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

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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-xL 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 dispersed in the cytoplasm and not on mitochondria or other organelles. After the death stimuli, Bcl-2 and Bcl-xL 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-xL 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 deregulation 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, A paf-1 promotes the autocalytic 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). A poptosis protease–activating factor 1 (Apaf-1) is probably a crucial target for its pro-survival members because A paf-1–deficient cells are refractory to several cytotoxic signals that are inhibitable by Bcl-2 (Cecconi et al., 1998; Yoshida et al., 1998). However, whether Bcl-2 and its close relatives control A paf-1 activity directly remains a central unresolved issue (Adams and Cory, 1998; Green and Reed, 1998; Gross et al., 1999).

In the widely discussed apoptosis model (Hengartner, 1997), first proposed for the nematode Caenorhabditis elegans, the Bcl-2–like proteins directly sequester A paf-1. Developmental cell death in the worm requires both the A paf-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

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

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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 (J.ames, 1997; Wu et al., 1997b).

The conservation of the apoptotic pathway would argue for analogous mammalian apoptosomes involving A paf-1, but complementation experiments have yielded conflicting data. Overexpressed A paf-1 was reported to associate with the pro-survival proteins Bcl-xL (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 complementation of any of the known mammalian pro-survival proteins with A paf-1 (orishi et al., 1999).

As the complementation data have given conflicting results, it is important to test the apoptosome model in alternative ways. The model strongly predicts that, if not all, A paf-1 molecules will be found at the sites where pro-survival molecules like Bcl-2 and Bcl-xL reside, namely on the cytoplasmic aspect of the mitochondrion, the nuclear envelope, and the ER (M.onaghan et al., 1992; Krajewski et al., 1993; Gonzalez-Garcia et al., 1994; Lithgow et al., 1994). A though A paf-1 was originally isolated from the soluble fraction of HL-60 cells (Zou et al., 1997) and was recently reported to behave as a soluble protein on subcellular fractionation (Zhivotovsky et al., 1999), its localization inside cells has not been definitively established. Therefore, to investigate how A paf-1 might be regulated and to test the apoptosome model, we have generated mAbs to A paf-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 A paf-1 is a soluble protein with a cytoplasmic localization distinct from that of pro-survival proteins such as Bcl-2 and Bcl-xL. 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 A paf-1 and, instead, must regulate its activity by the control of upstream events.

Materials and Methods

Monoclonal Rat Anti–A paf-1 Antibodies

Monoclonal rat anti-A paf-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 A paf-1 protein (residues 1-146; caspase recruitment domain [CA RD]) 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 (H A)-tagged full-length human A paf-1 (orishi et al., 1999). The lines were cloned twice and the antibodies were purified on protein G-Sepharose columns (A mersham Pharmacia).

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

A ll cell lines were cultured in the high glucose version of D ME medium supplemented with 13 M folic acid, 250 M 1- asparagine, 50 M 2-mercaptoethanol, and 10% FCS. Human T lymphoblasts were isolated and activated as described (J.ames, 1997). 293T, COS-7, or HL-60 cells were transiently transfected using Lipofectamine (Life Technologies; H uang et al., 1997b) and analyzed the next day. To generate Hela lines that stably overexpress Bcl-2 or Bcl-xL, cells transfected with vectors that also express the puromycin resistance gene were plated at a limiting dilution in the presence of 1 mg ml-1 puromycin (Sigma Chemical Co.). Clones expressing the relevant protein were identified by flow cytometric analysis (H uang et al., 1997a). Cell death was induced by treating cells with 1 nM- 1 M staurosporine (Sigma Chemical Co.), 0.1-1.0 mg ml-1 etoposide (VP-16; D ella West), or by UV irradiation (H ela cells 10-100 J m-2; H epG2 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 J M) stained cells. The expression constructs for human A paf-1, human Bcl-2, and human Bcl-xL have been described (Huang et al., 1997a; orishi 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, 125 mM NaCl, 1% Triton X-100, and 10% glycerol), supplemented with 0.5 mg ml-1 Pefabloc, 100 mg ml-1 soybean trypsin inhibitor, and 1 mg 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 H M K E E 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 H M K E E 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; K entte Glassware Corp.). A filter 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 H M K E E buffer, centrifuged (40,000 rpm for 2 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-Va-l-A-l-D-L-α-asp-fluoromethylketone; Bachem Bioscience Inc.) was used at 50 J M.

