveal any shift in Apaf-1 localization, even though they clearly had assumed an apoptotic morphology (data not shown).

Upon induction of apoptosis, Bcl-2 or Bcl-x_L conceivably might instead translocate to cytosolic Apaf-1. However, in accord with our previous finding that Bcl-2 and Bcl-x_L did not immunoprecipitate with Apaf-1 after induction of apoptosis (Moriishi et al., 1999), Bcl-2 remained in the insoluble fraction and, as noted previously (Hsu et al., 1997), the soluble Bcl-x_L molecules actually translocated into the insoluble fraction (Fig. 6, compare A with B). Thus, apoptotic stimuli did not provoke their association with Apaf-1.

Bcl-x_L Precluded Formation of Larger Apaf-1 Complexes In Vivo but Not In Vitro

Recent findings suggest that procaspase-9 activation involves the formation of higher order Apaf-1-containing multimers, a process which requires dATP and cytochrome *c* (Cain et al., 1999; Saleh et al., 1999; Zou et al., 1999). When HeLa cells were treated with UV, most of the endogenous Apaf-1 did enter larger complexes (Fig. 7 A),

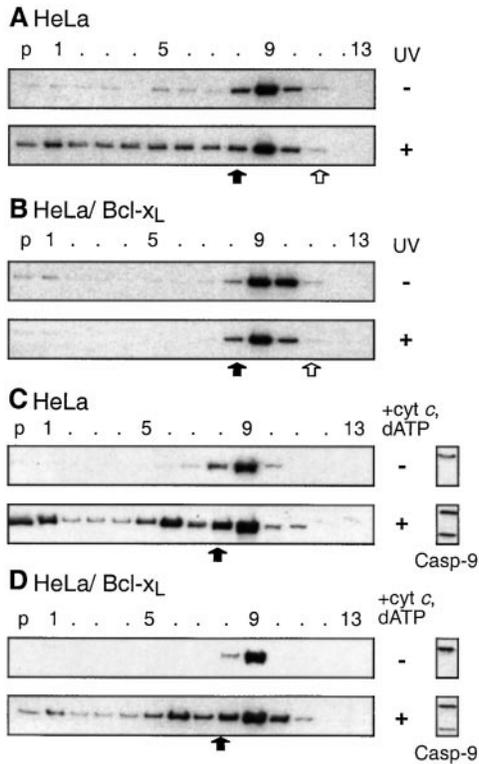


Figure 7. Apoptotic stimuli induce formation of larger Apaf-1 complexes in vivo and in vitro. (A) Larger Apaf-1-containing complexes formed in vivo in UV-irradiated cells. Lysates, prepared by Dounce homogenization in the presence of 50 μ M zVAD.fmk, from healthy HeLa cells (top) or 6 h after UV irradiation (100 J m^{-2} , bottom) were fractionated on 10–50% sucrose gradients, and the blots were probed with anti-Apaf-1 2E12. P represents pellet, which would include large protein aggregates as well as organelles. (B) Bcl- x_L blocks formation of larger Apaf-1-containing complexes. Lysates from HeLa/Bcl- x_L cells were prepared and analyzed as in A. In *in vitro* studies (C and D), addition of dATP and cytochrome *c* induced formation of larger Apaf-1-containing complexes in extracts of either parental HeLa cells (C) or a subline overexpressing Bcl- x_L (D). Equivalent amounts of the lysates were resolved on sucrose gradients after incubation in the absence (top) or presence of dATP/cytochrome *c* (bottom). Cleavage of pro-caspase-9 in the absence (C) or presence of Bcl- x_L overexpression (D) was detected by probing equivalent lysates with an antibody to caspase-9 (right panels). Markers included the 232-kD catalase (closed arrows) and the 158-kD aldolase (open arrows).

and this shift was precluded in cells that overexpress Bcl- x_L (Fig. 7 B) or Bcl-2 (data not shown). Because the pro-survival proteins also blocked cytochrome *c* release (Fig. 6 D), we tested how the addition of cytochrome *c* and dATP affected endogenous Apaf-1 in extracts of healthy cells. Addition of these cofactors caused endogenous Apaf-1 to form larger complexes and allowed its processing of endogenous procaspase-9 (Fig. 7 C). Importantly, both of these *in vitro* changes were equivalent in extracts of cells that overexpressed Bcl-2 or Bcl- x_L (Fig. 7 D and data not shown). These results suggest that Bcl-2 and Bcl- x_L do not directly prevent Apaf-1 activation, but instead function by controlling events upstream of cytochrome *c* release.

