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13. ABSTRACT *(Maximum 200 words)*

Dysregulation of the control of both cellular proliferation and cell death contributes to tumor growth. Consistent with this hypothesis, overexpression of genes that block apoptosis is observed in tumors. Furthermore, upregulation of genes that confer survival result in an increased resistance of tumors to apoptosis and thereby render them resistant to many chemotherapeutic drugs. The inhibitor of apoptosis proteins (IAP) are a family of anti-apoptotic proteins that are conserved across species. This family of proteins includes four human (c-IAP1/HIAP2;c-IAP2/HIAP1;XIAP/hILP;survivin), two *Drosophilia* (DIAP1;DIAP2), two yeast (SpBIR1;ScBIR2) and two *C. Elegans* homologs. I have shown that the IAPS are direct inhibitors of specific members of the cysteine family of proteases, caspases-3,-6,-7 and -9. The caspases play a central role as effectors in the apoptotic cascade and their inhibition contributes to tumor growth and resistance to chemotherapeutic agents. We have shown that IAPs can suppress different apoptotic pathways induced by various stimuli, by inhibiting distinct caspase members. These observations may contribute to the development of novel pharmacological agents that modulate these components to improve tumor response in carcinomas.

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✓ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

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Table of Contents

A. INTRODUCTION:

Apoptosis is a regulated cell suicide program that plays a critical role during developmental and adult homeostasis. Excessive apoptosis results in severe cell loss, such as that observed in degenerative disorders, while insufficient apoptosis contributes to the pathogenesis of disorders such as cancer (1). The Inhibitor of Apoptosis Proteins (IAPs) are a family of anti-apoptotic proteins that are conserved across species. The first human IAP to be identified, the neuronal apoptosis inhibitor protein (NAIP), was isolated based on its contribution to the neuromuscular disorder, spinal muscular atrophy (SMA) (2). This family of proteins now includes four human (c-IAP/HIAP2; c-IAP/HIAP1; XIAP/hILP;Survivin), two *Drosophilia* (DIAP1; ,DIAP2), two yeast (SpBIR1; ScBIR2) and two *c. elegans* homologs. The common structural feature of all family members is a motif termed the baculovirus IAP repeat (BIR) that is present in two or three copies. These motifs, at least for the mammalian, viral and *Drosophila* homologs, are sufficient to mediate cell survival. The human c-IAP1 and c-IAP2 proteins are unique in that they are recruited to the cytosolic domain of the tumor necrosis factor receptor II via their association with the TNF-associated factor (TRAF) proteins, TRAF-1 and TRAF-2. c-IAP has additionally shown to be a component of the TNFR-1 complex. However, the role that the IAPs play in these signal transduction pathways is not fully understood.

The cysteine family of proteases, named caspases, play a central role as effectors in the apoptotic cascade with apoptotic stimuli leading to proteolytic processing of upstream caspases that activate distal caspases either directly or indirectly through a mitochondrial step. These active caspases cleave key regulatory and structural proteins, thus leading to the demise of the cell. Disruption of mitochondrial homeostasis is central to the induction of cell death. Cytochrome c redistribution into the cytosol, calcium flux and loss of transmembrane potential are the biochemical perturbations that lead to downstream caspase activation and death. Cytochrome c release has been shown to precede the loss of mitochondrial transmembrane potential and caspase activation.

It is well established that tumor cells have a decreased ability to undergo apoptosis and possibly this occurs due to an upregulation of genes that confer survival. Chemotherapeutic drugs normally induce regression by activation of caspases, thus an upregulation of genes that inhibit these proteins renders tumors resistant to these treatments. The viral IAPs were isolated based on their ability to functionally complement the viral protein p35 (3). Subsequently, it was shown that p53 modulates apoptosis by potentiallly inhibiting the caspases. We reasoned that the IAPs could mediate survival by a similar mechanism.

B. BODY:

1. OBJECTIVES:

The originally proposed objectives remain applicable and are being systematically accomplished.

1. Analyze IAP expression in normal breast tissue, and breast tumor specimens and cell lines.

2. Determine the functions of IAPs in breast cancer cell lines.

3. Determine what other proteins IAPs bind.

4. Examine the effects of IAPs on caspase-family cell death proteases.

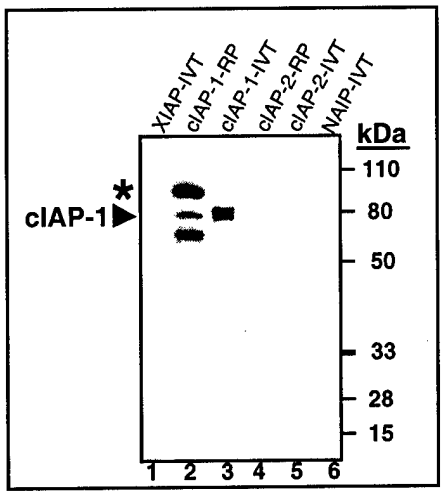
2. PROGRESS:

We have continued to make outstanding progress towards the originally proposed Objectives, which were intended to elucidated the patterns of expression and functions of IAP-family proteins in breast cancers and other types of cells.

TECHNICAL OBJECTIVE #1. Analyze IAP expression in normal breast tissue, and breast tumor specimens and cell lines.

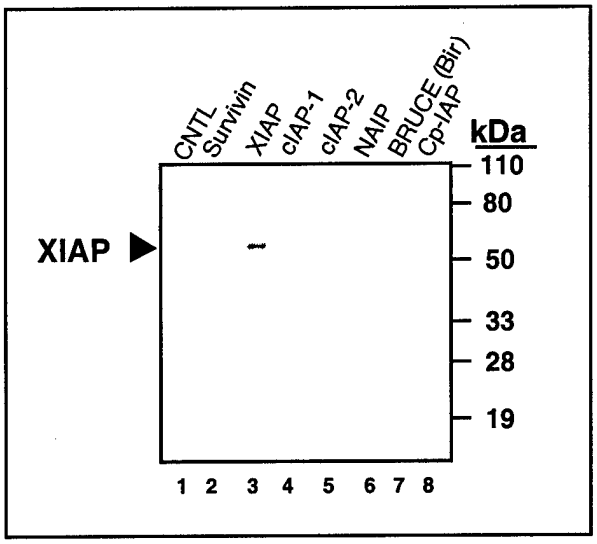
Task #1. Raise antibodies to IAPs.

We succeeded this year in producing highly specific antisera to three of the IAP-family proteins, cIAP1, XIAP, and Survivin. Attempts to produce antibodies to cIAP2 are still in progress, after two failed efforts. To accomplish this task, we produced fragments of the IAP proteins as GST-fusion proteins in bacteria, purified them, and immunized rabbits. The resulting antisera were then affinity-purified and tested for reactivity by immunoblotting with IAP family proteins. Previous attempts to generate high quality antisera using synthetic peptides failed.

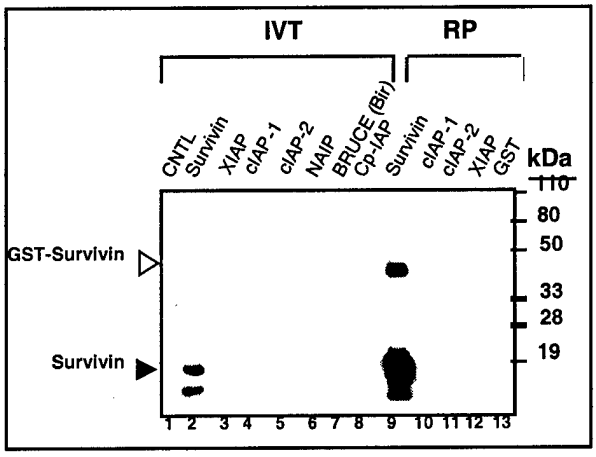


cIAP1: Figure 1 shows an immunoblot analysis performed with our anti-cIAP1 antibody. Here, various members of the IAP family of proteins were produced by in vitro translation (IVT) of their cDNAs or recombinant proteins (RP) we produced as GST fusions in

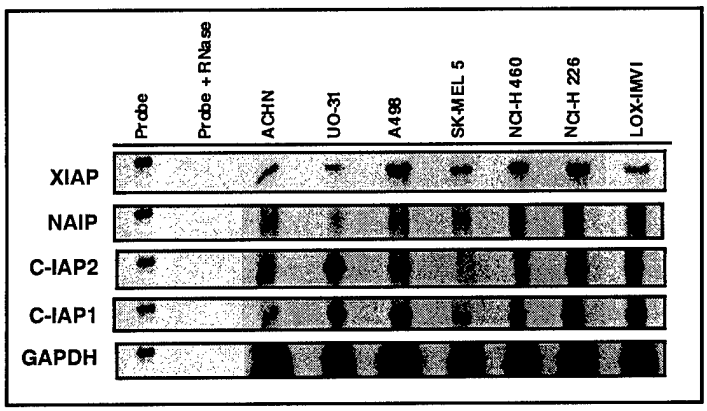
bacteria. These proteins were subjected to SDS-PAGE/immunoblot assay using our anti-cIAP1 antiserum. Note that the antibody reacts uniquely with cIAP1.



XIAP: Figure 2 shows an immunoblot analysis performed with our anti-XIAP antibody. Here again, various members of the IAP family of proteins were produced by in vitro translation of their cDNAs and subjected to SDS-PAGE/immunoblot assay using our anti-XIAP antiserum. Note that the antibody reacts uniquely with XIAP.



Survivin: Figure 3 shows an immunoblot analysis performed with our anti-Survivin antibody. Various members of the IAP family of proteins were produced by in vitro translation (IVT) of their cDNAs or expressed as recombinant proteins (RP) and subjected to SDS-PAGE/immunoblot assay using our anti-Survivin antiserum. Note that the antibody reacts uniquely with Survivin. This Survivin antibody also reacts with the expected ~17 kDa band in tumor cell lines but not in normal tissues.



Task #2. Northern blot and Western blot analysis of IAP expression in cancer cell lines.

An RNase protection assay was established for assessing the relative levels of IAP-family mRNAs in tumor cell lines. Using this assay, we analyzed the NCI 60 tumor cell line

screening panel. Figure 4 shows an example of some of the RNAse protection assay results. Note that mRNAs for XIAP, cIAP1, and cIAP2 were variably expressed among the tumor cell lines examined, whereas NAIP mRNA was undetectable. Controls showing that the probe was working and that roughly equal amounts of total RNA were input into the assays are also shown.

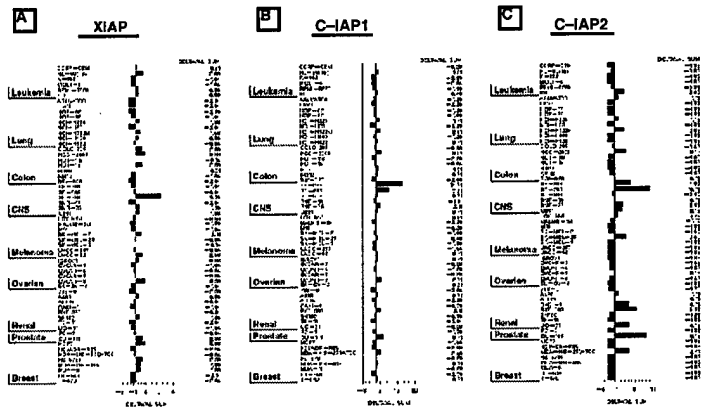


Figure 5 presents a quantitative summary of the results of the RNase protection assays, where the data (derived from densitometry of x-ray film autoradiograms) are expressed relative to the mean value determined for the entire set of 60 tumor cell lines. Correlations of these IAP mRNA levels with other biomarkers previously assessed in the NCI screening panel failed to reveal any correlations (not shown).

