# Failure of Bcl-2 family members to interact with Apaf-1 in normal and apoptotic cells

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## Abstract

**CED-9 blocks programmed cell death (apoptosis) in the nematode *C. elegans* by binding to and neutralizing CED-4, an essential activator of the aspartate-directed cysteine protease (caspase) CED-3. In mammals, the CED-9 homologs Bcl-2 and Bcl-x$_L$ also block apoptosis by interfering with the activation of CED-3-like caspases. However, it is unknown whether this occurs by binding to the CED-4 homolog Apaf-1. Whilst two groups previously detected an interaction between Bcl-x$_L$ and Apaf-1 in immunoprecipitates,[1,2] another group found no interaction between Apaf-1 and any of ten individual members of the Bcl-2 family using the same experimental approach.[3] In this study, we aimed to resolve this discrepancy by monitoring the binding of Apaf-1 to three Bcl-2 family members within cells. Using immunofluorescence and Western blot analysis, we show that whilst Apaf-1 is a predominantly cytoplasmic protein, Bcl-2, Bcl-x$_L$ and Bax mostly reside on nuclear/ER and mitochondrial membranes. This pattern of localization is maintained when the proteins are co-expressed in both normal and apoptotic cells, suggesting that Bcl-2, Bcl-x$_L$ or Bax do not significantly sequester cytoplasmic Apaf-1 to intracellular membranes. In addition, we confirm that Apaf-1 does not interact with Bcl-2 and Bcl-x$_L$ in immunoprecipitates. Based on these data, we propose that Apaf-1 is not a direct, physiological target of Bcl-2, Bcl-x$_L$ or Bax.** *Cell Death and Differentiation* (2000) **7,** 947–954.

**Abbreviations:** HEK, human embryonic kidney; R6, rat 6 embryo fibroblasts; CED, product of cell-death-abnormal gene; caspase, cysteinyl aspartate-specific proteinase; Apaf-1, apoptotic protease activating factor-1; BH, Bcl-2 homology domain; COX, cytochrome c oxidase; NP-40, Nonidet P-40; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate; ER, endoplasmic reticulum; IP, immunoprecipitation

## Introduction

Programmed cell death, or apoptosis, is the physiological process that removes superfluous and damaged cells from a multicellular organism.[4] Genetic studies in the nematode *C. elegans* revealed that programmed cell death during development is executed by the aspartate-directed cysteine protease (caspase) CED-3.[5] Activation of this protease occurs by binding an adaptor molecule CED-4[6,7] which oligomerizes and brings into proximity the inactive pro-form of CED-3 enabling it to autoprocess to the active protease.[8–10] Another protein, CED-9, blocks cell death by forming a complex with CED-4 and attenuating its ability to oligomerize and activate CED-3.[6,7,9,10,] The ability of CED-9, CED-4 and CED-3 to exist in a multiprotein complex has led to the 'apoptosome' model of cell-death regulation.[11]

Several counterparts of this genetic network have been identified in higher eukaryotes. Up to 14 mammalian CED-3-like caspases are known. Caspases-3 and -7 (and probably also caspase-6) function as executioner or effector caspases because they are activated in all types of apoptosis, their inhibition or chromosomal deletion interfere with apoptosis and/or they cleave most of the cellular substrates implicated in apoptosis.[12,13] The other caspases are so called initiator caspases which form a proteolytic cascade on selected signaling pathways (Fas/CD95, cytokine removal, etc.) to cleave and activate the inactive pro-forms of effector caspases.[12–14] The need for CED-4 homologs in caspase activation was not appreciated until the isolation of the first mammalian homolog Apaf-1.[15] Peculiarly, Apaf-1 does not directly activate effector caspases but forms oligomers to facilitate the activation of the initiator caspase-9 which in turn cleaves and activates effector caspases-3 and -7.[16–19] In addition, activation of Apaf-1 requires the binding of the mitochondrial component cytochrome c[15,20] which is released into the cytoplasm in response to many apoptotic stimuli.[21] Thus, like CED-4, Apaf-1 activates a CED-3-like caspase, but its function depends on mitochondria.

The mammalian homologs of CED-9 are a family of proteins that consists of anti-apoptotic (e.g. Bcl-2) and pro-apoptotic (e.g. Bax) members.[22–24] In analogy with *C. elegans*, anti-apoptotic proteins such as Bcl-2 and Bcl-x$_L$ might function by binding to CED-4 homologs such as Apaf-1 to prohibit the activation of CED-3-like effector caspases. In support of this notion, Bcl-2 and Bcl-x$_L$ have been shown to interfere with the activation of effector caspases,[25–27] and Bcl-x$_L$ has been reported to form a complex with CED-4 upon co-overexpression in mammalian cells.[6,28] Moreover, two recent studies have shown that Bcl-x$_L$ interacts with Apaf-1 in immunoprecipitates from lysates of cells overexpressing both proteins.[1,2] This interaction prevented caspase-9 activation either within a ternary caspase-9/Apaf-1/Bcl-x$_L$ complex[2] or by disrupting

the binding of Apaf-1 to caspase-9.[1] Although this finding is in agreement with the nematodal 'apoptosome' model, it is inconsistent with the modes of action that have so far been described for Bcl-2 and Bcl-x$_L$. In contrast to CED-9, Bcl-2 and Bcl-x$_L$ act by blocking the release of cytochrome c, i.e. at a step occurring before Apaf-1 activation.[29–31] Although the exact mechanism of this effect is yet unknown, it has