Discussion

If pro-survival Bcl-2 family members prevented the activation of caspases by sequestering the caspase-activator Apaf-1, as proposed in the apoptosome model (Hengartner, 1998), most if not all of the Apaf-1 molecules should localize with them on the organelles where they reside. Two novel mAbs, which recognize epitopes present on all known isoforms of Apaf-1 (Hu et al., 1999; Zou et al., 1999), allowed us to test this prediction with endogenous Apaf-1, thereby avoiding potential artifacts caused by its overexpression.

We found no convincing evidence for colocalization of Apaf-1 with either Bcl-2 or Bcl- x_L . On subcellular fractionation, in the presence or absence of detergent, Apaf-1 behaved almost entirely as a soluble monomeric protein, and very little comigrated with Bcl-2 or Bcl- x_L on sucrose gradients (Fig. 4 E). Apaf-1 is cytosolic (Figs. 1–4; Zhivotovsky et al., 1999) and remained so even when Bcl-2 or Bcl- x_L were overexpressed (Fig. 5) or after induction of apoptosis (Fig. 6; Zhivotovsky et al., 1999). In confocal microscopy, endogenous Apaf-1 gave a cytoplasmic pattern distinct from the nuclear membrane, mitochondria, and the ER sites where Bcl-2 and Bcl- x_L reside (Figs. 1–5). This pattern of Apaf-1 staining was observed in diverse cell types and with two antibodies that recognize different regions within Apaf-1. Our fractionation studies suggested that Apaf-1 is not bound within any intracellular membrane, and immunogold electron microscopy confirmed that Apaf-1 is dispersed over the cytoplasm and not associated with the organelles where the Bcl-2-like proteins reside (Fig. 3). Indeed, the EM studies ruled out any significant association with intracytoplasmic membranes. Thus, a combination of biochemical and imaging techniques allow us to conclude that Apaf-1 is a cytosolic protein, although it may associate with other regulatory proteins.

In support of our conclusion that endogenous Apaf-1 does not colocalize with Bcl-2 or Bcl- x_L , we recently reported that it failed to coimmunoprecipitate with any of the six known mammalian pro-survival Bcl-2 relatives in lysates of cells made either before or after induction of apoptosis (Moriishi et al., 1999). Thus, in contrast to the interaction and colocalization demonstrated for CED-9 and CED-4 by comparable experimental approaches (Chinnaiyan et al., 1997; James, 1997; Spector et al., 1997; Wu et al., 1997b), we have not detected analogous complexes between mammalian Bcl-2-like proteins and Apaf-1, even in the fraction where Apaf-1 and Bcl- x_L cosediment (data not shown). Collectively, our results strongly suggest that, contrary to the apoptosome model, the Bcl-2-like proteins do not control Apaf-1 activity by direct sequestration, but instead must govern its activation indirectly.

How might this be achieved? On induction of cell death (Fig. 6), Apaf-1 did not appear to translocate, as do the adapter FADD/MORT1 (Kischkel et al., 1995) and many of the pro-apoptotic members of the Bcl-2 family (Zha et al., 1996; del Peso et al., 1997; Hsu et al., 1997; Wolter et al., 1997; Puthalakath et al., 1999). Apaf-1, which is free from procaspase-9 in healthy cells (Fig. 4, E and F; Rodriguez and Lazebnik, 1999; Zou et al., 1999), on induction of cell death became part of larger complexes *in vivo* (Fig. 7 A; Zou et al., 1999). These complexes, which may reflect