SURVIVIN PROTEIN EXPRESSION IN THE NCI 60 (PLUS) CELL SCREEN PANEL

BREAST:		**LEUKEMIA/LYMPHOMA:**		**OVARIAN:**	
BT-549	0	CCRF-CEM	++	IGROV1	0
HS578T	0	HL-60	+++	OVCAR-3	0
MCF-7	0	K-562	0	OVCAR-4	0
MCF-7/ADR-RES	+	MOLT-4	++	OVCAR-5	++
MDA-MB-231	+	RPMI 8226	0	OVCAR-8	++++
MDA-MB-435	+/-	SR	+/-	SK-OV-3	0
MDA-N	0				
T47D	0	**LUNG:**		**PROSTATE:**	
BT-474	0	A549	0	DU-145	++++
A1N4	0	EKVX	0	PC-3	0
10A	0	HOP-62	0	PPC-1	+++
MDA-MB-468	+/-	HOP-92	0	ALVA-31	+++
ZR751	0	NCI-H322M	+	JCA-1	++++
BT-20	0	NCI-H226	0	LNCap	++++
		NCI-H23	0	Tsu-pri	++++
COLON:		NCI-H522	++		
COLO 205	0	NCI-H460	+	**RENAL:**	
HCT-15	0			786-0	++
HCT-116	0	**MELANOMA:**		A498	0
HCC-2998	0	LOX IMVI	+/-	ACHN	++
HT29	+	MALME-3M	0	CAKI-1	+/-
KM12	++	M14	0	RXF 393	0
SW-620	0	SK-MEL-2	++++	SN12C	+++
		SK-MEL-5	0	TK-10	0
CNS:		SK-MEL-28	0	UO-31	0
SF-268	0	UACC-62	+		
SF-295	0	UACC-257	0		
SF-539	0				
SNB-19	++				
SNB-75	0				
U251	0				

Using the antisera specific for cIAP1, XIAP, and Survivin, we phenotyped the NCI 60 tumor cell line screening panel, using a semi-quantitative immunoblotting method (4,5). The NCI tumor panel was supplemented in some instances with additional cell lines of our own, which increased the number of breast and prostate cancers in the analysis. Tables I, II, and III below present

cIAP-1 PROTEIN EXPRESSION IN THE NCI 60 (PLUS) CELL SCREEN PANEL

BREAST:		**LEUKEMIA/LYMPHOMA:**		**OVARIAN:**	
BT-549	0	CCRF-CEM	+	IGROV1	+
HS578T	+/-	HL-60	+	OVCAR-3	+
MCF-7	+	K-562	+/-	OVCAR-4	+/-
MCF-7/ADR-RES	+	MOLT-4	0	OVCAR-5	+++
MDA-MB-231	+	RPMI 8226	0	OVCAR-8	+/-
MDA-MB-435	++	SR	0	SK-OV-3	+++
MDA-N	+/-				
T47D	+++	**LUNG:**		**PROSTATE:**	
ZR751	0	A549	+	DU-145	0
A1N4	0	EKVX	0	PC-3	+
10A	0	HOP-62	0	PPC-1	+
MDA-MB-468	0	HOP-92	+/-	ALVA-31	++++
		NCI-H322M	+++	JCA-1	0
		NCI-H226	+	LNCap	+/-
COLON:		NCI-H23	+	Tsu-pri	+
COLO 205	+++	NCI-H522	+		
HCT-15	+++	NCI-H460	+	**RENAL:**	
HCT-116	0			786-0	0
HCC-2998	+/-	**MELANOMA:**		A498	0
HT29	+++	LOX IMVI	0	ACHN	0
KM12	+++	MALME-3M	0	CAKI-1	+
SW-620	+++	M14	0	RXF 393	0
		SK-MEL-2	++++	SN12C	0
CNS:		SK-MEL-5	+/-	TK-10	+
SF-268	+/-	SK-MEL-28	0	UO-31	++
SF-295	+/-	UACC-62	0		
SF-539	+/-	UACC-257	0		
SNB-19	+++				
SNB-75	+				
U251	+				

XIAP PROTEIN EXPRESSION IN THE NCI 60 (PLUS) CELL SCREEN PANEL

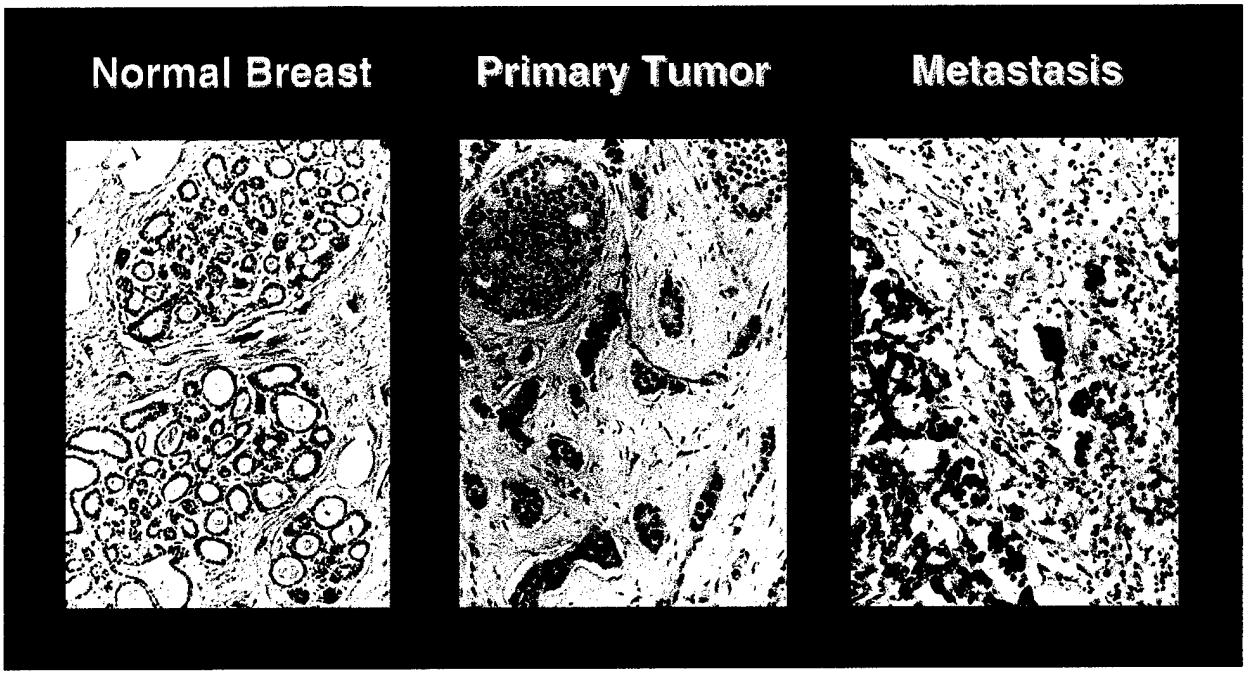
BREAST:		**LEUKEMIA/LYMPHOMA:**		**OVARIAN:**	
BT-549	+	CCRF-CEM	++	IGROV1	++
HS578T	+++	HL-60	+++	OVCAR-3	+++
MCF-7	+++	K-562	++	OVCAR-4	+/-
MCF-7/ADR-RES	++	MOLT-4	0	OVCAR-5	++++
MDA-MB-231	++	RPMI 8226	+	OVCAR-8	++
MDA-MB-435	+++	SR	+/-	SK-OV-3	++++
MDA-N	+				
T47D	+++	**LUNG:**		**PROSTATE:**	
BT-474	++	A549	++	DU-145	0
A1N4	+/-	EKVX	0	PC-3	++
10A	++++	HOP-62	0	PPC-1	+/-
MDA-MB-468	+/-	HOP-92	++	ALVA-31	+
ZR751	+/-	NCI-H322M	+++	JCA-1	+
BT-20	0	NCI-H226	+++	LNCap	0
		NCI-H23	+	Tsu-pri	0
COLON:		NCI-H522	++		
COLO 205	++	NCI-H460	+	**RENAL:**	
HCT-15	++			786-0	+++
HCT-116	0	**MELANOMA:**		A498	0
HCC-2998	+	LOX IMVI	+/-	ACHN	+/-
HT29	++	MALME-3M	+++	CAKI-1	+++
KM12	++	M14	+	RXF 393	+/-
SW-620	++	SK-MEL-2	++++	SN12C	+
		SK-MEL-5	+++	TK-10	+++
CNS:		SK-MEL-28	+/-	UO-31	+++
SF-268	+	UACC-62	++		
SF-295	++	UACC-257	++		
SF-539	+++				
SNB-19	+++				
SNB-75	++				
U251	++				

summaries of our data for cIAP1, XIAP, and Survivin, respectively. Note that variable expression of all of these IAP-family members is found. Having these data now positions us to select appropriate breast cancer cell lines for gene transfer experiments involving either over-expression or antisense ablation of cIAP1, XIAP, and Survivin.

Task #3. Immunohistochemistry of parrafin embedded breast carcinomas.

We have developed procedures for immunohistochemical analysis of XIAP and Survivin using our antisera and archival paraffin-embedded tumor specimens. Techniques for successfully employing our anti-cIAP1 antibody on paraffin-material have thus far been unsuccessful.

Figure 6 shows examples of Survivin immunostaining in normal mammary epithelium (Left), a primary tumor (Middle), and metastatic breast cancer (Right). Note that normal breast does not express Survivin. In contrast, intense Survivin immunostaining is present within the cytosol of essentially all the invasive breast cancer cells within the primary tumor specimen and in the metastatic breast cancer cells. Similar results have been obtained for 34 primary breast cancers and 6 metastatic lesions. We conclude, therefore, that tumor-specific deregulation of Survivin expression occurs commonly in breast cancers, suggesting an important role for this IAP-family member in this disease.



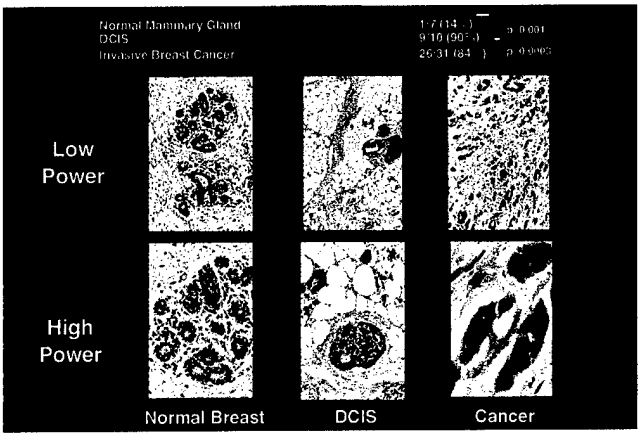


Figure 7 shows results from immunostaining of breast cancer specimens using our XIAP antibody. In contrast to Survivin which is entirely absent from normal breast, XIAP is expressed at low levels in mammary gland. However, comparisons of the intensity of immunostaining of normal mammary epithelial cells with ductal carcinomas in situ (DCIS) and invasive cancer revealed that XIAP levels are apparently upregulated in many breast neoplasms.

Task #4. Analyze bank of tumors previously typed for expression of p53, ER, Bcl-2, Bax and erbB2 for levels of IAP expression.

In progress.

TECHNICAL OBJECTIVE #2. Functional analysis of IAPs in breast cancer cell lines.

Task #5. Generation of IAP stable cell lines.

Several attempts to generate breast cancer cell lines over-expressing XIAP failed. The protein appears to be unstable and difficult to achieve accumulation in cells. Attempts to generate stably transfected cell lines overexpressing cIAP1, cIAP2, and Survivin are underway.

Task #6. Cell death assays with stable cell lines to assess effect of IAPs.

This will be done after stable transfectants are successfully produced.

TECHNICAL OBJECTIVE #3. Protein/Protein Interaction Studies.

Task #7. Yeast two hybrid assay to isolate interacting proteins.

Two cDNA libraries were screened with cIAP1 and cIAP2 (full-length) or the BIR-domain regions of these proteins as baits. Several positive clones were obtained, all representing components of the ubiquitin/protesome machinery. Attempts were made to examine whether IAPs somehow regulate the proteosome, using purified preparations of 26S proteosome and measuring proteosome-mediated degradation of a substrate peptide. No consistent effect of IAPs was observed. Our current hypothesis is that the levels of IAPs may be regulated in yeast by ubiquitination and

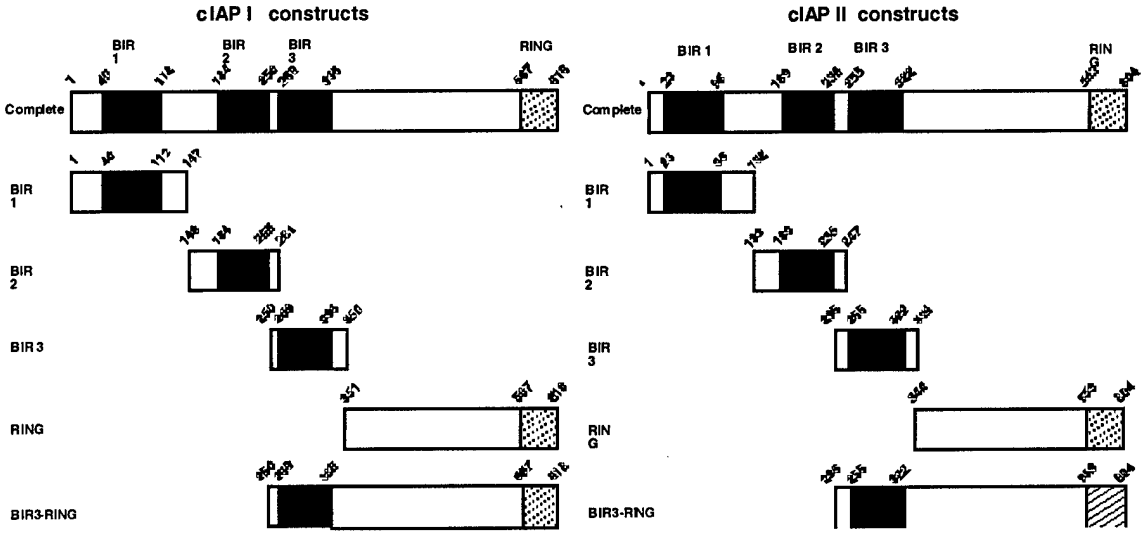
proteosome-mediated degradation, thus explaining why they interact with ubiquitin/proteosome cDNAs in two-hybrid assays. We are exploring whether IAPs are similarly regulated by ubiquitination/degradation in human cells.

We are also attempting to employ an alternative interaction cloning system for screening cDNA libraries (CytoTRAP; Stratagene, Inc.) in hopes of identifying novel IAP-interacting proteins.

Task #8. Isolation of associated proteins by co-immunoprecipitations.

We used a biochemical approach to identify IAP-binding proteins in cytosolic extracts from human cells, employing GST-cIAP1 and GST-cIAP2 fusion proteins as affinity selection agents. This resulted in the discovery that IAP family proteins tightly bind to and suppress caspase-family cell death proteases (6).These results were previously reported in earlier progress reports and will not be reiterated here.