been proposed that Bcl-2 and Bcl-x$_L$ directly regulate the permeability of the mitochondrial membrane due to their association with this organelle[23] and/or their presumed pore forming activity.[32,33] Alternatively, they may sequester a cytoplasmic, cytochrome c-release factor such as the Bcl-2 family member BID.[34,35] Based on these propositions, Moriishi *et al* have recently challenged the idea that Apaf-
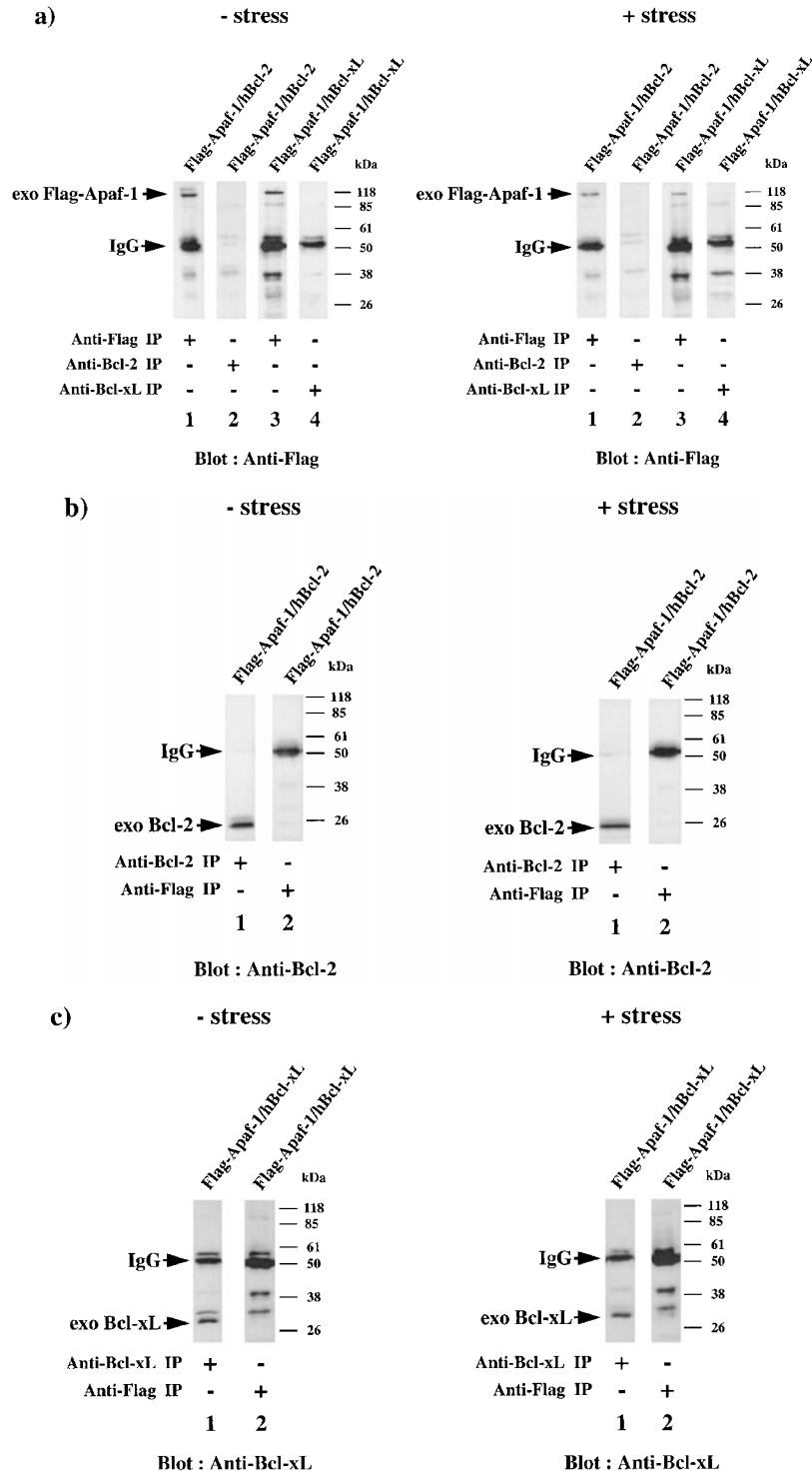
1 and Bcl-x$_L$ interact by showing that Bcl-x$_L$ or nine other Bcl-2 family members do not co-immunoprecipitate with Apaf-1 from lysates of mammalian cells.[3]

As Bcl-2 and Bcl-x$_L$ are both integral membrane proteins, they have to be solubilized by detergents for immunoprecipitation studies. It has however been reported that detergents can either promote or disrupt interactions between Bcl-2 family members.[36,37] We therefore needed to investigate the interaction between Apaf-1 and Bcl-2 family members in an experimental system that does not require detergent extraction. We chose an immunofluorescence analysis on fixed, permeabilized rat embryo fibroblasts overexpressing the proteins and show that three Bcl-2 family members Bcl-2, Bcl-x$_L$, or Bax cannot attract cytoplasmic Apaf-1 to membranes in normal and apoptotic cells. These data raise doubts that Apaf-1 is a direct target of Bcl-2, Bcl-x$_L$ or Bax in mammalian cells.

## Results

We first studied interactions between Apaf-1 and two anti-apoptotic (Bcl-2 and Bcl-x$_L$) and one pro-apoptotic (Bax) Bcl-2 family members using the previously reported immunoprecipitation approach.[1–3] For that purpose, N-terminally, Flag-tagged Apaf-1 was transiently co-transfected with either Bcl-2, Bcl-x$_L$ or Bax into human embryonic kidney 293 (HEK293) cells. In some cases, the cells were treated for different time periods with apoptotic agents such as the proteasomal inhibitor MG132 to determine whether interactions between Flag-Apaf-1 and Bcl-2 or Flag-Apaf-1 and Bcl-x$_L$ are only
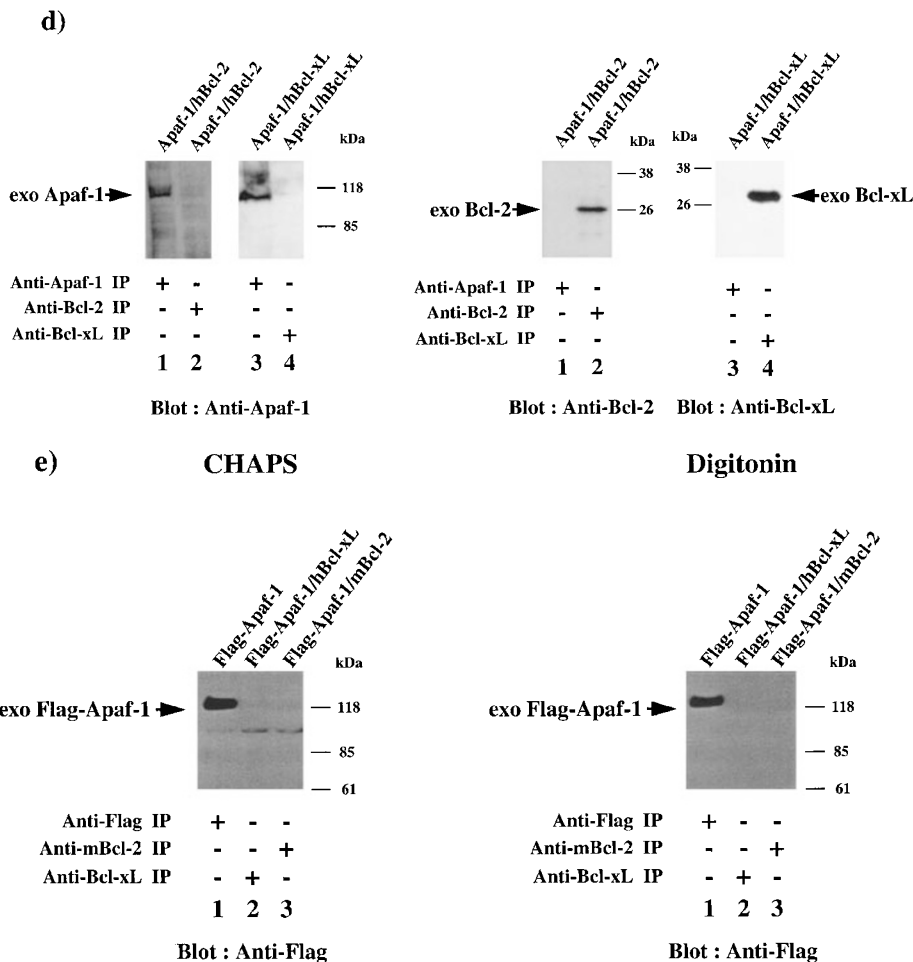


