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IAPs block apoptotic events induced by caspase-8 and cytochrome *c* by direct inhibition of distinct caspases

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Inhibitor of apoptosis (IAP) gene products play an evolutionarily conserved role in regulating programmed cell death in diverse species ranging from insects to humans. Human XIAP, cIAP1 and cIAP2 are direct inhibitors of at least two members of the caspase family of cell death proteases: caspase-3 and caspase-7. Here we compared the mechanism by which IAPs interfere with activation of caspase-3 and other effector caspases in cytosolic extracts where caspase activation was initiated by caspase-8, a proximal protease activated by ligation of TNF-family receptors, or by cytochrome *c*, which is released from mitochondria into the cytosol during apoptosis. These studies demonstrate that XIAP, cIAP1 and cIAP2 can prevent the proteolytic processing of pro-caspases -3, -6 and -7 by blocking the cytochrome *c*-induced activation of pro-caspase-9. In contrast, these IAP family proteins did not prevent caspase-8-induced proteolytic activation of pro-caspase-3; however, they subsequently inhibited active caspase-3 directly, thus blocking downstream apoptotic events such as further activation of caspases. These findings demonstrate that IAPs can suppress different apoptotic pathways by inhibiting distinct caspases and identify pro-caspase-9 as a new target for IAP-mediated inhibition of apoptosis.

Keywords: apoptosis/caspase/cytochrome *c*/IAP

Introduction

Apoptosis or programmed cell death is a normal physiological cell suicide program that is highly conserved among all animals (Vaux *et al.*, 1994; Steller, 1995). This regulated process of cell death plays a critical role during embryogenesis, tissue homeostasis and remodeling, and serves to remove unwanted cells such as self-reactive lymphocytes, tumor cells, cells with irreparable DNA damage or those infected with viruses. Insufficient apoptosis thus contributes to the pathogenesis of cancer, autoimmune disorders and sustained viral infection, while excessive apoptosis results in inappropriate cell loss and consequently degenerative disorders (Thompson, 1995).

Cell death proteases known as 'caspases' are integral components of apoptotic programs in diverse species (reviewed in Kumar, 1995; Whyte, 1996; Salvesen and Dixit, 1997; Thornberry *et al.*, 1997). Initially synthesized as inactive precursors (zymogens), caspases are activated by proteolytic processing that yields large and small subunits forming the active enzyme. In some cases, an N-terminal 'pro-domain' is subsequently removed by autocatalysis. The functional conservation of caspases in apoptotic programs throughout the animal kingdom make them likely targets for influencing the cell death decision.

We recently discovered that some human IAP family members (XIAP, cIAP1 and cIAP2) are potent caspase inhibitors (Deveraux *et al.*, 1997; Roy *et al.*, 1997). Originally identified in baculoviruses, IAPs were found to suppress the host cell death response, thereby allowing survival and propagation of the virus (Clem *et al.*, 1991; Clem and Miller, 1994). Subsequently, five human IAP relatives have been described (NAIP, cIAP1/HIAP-2/hMIHB, cIAP2/HIAP-1/hMIHC, XIAP/hILP and survivin) (Hay *et al.*, 1995; Rothe *et al.*, 1995; Roy *et al.*, 1995; Duckett *et al.*, 1996; Liston *et al.*, 1996; Uren *et al.*, 1996; Ambrosini *et al.*, 1997). Similar to their viral counterparts, ectopic expression of these human IAP genes can inhibit apoptosis induced by a variety of stimuli (Duckett *et al.*, 1996; Liston *et al.*, 1996). These observations suggest that IAPs block cell death at evolutionarily conserved steps in apoptosis—an idea that is consistent with our observations that IAPs inhibit caspases. However, of the caspases tested, XIAP, cIAP1 and cIAP2 are specific for caspases -3 and -7 but not caspases -1, -6, -8 or -10 (Deveraux *et al.*, 1997; Roy *et al.*, 1997 and unpublished data). The selectivity of these IAPs suggests that they may block some apoptotic pathways but not others.

In mammalian cells, activation of the caspases is achieved through at least two independent mechanisms which are initiated by distinct caspases, but result in activation of common executioner caspases. For example, several members of the tumor necrosis family (TNF) group of cytokine receptors recruit caspase-8 to their cytosolic domains upon binding cytokine ligands, resulting in proteolytic activation of this proximal caspase (reviewed in Wallach *et al.*, 1997). Once activated, caspase-8 can induce either directly or indirectly the activation of a number of distal caspases such as caspases -3, -6 and -7 (Srinivasula *et al.*, 1996a; Muzio *et al.*, 1997). Another pathway for caspase activation involves cytochrome *c*, which in mammalian cells is often released from the mitochondria into the cytosol during apoptosis (Liu *et al.*, 1996; Bossy-Wetzel *et al.*, 1998; Kharbanda *et al.*, 1997;

Kluck *et al.*, 1997b; Yang *et al.*, 1997). Upon entering the cytosol, cytochrome *c* induces the ATP- or dATP-dependent formation of a complex of proteins that results in the proteolytic activation of pro-caspase-3 and the apoptotic destruction of nuclei (Liu *et al.*, 1996). Among the members of this complex are the CED-4 homolog Apaf-1, and caspase-9 (Apaf-3) (Liu *et al.*, 1996; Li *et al.*, 1997; Zou *et al.*, 1997).

In this report we demonstrate that XIAP, cIAP1 and cIAP2 can block cytochrome *c*-induced activation of caspase-9, thus preventing the activation of caspases -3, -6 and -7. In contrast, caspase-8 induced proteolytic cleavage of pro-caspase-3 in cytosolic extracts was not blocked by IAPs. Nevertheless, the IAPs bound to and inhibited the enzymatic activity of caspase-3 following its activation, thereby arresting the proteolytic cascade initiated by caspase-8. These data suggest that IAPs can block differing apoptotic pathways by inhibiting distinct caspases and identify pro-caspase-9 as a new target for IAP-mediated inhibition of apoptosis.