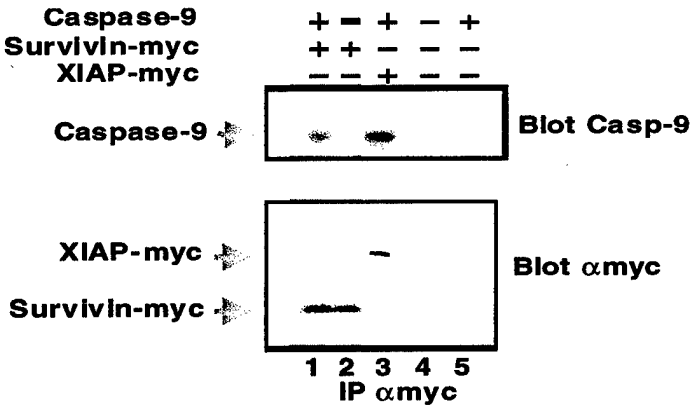
Attempts are currently underway to map the domain(s) in cIAP1 and cIAP2 which are responsible for binding caspases and inhibiting them. To this end, a series of plasmids have been generated which encode fragments of these IAP family proteins fused to GST for production in bacteria. Methods for production and purification of the proteins are currently being devised. Figure 8 depicts the fragments of cIAP1 and cIAP2 which have been generated.



Task #9. Isolation of full-length cDNAs from novel proteins.

Not applicable.

Task #10. Confirmation of interactions in vitro and in mammalian cells.



We confirmed that IAPs interact with selected caspases in cells, through co-immunoprecipitation assays. Interactions were most readily observable for caspase-9, the apical caspase in the cytochrome c pathway for apoptosis induction. Figure 9 shows an example of XIAP and Survivin co-immunoprecipitation with caspase-9 from 293 epithelial cancer cells which were transiently transfected with Myc epitope tagged XIAP or Myc-Survivin.

Task #11. Co-transfection assays with IAPs and novel proteins to determine functional consequence of interaction.

Not applicable. No novel IAP-binding proteins have been discovered. Co-transfection studies were performed where caspases were overexpressed and IAPs were shown to prevent caspase-induced apoptosis in transient transfection assays. Those findings were previously reported in progress reports and thus will not be reiterated here (6).

Task #12. Generation of stable cell lines expressing IAP-binding proteins.

Not applicable.

Technical OBJECTIVE #4. Explore effects of IAPs on caspase family proteases using caspase activity assays.

Task #13. Caspase assays with tetrapeptide substrates.

Comparison of Caspase Inhibitors
(K_i [nM])

	Caspase-1	Caspase-3	Caspase-6	Caspase-7	Caspase-8
XIAP	N.S.	0.7	N.S.	0.2	N.S.
cIAP1	N.S.	108	N.S.	42	N.S.
cIAP2	N.S.	35	N.S.	29	N.S.
CrmA	0.01	500	1.10	N.S.	0.95
p53	1.0	1.0	1.0	1.0	1.0

This goal was previously accomplished and previously reported in earlier progress reports. The inhibitory constants (Ki) for each of the IAPs were determined, against caspases-3 and -7 (6,7). The table on the left summarizes those findings.

3. PLANS.

Our plans remain essentially unchanged from the original proposal. We envision that most of the originally proposed Objectives should be accomplished within the final year of the grant.

4. PUBLICATIONS ATTRIBUTED TO THIS GRANT.

Roy, N., et al., *The c-IAP-1 and c-IaP-2 proteins are direct inhibitors of specific caspases.* EMBO J., 1997. **16**: p. 6914-6925.

Cardone, M., et al., *Regulation of cell death protease caspase-9 by phosphorylation.* SCIENCE, 1998. **282**: p. 1318-1320.

Deveraux, Q. L., et al., *IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases.* EMBO J., 1998. **17**: p. 2215-2223.

C. CONCLUSION

Insufficient apoptosis may result in tumor progression and consequently, the dysregulation of genes that normally inhibit the apoptotic cascade contributes to carcinogenesis. Moreover, tumor resistance to chemotherapeutic drugs has been associated with the upregulation of antiapoptotic genes. Chemotherapeutic drugs kill by initiating apoptosis and specifically lead to increases in caspase activity. We have shown that IAPs inhibit caspases. Pharmacological agents that induce cell death by the specific targeting of these should contribute to tumor regression by liberating caspases to induce apoptosis.

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The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases

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The inhibitor of apoptosis (IAP) family of proteins are highly conserved through evolution. However, the mechanisms by which these proteins interfere with apoptotic cell death have been enigmatic. Recently, we showed that one of the human IAP family proteins, XIAP, can bind to and potently inhibit specific cell death proteases (caspases) that function in the distal portions of the proteolytic cascades involved in apoptosis. In this study, we investigated three of the other known members of the human IAP family, c-IAP-1, c-IAP-2 and NAIP. Similarly to XIAP, *in vitro* binding experiments indicated that c-IAP-1 and c-IAP-2 bound specifically to the terminal effector cell death proteases, caspases-3 and -7, but not to the proximal protease caspase-8, caspases-1 or -6. In contrast, NAIP failed to bind tightly to any of these proteases. Recombinant c-IAP-1 and c-IAP-2 also inhibited the activity of caspases-3 and -7 *in vitro*, with estimated K_is of $\leq$0.1 μM, whereas NAIP did not. The BIR domain-containing region of c-IAP-1 and c-IAP-2 was sufficient for inhibition of these caspases, though proteins that retained the RING domain were somewhat more potent. Utilizing a cell-free system in which caspases were activated in cytosolic extracts by addition of cytochrome *c*, c-IAP-1 and c-IAP-2 inhibited both the generation of caspase activities and proteolytic processing of pro-caspase-3. Similar results were obtained in intact cells when c-IAP-1 and c-IAP-2 were overexpressed by gene transfection, and apoptosis was induced by the anticancer drug, etoposide. Cleavage of c-IAP-1 or c-IAP-2 was not observed when interacting with the caspases, implying a different mechanism from the baculovirus p35 protein, the broad spectrum suicide inactivator of caspases. Taken together, these findings suggest that c-IAP-1 and c-IAP-2 function similarly to XIAP by inhibiting the distal cell death proteases, caspases-3 and -7, whereas NAIP presumably inhibits apoptosis via other targets.
Keywords: apoptosis/caspase/cell death protease/inhibitor of apoptosis proteins

Introduction

It is now firmly established that cysteine proteases, termed caspases, related to the mammalian interleukin-1β converting enzyme (ICE or caspase-1) and to the nematode CED-3, play a central role as effectors of apoptosis (reviewed in Nicholson, 1996). Diverse stimuli which cause apoptosis result in activation of these cysteine proteases, implying that they play an essential role in the cell death pathway. The natural substrates of the caspases are key regulatory and structural proteins, including protein kinases and proteins involved in DNA repair and cytoskeleton integrity, thereby contributing to the demise of the cell (Casciola-Rosen *et al.*, 1994, 1996; Lazebnik *et al.*, 1994, 1995; Brancolini *et al.*, 1995; Emoto *et al.*, 1995; Martin *et al.*, 1995; Cardone *et al.*, 1997; Rudel and Bokoch, 1997). The caspases are synthesized as inactive precursors (zymogens) that are proteolytically processed to generate active subunits. Processing of the proenzymes and substrates usually occurs by cleavage at specific aspartic acid residues in the P_1 position. An enzymatic cascade is suggested by this observation and, indeed, several active caspases can process their zymogen forms or other members of the family, at least *in vitro* (Srinivasula *et al.*, 1996; Muzio *et al.*, 1997). Peptide inhibitors corresponding to the substrate P_4–P_1 residues of these cysteine proteases are potent inhibitors of apoptosis, substantiating the central role of caspases in mediating cell death (Enari *et al.*, 1995; Los *et al.*, 1995; Milligan *et al.*, 1995).

Ablation of caspase activity is attained by the viral proteins, p35 from baculovirus and CrmA from cowpox, which appear to be suicide inactivators, strongly inhibiting caspase enzymatic activity (Bump *et al.*, 1995; Xue and Horvitz, 1995; Bertin *et al.*, 1996; Zhou *et al.*, 1997). Overexpression of these viral caspase inhibitors in insect, nematode and mammalian cells results in resistance to apoptotic stimuli, confirming that components of the apoptotic pathway are highly conserved throughout evolution, and leading to the speculation that mammalian functional equivalents of these protease inhibitors may exist.

The inhibitor of apoptosis proteins (IAP) are a family of anti-apoptotic proteins that are conserved across several species. The baculovirus IAPs, Cp-IAP and Op-IAP, were the first members of this family to be identified based on their ability functionally to complement the cell death inhibitor, p35, in mutant viruses (Crook *et al.*, 1993; Clem and Miller, 1994). The first human IAP to be identified, the neuronal apoptosis inhibitory protein (NAIP), was isolated based on its contribution to the neurodegenerative disorder, spinal muscular atrophy (SMA) (Roy *et al.*, 1995). Deletions in *naip* are observed in many individuals with SMA, predominantly those with the most severe form of the disease, consistent with the hypothesis that the motor neuron depletion characteristic of this disorder occurs by the failure to inhibit apoptosis. Subsequently, four human (c-IAP-1/HIAP-2/hMIHB, c-IAP-2/HIAP-1/

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hMIHC, X-IAP/hILP, survivin) and two *Drosophila* IAP (DIAP-1, DIAP-2/dILP) homologs have been isolated, which have all been demonstrated to counter cell death (Hay *et al.*, 1995; Rothe *et al.*, 1995; Duckett *et al.*, 1996; Liston *et al.*, 1996; Uren *et al.*, 1996; Ambrosini *et al.*, 1997).

The common structural feature of all IAP family members is a motif termed the baculovirus IAP repeat (BIR) that is present in either two or three copies. The baculoviral IAPs and one of the *Drosophila* IAPs contain two BIR domains, while three of the human family members and a second *Drosophila* IAP contain three such domains. Survivin, in contrast, contains only one BIR domain, implying that a single BIR can be sufficient for anti-cell death activity (Ambrosini *et al.*, 1997). With the exceptions of NAIP and survivin, all other known IAP family members also contain a RING domain at their carboxy-terminus whose exact function remains elusive (Saurin *et al.*, 1996). The fact that the BIR motif is shared by all members suggests a central role for this domain in mediating cellular protection; however, this has not been demonstrated conclusively. It has been suggested, at least in the case of the baculoviral IAPs, that the RING finger domain is also required for suppression of apoptosis in insect cells (Clem *et al.*, 1994). However, overexpression of the *Drosophila* IAP BIR motifs in the absence of the RING domain suppresses cell death induced by X-irradiation, *hid* overexpression or naturally occurring developmental cell death in the eye of transgenic flies (Hay *et al.*, 1995). Moreover, transgenic flies overexpressing the RING domain alone exhibited increased cell death in the eye, suggesting that the RING domain may act as a negative regulator of cell death suppression. In addition, the human IAP family proteins NAIP and survivin are capable of inhibiting apoptosis, although they lack a RING finger domain, further suggesting that the BIR domains may be sufficient.

The human c-IAP-1 and c-IAP-2 proteins were identified originally as proteins that are recruited to the cytosolic domain of the p80 tumor necrosis factor receptor II (TNF-RII) via their association with the TNF-associated factor (TRAF) proteins, TRAF-1 and TRAF-2 (Rothe *et al.*, 1995). The c-IAP-1 protein additionally has been shown be a component of the p60 TNF-RI complex through its association with TRAF-2 (Shu *et al.*, 1996). Although associated with these receptor complexes, the precise function of c-IAP-1 and c-IAP-2 in TNFR family signal transduction cascades is at present unknown. Both c-IAP-1 and c-IAP-2 have been shown to inhibit apoptosis induced by several stimuli when overexpressed in mammalian cells, similar to other IAP family proteins. However, the mechanism by which these proteins suppress apoptosis has heretofore been enigmatic.

Mutant baculoviruses lacking the caspase inhibitor p35 can be genetically complemented by the IAPs, suggesting that the IAP family of proteins may function at the same level within the apoptotic cascade to suppress cell death. Consistent with this hypothesis, we recently have shown that the human family member XIAP can directly inhibit two members of the cell death protease family, caspases-3 and -7 (Deveraux *et al.*, 1997). These particular cell death proteases function in the distal portions of the cell death pathway, downstream of proximal caspases such as caspase-8 (FLICE/MACH-1/Mch5) which bind to the TNF-RI and Fas/APO-1 receptor complexes (Boldin *et al.*, 1996; Muzio *et al.*, 1996, 1997; Medema *et al.*, 1997). The association of c-IAP-1 and c-IAP-2 with TRAF-1 and TRAF-2, however, suggests that these IAP family members may act at a different level of the apoptotic cascade and/or have alternative functions. Here, we report that, despite their ability to bind TRAF-1 and TRAF-2, the c-IAP-1 and c-IAP-2 proteins are direct inhibitors of the downstream proteases caspase-3 (CPP32/Yama) and caspase-7 (Mch-3/ICE-Lap3) but not the upstream protease caspase-8. Taken together, our data substantiate a model whereby the biochemical mechanism of suppression of apoptosis by these cellular IAP family proteins involves inhibition of caspases that operate in the distal portions of the cell death pathway.