**Figure 1** Flag-Apaf-1 or Apaf-1 do not interact with Bcl-2 or Bcl-x$_L$ in immunoprecipitates. Anti-Flag (**a,e**), anti-Bcl-2 (**b,d**), anti-Bcl-x$_L$ (**c,d**), or anti-Apaf-1 (**d**) Western blots of anti-Flag, anti-Bcl-2, anti-Bcl-x$_L$ or anti-Apaf-1 immunoprecipitates (IP) from 0.2% NP-40 (**a–d**), 0.2% CHAPS (**e**) or 0.5% digitonin (**e**) lysates of HEK293 cells transfected with Flag-Apaf-1 alone or co-transfected with Flag-Apaf-1 and human Bcl-2 (hBcl-2), Flag-Apaf-1 and mouse Bcl-2 (mBcl-2), Flag-Apaf-1 and human Bcl-x$_L$ (hBcl-x$_L$), Apaf-1 and human Bcl-2, or Apaf-1 and human Bcl-x$_L$, treated (+stress) or not (−stress) with 1 $\mu$M of the proteasome inhibitor MG132 for 24 h. Note that exogenous Flag-Apaf-1 is present in anti-Flag (**a**, lanes 1 and 3, **e**, lanes 1) but not in anti-Bcl-2 (**a**, lanes 2, **e**, lanes 3) or anti-Bcl-x$_L$ (**a**, lanes 4, **e**, lanes 2) IPs from co-transfected cells. Similarly, Apaf-1 is present in anti-Apaf-1 (**d**, lanes 1 and 3, left panel), but not in anti-Bcl-2 (**d**, lane 2, left panel) or anti-Bcl-x$_L$ (**d**, lane 4, left panel) IPs. By contrast, exogenous Bcl-2 is present in anti-Bcl-2 (**b**, lanes 1, **d**, lane 2, middle panel) but not in anti-Flag (**b**, lanes 2) or anti-Apaf-1 (**d**, lane 1, middle panel) IPs and exogenous Bcl-x$_L$ is present in anti-Bcl-x$_L$ (**c**, lanes 1, **d**, lane 4, right panel) but not in anti-Flag (**c**, lanes 2) or anti-Apaf-1 (**d**, lane 3, right panel) IPs from co-transfected cells. The two bands at 32 and 38 kDa detected in anti-Flag IPs (**c**, lanes 2) correspond to cross-reactive proteins as they are not detected in anti-Apaf-1 IPs (**d**, lane 3, right panel). Sometimes the secondary antibody reacted with the heavy IgG (ca. 50 kDa) of the primary antibody

formed when the latter two proteins act as death suppressors.[37] Cell lysates prepared in the presence of the detergent Nonidet P-40 (NP-40) were subjected to anti-Flag, anti-Bcl-2 or anti-Bcl-x$_L$ immunoprecipitations, and the immunoprecipitates were Western-blotted for the detection of co-precipitating proteins. As shown in Figures 1 and 3, the proteins were similarly co-expressed so that interactions should have been