Results

XIAP differentially inhibits processing and activation of pro-caspase-3 in extracts treated with caspase-8 versus cytochrome c

We employed a cell-free system based on the ability of exogenously added active caspase-8 or cytochrome *c* to induce proteolytic processing of pro-caspase-3 in cytosolic extracts (Liu *et al.*, 1996; Deveraux *et al.*, 1997; Muzio *et al.*, 1997). Caspase-8 induced proteolytic processing of pro-caspase-3 into its signature p20 and p17 forms (the small p12 subunit of caspase-3 is undetectable with the anti-caspase-3 antibody used for these studies). As a control, recombinant purified CrmA was added to the extracts concurrently with active caspase-8. The cowpox CrmA protein is a serpin that binds tightly and potently inhibits the proximal cell death protease caspase-8, but is far less active against caspase-3 and other downstream effector caspases (Komiyama *et al.*, 1994; Srinivasula *et al.*, 1996a; Orth and Dixit, 1997; Zhou *et al.*, 1997). Addition of recombinant CrmA completely prevented caspase-8-induced processing of pro-caspase-3. However, subsequent addition of cytochrome *c* and dATP bypassed the CrmA-mediated inhibition of pro-caspase-3 processing (Figure 1A). Even relatively large quantities of CrmA (10 μM) failed to suppress substantially the cytochrome *c*-induced processing of pro-caspase-3, whereas 0.1 μM of CrmA completely inhibited caspase-8-induced processing of pro-caspase-3. Thus, CrmA is a relatively potent inhibitor of caspase-8-induced processing of pro-caspase-3 but is far less effective against the cytochrome *c*-mediated activation of pro-caspase-3. In contrast, addition of recombinant XIAP (0.1–0.2 μM) effectively abolished cytochrome *c*-induced processing of pro-caspase-3 in cytosolic extracts. Similar results were obtained when caspase activity was measured in cytosolic extracts by measuring the rate of Ac-DEVD-AFC hydrolysis (Figure 1B). These data suggest that caspase-8 is upstream or independent of the cytochrome *c* pathway. Regardless, XIAP functioned downstream of cytochrome *c* by inhibiting pro-caspase-3 processing, as previously demonstrated (Deveraux *et al.*, 1997).

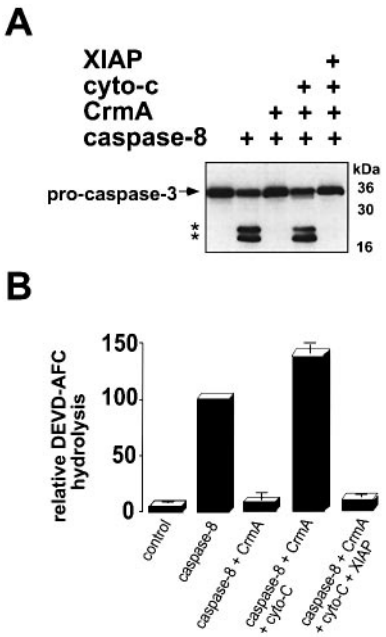

Fig. 1. CrmA and XIAP inhibition of caspase-8 and cytochrome *c*-induced processing and activation of pro-caspase-3 in cytosolic extracts. Recombinant purified and active caspase-8 (0.1 μM) was added to cytoplasmic extracts from 293 cells in the absence or presence of various combinations of CrmA (0.5 μM), cytochrome *c* (10 μM) and dATP (1 mM), or XIAP (0.2 μM). Samples were incubated at 30°C for 30 min. (**A**) Extracts were then separated in SDS–PAGE gels, transferred to nitrocellulose and incubated with antisera specific for the zymogen and large subunit (asterisks) of caspase-3. (**B**) Alternatively, samples were assayed for DEVD-AFC cleavage activity. Data represent mean ± SE ($n = 2$). Molecular weight standards are depicted to the right of (A).

To explore further the differences in between caspase-8 and cytochrome *c*-mediated proteolytic processing of caspases in these extracts we examined by immunoblot analysis, the processing of pro-caspases -3, -6 and -7 in the presence or absence of recombinant XIAP (Figure 2). Addition of (i) cytochrome *c* with dATP or (ii) active caspase-8, to cytosolic extracts in the absence of XIAP resulted in the proteolytic processing of caspases -3, -6 and -7 as indicated by the conversion of their zymogen forms. In contrast, addition of XIAP to cytochrome *c*-treated extracts inhibited processing of pro-caspases-3, -6 and -7. Note that a small amount of the large subunit of caspase-3 was detected in cytochrome *c*-treated extracts containing XIAP, but most of the protein remained unprocessed (~36 kDa) (Figure 2A). In extracts treated with caspase-8, processing of pro-caspases-6 and -7 was also blocked by XIAP; however, pro-caspase-3 was cleaved into large and small subunits. Note that the ~36 kDa zymogen of caspase-3 (~36 kDa) was almost completely consumed while a ~24 kDa form of the large subunit of caspase-3 accumulated in extracts treated with caspase-8 and XIAP. Little or none of the mature ~20 kDa and ~17 kDa forms of the large subunit were observed in these XIAP-inhibited extracts (Figure 2A).

Previous studies have shown that processing of pro-caspase-3 involves an initial cleavage that generates the p12 small subunit, and the p24 partially processed large subunit (Martin *et al.*, 1996). The p24 large subunit is further processed by autocatalytic removal of its N-terminal pro-domain to generate either p20 or p17 forms

of the large subunit (Martin *et al.*, 1996). Thus, XIAP blocked only the autocatalytic processing of the large subunit of caspase-3, but did not inhibit the initial cleavage of pro-caspase-3 by caspase-8. In contrast, XIAP strongly suppressed the initial processing of pro-caspase-3 into large and small subunits in cytochrome *c*-treated extracts.

We recovered the GST-XIAP protein from the same extracts described above using glutathione–Sepharose, and analyzed by immunoblotting whether processed caspase-3 was bound to XIAP (Figure 2A, right panel, lane 1). Removal of the GST-XIAP protein from cytochrome *c*-treated extracts revealed no associated caspase-3 molecules. In contrast, in extracts treated with caspase-8, GST-XIAP bound mostly the p24 form of the large subunit of caspase-3 (Figure 2A, lane 2). As a control, GST-XIAP was also added to extracts that had previously been treated with cytochrome *c* for 1 h and then recovered on glutathione–Sepharose (lane 3), demonstrating that active caspase-3 in these extracts bound to GST-XIAP and that most of the large subunit of the protease had been processed to p17 and p20 forms, with only a small proportion of partially processed p24 present. Similar results were obtained when GST-cIAP-1 or GST-cIAP-2 was substituted for GST-XIAP (data not presented).

XIAP was also found complexed with the p24 form of partially processed caspase-3 in cells overexpressing Fas (CD95), a known activator of caspase-8. As shown in Figure 2B, for example, when 293 cells were co-transfected with plasmids encoding Fas and myc-epitope tagged XIAP, Fas-induced apoptosis was markedly suppressed. Immunoprecipitation of the myc-XIAP protein from lysates obtained from Fas-overexpressing 293 cells revealed associated p24–caspase-3 (Figure 2B, right panel, lane 4). In contrast, when Bax overexpression, which is known to induce cytochrome *c* release from mitochondria (Rosse *et al.*, 1998), is used as the stimulus for inducing apoptosis, it has been shown that pro-caspase-3 processing is completely prevented and no p24 or other forms of processed caspase-3 can be co-immunoprecipitated with XIAP (Deveraux *et al.*, 1997).