Results

c-IAP-1 and c-IAP-2 but not XIAP and NAIP bind to TRAF family proteins

Previous studies have indicated that c-IAP-1 and c-IAP-2 can bind to the TRAF family proteins, TRAF-1 and TRAF-2 (Rothe *et al.*, 1995). However, it is unknown whether the ability to bind TRAF family proteins is a general characteristic of IAPs. To address this question, c-IAP-1, c-IAP-2, XIAP and the BIR domain-containing region of NAIP were expressed as GST fusion proteins in bacteria and tested for binding to *in vitro* translated [^{35}S]TRAF-1, TRAF-2, TRAF-3, TRAF-4, TRAF-5 and TRAF-6. As shown in Figure 1, TRAF-1 and TRAF-2 bound to GST–c-IAP-1 and GST–c-IAP-2, as anticipated. In contrast, TRAF-1 and TRAF-2 did not bind to GST–XIAP or GST–NAIP. Moreover, the TRAF-3, TRAF-4, TRAF-5 and TRAF-6 proteins failed to bind to all IAPs tested. These *in vitro* translated TRAF family proteins, however, did bind to GST fusion proteins containing the cytosolic domains of either CD40 or the lymphotoxin-β receptor (LTβR), as expected from prior reports (Figure 1 and data not shown). Thus, c-IAP-1 and c-IAP-2 are unique among these four members of the human IAP family in their ability to bind selected TRAF family proteins.

c-IAP-1 and c-IAP-2 inhibit specific caspases in vitro

Though we have reported recently that XIAP can bind to and inhibit certain caspases which participate in the distal portions of the proteolytic cascade involved in apoptosis (Deveraux *et al.*, 1997), it was unknown whether other IAP family proteins could also function as caspase inhibitors. Moreover, the finding that c-IAP-1 and c-IAP-2 uniquely interact with the TNF-R family-binding proteins TRAF-1 and TRAF-2 raised the possibility that these proteins, if they were caspase inhibitors, might target more proximal proteases such as caspase-8, which is known to associate with the TNF-RI and Fas/APO-1 receptor complexes (Boldin *et al.*, 1996; Muzio *et al.*, 1996; Medema *et al.*, 1997).

To address this issue, we tested the ability of recombinant IAPs to repress activity of five different purified active recombinant caspases. Some of these caspases are believed to function at proximal portions of cell death pathways

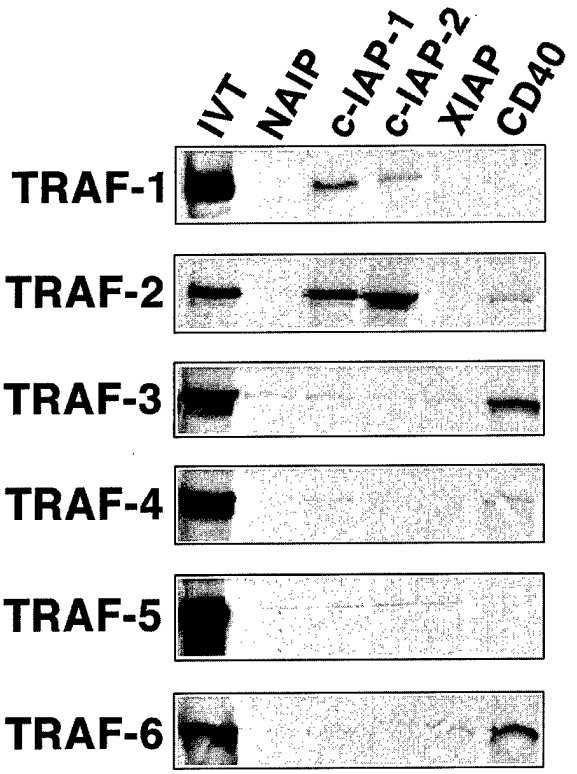

Fig. 1. c-IAP-1 and c-IAP-2 are unique in their ability to bind TRAF family proteins. GST–c-IAP-1, c-IAP-2, XIAP, NAIP (residues 1–368) and CD40 (cytosolic domain) fusion proteins (~3 μM) were immobilized on glutathione–Sepharose and incubated with 15 μl of reticulocyte lysates containing *in vitro* translated ^{35}S-labeled TRAF-1, TRAF-2, TRAF-3, TRAF-4, TRAF-5 or TRAF-6. After extensive washing, bound proteins were analyzed by SDS–PAGE/ autoradiography. The first lane (left side) contains 3 μl of the *in vitro* translation reaction, included as a positive control.

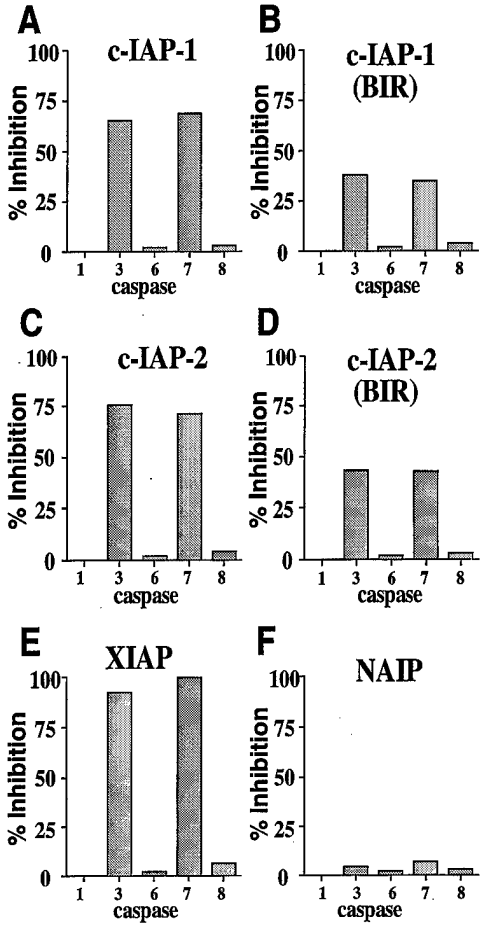

Fig. 2. c-IAP-1 and c-IAP-2 inhibit caspases-3 and -7 but not caspases-1, -6 and -8. Enzyme activity was measured by the release of the AFC fluorophore in the absence or presence of either (**A**) GST–c-IAP-1, (**B**) c-IAP-1 (BIR), (**C**) c-IAP-2, (**D**) c-IAP-2 (BIR) (**E**) XIAP or (**F**) NAIP. The rate of substrate hydrolysis in the presence of inhibitor (V_i) versus the rate in the absence of inhibitor (V_o) was calculated after allowing reactions to reach steady-state and is shown as percentage inhibition. Purified caspases (0.1–1 nM) incubated with a 100- to 5000-fold molar excess of GST–IAP proteins (0.1–0.5 μM) in the presence of substrate (100 μM).

activated by TNF family cytokines (caspases-2 and -8), whereas others appear to act at distal points in cell death pathways (caspases-3, -6 and -7) (Berges *et al.*, 1993; Boldin *et al.*, 1996; Muzio *et al.*, 1996, 1997; Orth *et al.*, 1996; Medema *et al.*, 1997). Moreover, evidence has been presented suggesting that caspase-6 (Mch2) lies at an intermediate position within the apoptotic proteases cascade upstream of the terminal effector caspases-3 and -7 (Liu *et al.*, 1996a; Orth *et al.*, 1996).

For these experiments, c-IAP-1 and c-IAP-2 were expressed as GST fusion proteins in bacteria, affinity purified, and then incubated with active caspase-1, -3, -6, -7 or 8, in the presence of either the fluorogenic tetrapeptide substrate, Z-YVAD-AFC for caspase-1, or Z-DEVD-AFC for the other caspases. Residual enzyme activity was measured by spectrofluorimetry. As shown in Figure 2, c-IAP-1 and c-IAP-2 inhibited caspases-3 and -7 by ~65 and ~75%, respectively, when used at ~0.5 μM (representing a 100- to 1000-fold molar excess of inhibitor relative to enzyme). In contrast, c-IAP-1 or c-IAP-2 did not alter the activity of caspases-1, -6 or -8, even at this molar excess. Several control proteins including GST, GST–Bcl-2, GST–Bax and GST–CD40 had no effect on the activities of these caspases (data not shown), thus confirming the specificity of the IAPs. Truncated forms of c-IAP-1 (amino acids 1–351) and c-IAP-2 (amino acids 1–336) encoding the three BIR motifs but lacking the C-terminal RING domain were also assayed for their ability to inhibit active recombinant caspases *in vitro*. The c-IAP-1 (BIR) and c-IAP-2 (BIR) proteins both decreased caspase-3 and -7 activity by ~40%. Thus, the c-IAP-(BIR) and c-IAP-2-(BIR) proteins retained the ability to inhibit caspases, albeit with somewhat reduced potency compared with full-length c-IAP-1 and c-IAP-2. These data indicate that c-IAP-1 and c-IAP-2 are selective inhibitors of certain caspases, and demonstrate furthermore that their inhibitory function can be ascribed to or within their BIR motifs.

Similarly to c-IAP-1 and c-IAP-2, a GST fusion protein encoding the IAP family member XIAP (~0.1 μM) potently inhibited caspases-3 and -7 but not caspases-1, -6 and -8, as expected from previous results (Figure 1E) (Deveraux *et al.*, 1997). In contrast, a GST–NAIP fusion protein corresponding to the three BIR motifs did not display any inhibitory effect on any of the caspases tested, including caspases-3 and -7 (Figure 1F). We cannot, however, rule out the possibility that this recombinant protein is inactive.

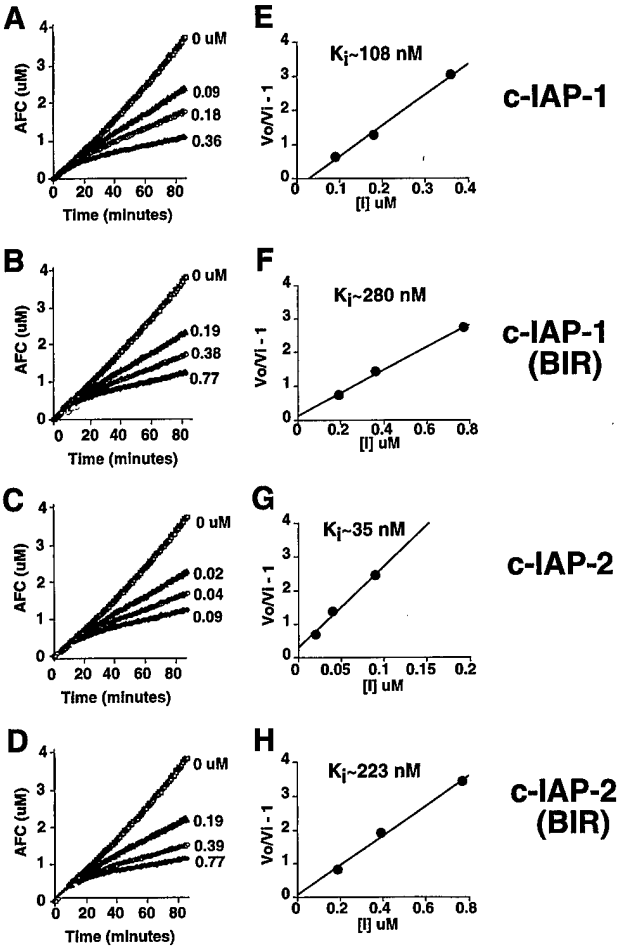

Fig. 3. Concentration-dependent inhibition of caspase-3 by c-IAP-1 and c-IAP-2. Representative progress curves are presented, demonstrating the inhibition of caspase-3 by (**A**) c-IAP-1, (**B**) c-IAP-1 (BIR), (**C**) c-IAP-2 and (**D**) c-IAP-2 (BIR). Caspase-3 was added to a final concentration of 7 pM with 100 μM of DEVD-AFC and the indicated concentrations of active IAP protein (μM). Enzyme activity was determined by the release of the AFC fluorophore (μM) over time. The inhibition constants (K_is) for caspase-3 by (**E**) c-IAP-1, (**F**) c-IAP-1 (BIR), (**G**) c-IAP-2 and (**H**) c-IAP-2 (BIR) were determined by plotting V_o/V_i-1 against inhibitor concentration (I).