seen in immunoprecipitates if they indeed occurred. An anti-Flag Western blot showed that Flag-Apaf-1 was present in anti-Flag (Figure 1a, lanes 1 and 3) but not in anti-Bcl-2 (Figure 1a, lane 2) or anti-Bcl-x$_L$ (Figure 1a, lane 4) immunoprecipitates of HEK293 cell lysates co-overexpressing Flag-Apaf-1 and Bcl-2 or Flag-Apaf-1 and Bcl-x$_L$, respectively. Similarly, whilst Bcl-2 or Bcl-x$_L$ could be
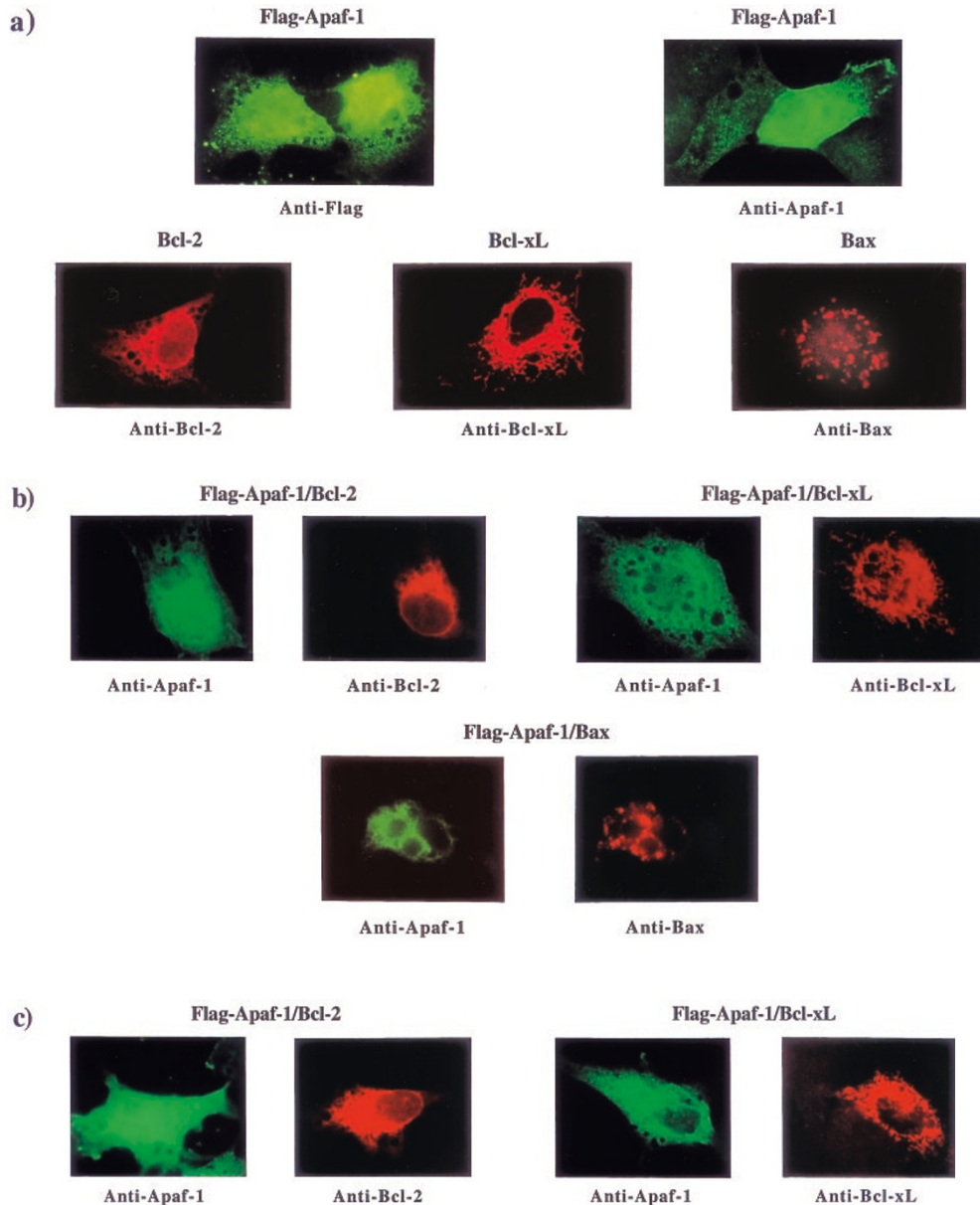


**Figure 2** Bcl-2, Bcl-x$_L$ or Bax cannot attract cytoplasmic Flag-Apaf-1 to intracellular membranes in both normal and apoptotically stressed cells. (**a**) Anti-Flag or anti-Apaf-1 (fluorescein), and anti-Bcl-2, anti-Bcl-x$_L$ or anti-Bax (all Texas Red) immunofluorescence analysis on rat embryo fibroblasts (R6) transfected with Flag-Apaf-1, hBcl-2, hBcl-x$_L$ or hBax for 15 h, respectively. (**b**) Anti-Apaf-1 (fluorescein) and anti-Bcl-2, anti-Bcl-x$_L$ or anti-Bax (all Texas Red) immunofluorescence analysis on R6 cells co-transfected with Flag-Apaf-1 and hBcl-2 or Flag-Apaf-1 and hBcl-x$_L$ or Flag-Apaf-1 and hBax for 15 h, respectively. (**c**) Immunofluorescence analysis of co-transfected R6 cells as in (**b**) but after treating the cells with 1 $\mu$M MG132 for 1 h (out of 15 h total transfection time). Note that Flag-Apaf-1 and Apaf-1 show a diffuse, cytoplasmic/nuclear staining irrespective of whether it is co-transfected with Bcl-2 (nuclear envelope/endoplasmic reticulum), Bcl-x$_L$ (mitochondria, elongated) or Bax (mitochondria, punctuated) and/or whether the cells are stressed with the apoptotic agent MG132. The cells expressing Bax undergo apoptosis (nuclear fragmentation) (data not shown)

specifically immunoprecipitated with anti-Bcl-2 or anti-Bcl-x$_L$ antibodies, respectively (Figure 1b, c, lanes 1), they did not co-immunoprecipitate with anti-Flag antibodies from the same cell extracts (Figure 1b, c, lanes 2). This was also the case for extracts prepared from cells exposed to MG132 for 0 – 24 h (Figure 1, compare − stress and +stress, 24 h). The anti-Flag antibody seemed to co-immunoprecipitate two proteins at 32 and 38 kDa which were detected by the anti-Bcl-x$_L$ antibody (Figure 1c). However, upon close examination, the same proteins were also detected by anti-Flag and anti-Bcl-2 antibodies (Figure 1a, lanes 1 and 3 and Figure 1b, lane 2). By contrast, a highly specific anti-Apaf-1 antibody did not co-immunoprecipitate these proteins (Figure 1d, lane 3 in the anti-Bcl-x$_L$ panel). This indicates that the 32 and 38 kDa proteins are most likely degradation products of IgG heavy chains of the anti-Flag antibody rather than modified (i.e. phosphorylated) forms of Bcl-x$_L$. The lack of interaction between Flag-Apaf-1 and Bcl-2 or Bcl-x$_L$ was not due to the Flag-tag, as the same results were obtained by transfecting non-tagged Apaf-1 and using an anti-Apaf-1 specific antibody (Figure 1d). The latter experiment was also instructive because it did not reveal any interaction between Bcl-2 or Bcl-x$_L$ and endogenous Apaf-1 (Figure 1d, lanes 2 and 4 in anti-Apaf-1 panel). Moreover, the failure of these molecules to

form complexes was not due to the experimental conditions as anti-Bcl-2 effectively co-immunoprecipitated endogenous Bax, a known physiological partner of Bcl-2.[37,38] It was also highly unlikely that the putative Apaf-1/Bcl-2 complex was disrupted by the immunoprecipitation antibodies, because we used both polyclonal (data not shown) and monoclonal (Figure 1) antibodies to Bcl-x$_L$ and Bcl-2. We also used two other detergents (digitonin, CHAPS) for cell lysis and immunoprecipitation and still did not find interactions between Apaf-1 and Bcl-2 or Apaf-1 and Bcl-x$_L$ (Figure 1e). Unfortunately, we were unable to monitor the interaction between Apaf-1 and Bax as this combination killed most of the co-expressing cells before they could be lysed. Thus, our data confirm the recent report by Moriishi et al[3] that Apaf-1 does not form a stable complex with Bcl-2 or Bcl-x$_L$ under the conditions used for immunoprecipitations.