Taken together, the data in Figure 2A and B suggest that XIAP inhibits the cytochrome *c* pathway upstream of caspases -3, -6 and -7, since little or no processing of these caspases occurs in the presence of XIAP. In contrast, XIAP inhibits the caspase-8 apoptotic pathway at the level of caspase-3, allowing caspase-8 to induce processing of caspase-3 but preventing the subsequent autocatalytic maturation by directly binding to and inhibiting the partially processed enzyme. Caspases -6 and -7 appear to be downstream of caspase-3 in the caspase-8 apoptotic pathway, since they remain mostly in their zymogen forms when XIAP is present.

On the basis of these observations, a model can be invoked whereby caspase-8 or cytochrome *c* activate pro-caspase-3 independently with each pathway inhibited by XIAP at distinct points (Figure 2C). The model predicts that XIAP blocks the caspase-8-induced apoptotic program by directly inhibiting caspase-3, thereby preventing the activation of downstream caspases -6 and -7. In contrast,

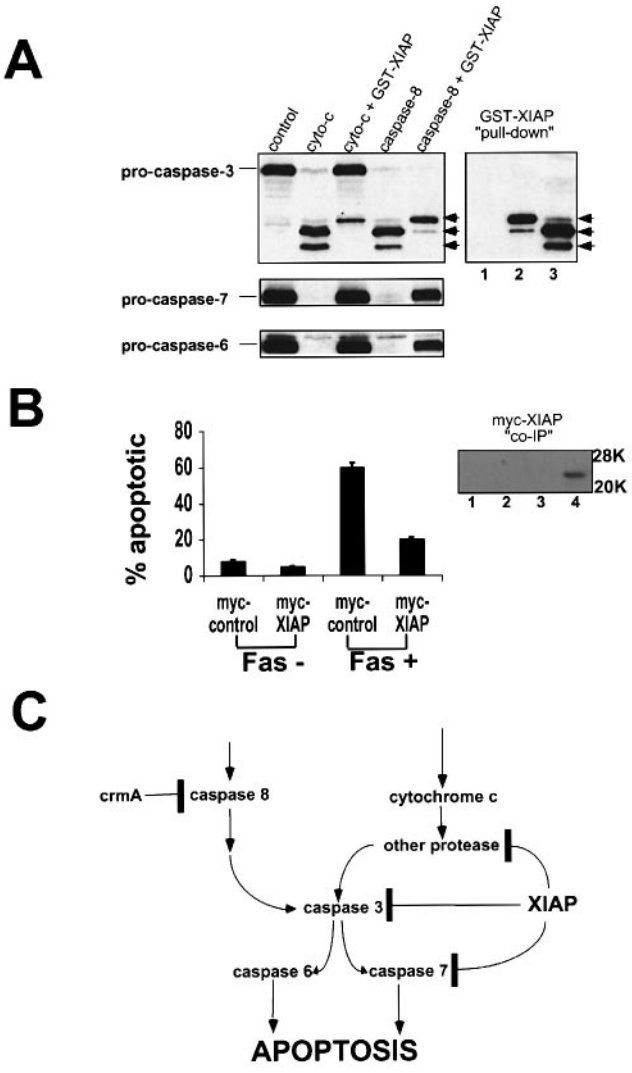

Fig. 2. XIAP-mediated inhibition of pro-caspases 3, -6 and -7 processing in cytochrome *c*- and caspase-8-treated extracts. (**A**) Cytochrome *c* (10 μM) together with dATP (1 mM) or active caspase-8 (0.1 μM) were added to cytosolic extracts from 293 cells with or without GST-XIAP (0.2 μM). Extracts were incubated at 30°C for 1 h and then analyzed by immunoblot analysis for the zymogen and large subunits of caspase-3 (upper left panel) or for the zymogen forms of caspases -7 and -6 (lower left panels). In the upper right panel, samples of extracts containing GST-XIAP were also incubated with glutathione–Sepharose beads. Resulting bound proteins were analyzed by SDS–PAGE and immunoblotting using anti-caspase-3 antiserum. Lane 1: glutathione beads were incubated with extracts containing cytochrome *c*, dATP and GST-XIAP. Lane 2: glutathione beads were incubated with extracts containing caspase-8 and GST-XIAP. Lane 3: GST-XIAP glutathione beads were incubated with extracts that had been previously treated with cytochrome *c* and dATP for 1 h. In experiments with GST and other control GST-fusion proteins, neither inhibition of caspase processing nor caspase binding was observed (not shown). (**B**) 293 cells in 60 mm dishes were transiently transfected with 6 μg of pcDNA-myc-tag control or pcDNA-myc-XIAP plasmids, and either 2 μg of pCMV5 or pCMV5-Fas plasmid DNA. All transfections included 0.5 μg of pEGFP as a marker and were normalized for total DNA content. The percentage of GFP-positive cells with apoptotic morphology and nuclear changes consistent with apoptosis were enumerated by DAPI-staining (mean ± SD; $n = 3$) at 36 h. Alternatively, cell lysates were prepared and immunoprecipitates collected using anti-myc monoclonal antibody with protein G–Sepharose, followed by SDS–PAGE immunoblot assay using anti-caspase-3 antiserum (Krajewska *et al.*, 1997) to reveal the XIAP-associated p24 isoform of partially processed caspase-3. Lanes correspond to cells transfected with: (1) control plasmid; (2) myc-XIAP; (3) Fas plus myc-control; and (4) Fas plus myc-XIAP. (**C**) Proposed model for XIAP-mediated inhibition of either caspase-8 or cytochrome *c*-induced activation of pro-caspases -3, -6 and -7.

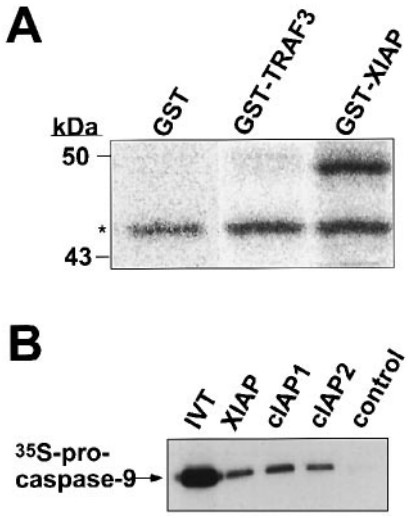

Fig. 3. Pro-caspase-9 binds to XIAP, cIAP1 and cIAP2. (**A**) GST-XIAP was incubated in lysates from U937 cells that had been cultured in [^{35}S]L-methionine-containing media. Lysates were incubated at 4°C for 1.5 h in GST, GST-TRAF-3 (1-357), or GST-XIAP. Proteins were separated on SDS–PAGE gels and analyzed by autoradiography. The asterisk indicates a background band which was non-specifically recovered with the beads and serves as a loading control. Similar results were obtained using extracts from 293 cells (not shown). (**B**) GST-XIAP, cIAP1, cIAP2 or a GST-control fusion protein (~2 μM) immobilized on glutathione–Sepharose was incubated with 10 μl of reticulocyte lysates containing *in vitro*-translated ^{35}S-labeled pro-caspase-9. After extensive washing, bound proteins were analyzed by SDS–PAGE/autoradiography; 1.5 μl of the *in vitro*-translated reaction (IVT) is included as a positive control.