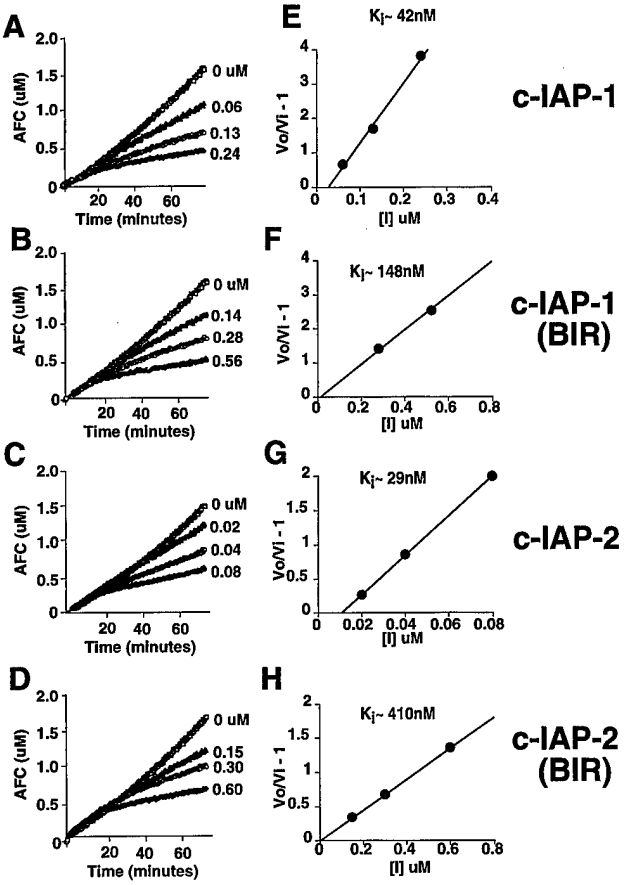

Fig. 4. Concentration-dependent inhibition of caspase-7 by c-IAP-1 and c-IAP-2. Representative progress curves are presented, demonstrating the inhibition of caspase-7 by (**A**) c-IAP-1, (**B**) c-IAP-1 (BIR), (**C**) c-IAP-2 and (**D**) c-IAP-2 (BIR). Caspase-7 was added to a final concentration of 150 pM with 100 μM of DEVD-AFC and the indicated concentrations of active IAP protein (μM). Enzyme activity and inhibition constants (K_is) were measured as described for Figure 3.

Determination of inhibition constants (K_is) for c-IAP-1, c-IAP-1 (BIR), c-IAP-2 and c-IAP-2 (BIR)

We next sought to determine the kinetics of inhibition of caspases-3 and -7 by c-IAP-1 and c-IAP-2. Representative progress curves for the inhibition of caspases-3 and -7 are shown in Figures 3A–D and 4A–D respectively, using a range of c-IAP-1, c-IAP-1 (BIR), c-IAP-2 and c-IAP-2 (BIR) concentrations. In all cases, the inhibition was concentration dependent. Steady-states of product formation in the absence or presence of inhibitor were attained and utilized to calculate the apparent inhibition constants (K_i) by plotting V_o/V_i-1 (Figures 3E–H and 4E–H), as described (Zhou *et al.*, 1997). The inhibition of caspases-3 and -7 by the IAPs appears to be reversible, given that final steady-states of substrate hydrolysis were attained in the presence of a large excess of the inhibitor. Prior to steady-state rates of substrate hydrolysis, variable relaxation times are observed, indicating binding of the inhibitor to the caspase. This association, however, appears to be quite slow. The c-IAP-1 and c-IAP-2 proteins inhibited caspase-3 with estimated K_is of 108 and 35 nM, respectively. The K_is for caspase-7 were estimated at 42 and 29 nM, respectively. The c-IAP-1 (BIR) and c-IAP-2 (BIR) proteins inhibited caspases-3 and -7, although less potently, with apparent K_is of 280 and 223 nM, respectively, for caspase-3 and of 148 and 410 nM, respectively, for caspase-7.

c-IAP-1 and c-IAP-2 inhibit caspase-like protease activity and proteolytic processing of caspase-3 in cytochrome c-treated cytosols

Cytochrome *c* recently has been identified as a factor which is released from mitochondria into the cytosol of cells undergoing apoptosis, resulting in the activation of caspase-3 and probably other DEVD-specific caspases by an unknown mechanism (Liu *et al.*, 1996b; Kluck *et al.*, 1997; Yang *et al.*, 1997). We used a cell-free system based on the ability of exogenously added cytochrome *c* to induce activation of the caspases in cytosolic extracts to ask whether recombinant c-IAP-1 and c-IAP-2 could inhibit cellular caspases, in the context of a more physiologic milieu. Cytosols from 293 cells were incubated with 10 μM of cytochrome *c* and 1 mM dATP for 30 min, and DEVD-AFC hydrolysis was measured. Striking increases

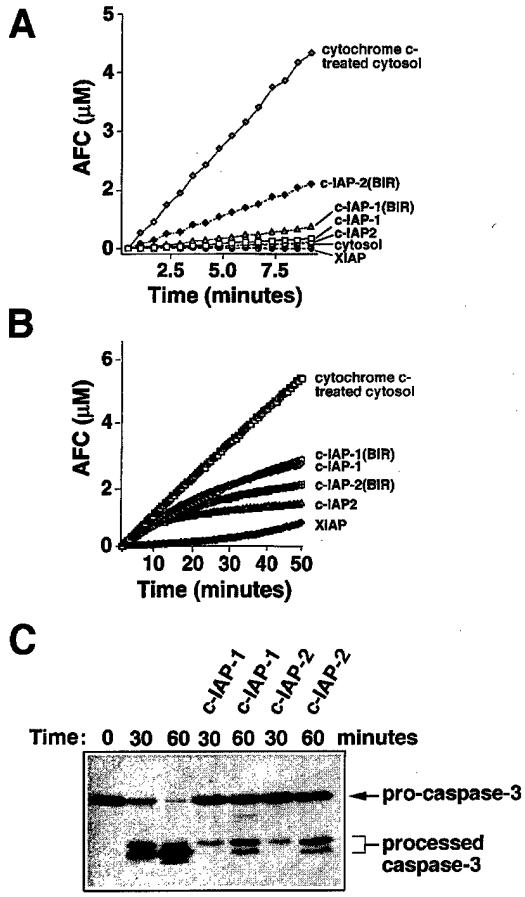

Fig. 5. Inhibition of cytochrome *c*-induced caspase-3 processing and DEVD activity by c-IAP-1 and c-IAP-2. (**A**) Z-DEVD-AFC hydrolysis was measured in cytosolic extracts from 293 cells into which ~0.3 μM GST–c-IAP-1, c-IAP-1 (BIR), c-IAP-2 or c-IAP-2 (BIR) was added prior to activation for 30 min at 37°C by the addition of cytochrome *c* (10 μM) and dATP (1 mM). Untreated cytosolic extracts (used as a control) were similarly incubated but without cytochrome *c* and dATP. (**B**) Cytosolic extracts from 293 cells were pre-activated for 30 min with cytochrome *c* and dATP, and Z-DEVD-AFC hydrolysis was then measured in the presence of the IAPs as indicated. (**C**) Immunoblot analysis of caspase-3 in cytosolic extracts activated for the indicated times with cytochrome *c* and dATP, either in the presence or absence of c-IAP-1 or c-IAP-2 (0.3 μM). The positions of the unprocessed (pro) form and processed (activated) forms of caspase-3 are shown.

in DEVD-specific protease activity were observed in cytochrome *c*-treated extracts, in contrast to control untreated extracts which exhibited negligible amounts of caspase-like activity (Figure 5A). The addition of 0.3 μM purified recombinant c-IAP-1 or c-IAP-2 prior to cytochrome *c* resulted in significant suppression of the DEVD-cleaving activity in these extracts (Figure 5A). Similarly, c-IAP-1 (BIR) or c-IAP-2 (BIR) also inhibited cytochrome *c*-induced DEVD-cleaving activity. In contrast, several control proteins, including GST, GST–Bcl-2, GST–Bax and GST–CD40, had no effect. We conclude, therefore, that both c-IAP-1 and c-IAP-2, as well as truncation mutants of these proteins lacking the RING domains, can interfere specifically with the cytochrome *c*-induced generation of caspase-like protease activities in cytosolic extracts.

In addition to experiments where c-IAP-1 and c-IAP-2 were added to cytosolic extracts prior to cytochrome *c*, we also examined whether the DEVD-cleaving activity in these extracts could be suppressed if the extract was treated with cytochrome *c* for 30 min prior to the addition of c-IAP-1 and -2. As shown in Figure 5B, c-IAP-1, c-IAP-1 (BIR), c-IAP-2 and c-IAP-2 (BIR) all caused reductions in DEVD-cleaving activity in extracts which had been pre-treated with cytochrome *c*.

The ability of c-IAP-1 and c-IAP-2 to inhibit the activities of DEVD-cleaving caspases could be due either to suppression of the active proteins, interference with the processing events that activate their zymogens, or both. To explore this possibility, we performed immunoblot analysis of cytosolic extracts treated with cytochrome *c* and dATP. The zymogen of caspase-3 (32 kDa) is processed initially by cleavage at aspartic acid residue 180 (D175) located between its large and small subunits, followed by autocatalytic removal of the N-terminal prodomain (Nicholson *et al.*, 1995; Han *et al.*, 1997). Whereas the smaller subunit is not recognized by our antibody (Krajewska *et al.*, 1997; Krajewski *et al.*, 1997), the larger subunit is and can exist either as a form that includes the N-terminal prodomain, or as smaller forms produced by removal of part or all of the prodomain (Nicholson *et al.*, 1995; Han *et al.*, 1997). Figure 5C demonstrates that treatment of extracts with cytochrome *c* results in loss of the 32 kDa precursor form of caspase-3 and the appearance of two versions of the large subunit. In contrast, caspases-1 and -2 remained as unprocessed zymogens (data not shown), suggesting that they act proximal to cytochrome *c*-initiated events. This observation also provides evidence that non-specific catalysis of all caspases had not occurred upon addition of cytochrome *c*, implying processing solely of downstream caspases.

Addition of c-IAP-1 or c-IAP-2 to extracts prior to cytochrome *c* and dATP resulted in nearly complete inhibition of pro-caspase-3 processing, with retention of the 32 kDa zymogen at levels comparable with control cytosols (Figure 5C). Thus c-IAP-1 and c-IAP-2 appear to prevent the initial cleavage of pro-caspase-3 at D180. Control GST fusion proteins added at similar concentrations to the extracts did not inhibit processing of pro-caspase-3. GST–NAIP also failed to inhibit cytochrome *c*-induced processing of pro-caspase-3 (not shown). Taken together, these data indicate that c-IAP-1 and c-IAP-2 can inhibit the cytochrome *c*-induced proteolytic processing events which lead to caspase-3 activation and can also inhibit the active caspase-3 enzyme after processing. The inhibition of pro-caspase-3 processing presumably occurs by either inhibiting the actions of an upstream protease that cleaves pro-caspase-3 at the IETDS site (amino acids 172–176) or by interfering with caspase-3 mediated 'trans-processing' of pro-caspase-3.

c-IAP-1 and c-IAP-2 inhibit apoptosis and caspase-like DEVD-cleaving activity in etoposide-treated intact cells

The inhibitory effect of c-IAP-1 and c-IAP-2 on cytochrome *c*-induced caspase activity in a cell-free system suggested that these inhibitors could also protect intact cells from apoptosis-inducing stimuli that cause the release of cytochrome *c* from mitochondria. The anticancer drug etoposide inhibits topoisomerase II, resulting in DNA damage and apoptosis (Liu, 1989; Dubrez *et al.*, 1995).

This cell death is accompanied by caspase activation which has DEVD-specific activity (Dubrez *et al.*, 1996; Martins *et al.*, 1997). Additionally, etoposide treatment of cells has been shown to induce release of cytochrome *c* into the cytosol, followed by caspase activation and apoptosis (Yang *et al.*, 1997).

Human kidney epithelial 293T cells were treated with etoposide, and the effect of overexpression of c-IAP-1 and c-IAP-2 on cell survival and caspase activity was examined. Cells were co-transfected with pEGFP as a transfection marker and either pcDNA3-myc-c-IAP-1, pcDNA3-myc-c-IAP-2 or, as a control, pcDNA3-myc. Treatment of 293T cells with 30 μM etoposide for 2 days resulted in substantial DEVD-specific activity, compared with untreated cells (Figure 6A). Similarly, 293T cells that had been transfected with the control plasmid, pcDNA-3-myc, accumulated DEVD-cleaving activity to approximately the same extent as untransfected 293T cells. In contrast, 293T cells transfected with plasmids encoding myc-tagged c-IAP-1 or c-IAP-2 accumulated much less DEVD-cleaving activity after treatment with etoposide. The proteolytic processing of caspase-3 was also inhibited in cells expressing c-IAP-1 or c-IAP-2, as determined by immunoblot analysis of the same cell lysates used for the enzyme assays (Figure 6B). As shown, etoposide induced consumption of the 32 kDa pro-caspase-3 protein, presumably as a result of processing to active protease, in control but not c-IAP-1- or c-IAP-2-transfected cells. Unlike the situation where cytochrome *c* was added to cytosolic extracts, it was difficult to detect the p17 subunit of processed caspase-3 under the conditions of these assays, probably due to the short half-life of the active protease in many types of cells. Finally, the effects of c-IAP-1 and c-IAP-2 overexpression on etoposide-induced apoptosis were determined by examining the morphology of 293T nuclei using the DNA-binding fluorochrome 4′,6′-diamidino-2-phenylindole (DAPI). As shown in Figure 6C, etoposide induced apoptosis of ~65% of the control transfected 293T cells under the conditions of these experiments. In contrast, the percentage of cells undergoing apoptosis was reduced substantially in cultures of c-IAP-1- and c-IAP-2-transfected cells following exposure to etoposide. Similar effects of c-IAP-1 and c-IAP-2 on caspase-3 processing and apoptosis were observed in 293 cells when overexpression of Fas (CD 95) was employed as the apoptotic stimulus (data not shown).