Immunoprecipitations were performed as described (orishi et al., 1999). Total cell lysates, immunoprecipitates, or fractionated samples were resolved by SD S-PA GE (Novex) and electrobotted onto nitrocellulose membranes (A mersham Pharmacia). Nonspecific binding was blocked by incubating the filters in PBS containing 5% skimmed milk (Di ploma), 1% casein (Sigma Chemical Co.), and 0.1% Tween 20 (Sigma Chemical Co.) for >1 h before incubation with the antibody. Mouse mAbs included anti-HA 16B12 (H A.11; BA DC0), anti-HSP60 and anti-HSP90 (both from Stressgen), antipornin/νDA C (Calbiochem-Novabiochem Corp.), anti-human Bcl-2 (Bcl-2,100), anti–Bcl-x (7B2.5; a gift from Y. Lazebnik, Cold Spring Harbor Laboratory, NY), anti–Golgi S8-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 (2-22; a gift from Y. Lazebnik, Cold Spring Harbor Laboratory, NY).

Immunofluorescence Staining and Confocal Microscopy

To stain for A paf-1, cells were grown on glass coverslips (10-mm diam; L omb Scientific) or in chamber slides (Becton Dickinson & Co.). Nonadherent cells or cells treated with apoptotic stimuli were attached using poly-L-lysine or Cell T AK (Becton Dickinson & Co.), fixed with 30% acetic acid/30% methanol for 10 min at room temperature, and permeabilized

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with 0.5% Tween 20. COS cells transiently expressing A pa-f1 HA were incubated overnight at 4°C with the primary antibodies (mouse anti-HA and rat anti-A paf-1), washed with 0.2% Tween 20 in PBS, and incubated with FITC-conjugated goat anti-mouse Ig (Southern Biotechnology A ssoicates 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 A pa-f1 required amplification of the immunofluorescence signal by tyramide signal amplification (NES), 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. A pa-f1 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 A ssociates Inc.). A first tyramide amplification for 7 min (with mAbs 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 A ssociates Inc.). Controls included staining with an isotype-matched antibody (anti-rat IgG2α) overnight at 4°C.

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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). A first several washes with PBS, the cells were scraped off the dishes, gently pelleted, and resuspended in 0.1 M NaPO4, pH 7.4, containing 10% gelatin. The pellets were cooled on ice until solid, and smaller cubes were cryoprotected in 15% polyvinyl-pyrolidone 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 FCSU Inract S. The ultra-thin sections were collected onto Formvar-coated nickel grids in 1% methacrylcellose, and 1.5 M sucrose and stored at 4°C (Kleijmeer et al., 1997). The grids were floated on 2% gelatin in 0.1 M NaPO4 for 30 min. A ldehyde groups and nonspecific binding were blocked by incubating the samples first in 50 mM glycerine in PBS, then in incubation buffer (10 mM NaPO4, 0.1% cold fresh water fish gelatin, 0.5% ovalbumin, 150 mM NaCl, 20 mM Na3PO4 containing 5% normal goat serum. The samples were incubated with 2 μg ml−1 anti-A pa-f1 2E12 or rat IgG2α κ R35-95 in IB, washed, and then treated with the secondary antibody (10 nM gold-conjugated goat anti-rat IgG; British Biocell) diluted 1:40 in IB. A first 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 A GFA SN A PScan 1236 scanner and Fotolook SA. 2.09.6 software. These and the confocal scanning images were edited by using A dobe Photoshop or FreeHand software.

Results
Novel Monoclonal Anti–A pa-f1 Antibodies
To generate mAbs against human A pa-f1, myeloma cells were fused with spleen cells from rats immunized with truncated human A pa-f1 recombinant protein containing the NH2-terminal CARD and CE D-4 homologous regions (Zou et al., 1997). Clones secreting antibodies specific for A pa-f1 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, 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) A pa-f1. Bals, 10 μm.

A pa-f1 was 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 A paf-1 transiently expressed in the 293T cells, though the overexpressed protein also yielded an ~60-kD NH$_2$-terminal proteolytic fragment (Fig. 1 A). In confirmation of the specificity of the antibodies, their staining pattern on COS cells expressing human A paf-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 A paf-1 from several species, worked in all applications tested except for immunoblotting (Fig. 1 C). The 2E12 epitope mapped to the NH$_2$-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 A paf-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 A paf-1 with either antibody required tyramide signal amplification (Bobrow et al., 1992; see Materials and Methods).

In all cell types examined, endogenous A paf-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 A paf-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 A paf-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 A paf-1 pattern differed from that of mitochondria, we compared its staining, in human

![Figure 2](image-url). Confocal images showing A paf-1 localized to the cytoplasm but not significantly to the mitochondria of epithelial cells. Staining for endogenous A paf-1 in HeLa (A ), MCF-7 (B ), or HepG2 (C ) cells with anti-A paf-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-A paf-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 μm.
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 A paf-1 appeared distinct from mitochondria, as revealed by costaining with either MitoTracker red (Fig. 2 C) or for HSP60 (Fig. 2 D). A paf-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 (NIH 3T3, R at-1A) and lymphocytes (Jurkat human T-lymphoma, activated primary human T cells; data not shown). A paf-1 also failed to colocalize with markers for the Golgi apparatus (Golgi p58) or lysosomes (LysoTracker; data not shown).