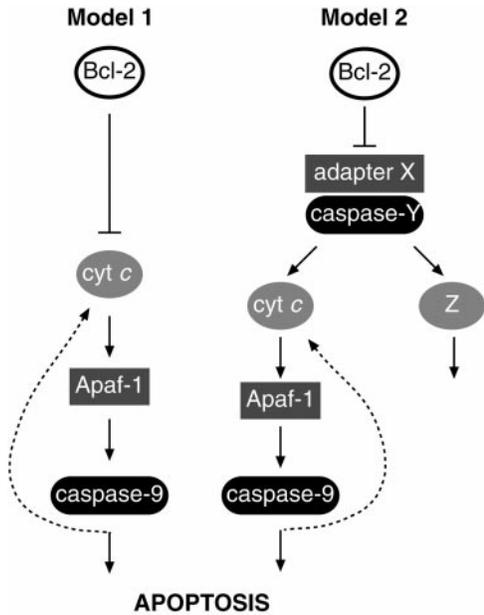


Figure 8. Two models for the regulation of Apaf-1 activity by Bcl-2 pro-survival family members (see text also). In model 1, Bcl-2 acts as a gatekeeper for mitochondria (and other organelles). Its inactivation following an apoptotic signal, probably mediated by pro-apoptotic BH3-only members of the family, leads to release of cytochrome *c* (and other pro-apoptotic molecules) to trigger activation of Apaf-1, probably by promoting its formation of large protein complexes. In model 2, Bcl-2 regulates a novel apoptosome complex, consisting of an unknown adapter X and caspase-Y, which lies upstream of Apaf-1. Activation of X and Y results in the release of cytochrome *c* and activation of Apaf-1; it may also trigger other novel activities, denoted by Z. In either model, more cytochrome *c* may be released after activation of cytosolic caspases, providing a positive feedback loop (dotted lines; Cosulich et al., 1999).

self-association and/or binding to other molecules, presumably lead to the activation of procaspase-9 (Li et al., 1997; Srinivasula et al., 1998), probably by the generation of an active holoenzyme (Rodriguez and Lazebnik, 1999). The formation of larger Apaf-1-containing complexes in apoptotic cells could be readily recapitulated *in vitro* by addition of cytochrome *c* and dATP to lysates of healthy cells (Fig. 7 C), which is consistent with other recent evidence that these Apaf-1 cofactors participate in the formation of such complexes (Cain et al., 1999; Saleh et al., 1999; Zou et al., 1999).

Because overexpressed Bcl- x_L or Bcl-2 inhibited both the cytochrome *c* release and the formation of larger Apaf-1 complexes *in vivo* after cytotoxic treatment (Figs. 6 D and 7 B), but did not prevent its aggregation after addition of exogenous dATP and cytochrome *c* (Fig. 7 D), their function may include maintenance of mitochondrial integrity to prevent Apaf-1 activation (Kluck et al., 1997; Yang et al., 1997; Bossy-Wetzel et al., 1998), as in model 1 of Fig. 8. For example, Bcl-2 might control the release of cytochrome *c* and other pro-apoptotic molecules by regulating the integrity of the mitochondrial membrane (Vander Heiden et al., 1997) or by its association with mitochondrial pores (Marzo et al., 1998; Shimizu et al., 1999). Such

a model does not preclude the possibility that Bcl-2 performs more than one function (Swiss army knife model; Hengartner, 1998), as suggested by its multiple reported biological activities and binding partners (Kroemer, 1997; Reed, 1997).

Nevertheless, given the evolutionary conservation of cell death pathways, a variation of the apoptosome model remains an attractive possibility. Although our data strongly argue against an apoptosome containing Bcl-2 or Bcl- x_L together with Apaf-1 (Moriishi et al., 1999; and this paper), Bcl-2 may instead function by direct interaction with an as-yet-unidentified mammalian CED-4 homologue, adapter X in model 2, that acts upstream of Apaf-1 (Fig. 8). The possibility that such a CED-4 homologue remains to be found is suggested by the observations that mammalian Apaf-1 and its *Drosophila* counterpart DARK (Rodriguez et al., 1999) contain a COOH-terminal regulatory region absent from the *C. elegans* protein, and that the latter does not require cytochrome *c* as a cofactor (Yang et al., 1998). In this model, the authentic mammalian CED-4 regulates an initiator procaspase (Y) whose activation results in the release of cytochrome *c* and other apoptotic factors from organelles. Apaf-1 and procaspase-9 might serve primarily to amplify the apoptotic pathway downstream of the step regulated by Bcl-2. The ability of Bcl-2 to regulate a membrane-bound procaspase (Krebs et al., 1999) would be consistent with such a model.

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