c-IAP-1 and c-IAP-2 bind directly to activated caspases-3 and -7

A characteristic property of proteinase inhibitors is their ability to form tight complexes with their target enzymes. We therefore examined the ability of c-IAP-1 and c-IAP-2 to bind caspase-3 and caspase-7, *in vitro*. GST fusion proteins encoding c-IAP-1, c-IAP-1 (BIR), c-IAP-2, c-IAP-2 (BIR), NAIP, XIAP or the cytosolic domain of CD-40 (used as a control) were immobilized on glutathione–Sepharose and incubated with purified recombinant active caspases-3, -6 or -7. The c-IAP-1 and c-IAP-2 fusion proteins, as well as their truncated forms encoding only the three BIR motifs, exhibited specific binding to caspase-3 and caspase-7 but not to caspase-6 (Figure 7A), consistent with our enzyme inhibition data. Though GST–c-IAP-2 (BIR) appeared to bind somewhat less efficiently

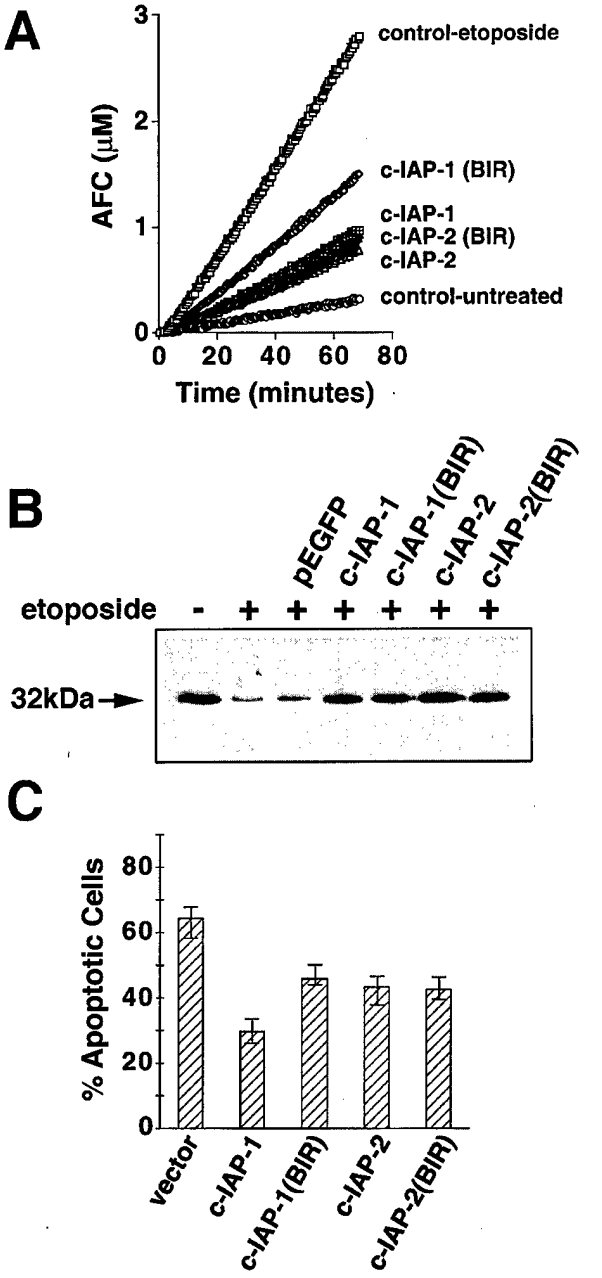

Fig. 6. c-IAP-1 and c-IAP-2 inhibit etoposide-induced apoptosis, processing of caspase-3 and generation of caspase-like protease activity in cells. 293T cells were either used directly or transiently co-transfected with a green fluorescent protein (GFP) marker plasmid pEGFP and either a control plasmid pcDNA-3-myc or expression plasmids encoding myc-tagged c-IAP-1, c-IAP-2, c-IAP-1 (BIR) or c-IAP-2 (BIR). After 6 h, etoposide (30 μM) was added to cultures as indicated (+). Lysates were prepared and apoptosis was assessed at 48 h post-transfection. Immunoblot analysis confirmed production of comparable amounts of the myc-tagged c-IAP-1, c-IAP-2, c-IAP-1 (BIR) and c-IAP-2 (BIR) proteins (not shown). (**A**) Z-DEVD-AFC hydrolysis was measured using lysates from the transfected 293T cells. Representative progress curves are shown. (**B**) Immunoblot analysis of pro-caspase-3 levels was performed using lysates from the transfected cells cultured with (+) or without (–) etoposide. (**C**) The percentage of apoptotic cells was determined by microscopic evaluation of DAPI-stained nuclei in GFP-positive cells. Data represent mean ±SE (n = 3).

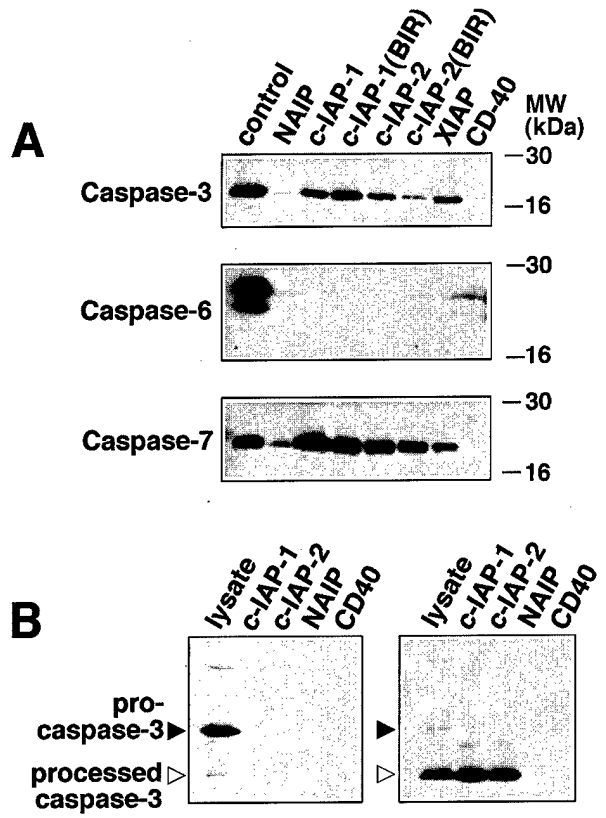

Fig. 7. c-IAP-1 and c-IAP-2 bind caspase-3 and caspase-7 but not caspase-6. (**A**) GST–c-IAP-1, c-IAP-1 (BIR), c-IAP-2, c-IAP-2 (BIR), NAIP, XIAP or CD40 fusion proteins (~3 μM) immobilized on glutathione–Sepharose were assayed for binding *in vitro* to active recombinant caspase-3, caspase-6 and caspase-7 (0.5 μg) in 400 μl of caspase buffer. Caspases were detected by immunoblotting. (**B**) GST–c-IAP-1, c-IAP-2, NAIP or CD40 immobilized on glutathione–Sepharose were incubated with 293 cytosolic extracts (45 μl) that had been treated with (right panel) or without (left panel) cytochrome *c*/dATP for 30 min. Bound caspase-3 was detected by immunoblotting. An equivalent volume of cell lysate was run directly (first lane), showing the pro-caspase-3 zymogen (dark arrow) and processed large subunit (open arrow).

than full-length c-IAP-2 in the experiment presented in Figure 7A, this was due to loading of less intact GST fusion protein on the glutathione–Sepharose beads. GST–XIAP also bound to caspase-3 and caspase-7, as expected from our previous studies of this member of the IAP family (Deveraux *et al.*, 1997). In contrast to c-IAP-1, c-IAP-2 and XIAP, however, far less binding of caspase-3 and caspase-7 to GST–NAIP was detected (Figure 7A). Although based on a semi-quantitative assay, the less efficient interaction of NAIP with these caspases is in accord with our enzyme inhibition data and suggests that NAIP either has specificity for other caspases not tested here, or that it operates through a different mechanism to inhibit cell death.

We next determined whether c-IAP-1 and c-IAP-2 could bind the inactive zymogens of caspase-3, since we had observed inhibition of their processing. Untreated 293 cytosolic extracts containing unprocessed pro-caspase-3 and cytochrome *c*-treated extracts containing processed caspase-3 were incubated with GST–c-IAP-1 or GST–c-IAP-2 fusion proteins and the bound proteins were analyzed by immunoblotting. As shown in Figure 7B, no binding of the 32 kDa unprocessed form of caspase-3 to c-IAP-1 or c-IAP-2 was detected. However, the processed large subunits of active caspase-3, produced by the addition of cytochrome *c*/dATP, bound to both c-IAP-1 and c-IAP-2 but not to the control protein, CD40. As before, little or no binding of processed caspase-3 to GST–NAIP was observed (Figure 7B), further confirming the difference in the specificity of this member of the IAP family.

c-IAP-1 and c-IAP-2 act as inhibitors but not as substrates of the caspases

The viral proteins p35 and CrmA are potent inhibitors of several caspases, and appear to act by a suicide mechanism that includes peptide bond hydrolysis at the inhibitory site. We examined if the inhibition by c-IAP-1 and c-IAP-2 occurs by a similar mechanism. c-IAP-1, c-IAP-2 and p35 were *in vitro* translated and transcribed in the presence of [^{35}S]L-methionine and then incubated with either caspase-3, -6, -7 or -8 for 1 h at 37°C. The resulting products were resolved by SDS–PAGE and detected by fluorography. As shown in Figure 8, p35 was cleaved efficiently by all the caspases, with nearly complete loss of the full-length p35 protein and the appearance of a smaller 25 kDa fragment. In contrast, neither c-IAP-1 nor c-IAP-2 underwent any significant cleavage in the presence of caspase-3 or -7, consistent with our kinetic data which demonstrate that these proteins exhibit reversible inhibition. Our data therefore imply that the mechanism of inhibition of caspases by c-IAP-1 and c-IAP-2 differs from that observed for p35. Thus, in contrast to p35 and CrmA, which are apparently suicide inactivators, the IAPs do not seem to require peptide bond hydrolysis as part of their inhibitory mechanism.

Discussion

The IAPs are an evolutionary conserved family of proteins which prevent cell death across species, implying that they act at a central, highly conserved point in the cell death cascade. We report that c-IAP-1 and c-IAP-2 directly interact with and inhibit two members of the caspase protease family, caspases-3 and -7. c-IAP-1 and c-IAP-2, however, did not bind to or inhibit caspases-1, -6 or -8. Thus, c-IAP-1 and c-IAP-2 displayed similar specificities to XIAP for the caspases (Deveraux *et al.*, 1997).

The c-IAP-1 and c-IAP-2 proteins inhibited caspases-3 and -7 with K_is in the low nanomolar range (~30–120 nM), significantly lower than CrmA which has been reported to inhibit caspase-3 with a K_i of ~500 nM (Zhou *et al.*, 1997). Despite their potent inhibition of caspases-3 and -7, however, XIAP inhibited these same caspases with K_is of 0.2–0.7 nM, representing 2–3 logs greater potency. This observation suggests that notwithstanding their overall homology, structural differences do exist between XIAP and the c-IAP-1 and c-IAP-2 proteins that affect how well they bind to and inhibit specific caspases. Thus while the K_is obtained for c-IAP-1 and c-IAP-2 suggest that they are physiologically relevant inhibitors of caspases-3 and -7, presumably it would be necessary for these proteins to be present at higher concentrations than XIAP to achieve the same level of protection against caspases-3 and -7. Moreover, the measured differences in the K_is for XIAP compared with c-IAP-1 and c-IAP-2 raise the

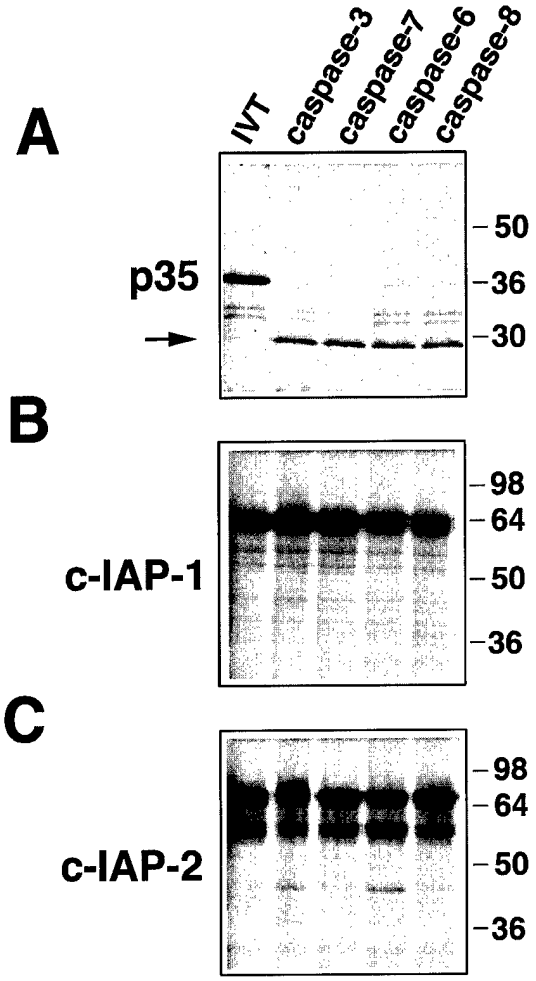

Fig. 8. c-IAP-1 and c-IAP-2 are not cleaved by caspases. *In vitro* translated ^{35}S-labeled p35 (**A**), c-IAP-1 (**B**) or c-IAP-2 (**C**) were incubated in the presence or absence of purified recombinant caspase-3, -6, -7 or -8 for 1 h, resolved by SDS–PAGE and analyzed by audioradiography. The 25 kDa cleavage product of p35 is indicated. The first lane (left side) represents untreated [^{35}S]p35, c-IAP-1 and c-IAP-2. The faint band at ~45 kDa in some c-IAP-2 lanes (C) represents a non-specific cleavage product that was variably present regardless of which caspases were added.

possibility that c-IAP-1 and c-IAP-2 may differ from XIAP in their ability to inhibit other caspases not studied here. Hence, whereas XIAP is a superior inhibitor of caspases-3 and -7, it is conceivable that c-IAP-1 and c-IAP-2 are better inhibitors of other as yet untested caspases. By analogy, NAIP theoretically could be a potent inhibitor of certain caspases, though having little apparent affinity for caspases-3 and -7. Nevertheless, the data reported here confirm that at least three of the currently known human members of the IAP family are direct inhibitors of select caspases and, therefore, establish inhibition of active cell death proteases as at least one mechanism by which this family of evolutionary conserved proteins suppresses apoptosis.