The A paf-1 confocal pattern still might conceivably reflect its association with an organelle. Alternatively, as only a few hundred A paf-1 molecules would be expected in any optical plane, the staining might simply reflect the amplified signals from individual or small clusters of A paf-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 A paf-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 A paf-1 staining (Fig. 3), and identical results were obtained with HeLa cells (data not shown). Thus, in diverse cell types, A paf-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, A paf-1 is located diffusely throughout the cytoplasm.

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

It is important to establish the A paf-1 localization using different fractionation techniques, because some can generate artifacts (Hsu and Youle, 1997). To confirm that A paf-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 A paf-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-xL resided in both fractions, possibly reflecting a weaker interaction with intracytoplasmic membranes. A paf-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 A paf-1 behavior, we also prepared HeLa cell lysates by Dounce homogenization.
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-xL was more dispersed. Importantly, however, the fraction containing the vast majority of A paf-1 (fraction 5) had very little Bcl-xL (<1% by densitometric measurements) and no detectable Bcl-2. Thus, even in the absence of detergent, most A paf-1 was not associated with these pro-survival molecules. Nor was A paf-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 A paf-1 (Fig. 4 E). The communo-precipitation 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), A paf-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-xL and were thereby rendered refractory to death induced by UV irradiation, staurosporine, or etoposide (data not shown). The A paf-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-xL (Fig. 5 B). In contrast to the pan-cytoplasmic distribution of A paf-1, Bcl-2 was predominantly perinuclear, with smaller amounts on the mitochondria (Fig. 5 A and B). Moreover, in fractionated lysates of HeLa cells overexpressing either Bcl-2 or Bcl-xL, A paf-1 remained cytosolic (Fig. 5, right panels). Furthermore, even an overexpression of A paf-1 in HeLa cells that overexpress Bcl-2 or Bcl-xL 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, A paf-1 might also shift location as apoptosis is initiated. Hence, we examined A paf-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
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-xL efficiently inhibited cytochrome c release, procaspase-9 activation, and cell death, 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-xL conceivably might instead translocate to cytosolic Apaf-1. However, in accord with our previous finding that Bcl-2 and Bcl-xL 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-xL 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-xL 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 A paf-1 did enter larger complexes (Fig. 7 A),
equivalent lysates with an antibody to caspase-9 (right panels).

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 μM 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-xL blocks formation of larger Apaf-1-containing complexes. Lysates from HeLa/Bcl-xL 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-xL (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-xL 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).

Discussion

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

We found no convincing evidence for colocalization of A paf-1 with either Bcl-2 or Bcl-xL. On subcellular fractionation, in the presence or absence of detergent, A paf-1 behaved almost entirely as a soluble monomeric protein, and very little comigrated with Bcl-2 or Bcl-xL on sucrose gradients (Fig. 4 E). A paf-1 is cytosolic (Figs. 1–4; Zhivotovsky et al., 1999) and remained so even when Bcl-2 or Bcl-xL were overexpressed (Fig. 5) or after induction of apoptosis (Fig. 6; Zhivotovsky et al., 1999). In confocal microscopy, endogenous A paf-1 gave a cytoplasmic pattern distinct from the nuclear membrane, mitochondria, and the ER sites where Bcl-2 and Bcl-xL reside (Figs. 1–5). This pattern of A paf-1 staining was observed in diverse cell types and with two antibodies that recognize different regions within A paf-1. Our fractionation studies suggested that A paf-1 is not bound within any intracellular membrane, and immunogold electron microscopy confirmed that A paf-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 A paf-1 is a cytosolic protein, although it may associate with other regulatory proteins.

In support of our conclusion that endogenous A paf-1 does not colocalize with Bcl-2 or Bcl-xL, we recently reported that it failed to colocalize with CED-9 or 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 A paf-1, even in the fraction where A paf-1 and Bcl-xL cosediment (data not shown). Collectively, our results strongly suggest that, contrary to the apoptosome model, the Bcl-2-like proteins do not control A paf-1 activity by direct sequestration, but instead must govern its activation indirectly.

How might this be achieved? On induction of cell death (Fig. 6), A paf-1 did not appear to translocate, as do the adapter FA D D 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). A paf-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
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-xL 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 dories (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, 1999).

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-xL together with Apaf-1 and pro-caspase-9, 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 apoptogenic 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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