XIAP inhibits another protease that lies upstream of caspases -3, -6 and -7 in the cytochrome *c* apoptotic program.

IAPs associate with caspase-9 in cytochrome c-treated cytosolic extracts

To identify the protease(s) that XIAP inhibits in the cytochrome *c* pathway, we prepared cytosolic extracts from 293 cells that were cultured in the presence of [^{35}S]L-methionine. GST-XIAP or various control GST proteins were then added to the metabolically labeled extracts and subsequently recovered using glutathione–Sepharose. Separation of bound proteins by SDS–PAGE revealed an ~50 kDa ^{35}S-labeled protein that associated specifically with GST-XIAP (Figure 3A).

Only two known caspases have a molecular mass of ~50 kDa: caspase-2 and caspase-9. Since caspase-2 does not appear to be activated in cytochrome *c*-containing extracts (Roy *et al.*, 1997), we asked whether caspase-9 might associate with XIAP. Pro-caspase-9 was *in vitro*-translated in the presence of [^{35}S]L-methionine and incubated with (GST-XIAP, GST-cIAP1, GST-cIAP2) or GST control proteins that fail to prevent caspase activation by cytochrome *c* (Deveraux *et al.*, 1997; Roy *et al.*, 1997). GST-XIAP, GST-cIAP1 and GST-cIAP2, but not GST-control proteins, associated with pro-caspase-9 (Figure 3B and data not shown). Taken together, these results indicate that XIAP, cIAP1 and cIAP2 can associate with the zymogen of caspase-9. In contrast, only the active forms of caspases -3 and -7 bind to these IAPs (Deveraux *et al.*, 1997; Roy *et al.*, 1997).

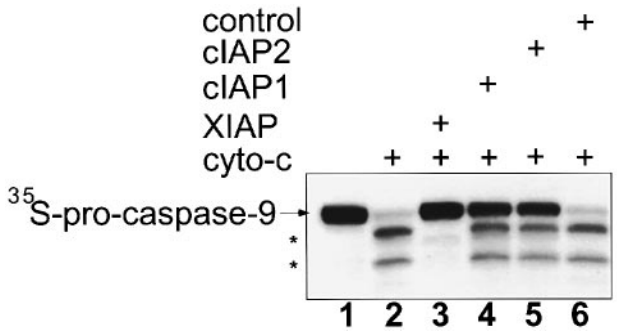

Fig. 4. Inhibition of cytochrome *c*-induced caspase-9 processing by XIAP, cIAP1 and cIAP2. *In vitro*-translated ^{35}S-labeled pro-caspase-9 was added to cytosolic extracts from 293 cells which were then incubated for 30 min at 30°C with (lanes 2–6) or without (lane 1) cytochrome *c* (10 μM) and dATP (1 mM) in the presence or absence of 0.2 μM GST-IAP proteins or a GST control protein. Cytochrome *c*-induced processing of pro-caspase-9 was then monitored by SDS–PAGE and autoradiography. The positions of the processed subunits of caspase-9 are indicated by asterisks.

IAPs block pro-caspase-9 processing in cytosolic extracts treated with cytochrome c

The observation that XIAP, cIAP1 and cIAP2 can bind pro-caspase-9 *in vitro* suggested that they could inhibit its activation. We therefore determined whether addition of cytochrome *c* to cytosols resulted in processing of *in vitro*-translated ^{35}S-labeled pro-caspase-9 and asked whether its processing could be blocked by the IAPs. As shown in Figure 4, pro-caspase-9 remained unprocessed when incubated with cytosolic extracts; however, upon addition of cytochrome *c*, pro-caspase-9 was cleaved into fragments characteristic of the active subunits of the enzyme. Addition of XIAP nearly completely abolished pro-caspase-9 processing while cIAP1 and cIAP2 exhibited slightly less inhibition. These results are consistent with the data presented in Figure 3 which demonstrated binding of pro-caspase-9 by XIAP, cIAP1 and cIAP2.

Reconstitution of caspase-9 processing in vitro: inhibition by the IAPs

To explore further the effects of IAP-family proteins on cytochrome *c*-induced processing of pro-caspase-9, an *in vitro* reconstitution system was employed using cytochrome *c* and dATP, *in vitro*-translated Apaf-1 and ^{35}S-labeled caspase-9 zymogen. Incubation of Apaf-1 with pro-caspase-9 did not result in processing unless cytochrome *c* and dATP were also present (Figure 5A). Addition of XIAP, cIAP1 and cIAP2 to reactions containing Apaf-1 together with cytochrome *c* and dATP completely blocked pro-caspase-9 processing. Conversely, various control GST-fusion proteins failed to inhibit the cytochrome *c*-induced cleavage of pro-caspase-9 under these conditions. The addition of cytochrome *c* and dATP to pro-caspase-9 in the absence of *in vitro*-translated Apaf-1 revealed no processing of the zymogen (Figure 5A). Conversely, incubation of Apaf-1 with cytochrome *c* and the pro-form of caspase-3 in the absence of pro-caspase-9 did not result in activation of pro-caspase-3, establishing the requirement for caspase-9 in this mechanism (data not shown), consistent with recent observations (Liu *et al.*, 1996; Li *et al.*, 1997; Zou *et al.*, 1997).

Unlike the IAPs, recombinant Bcl-X_L protein did not

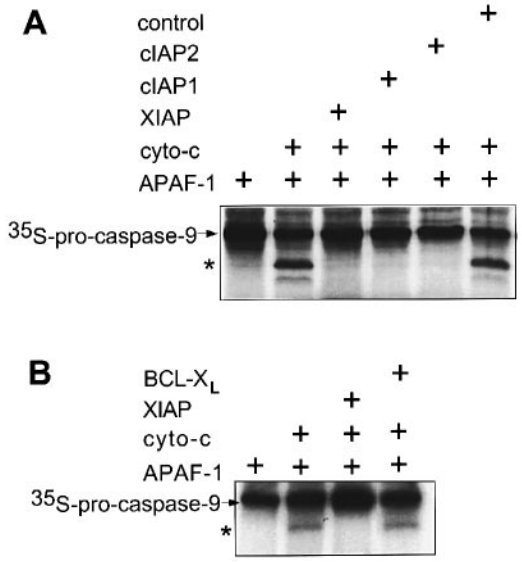

Fig. 5. Pro-caspase-9 processing requires Apaf-1 and cytochrome *c* and is inhibited by XIAP, cIAP1 and cIAP2. *In vitro*-translated ^{35}S-labeled pro-caspase-9 and Apaf-1 were incubated individually or together with cytochrome *c* (10 μM) and dATP (1 mM). Processing of pro-caspase-9 in the absence or presence of (**A**) GST-IAPs (0.1 μM) or (**B**) Bcl-X_L (0.1 μM) was then monitored by SDS–PAGE and autoradiography. Asterisks indicate the position of the processed large subunit of caspase-9. Similar results were obtained with as much as 2 μM Bcl-X_L addition to cytochrome *c*-stimulated cytosolic extracts (not shown).