In contrast to the viral protein, p35, which functions as a broad specificity inhibitor of phylogenetically diverse caspases, the c-IAP-1, c-IAP-2 and XIAP proteins are selective inhibitors. The specificity presumably lies in differences in the substrate contact region of the caspases, which is seen when the three-dimensional structures of caspases-1 and -3 are compared (Wilson *et al.*, 1994; Rotonda *et al.*, 1996). Though of overall similar folds, an additional 10 residues (248–257) are present in caspase-3 that form an extra loop which guards the entrance to the active site of this protease. This loop contributes residues that form part of the S_4 pocket. The size and shape of the S_4 subsite consequently are determined at least in part by these residues. The extra loop region, although conserved in the caspase-3 subfamily, is most similar between caspases-3 and -7. One could speculate, therefore, that this loop is involved in IAP binding. Furthermore, caspases-3 and -7 have the highest overall identity (53%) among the 10 known members of the caspase family, suggesting that additional residues outside the loop may also confer specificity, leading to selective inhibition by c-IAP-1, c-IAP-2 and XIAP.

Suppression of apoptosis induced by gene transfer-mediated overexpression of caspases has been documented for several of the IAPs. The human XIAP and baculovirus Op-IAP, for example, were both shown to inhibit apoptosis induced by overexpression of caspase-1 in mammalian cells (Hawkins *et al.*, 1996; Uren *et al.*, 1996). Op-IAP, however, was unable to protect against caspase-2- or caspase-7-induced apoptosis. Op-IAP may thus inhibit caspases more proximal to caspase-7 but downstream of those, such as caspase-2, which are thought to function at upstream points in protease cascades due to their association with components of plasma membrane receptor complexes (Ahmad *et al.*, 1997; Duan and Dixit, 1997). Moreover, recent evidence suggests that Op-IAP may function upstream of p35 to prevent apoptosis, possibly inhibiting a protease (Manji *et al.*, 1997). This would support a model where the IAPs have specificity for different caspases, a phenomenon that may have evolved over time. If true, it also implies that one of the human homologs may target more upstream caspases. However, we cannot exclude the possibility that Op-IAP does inhibit caspase-7, like c-IAP-1, c-IAP-2 and XIAP, but that within the context of experiments where caspase overexpression is used to induce apoptosis, the amount of caspase-7 produced overwhelmed Op-IAP-mediated protection.

Our data demonstrating that c-IAP-1 and c-IAP-2 target the downstream caspases-3 and -7 are in accordance with their reported ability to inhibit apoptosis induced by the pro-apoptotic Bcl-2 family proteins, Bik and Bak (Orth and Dixit, 1997). Bcl-2 family proteins have been shown to regulate apoptosis at a mitochondria-dependent step, with the anti-apoptotic members such as Bcl-2 preventing release of cytochrome *c* and activation of caspases, and the pro-apoptotic members such as Bax inducing mitochondrial permeability transition and processing of distal caspases including caspases-3, -6 and -7 (Kroemer *et al.*, 1996; Susin *et al.*, 1996; Kluck *et al.*, 1997; Yang *et al.*, 1997). Bcl-2 family proteins, in contrast, do not appear to modulate the processing of upstream proteases such as caspase-8 (Boise and Thompson, 1997). Reports that c-IAP-1 and c-IAP-2 inhibit apoptosis induced by Bik and Bak, and that XIAP inhibits apoptosis triggered by Bax, therefore support the hypothesis that the IAP family proteins function at the level of distal caspases (Deveraux *et al.*, 1997; Orth *et al.*, 1997). These experiments, however, do not exclude the possibility of effects of these IAP family proteins on other caspases not tested here

which operate more proximally in the proteolytic cascades. Moreover, it should be noted that while IAP-mediated inhibition of caspases can prevent apoptosis, this may not necessarily stop cell death from occurring by other caspase-independent pathways.

Cytochrome *c* release from mitochondria into the cytosol represents a recently recognized event associated with caspase activation and apoptosis. Addition of cytochrome *c* to cytosolic extracts, for example, results in the processing of specific caspases, such as caspase-3 (Liu *et al.*, 1996b; Kluck *et al.*, 1997; Yang *et al.*, 1997). In intact cells, the release of cytochrome *c* from mitochondria upon treatment with various apoptotic stimuli, including chemotherapeutic drugs such as etoposide, has been observed and precedes the processing and activation of caspases (Yang *et al.*, 1997). Cytochrome *c* has been reported to induce the generation of caspase-3-like DEVD-specific cleaving activity but not caspase-1-like YVAD-cleaving proteases (Dubrez *et al.*, 1996). Moreover, apoptotic-like destruction of nuclei added to cytosolic extracts derived from etoposide-treated cells can be inhibited by the peptidyl inhibitors containing the sequence DEVD but not YVAD, consistent with the observation that DEVD-specific caspases are activated by cytochrome *c* (Dubrez *et al.*, 1996). Using cytochrome *c*-treated cytosolic extracts, we observed that c-IAP-1 and c-IAP-2 can inhibit DEVD-specific caspases. Furthermore, etoposide-induced DEVD-cleaving activity was also inhibited in intact cells by c-IAP-1 and c-IAP-2. These observations are consistent with a model where c-IAP-1 and -2 can inhibit caspases in more distal portions of the cell death pathway, downstream of cytochrome *c*.

We found that c-IAP-1 and c-IAP-2 proteins can bind to the enzymatically active forms of caspases-3 and -7 but not to their inactive zymogens. Nevertheless, the IAPs can block processing of pro-caspase-3, suggesting that they may inhibit a proximal caspase which is responsible for activating caspase-3, but which lies downstream of cytochrome *c*. Liu *et al.* (1996b) indicated that other cytosolic factors are required for caspase-3 activation by cytochrome *c*. More recently, it has been reported that two enzymatic activities are required for caspase-3 processing (Han *et al.*, 1997). The protease mediating the cleavage between the small and the large subunits is sensitive to a tetrapeptide inhibitor containing the sequence IETD, but not DEVD, whereas the protease that mediates cleavage events removing the prodomain is both ESMD and DEVD sensitive (Han *et al.*, 1997). Maintenance of caspase-3 in its 32 kDa unprocessed form in the presence of c-IAP-1 or c-IAP-2, despite cytochrome *c* treatment, suggests that these proteins may inhibit an IETD-sensitive protease or a protease upstream of it. We cannot exclude the possibility, however, that failure to observe processing of pro-caspase-3 results from a need for a small initial amount of caspase-3 activation through a positive feedback mechanism in which active caspase-3 cleaves pro-caspase-3, not unlike what occurs when pro-caspase-3 is expressed in bacteria where it typically is recovered as fully processed active protease.

At present, the role of c-IAP-1 and -2 recruitment to TNF-RI or TNF-RII upon ligand stimulation is unknown. One possibility is that c-IAP-1 and c-IAP-2 may be sequestered at the receptor and thus unable to reach their downstream target caspases. Alternatively, the recruitment of IAP family proteins to TNF-R complexes may promote their interaction with caspases that they otherwise would not have opportunities to regulate. Preliminary experiments, however, have failed to reveal an effect of TRAF-1 or TRAF-2 or their combination on c-IAP-1- and c-IAP-2-mediated suppression of cell death induced by either TNF-RI overexpression or etoposide (N.Roy, J.Reed and T.Van Arsdale, unpublished observations). Moreover, the finding that other members of the IAP family, such as XIAP and NAIP, fail to interact with TRAF family proteins suggests that binding to TRAFs is not essential for the caspase-inhibitory function of IAP family proteins.

We have shown that the *in vitro* caspase inhibitory activity of c-IAP-1 and c-IAP-2 resides within their BIR motifs. Consistent with this finding, the death-preventing activity of these IAP family proteins was also retained by overexpression of these BIR motifs. Thus, the preponderance of data available to date support the hypothesis that it is the BIR domains that represent the minimal functional region, at least among the cellular homologs of these proteins. It is of interest, however, that truncation mutants of c-IAP-1 and c-IAP-2 lacking the RING domain were less potent inhibitors of caspases-3 and -7 *in vitro* relative to their full-length forms. Thus, while not essential, the RING domain may make some contributions to c-IAP-1 and c-IAP-2 function, even if indirectly by perhaps stabilizing bioactive conformations of the BIR domains.

Although the mechanism of caspase inhibition by the IAPs remains to be elucidated, it appears to be different from p35. Unlike inhibition by p35 which involves proteolytic cleavage, we did not observe any significant cleavage of c-IAP-1 or c-IAP-2 in the presence of purified caspases. These data suggest that c-IAP-1 and -2 do not require cleavage for their inhibitory activity. Most natural inhibitors are active site-directed, in that the inhibitor directly blocks the active site of the protease. A survey of the known families of natural protease inhibitors reveals a common mechanistic theme. These inhibitors, such as kunitz, kazal and eglin families that inhibit serine proteases, contain a loop of defined structure that is preformed to adapt to the substrate groove of the protease (Bode and Huber, 1991). Similarly, the cystatins contain loops that fit the substrate groove of members of the papain family of cysteine proteases (Turk and Bode, 1991). In all cases, inhibition is caused by a lock-and-key type interaction of the pre-formed inhibitory loops with the protease substrate groove, and no peptide bond cleavage takes place. Peptide bond cleavage is rare in protease–protease inhibitor interactions, and is only observed for p35 and serpins (of which CrmA is a member), and the unusual α-macroglobulins that act as protease cages (Barrett and Starkey, 1973). Exactly how a protein can be a substrate yet still an inhibitor is still controversial, but these types of inhibitors are usually called suicide inactivators to indicate that the inhibitor is consumed during the process of inhibition (Patston *et al.*, 1991). Consequently, since the IAPs are not cleaved during inhibition, it is likely that they will operate by a mechanism similar to the conventional lock-and-key inhibitors, and studies are underway in our laboratories to test this hypothesis.

At least two roles can be envisaged for how IAP family

proteins might function physiologically as suppressors of downstream caspases. First, IAPs could be intended to inhibit the small amounts of adventitial caspase activation that surely must occur during the normal functions of healthy cells. In this capacity, IAPs potentially could prevent inappropriate induction of apoptosis which might otherwise be stimulated by small amounts of active proteases amplifying their effects through cascades of proteolytic processing of additional pro-caspases. Second, IAPs conceivably could prevent apoptosis induced by a broad array of cell death stimuli, provided that the IAPs are expressed at sufficiently high concentrations within cells. High levels of IAP protein production might occur normally under some circumstances during cell differentiation, as well as pathologically, such as in tumors. In this regard, striking overexpression of one of the IAP family proteins, survivin, has been reported recently in a wide variety of human tumors (Ambrosini *et al.*, 1997). Regardless of their intended roles, the findings presented here provide further evidence that the major mechanism by which the IAPs promote cell survival is by interfering with specific members of the caspase family of cell death proteases.

Materials and methods

Expression and purification of recombinant IAPs and caspases

c-IAP-1 and c-IAP-2 cDNAs were obtained by RT–PCR of RNA derived from Jurkat T-cells with the following primers for c-IAP-1 (forward, 5′-AGGGAATTCATGCACAAAACTGCCTCCCA-3′; reverse, 5′-CTCCTCGAGGATGGCTTCAAGTGTTCAAC-3′) and for c-IAP-2 (forward, 5′-AGGGAATTCATGAACATAGTAGAAAACAGCA-3′; reverse, 5′-CTCCTCGAGAGATGATGTTTTGGTTCTTCTT-3′). NAIP constructs encoding the three BIR motifs were generated by PCR from a full-length cDNA in pBluescript (Stratagene) with forward primer 5′-AGGGAATTCATGGCCACCCAGCAGAAA-3′ and reverse primer 5′-CTCCTCGAGCAGTAATTGAGAAAGTTCACC-3′. PCR products were digested with *Eco*RI and *Xho*I and ligated into pGEX4T and pcDNA3-myc. c-IAP-1 (BIR) and c-IAP-2 (BIR) constructs were generated by PCR from the full-length constructs with reverse primers 5′-CTCCTCGAGGATCTAACCTTGAATCTCATCAACAAAC-3′ and 5′-CTCCTCGAGGATCTACTTGAACTTGACGGATGATGAAC-3′ respectively, and the forward primers described above. All IAPs were expressed in *Escherichia coli* strain BL21(DE3) containing the plasmid pT-Trx (Yasukawa *et al.*, 1995). Bacteria were grown at 30°C to an optical density of 0.6, and fusion protein production was induced at 30°C with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h, with the exception of GST–c-IAP-2 which was induced for 1 h at room temperature. Fusion proteins were obtained from the soluble fraction and affinity purified on glutathione–Sepharose by standard methods. Eluted proteins were dialyzed against phosphate-buffered saline (PBS). GST–XIAP was prepared as described (Deveraux *et al.*, 1997).