Although we tested different detergents (NP-40, CHAPS, digitonin) for our in vitro experiments, it was still possible that Bcl-2 and Bcl-x$_L$ interacted with Apaf-1 in cells, but that this interaction was disrupted during cell lysis and/or immuno-precipitation. To resolve this issue, we performed an immunofluorescence analysis on rat embryo fibroblasts (R6), Hela, HEK293 and SW480 human colon carcinoma cell lines which had previously been co-transfected with Flag-
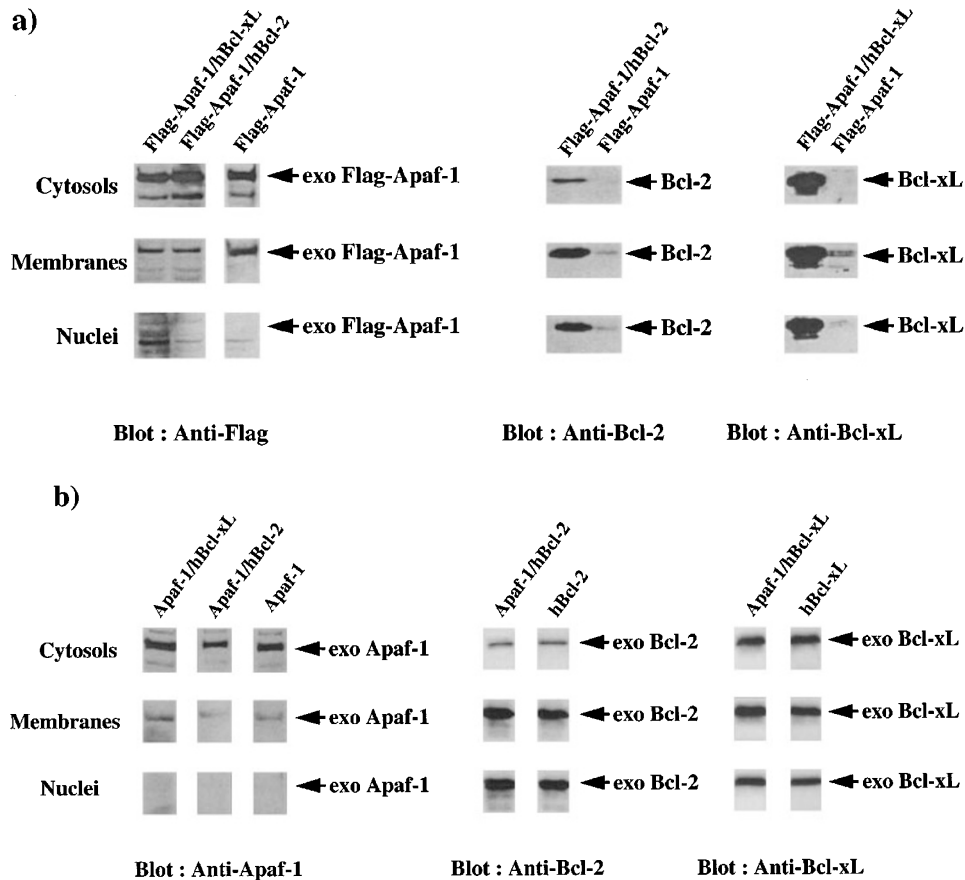


Figure 3 Apaf-1 is predominantly cytoplasmic while Bcl-2 and Bcl-x$_L$ are mainly found in nuclear and membrane fractions. (a) Anti-Flag, anti-Bcl-2 or anti-Bcl-x$_L$ Western blots of cytosolic, nuclear and membrane fractions of HEK293 cells transfected with Flag-Apaf-1 or co-transfected with Flag-Apaf-1 and hBcl-2 or Flag-Apaf-1 and hBcl-x$_L$. (b) Anti-Apaf-1, anti-Bcl-2 or anti-Bcl-x$_L$ Western blots of cytosolic, nuclear and membrane fractions of HEK293 cells transfected with Apaf-1, hBcl-2 or hBcl-x$_L$ or co-transfected with Apaf-1 and hBcl-2 or Apaf-1 and hBcl-x$_L$

Apaf-1 and Bcl-2, Flag-Apaf-1 and Bcl-$x_L$ or Flag-Apaf-1 and Bax. Here we show the results in R6 cells but similar results were obtained in the other cell lines. After transient transfection, Flag-Apaf-1 displayed a diffuse immunostaining in the cytoplasm with both anti-Flag and anti-Apaf-1 antibodies (Figure 2a, Flag-Apaf-1). This staining was specific as non-transfected cells revealed only a low background staining with both antibodies (see neighboring cell in Figure 2a, anti-Apaf-1). The nuclear staining of Flag-Apaf-1 was probably due to the non-confocal type of immunofluorescence method used here as no Flag-Apaf-1 was detected in nuclear fractions isolated from Flag-Apaf-1-transfected cells (Figure 3a, nuclei). In contrast to the diffuse immunostaining of Apaf-1, Bcl-$x_L$ was observed on elongated, 'spaghetti-like' structures that co-localized with the mitochondrial marker cytochrome c oxidase (COX) (Figure 2a, Bcl-$x_L$, and data not shown). Bax was also clearly mitochondrial, but in a punctated pattern, and the cells containing high levels of Bax were dying by apoptosis (Figure 2a, Bax, and ref[39]). Finally, Bcl-2 showed a broad subcellular localization pattern that included the nuclear envelope and the endoplasmic reticulum as previously reported (Figure 2a, Bcl-2).[40] Thus, if Flag-Apaf-1 interacted with Bcl-2 or Bcl-$x_L$ upon co-overexpression, it should migrate to their sites of localization. However, despite effective and equal co-overexpression with Bcl-2, Bcl-$x_L$ or Bax, Flag-Apaf-1 continued to display a predominantly cytoplasmic localization (Figure 2b). The same result was seen when the cells were stressed with MG132 or other apoptotic stimuli for different time periods (Figure 2c, 1 $\mu$M MG132 for 1 h is shown here). Thus, Apaf-1 does not seem to be attracted to intracellular sites where Bcl-2, Bcl-$x_L$ or Bax reside.