suppress the *in vitro* processing of pro-caspase-9-induced by the combination of Apaf-1, cytochrome *c* and dATP (Figure 5B). Bcl-X_L also did not inhibit the cytochrome *c*-induced activation of caspases in cytosols (not shown). This same preparation of recombinant Bcl-X_L protein, however, was fully functional in ion-channel formation assays using KCl-loaded liposomes (Schendel *et al.*, 1997) and competent at dimerizing with other Bcl-2 family proteins (not shown). Thus, Bcl-X_L does not block pro-caspase-9 processing mediated by cytochrome *c* and Apaf-1, at least under these *in vitro* conditions. These data are consistent with recent observations that have positioned Bcl-X_L and Bcl-2 upstream or at the level of cytochrome *c* release (Deveraux *et al.*, 1997; Kharbanda *et al.*, 1997; Kluck *et al.*, 1997a; Duckett *et al*., 1998).

XIAP inhibits active caspase-9

We next compared the ability of XIAP to block pro-caspase-9 processing in cytochrome *c*- and dATP-treated cytosols with two well-characterized caspase inhibitors, namely Ac-DEVD-fmk and ZVAD-fmk, which have been used extensively to address the role of caspases in cell death (reviewed in Jacobson and Evan, 1994; Martin and Green, 1995; Patel *et al.*, 1996). Of the three molecules, XIAP is a more potent inhibitor of cytochrome *c*-mediated processing of pro-caspase-9 in cytosolic extracts than either Ac-DEVD-fmk or Z-VAD-fmk (Figure 6). In these assays, less than 0.2 μM of recombinant XIAP was typically sufficient to abolish completely the processing of pro-caspase-9, whereas at least 5 μM of zVAD-fmk or Ac-DEVD-fmk was required for similar inhibition. XIAP was also ~5-fold more potent than baculovirus p35 protein at inhibiting cytochrome *c*-induced processing of pro-caspase-9 in these assays (not shown).

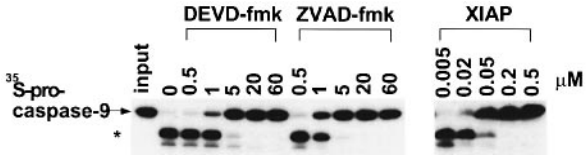

Fig. 6. Comparison of pro-caspase-9 inhibition by Ac-DEVD-fmk, zVAD-fmk and XIAP. *In vitro*-translated ^{35}S-labeled pro-caspase-9 was added to cytosolic extracts from 293 cells containing 10 mM cytochrome *c* and 1 mM dATP. Samples were incubated at 30°C for 30 min in the presence of the indicated concentrations of inhibitors. Proteins were separated on SDS–PAGE gels, dried directly and exposed to film. The asterisks denotes the processed large subunit of caspase-9.

We next purified recombinant active caspase-9 that was expressed in *Escherichia coli* and determined whether IAPs directly inhibit its activity. Recombinant caspase-9 was found to be extremely sensitive to dilution and the fluorogenic tetrapeptides typically used for caspase assays proved to be poor substrates for this enzyme. We therefore used recombinant pro-caspase-3 as the substrate for monitoring the activity of caspase-9. Incubation of caspase-9 with purified pro-caspase-3 resulted in proteolytic processing of pro-caspase-3 as determined by immunoblot analysis (Figure 7A). Addition of an equimolar concentration of XIAP, relative to caspase-9, strongly inhibited cleavage of pro-caspase-3. Activity of caspase-9 was also measured in a coupled reaction based on hydrolysis of Ac-DEVD-AFC as a result of caspase-3 activation *in vitro*. XIAP, cIAP1 and cIAP2 efficiently inhibited pro-caspase-3 activation and cleavage of the tetrapeptide substrate whereas various GST control proteins had no significant effect on pro-caspase-3 activation by caspase-9 (Figure 7B and data not shown).

Because active caspase-3 has been reported to cleave and activate pro-caspase-9 (Srinivasula *et al.*, 1996b), we were concerned about the possibility of a feedback loop in these experiments. To eliminate this possibility, therefore, we tested XIAP for inhibition of bacterially produced active caspase-9 using *in vitro*-translated and purified ^{35}S-labeled pro-caspase-9 as a substrate. As shown in Figure 7C, GST-XIAP protein potently inhibited processing of pro-caspase-9 in these *in vitro* reactions, whereas GST-control protein had little or no effect. Taken together, these data therefore demonstrate that XIAP is a direct inhibitor of caspase-9.

XIAP, cIAP1 and cIAP2 inhibit caspase-9-induced processing of pro-caspase-3 in intact cells

The inhibitory effect of XIAP, cIAP1 and cIAP2 on pro-caspase-9 activation *in vitro* suggested that these inhibitors could also protect against caspase-9-induced apoptosis in intact cells and inhibit downstream events such as processing of pro-caspase-3. Overexpression of caspases *in vivo* often results in apoptosis (reviewed in Jacobson *et al.*, 1994; Martin *et al.*, 1995; Patel *et al.*, 1996); thus, to explore the effect of IAPs on caspase-9 activation *in vivo*, we transfected 293T cells with an epitope-tagged FLAG–caspase-9 alone or in combination with myc-tagged IAPs. Lysates were collected 1 day following transfection and the proteolytic processing of pro-caspase-3 was examined by immunoblot analysis. As shown in Figure 8A, overexpression of caspase-9 resulted

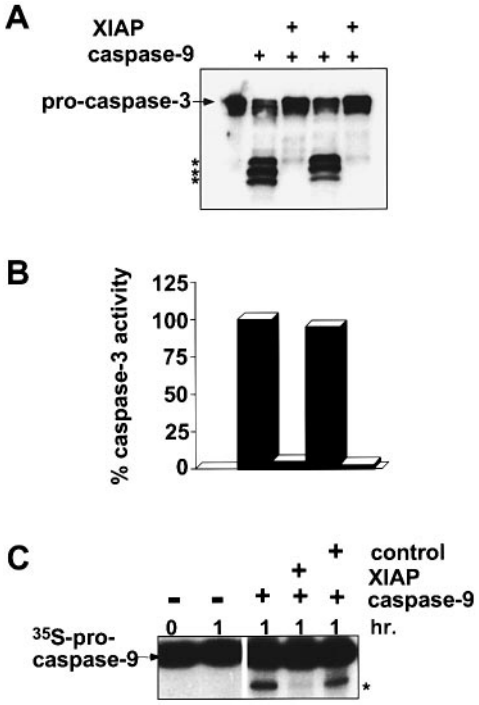

Fig. 7. Inhibition of purified active caspase-9 by XIAP. Active caspase-9 was produced in bacteria and purified as a His_6-tagged protein. Caspase-9 activity was measured by monitoring the processing (A) and activity (B) of the purified recombinant zymogen form of caspase-3 that was produced in bacteria. Active caspase-9 (0.1 μM) was incubated with pro-caspase-3 (0.5 μM) in the presence or absence of GST-XIAP (0.1 μM). Experiments were performed with two independent preparations of active caspase-9. (**A**) Samples were analyzed for pro-caspase-3 processing by immunoblot analysis. Asterisks denote the processed forms of the large subunit of caspase-3. (**B**) Samples were simultaneously assayed for release of the AFC fluorophore from DEVD-AFC. Activity was arbitrarily designated as 100% for one of the two preparations of active caspase-9. (**C**) Pro-caspase-9 was *in vitro*-translated in reticulocyte lysates in the presence of [^{35}S]L-methionine and then purified by metal chromatography and 2 μl of the resulting samples were either immediately boiled in an equal volume of Laemmli buffer or incubated at 30°C for 1 h alone (–) or with 0.1 μM recombinant active caspase-9 (+) in the presence of absence of 0.1 μM GST-XIAP or a GST control protein. Proteins were analyzed by SDS–PAGE/autoradiography (asterisk denotes the processed form of caspase-9). Recombinant GST control proteins had little or no effect upon caspase-9 activity in these assays (not shown).

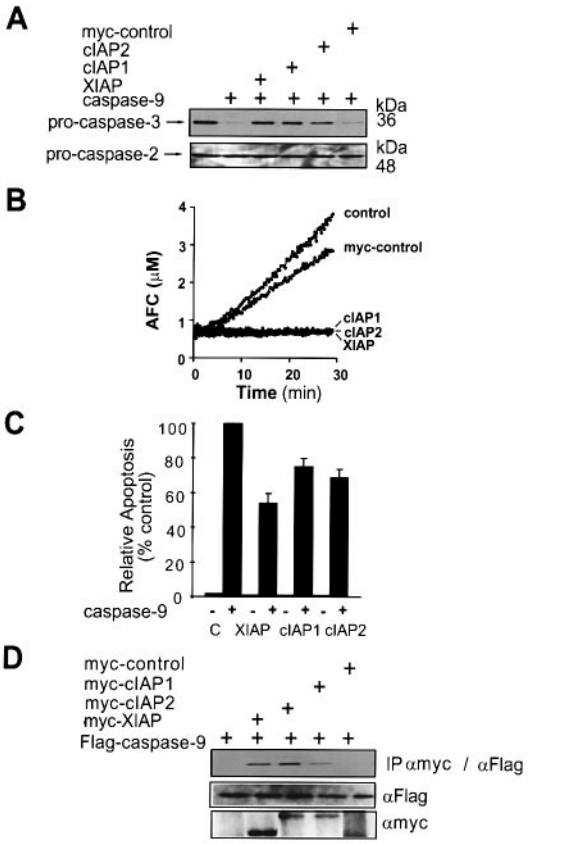

Fig. 8. XIAP, cIAP1 and cIAP2 bind pro-caspase-9 *in vivo* and inhibit caspase-9-induced processing of caspase-3. 293T cells were transfected with either FLAG-tagged pro-caspase-9 or pcDNA-myc-tag control plasmid DNA alone or in combination with myc-tagged XIAP, cIAP1, cIAP2 or a myc-tagged control protein. Cell lysates were prepared 16 h later for either (A) immunoblot analysis of caspase-3 or (B) Ac-DEVD-AFC. (**A**) Immunoblot analysis of pro-caspase-3 was performed using lysates from cells induced to undergo apoptosis by overexpressing pro-caspase-9 in the absence or presence of the IAPs. (**B**) Lysates were normalized for total protein content and assayed for hydrolysis of Ac-DEVD-AFC. (**C**) Apoptosis was scored at 1.5–2 days after transfection by DAPI-staining (mean ± SE; $n = 3$) for 293T cells co-transfected with pGFP and FLAG–control (–) or FLAG–pro-caspase-9 (+) and either pcDNA3-myc-tag control plasmid, pcDNA3-myc-XIAP, pcDNA3-myc-IAPI or pcDNA3-myc-cIAP2. (**D**) IAP proteins were immunoprecipitated with anti-myc antibody immobilized on protein G–Sepharose at ~16 h post-transfection. Immunoblot analysis with anti-FLAG was employed for detection of pro-caspase-9 in the resulting immune complexes. Lysates from the same cells (50 μg/lane) were also analyzed by immunoblotting using anti-Flag and anti-Myc antibodies to verify expression of IAPs and caspase-9, respectively.

in the complete conversion of the caspase-3 zymogen and an increase in Ac-DEVD-AFC cleavage activity (Figure 8B). In contrast, caspase-9-induced proteolytic cleavage of pro-caspase-3 and Ac-DEVD-AFC cleavage activity was markedly reduced in 293T cells that had been co-transfected with XIAP, cIAP1 and cIAP2. The observed inhibition of pro-caspase-3 processing by XIAP, cIAP1 and cIAP2 was accompanied by a reduction in the number of apoptotic 293T cells (Figure 8C). The more extensive suppression of DEVD-cleaving activity than of apoptosis may be attributable to an eventual breakthrough of caspase-9-induced protease activation due to the generally short half-life of IAP-family proteins (unpublished observations).

Given that the zymogen form of caspase-9 binds to XIAP, cIAP1 and cIAP2 *in vitro*, we next sought to determine whether these IAP family proteins could be co-immunoprecipitated with caspase-9 in 293T cells. Using 293T cells co-transfected with FLAG–pro-caspase-9 and myc-epitope-tagged IAP proteins, immunoprecipitations were performed with anti-myc antibody and the resulting immunecomplexes analyzed by immunoblotting using antisera specific for the FLAG epitope. As shown in Figure 8D, the zymogen form of caspase-9 co-immunoprecipitated with XIAP, cIAP1 and cIAP2 (Figure 8) but not with various control proteins (not shown). Thus, XIAP, cIAP1 and cIAP2 bind to pro-caspase-9 *in vivo* preventing its activation, thereby blocking activation of pro-caspase-3 and consequently apoptosis.