To confirm that Apaf-1 was mostly cytoplasmic and Bcl-2 or Bcl-$x_L$ were mostly membrane-bound even in co-transfected cells, we performed Western blots of cytosolic, membrane and nuclear fractions of HEK293 cells either transfected with Flag-Apaf-1, Apaf-1, Bcl-2 or Bcl-$x_L$ alone or co-transfected with Flag-Apaf-1 (or Apaf-1) and Bcl-2 or Flag-Apaf-1 (or Apaf-1) and Bcl-$x_L$. Although some Flag-Apaf-1 and Apaf-1 were detected in the membrane fraction, most of these proteins resided in the cytosol, irrespective of the co-presence of overexpressed Bcl-2 or Bcl-$x_L$ (Figure 3a, b). By contrast, both Bcl-2 and Bcl-$x_L$ localized to membranes or nuclei, and this distribution did not change in the presence of overexpressed Flag-Apaf-1 (Figure 3a) or Apaf-1 (Figure 3b). Thus, Apaf-1 has a cytoplasmic localization distinct from Bcl-2 or Bcl-$x_L$.

## Discussion

Despite intensive research, it has remained unknown how anti-apoptotic Bcl-2 family members prevent caspase activation. Two modes of action have been proposed, one which indirectly and another which directly affects Apaf-1 function. The indirect model suggests that Bcl-2 and/or Bcl-$x_L$ act on the outer mitochondrial membrane to prevent the release of cytochrome c,[29–31] a necessary co-factor for Apaf-1 oligomerization and the subsequent activation of caspase-9 and -3.[15–17,20] The direct model suggests a physical interaction between Bcl-2 and Apaf-1 or Bcl-$x_L$ and Apaf-1.

Heterologous overexpressions of the *C. elegans* death regulators in mammalian cells have shown that CED-9, CED-4 and CED-3 exist in a multiprotein complex, dubbed the apoptosome, in which CED-9 inhibits apoptosis by binding to CED-4, thereby preventing it from activating CED-3.[6,7,9–11] Interestingly, such interactions have not only been detected by co-immunoprecipitation but also using immunofluorescence analysis where soluble CED-4 was sequestered to the perinuclear region by CED-9.[28] The latter study showed that it should be possible to detect a similar attraction between Apaf-1 and Bcl-2 or Bcl-$x_L$ if the analogous mammalian apoptosome indeed consisted of these factors.

Our data disfavor the notion that Apaf-1 forms a mammalian apoptosome with Bcl-2 or Bcl-$x_L$. Why are our results in conflict with two previous studies that have reported direct interactions between Bcl-$x_L$ and Apaf-1?[1,2] Both studies used immunoprecipitation approaches to determine protein-protein interactions. Although Moriishi *et al*[3] and we have used immunoprecipitation protocols that closely replicated those reported[1,2] it may be difficult to reach the exact same experimental conditions for reproducing the results. For example, we noticed that, when in abundance, Apaf-1, Bcl-2 and Bcl-$x_L$ all bind non-specifically to Sepharose beads (data not shown), and thus part of the presumed Apaf-1/Bcl-2 or Apaf-1/Bcl-$x_L$ complexes may originate from such artefacts. In addition, there remains the possibility that the quality of the detergents influences complex formations during cell lysis and immunoprecipitation. Our use of immunofluorescence to study the interaction between Apaf-1 and Bcl-2 family members within cells circumvents these in vitro problems. In several different cell types co-transfected with Flag-Apaf-1, Apaf-1, Bcl-2, Bcl-$x_L$ or Bax and exposed to different apoptotic stimuli for various time periods, we have not seen any evidence that the diffusely stained, cytoplasmic Apaf-1 is sequestered to nuclear or mitochondrial membranes in a similar fashion that CED-9 sequesters CED-4.[28] Is it possible that Apaf-1 was recruited to Bcl-2 or Bcl-$x_L$ at levels undetectable by immunofluorescence? Although such a possibility cannot be entirely excluded, we think that we should have seen a major sequestration by this technique as the apoptosome model in *C. elegans* accounts for a tight complex formation between CED-9 and CED-4 in healthy cells.[28,41] In addition, Western blot analysis of subcellular fractions of HEK293 cells co-transfected with Flag-Apaf-1/Bcl-2 or Flag-Apaf-1/Bcl-$x_L$ revealed that both Apaf-1 and Flag-Apaf-1 were predominantly present in the cytosol rather than in membrane fractions. Thus, instead of being sequestered to membrane-associated Bcl-2 or Bcl-$x_L$, Apaf-1 seems to constitutively reside in the cytoplasm to await the release of cytochrome c for the activation of caspase-9. A novel CED-4 homolog, named Nod1/CARD4[42,43] has recently been identified, and it will be interesting to determine whether it fulfills the criteria to form an apoptosome with Bcl-2 or Bcl-$x_L$. Based on the findings that Bcl-2 and Bcl-$x_L$ prevent the release of cytochrome c, it is expected that these proteins bind to and inhibit a CED-4 homolog that functions upstream of mitochondria to initiate the release of cytochrome c and other apoptogenic factors.