Discussion

XIAP, cIAP1 and cIAP2 suppress apoptosis induced by stimuli known to cause release of cytochrome *c* from mitochondria and can inhibit caspase activation induced by cytochrome *c in vitro* (Deveraux *et al.*, 1997; Roy *et al.*, 1997). Similarly, in cell microinjection experiments, XIAP has been reported to block cytochrome *c*-induced apoptosis (Duckette *et al.*, 1998). Here we build upon those observations by demonstrating that XIAP, cIAP1 and cIAP2 block two differing pathways of caspase activation by inhibiting distinct caspases and identify a new caspase target for IAP-mediated inhibition. Caspase-8-induced protease activation was suppressed by XIAP, cIAP1 and cIAP2 at the level of caspase-3 by inhibiting active caspase-3, following its initial cleavage to p24 and p12 subunits. In contrast, IAP-mediated inhibition of cytochrome *c*-induced activation occurs upstream of caspase-3 through direct inhibition of pro-caspase-9 processing. XIAP, cIAP-1 and cIAP-2 were shown to bind pro-caspase-9 *in vitro* and could be co-immunoprecipitated with pro-caspase-9 in cell lysates. The IAPs also blocked the proteolytic processing of pro-caspase-9 induced by cytochrome *c* in cytosolic extracts as well as in an *in vitro*-reconstituted system containing cytochrome *c* and dATP, Apaf1 and pro-caspase-9. Moreover, XIAP, cIAP-1 and cIAP-2 directly inhibited active caspase-9 *in vitro*. Because caspase-9 can be activated by Apaf1 in combination with cytochrome *c* and dATP, it is likely to be at least one of the initial caspases in the cytochrome *c* pathway. Thus, the data presented here support recent observations that pro-caspase-9 is a vital component of the cytochrome *c* apoptotic initiator complex (Li *et al.*, 1997).

Although the mechanistic details of pro-caspase-9 activation by Apaf-1, cytochrome *c* and dATP remain to be elucidated, some IAP family members clearly interfere with this reaction. The initial cleavage of pro-caspase-9 is likely to be autocatalytic and facilitated by Apaf-1 via its putative ATPase domain—possibly akin to the chaperone-mediated conformational alterations in target proteins. The observation that IAPs can both bind to the zymogen form of caspase-9 and directly inhibit active caspase-9 is consistent with this idea. We cannot, however, exclude the possibility that IAPs bind to pro-caspase-9 thereby preventing its association with the Apaf-1 and cytochrome *c* complex. However, the ability of the IAPs to inhibit active recombinant caspase-9 suggests that prevention of pro-caspase-9 recruitment is not the exclusive mechanism of inhibition by the IAPs.

Interestingly, caspase-3 has been reported to cleave and activate pro-caspase-9 *in vitro* (Srinivasula *et al.*, 1996c). Though not presented here, we found that caspase-3, following its activation by caspase-9, appears to participate in either enhancing the rate of caspase-9 activation or participating in its maturation since addition of the purified zymogen form of caspase-3 together with Apaf-1, cytochrome *c* and dATP resulted in greater abundance of the processed caspase-9. XIAP, cIAP1 and cIAP2 would therefore also be expected to interfere with this positive feedback mechanism. Thus, IAPs may suppress caspase-9 processing by two mechanisms: (i) direct inhibition of auto-activation of pro-caspase-9 induced by Apaf-1, cytochrome *c* and dATP; and (ii) by blocking the cleavage of pro-caspase-9 by active caspase-3.

The common structural feature shared by all IAP family members is a motif termed the baculovirus IAP repeat (BIR) that is present in either one to three copies. We demonstrated previously that the BIR domains are sufficient to inhibit active caspases-3 and -7 and can also prevent caspase activation induced by cytochrome *c*- and dATP-treated cytosol (Deveraux *et al.*, 1997; Roy *et al.*, 1997). These observations predict that the BIR motifs of XIAP, cIAP1 and cIAP can suppress cytochrome *c*- and dATP-induced processing of pro-caspase-9. Indeed, we have found that a single BIR domain of XIAP is sufficient to block cytochrome *c*- and dATP-induced processing of pro-caspase-9, whereas the *c*-terminal RING domain of the IAPs had no effect (Takahashi *et al.*, 1998). Caspases are the effectors of apoptosis in species ranging from nematodes to humans. The discovery that at least some human IAP family members can interact with and inhibit specific caspases is consistent with the previous observations that invoked the idea that IAPs must inhibit cell death programs at evolutionarily conserved steps (Clem and Miller, 1994; Liston *et al.*, 1996). We found previously that XIAP inhibited caspases -3 and -7 with K_is of 0.2–0.7 nM, whereas cIAP1 and cIAP2 inhibit these caspases with K_is in the low nM range (30–120 nM), representing 2–3 logs lower potency. These data suggest that significant structural differences exist between XIAP and the cIAP1 and cIAP2 proteins despite their substantial amino acid sequence similarity within the BIR domains. Nevertheless, XIAP, cIAP1 and cIAP2 do inhibit caspases -3, -7 and -9, indicating substantial functional overlap. It is interesting to speculate, therefore, why humans evolved multiple IAPs. Possibly IAPs have functions other than caspase inhibition or effect cellular events distinct from apoptosis. Moreover, different IAPs may have specificity for distinct caspases. Of the 10 human caspases reported to date, only a subset have been tested for inhibition by IAPs.

Many questions remain concerning the role of cellular IAPs. Do they function, for example, to continually suppress low levels of adventitious caspase activity that may arise during the normal course of cellular life, thereby preventing accidental apoptosis? Is the expression of IAP family protein constitutive or is it highly dynamic, playing a role for instance in differentiation, where some types of differentiated cells enjoy long lifespans while others are programmed to die rapidly? Certainly recent studies of the expression of the IAP-family protein survivin demonstrate that at least some members of this family of apoptosis-suppressors can be regulated in profound ways both during normal mammalian development and during oncogenesis (Ambrosini *et al.*, 1997; Adida *et al.*, 1998). In addition, the range of stimuli that IAPs can provide protection against *in vivo* may vary, depending on whether irreversible damage to mitochondria results in the eventual demise of cells due to a lack of ATP production, even though IAPs have prevented caspase activation and apoptosis (Reed, 1997). In this regard, whether IAPs block cell death may depend not only upon the type of apoptotic stimuli but also its magnitude. For instance, low levels of active caspase-8 may require mitochondria and cytochrome *c* release as an amplification step for efficient activation of downstream caspases such as caspase-3, whereas stronger apoptotic stimuli might allow caspase-8 to activate independently pro-caspase-3, as we have

observed in the cell-free system. Further studies should continue to elucidate the relationship between the IAP and caspase families of proteins and the role that this relationship plays in the physiological and pathological events that control apoptosis in different cellular contexts.

Materials and methods

Expression and purification of recombinant IAPs and caspases

GST-XIAP, cIAP1 and cIAP2 were purified as described (Deveraux *et al.*, 1997; Roy *et al.*, 1997). Control GST proteins used for these experiments included GST non-fusion, various GST fusions such as GST-CD40, GST-Bcl-2 and GST-TRAF-3, and a GST-NAIP fusion protein in which the NAIP protein fragment fails to fold properly, as determined by circular dichroism. Caspases-3, -6 and -7 containing *c*-terminal His_6-tags and caspase-8 containing an N-terminal His_6-tag were purified as described previously (Orth *et al.*, 1996; Quan *et al.*, 1996; Muzio *et al.*, 1997; Zhou *et al.*, 1997). Full-length N-terminally tagged caspase-9 was subcloned from pcDNA3 (Duan *et al.*, 1996; kindly provided by Dr Vishva Dixit) into the *Nco*I–*Xho*I sites of pET-23d as a *Nco*I–*Xba*I fragment by blunt ending of the *Xho*I and *Xha*I sites. The resulting vector was introduced into BL21 (DE3) and fully processed enzyme was obtained when induced by 0.2 mM IPTG at OD_{600} = 0.6 for 4 h. The zymogen form of caspase-3 was obtained by expression as described previously by reducing the expression time to 30 min. Pro-caspase-3 and processed caspase-9 were isolated using Ni-chelate Sepharose (Pharmacia, Sweden) chromatography according to the manufacturer's recommendations and eluting with an imidazole gradient from 0–200 mM in 10 mM Tris, 100 mM NaCl, pH 8.0. The concentrations of the purified enzymes were determined from the absorbance at 280 nm based on the molar absorption coefficients for the caspases calculated from the Edelhoch relationship (Edelhoch, 1967); caspase-3 (ε_{280} = 26 000 M^{-1} cm^{-1}), caspase-9 (ε_{280} = 30 010 M^{-1} cm^{-1}).

Caspase activation in cytosolic extracts

Cytosolic extracts were prepared essentially as described (Liu *et al.*, 1996), with several modifications (Deveraux *et al.*, 1997), using 293 embryonic kidney cells. Briefly, cells were washed with ice-cold buffer A (20 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA and 1 mM DTT) and suspended in 1 volume of buffer A. Cells were incubated on ice for 20 min and then disrupted by 15 passages through a 26-gauge needle. Cell extracts were clarified by centrifugation at 16 000 *g* for 30 min and the resulting supernatants were stored at –80°C. For initiating caspase activation, either 10 μM horse heart cytochrome *c* (Sigma) together with 1 mM dATP or 100 nM of purified recombinant caspase-8 was added to extracts (10–15 mg total protein/ml).

Enzyme assays

Caspase activity was assayed by release of amino-4-trifluoromethyl-coumarin (AFC) or *p*-nitroanilide (pNA) (Enzyme System Products) from YVAD- or DEVD-containing synthetic peptides using continuous-reading instruments as described (Quan *et al.*, 1995; Stennicke and Salvesen, 1997). Tetrapeptide inhibitors were purchased from Calbiochem.

Caspase-9 activation in vitro

One μg of plasmids containing cDNAs encoding pro-caspase-9 [pET21(b)-Mch-6] or Apaf-1 (pcDNA3-Apaf-1) was *in vitro*-transcribed and translated in the presence of [^{35}S]L-methionine using a coupled transcription/translation TNT kit (Promega) according to manufacturer's instructions. Proteins were desalted and exchanged into Buffer A with Bio-spin P-6 columns (Bio-Rad). Caspase-9 (2 μl) was combined with Apaf-1 (6 μl) and cytochrome *c*/dATP in a total volume of 10 μl with either Buffer A or an equal volume of GST-XIAP, GST-cIAP1, GST-cIAP2 or GST-NAIP and incubated for 1 h at 30°C. The reactions were analyzed by SDS–PAGE and autoradiography. For some experiments, *in vitro*-translated His_6-caspase-9 was purified by metal chromatography.

GST pull-down assays

U937 or 293 cells were cultured in methionine-free RPMI or DMEM containing dialyzed 5% FBS and 50 μCi/ml [^{35}S]L-methionine for 3 h before extraction into TBS containing 1% Triton X-100 and 1 mM DTT. Lysates were pre-cleared by addition of glutathione–Sepharose beads and incubation for 1 h at 4°C. Glutathione beads were then removed by centrifugation and washed twice with TBS containing 1% Triton X-100 and 1 mM DTT. Bound proteins were resolved in SDS–PAGE gels.

Co-immunoprecipitations and immunoblot assays

Human embryonic kidney 293T cells were maintained in DMEM supplemented with 10% fetal bovine serum, 1 mM L-glutamine and antibiotics. 2×10^6 cells were plated in 10 mm dishes and 24 h later transiently co-transfected with 2 μg of either pFLAG–CMV2-caspase-9 or pCMV-Fas and 6–8 μg of either pcDNA3myc-XIAP, pcDNA3myc-cIAP1, pcDNA3myc-cIAP2 or pcDNA3myc-control plasmid DNA by a calcium phosphate precipitation method (Deveraux *et al.*, 1997; Roy *et al.*, 1997; Takahashi *et al.*, 1998). Cells were collected 24–48 h later by centrifugation, washed in ice-cold PBS and lysed for 20 min in lysis buffer (10 mM HEPES, 142 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.2% NP-40). Lysates were cleared by centrifugation at 16 000 *g* for 30 min. Myc-tagged IAP proteins were immunoprecipitated with 40 μl of anti-myc (9E10) antibody immobilized on Protein G–Sepharose (Santa Cruz) for 2 h. Immunoprecipitates were washed three times with lysis buffer and bound proteins separated by SDS–PAGE and analyzed by immunoblotting using antibodies specific for FLAG epitope (Kodak, Inc.), myc-epitope or caspase-3.

Immunoblotting for caspases was performed as described (Deveraux *et al.*, 1997), using 750 mM Tris–12% polyacrylamide gels, after normalizing cell lysates for protein. Antisera specific for caspases-3, -6 and -7 were prepared as described (Orth *et al.*, 1996; Srinivasula *et al.*, 1996c; Krajewski *et al.*, 1997).

Apoptosis assays

A total of 293 cells were transfected as described above, except that 0.5 mg pEGFP plasmid DNA was included. Both floating and adherent cells were recovered 24–36 h later and the percentage of GFP-positive cells that exhibited apoptotic morphology as determined by staining with 0.1 mg/ml DAPI (Deveraux *et al.*, 1997; Roy *et al.*, 1997; Takahashi *et al.*, 1998).

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