# Materials and Methods

## Subcellular fractionation, immunoprecipitations and Western blot analysis

HEK293 cells were co-transfected with 5 $\mu$g of each human Flag-Apaf-1/pcDNA3 (kindly provided by V Dixit), human Apaf-1/pcDNA3.1($-$) (kindly provided by X Wang), human Bcl-2/pcDNA3, mouse Bcl-2/pcDNA3 or human Bcl-x$_L$/pcDNA3 (kindly provided by G Nuñez) using 25 $\mu$l of Superfect (Qiagen) as described by the manufacturer. In some cases, the cells were treated with 1 $\mu$M of MG132 (Alexis) for 0–24 h. The cells were lysed in 0.2% NP-40, 0.2% CHAPS or 0.5% digitonin and subjected to immunoprecipitation using 3 $\mu$l of mouse monoclonal anti-hBcl-2/100 (Neomarker), 5 $\mu$l of rabbit polyclonal anti-m/rBcl-2/27-6,[37] 3 $\mu$l of mouse monoclonal anti-Bcl-x$_L$/2H12 (Zymed), 2.5 $\mu$l of mouse monoclonal anti-Flag/M5 (Sigma) or 5 $\mu$l of rabbit polyclona anti-Apaf-1/IP14 (Zymed) and 50 $\mu$l of 50% (v/v) protein G-Sepharose (PharmaciaAmersham) or protein A-Sepharose (Sigma) as previously described.[37] Immunocomplexes were subjected to Western blot analysis using either anti-hBcl-2/100 at a titer of 1 : 200, anti-Bcl-x$_L$/2H12 at a titer of 1 : 1000, anti-Flag/M5 at a titer of 1 : 1000 or anti-Apaf-I/IP14 at a titer of 1 : 1000. Secondary antibodies were Fc$\gamma$-specific, peroxidase-coupled goat anti-rabbit or anti-mouse antibodies (Jackson ImmunoResearch Laboratories), and the detection system was enhanced chemiluminescence (ECL, AmershamPharmacia). For subcellular fractionation analysis, the co-transfected HEK293 cells were lysed in buffer A/sucrose (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl$_2$, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.25 M sucrose, 10 $\mu$g/ml leupeptin, 2 $\mu$g/ml aprotinin, 0.4 mM Pefabloc, 1 $\mu$g/ml pepstatin) using a tight fitting Dounce homogenizer. The lysates were centrifuged at $750 \times g$ to obtain a nuclear pellet. The postnuclear supernatant was further centrifuged at $100\ 000 \times g$ to obtain a membrane pellet and the cytosol. Thirty $\mu$g of each fraction was loaded on polyacrylamide gels for Western blot analysis.

## Immunofluorescence analysis

R6 cells were transfected with 0.8 $\mu$g of each Flag-hApaf-1/pcDNA3, hBcl-2/pcDNA3, hBcl-x$_L$/pcDNA3 or hBax/pcDNA3 in 2.4 $\mu$l (single transfections) or 4.8 $\mu$l (double transfections) Superfect as described by the manufacturer (Qiagen). The cells were fixed in 4% paraformaldehyde, and permeabilized with 0.05% saponin and acetone as previously described.[39] The cells were treated with mouse monoclonal anti-Flag (1 : 100), rabbit polyclonal anti-Apaf-1/IP14 (1 : 50), mouse monoclonal anti-hBcl-2/100 (1 : 100), mouse monoclonal anti-Bcl-x$_L$/2H12 (1 : 100) or mouse monoclonal anti-hBax/2D2 (1 : 100) (Zymed) for 1 h followed by incubations with fluorescein- and Texas Red-conjugated goat anti-rabbit and anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratories). After postfixation in 4% paraformaldehyde, the antifading agent Slowfade (Molecular Probes) was added, and the cells were viewed under a regular Zeiss Axiovert fluorescence microscope at a magnification of $1000 \times$. Pictures were taken with a Contax 167 MT camera.

# Note added in proof

While this paper was under review, Hausmann *et al*[44] published that Apaf-1 does not colocalize with Bcl-2 or Bcl-x$_L$ when analyzed by immunofluorescence and immunogold electron microscopy. Moreover, Newmeyer *et al*[45] and Haraguchi *et al*[46] used *in vitro* assays and cells deficient in Apaf-1, respectively, to show that Bcl-2 and Bcl-x$_L$ do not require Apaf-1 for their caspase-inhibiting and death protective function.

# Acknowledgements

# References

1. Hu Y, Benedict MA, Wu D, Inohara N and Nuñez G (1998) Bcl-x$_L$ interacts with Apaf-1 and inhibits Apaf-1-dependent caspase-9 activation. Proc. Natl. Acad. Sci. U.S.A. 95: 4386–4391

2. Pan G, O'Rourke K and Dixit VM (1998) Caspase-9, Bcl-x$_L$, and Apaf-1 form a ternary complex. J. Biol. Chem. 273: 5841–5845

3. Moriishi K, Huang DC, Cory S and Adams JM (1999) Bcl-2 family members do not inhibit apoptosis by binding the caspase activator Apaf-1. Proc. Natl. Acad. Sci. U.S.A. 96: 9683–9688

4. Vaux DL and Korsmeyer SJ (1999) Cell death in development. Cell 96: 245–254

5. Shaham S and Horvitz HR (1996) Developing *Caenorhabditis elegans* neurons may contain both cell-death protective and killer activities. Genes Dev. 10: 578–591

6. Chinnaiyan AM, O'Rourke K, Lane BR and Dixit VM (1997) Interaction of CED-4 with CED-3 and CED-9: a molecular framework for cell death. Science 275: 1122–1126

7. Wu D, Wallen HD, Inohara N and Nuñez G (1997) Interaction and regulation of the *C. elegans* death protease CED-3 by CED-4 and CED-9. J. Biol. Chem. 272: 21449–21454

8. Chinnaiyan AM, Chaudhary D, O'Rourke K, Koonin EV and Dixit VM (1997) Role of CED-4 in the activation of CED-3. Nature 388: 728–729

9. Seshagiri S and Miller LK (1997) *Caenorhabditis elegans* CED-4 stimulates CED-3 processing and CED-3-induced apoptosis. Curr. Biol. 7: 455–460

10. Yang X, Chang HY and Baltimore D (1998) Essential role of CED-4 oligomerization in CED-3 activation and apoptosis. Science 281: 1355–1357

11. Hengartner MO (1997) CED-4 is a stranger no more. Nature 388: 714–715

12. Earnshaw WC, Martins LM and Kaufmann SH (1999) Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu. Rev. Biochem. 68: 383–424

13. Nicholson DW (1999) Caspase structure, proteolytic substrates, and function during apoptotic cell death. Cell Death Differ. 6: 1028–1042

14. Kumar S (1999) Mechanisms mediating caspase activation in cell death. Cell Death Differ. 6: 1060–1066

15. Zou H, Henzel WJ, Liu X, Lutschg A and Wang X (1997) Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspases. Cell 90: 405–413

16. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES and Wang X (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91: 479–489

17. Srinivasula SM, Ahmad M, Fernandes-Alnemri T and Alnemri ES (1998) Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. Mol. Cell 1: 949–957

18. Cain K, Brown DG, Langlais C and Cohen GM (1999) Caspase activation involves the formation of the aposome, a large ($\sim$700 kDa) caspase-activating complex. J. Biol. Chem. 274: 22686–22692

19. Cecconi F (1999) Apaf-1 and the apoptotic machinery. Cell Death Differ. 6: 1087–1098

20. Hu Y, Benedict MA, Ding L and Nuñez G (1999) Role of cytochrome c and dATP/ATP hydrolysis in Apaf-1-mediated caspase-9 activation and apoptosis. EMBO J. 18: 3586–3595

21. Green DR and Reed JC (1998) Mitochondria and apoptosis. Science 281: 1309–1312

22. Adams JA and Cory S (1998) The Bcl-2 protein family: arbiters of cell survival. Science 281: 1322–1326

23. Reed J (1998) Bcl-2 family proteins. Oncogene 17: 3225–3236

24. Kelekar A and Thompson CB (1998) Bcl-2 family proteins: the role of the BH3 domain in apoptosis. Trends Cell Biol. 8: 324–330

25. Monney L, Otter I, Olivier R, Ravn U, Mirzasaleh H, Fellay I, Poirier GG and Borner C (1996) Bcl-2 overexpression blocks activation of the death protease CPP32/Yama/Apopain. Biochem. Biophys. Res. Commun. 221: 340 – 345

26. Chinnaiyan AM, Orth K, O'Rourke K, Duan H, Poirier GG and Dixit VM (1996) Molecular Ordering of the cell death pathway: Bcl-2 and Bcl-xL function upstream of the CED-3-like apoptotic proteases. J. Biol. Chem. 271: 4573 – 4576

27. Boulakia CA, Chen G, Ng FWH, Teodore JG, Branton PE, Nicholson DW, Poirier GG and Shore GC (1996) Bcl-2 and adenovirus E1B 19 kDA protein prevent E1A-induced processing of CPP32 and cleavage of poly(ADP-ribose) polymeras. Oncogene 12: 529 – 535

28. Wu D, Wallen HD and Nuñez G (1997) Interaction and regulation of subcellular localization of CED-4 by CED-9. Science 275: 1126 – 1129

29. Kluck RM, Bossy-Wetzel E, Green DR and Newmeyer DD (1997) The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 275: 1132 – 1136

30. Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng T-I, Jones DP and Wang X (1997) Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science 275: 1129 – 1132

31. Kharbanda S, Pandey P, Schofield L, Israels S, Roncinske R, Yoshida K, Bharti A, Yuan Z-M, Saxena S, Weichselbaum R, Nalin C and Kufe D (1997) Role for Bcl-xL as an inhibitor of cytosolic cytochrome c accumulation in DNA damage-induced apoptosis. Proc. Natl. Acad. Sci. U.S.A. 94: 6939 – 6942

32. Minn AJ, Vélez P, Schendel SL, Liang H, Muchmore SW, Fesik SW, Fill M and Thompson CB (1997) Bcl-$x_L$ forms an ion channel in synthetic lipid membranes. Nature 385: 353 – 357

33. Schendel SL, Xie Z, Montal MO, Matsuyama S, Montal M and Reed JC (1997) Channel formation by antiapoptotic protein Bcl-2. Proc. Natl. Acad. Sci. U.S.A. 94: 5113 – 5118

34. Luo X, Budihardjo I, Zou H, Slaughter C and Wang X (1998) Bid, a Bcl-2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell 94: 481 – 490

35. Li H, Zhu H, Xu C-J and Yuan J (1998) Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway to apoptosis. Cell 94: 491 – 501

36. Hsu Y-T and Youle RJ (1997) Nonionic detergents induce dimerization among members of the Bcl-2 family. J. Biol. Chem. 272: 13829 – 13834

37. Otter I, Conus S, Ravn U, Rager M, Olivier R, Monney L, Fabbro D and Borner C (1998) The binding properties and biological activities of Bcl-2 and Bax in cells exposed to apoptotic stimuli. J. Biol. Chem. 273: 6110 – 6120

38. Oltvai ZN, Milliman CL and Korsmeyer SJ (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programed cell death. Cell 74: 609 – 619

39. Rossé T, Olivier R, Monney L, Rager M, Conus S, Fellay I, Jansen B and Borner C (1998) Bcl-2 prolongs cell survival after Bax-induced release of cytochrome c. Nature 391: 496 – 499

40. Givol I, Tsarfaty I, Resau J, Rulong S, Pinto da Silva P, Nasioulas G, DuHadaway J, Hughes SH and Ewert DL (1994) Bcl-2 expressed using a retroviral vector is localized primarily in the nuclear membrane and the endoplasmic reticulum of chicken embryo fibroblasts. Cell Growth Differ. 5: 419 – 424

41. Spector MS, Desnoyers S, Hoeppner DJ and Hengartner MO (1997) Interaction between the *C. elegans* cell-death regulators CED-9 and CED-4. Nature 385: 653 – 656

42. Inohara N, Koseki T, del Peso L, Hu Y, Yee C, Chen S, Carrio R, Merino J, Liu D, Ni J and Nuñez G (1999) Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-kB. J. Biol. Chem. 274: 14560 – 14567

43. Bertin J, Nir W-J, Fischer CM, Tayber OV, Errada PR, Grant JR, Keilty JJ, Gosselin ML, Robison KE, Wong GHW, Glucksmann AM and DiStefano PS (1999) Human CARD4 protein is a novel CED-4/Apaf-1 cell death family member that activates NF-kB. J. Biol. Chem. 274: 12955 – 12958

44. Hausmann G, O'Reilly LA, van Driel R, Beaumont JG, Strasser A, Adams JM and Huang DCS (2000) Pro-apoptotic apoptosis protease-activating factor 1 (Apaf-1) has a cytoplasmic localization distinct from Bcl-2 and Bcl-$x_L$. J Cell Biol. 149: 623 – 633

45. Newmeyer DD, Bossy-Wetzel E, Kluck RM, Wolf BB, Beere HM and Green DR (2000) Bcl-xL does not inhibit the function of apaf-1. Cell Death Differ 7: 402 – 407

46. Haraguchi M, Torii S, Matsuzawa S-I, Xie Z, Kitada S, Krajewski S, Yoshida H, Mak TW and Reed JC (2000) Apoptotic protease activating factor (Apaf-1)-independent cell death suppression by Bcl-2. J. Exp. Med. 191: 1709 – 1720