Caspases-3, -6 and -7 containing C-terminal His_6 tags and caspase-8 containing an N-terminal His_6 tag were purified as previously described (Orth *et al.*, 1996; Quan *et al.*, 1996; Muzio *et al.*, 1997; Zhou *et al.*, 1997).

In vitro protease assays

Caspase activities were assayed at 37°C using a fluorometric plate reader (Perkin-Elmer, LS50B) in the kinetic mode with excitation and emission wavelengths of 400 and 505 nm, respectively. Activity was measured by the release of 7-amino-4-trifluoromethyl-coumarin (AFC) from the synthetic substrate benzyloxycarbonyl-Asp-Glu-Val-Asp (Z-DEVD-AFC). Inhibition rates and equlibria were calculated from progress curves where substrate hydrolysis (100 μM) by caspase-3 (7 pM), caspase-6 (100 pM), caspase-7 (150 pM) or caspase-8 (125 pM) was measured in the presence of a range of inhibitor concentrations (0.025–1.5 μM) in caspase buffer [50 mM HEPES, 100 mM NaCl, 1 mM EDTA, 0.1% CHAPS, 10% sucrose and 5 mM dithiothreitol (DTT)]. The observed inhibition constant, K_i, was calculated as described (Zhou *et al.*, 1997). Given that the mechanism of inhibition is unknown, K_is were determined without taking into account substrate concentration. Additionally, recombinant c-IAP-1 and c-IAP-2 proteins were titrated against caspase-7 to determine the amount of active protein in our preparations, which ranged from ~25 to 45%, assuming that inhibitor and enzyme form equimolar complexes.

For cell-free experiments, 293 cytosolic extracts were incubated with GST fusion proteins (0.3 μM) and activated by the addition of cytochrome *c* (10 μM) and dATP (1 mM) for 30 min at 37°C. For protease assays, 1 μl of cytosolic extracts was added to 100 μl of caspase buffer in the presence of 100 μM substrate, and DEVD hydrolysis was measured. Alternatively, extracts were activated for 30 min with cytochrome *c*/dATP, and DEVD hydrolysis was measured in the absence or presence of GST fusion proteins (0.3 μM) and substrate (100 μM).

Preparation of cytosolic extracts

293 cells were harvested by centrifugation at 1800 *g* for 10 min and washed with ice-cold buffer A [20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)]. The cell pellet was resuspended in 1 volume of buffer A and incubated on ice for 20 min. Cells were broken by passing 15 times though a 26 gauge needle, and the extracts were clarified by centrifugation at 16 000 *g* for 30 min. NaCl was then added to a final concentration of 50 mM to the resulting supernatants which were stored at -80°C.

Protein-binding assays

Purified recombinant caspase-3, -6 or -7 (0.5 μg) was incubated with GST fusion proteins (~3 μM) immobilized on glutathione–Sepharose beads in 400 μl of caspase buffer supplemented with bovine serum albumin (BSA) to a final concentration of 1%. The beads were washed three times with 1 ml of wash buffer [50 mM Tris (pH 7.5), 150 mM KCl, 2 mM DTT, 0.1% Triton X-100]. To test binding in cytosolic extracts, GST fusion proteins were incubated for 2 h with 45 μl of cytosolic extract that had been either unactivated or activated with cytochrome *c*/dATP and then supplemented to 400 μl with binding buffer (10 mM HEPES, 142 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.2% NP-40). TRAF proteins were *in vitro* transcribed and translated in the presence of [^{35}S]methionine and were incubated with IAP–GST fusion proteins (3 μM) in binding buffer for 3 h. Beads were washed three times in binding buffer; bound proteins were eluted by boiling in Laemmli sample buffer and subjected to SDS–PAGE and immunoblot analysis.

Immunoblot analysis

Proteins were separated by SDS–PAGE using 750 mM Tris–12% polyacrylamide gels and transferred to PVDF membranes. A monoclonal antibody against human caspase-2 was purchased from Transduction laboratories, and polyclonal antibodies against the other caspases were utilized as previously described (Orth *et al.*, 1996; Krajewska *et al.*, 1997; Krajewski *et al.*, 1997). Antibody detection was performed using an enhanced chemiluminescence detection kit (Amersham).

Cell culture

Human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Cells were transiently transfected with 12 μg of pcDNA plasmid DNA and 0.5 μg of pEGFP (Clontech) by a calcium phosphate precipitation method. Cells were incubated for 6 h with the transfection solution, washed three times with PBS, and then returned to culture with fresh media with or without 30 μM etoposide (Sigma). Cells were collected by centrifugation at 48 h post-transfection, and washed in ice-cold PBS. For protease-cleaving assays, cells were lysed in binding buffer and incubated on ice for 20 min. Lysates were cleared by centrifugation at 16 000 *g* for 30 min and supernatants stored at –80°C. To determine cell death, cells were fixed with 4% paraformaldehyde, rinsed with PBS and stained with 0.1 μg/ml DAPI. Nuclear morphology of cells was analyzed by fluorescence microscopy.

Protease inhibitor cleavage assays

Full-length cDNAs encoding c-IAP-1, c-IAP-2 and p35 in pcDNA3 were *in vitro* transcribed and translated in the presence of [^{35}S]methionine using the coupled transcription/translation TNT kit (Promega) according to the manufacturer's instructions. Five μl of the translation products were incubated with 2 μl of either caspase-1, -3, -6, -7 or 8 in caspase buffer in a total volume of 12 μl and incubated for 1 h at 37° C. The reactions were analyzed by SDS–PAGE and autoradiography.

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SCIENCE

Regulation of Cell Death Protease Caspase-9 by Phosphorylation

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Regulation of Cell Death Protease Caspase-9 by Phosphorylation

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Caspases are intracellular proteases that function as initiators and effectors of apoptosis. The kinase Akt and p21-Ras, an Akt activator, induced phosphorylation of pro–caspase-9 (pro-Casp9) in cells. Cytochrome c–induced proteolytic processing of pro-Casp9 was defective in cytosolic extracts from cells expressing either active Ras or Akt. Akt phosphorylated recombinant Casp9 in vitro on serine-196 and inhibited its protease activity. Mutant pro-Casp9(Ser196Ala) was resistant to Akt-mediated phosphorylation and inhibition in vitro and in cells, resulting in Akt-resistant induction of apoptosis. Thus, caspases can be directly regulated by protein phosphorylation.

Many apoptotic stimuli induce release of cytochrome c (cyto c) from mitochondria into the cytosol, where it binds to the CED-4 homolog Apaf-1, inducing binding to pro-Casp9 and resulting in proteolytic processing and activation of pro-Casp9. Active Casp9 then directly cleaves to and activates pro-Casp3, initiating a cascade of additional caspase activation that culminates in apoptosis. Cyto c induces caspase activation when added to cytosolic extracts in vitro with deoxyadenosine triphosphate (dATP) (*1*). We noticed that epithelial cell lines 267 (prostate) and MCDK (kidney) transfected with transforming Ki-Ras(Val12) or Ha-Ras(Val12) displayed resistance to apoptotic stimuli that are known to cause cyto c release (*2*), such as staurosporine and etoposide (VP16) (Fig. 1, A and B), and that cytosolic extracts derived from these cells were resistant to cyto c–induced caspase activation, as measured by cleavage of Ac-DEVD-AFC (*3*) (Fig. 1C) (*4*). Reduced caspase activity was not due to lower concentrations of pro-Casp3 but correlated with inhibition of proteolytic processing of pro-Casp3 (Fig. 1B) (*4*), implying a defect at or upstream of this caspase. Ras extracts, however, were not resistant to caspase activation induced by granzyme B (GraB) (*5*), which implies that other routes of protease activation were intact (Fig. 1D).

A farnesyl transferase inhibitor (FTI) reversed the resistance of Ras cytosolic extracts to cyto c (Fig. 1E), which suggests that the phenomenon is not due to secondary genetic changes in these cells. Moreover, resistance to cyto c–mediated activation of caspases was not an artifact of over-expressing oncogenic Ras, because extracts from DLD-1 colon cancer cells, which contain an endogenous activated Ki-Ras gene, displayed similar resistance to cyto c. Resistance was reverted by targeted disruption of the mutant Ras allele in two independent clones, DKO3 and DKS8 (Fig. 1F) (*6*).

The only proteins known to be required for cyto c–induced processing of pro-Casp3 are Apaf-1 and pro-Casp9 (*7*). Examination of Apaf-1 and pro-Casp9 mRNA and protein levels in control and Ras(V12)-expressing cells revealed no differences in their expression (*4*). Additionally, no difference in expression of Bcl-X_L or products of inhibitor of apoptosis (IAP) family genes (cIAP-1, cIAP-2, XIAP, NAIP, and survivin) was detected, excluding elevations in the concentration of these proteins that can directly or indirectly bind and inhibit some caspases within the cyto c pathway (*8*). Although pro-Casp9 protein was present at normal concentrations, its proteolytic processing in response to cyto c was impaired in cytosolic extracts derived from Ras(V12)-expressing cells (Fig. 2A) but not by GraB (*4*). Further, treatment of the extracts with a protein phosphatase (CIP) restored cyto c–induced processing of pro-Casp9 and accumulation of Ac-DEVD-AFC–cleaving caspases (Fig. 2, A and B), which implies that protein phosphorylation is required for Ras(V12)-mediated resistance to cyto c–induced processing of pro-Casp9.

Among the effectors of Ras is phosphatidylinositol 3-kinase (PI3K), which generates phosphoinositol phospholipid second messengers that activate Akt, a serine-threonine protein kinase previously implicated in apoptosis suppression (*9*). In vitro kinase assays revealed elevated Akt activity in Ras(V12)-transfected 267 cells as compared to control 267 cells (*4*). Treatment of Ras(V12)-expressing cells with the PI3K inhibitor Ly294002 (Fig. 2C) or with wortmannin (*4*) before (but not after) preparation of cytosolic extracts restored in vitro sensitivity to cyto c, resulting in pro-Casp9 processing and accumulation of Ac-DEVD-AFC–cleaving caspase activity. Further evidence of involvement of the PI3K/Akt pathway was obtained by expressing active v-akt in cells (*10*), resulting in suppression of cyto c–induced processing of pro-Casp9 and reduced caspase activity (Fig. 2D).

We therefore explored whether active Ras(V12) and active Akt induced phosphorylation of pro-Casp9 in cells. Immunoprecipitation of pro-Casp9 from cells metabolically labeled with $^{32}PO_4$ revealed increased radio incorporation into this protein [but not Apaf-1 (*4*)] in cells transfected with Ki-Ras(V12) or active Akt as compared to control cells (Fig. 3, A and B) (*11, 12*). Conversely, in cells transfected with a dominant negative (dn) kinase-inactive mutant of Akt(K178M), serum- and Ki-Ras(V12)–induced phosphorylation of pro-Casp9 was reduced (Fig. 3, C and D). The observation that Akt(K178M) only partially suppressed ^{32}P labeling of pro-Casp9 may indicate that it only partly blocks endogenous Akt or that other kinases can also phosphorylate pro-Casp9.

Pro-Casp9 contains sites that conform to the consensus Akt phosphorylation motif RXRXXS/T at Ser^{183} (RTRTGS) and Ser^{196} (RRRFSS) (*3, 13, 14*). To explore whether Akt can directly phosphorylate Casp9, active Akt was immunoprecipitated from Akt-transfected 293T human epithelial kidney (HEK) cells (*15*), and in vitro kinase assays were performed with [$\gamma^{32}P$]ATP and recombinant, purified, unprocessed Casp9 or processed Casp9 as candidate substrates (*16, 17*). As controls, Akt immune complexes were also incubated with a known substrate histone 2B (positive control) or with an irrelevant protein (negative control) that is not phosphorylated by Akt. Akt immune complexes induced phosphorylation of both unprocessed recombinant Casp9 and the large subunit of processed Casp9 in vitro (Fig. 4A). In contrast, a variety of control kinase complexes did not cause in vitro phosphorylation of either pro-Casp9 or processed Casp9 (*4*). Similar results were obtained with baculovirus-produced, recom-

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binant active GST-Akt (*18*), resulting in phosphorylation in vitro of Casp9 but not of other recombinant caspases such as Casp3 or Casp8 (Fig. 4B). Thus, Akt can directly phosphorylate both unprocessed and processed Casp9 in vitro. Moreover, the protease activity of bacteria-produced processed Casp9 was consistently reduced after in vitro treatment with Akt (Fig. 4, A and B) (*19*).

To explore whether Ser^{183} or Ser^{196} was phosphorylated by Akt in vitro, these sites were individually mutated to alanine. His_6-tagged versions of these mutant pro-Casp9 proteins were produced in bacteria and affinity purified for use as in vitro substrates of GST-Akt (*20*). Casp9(S196A) was not phosphorylated when treated with GST-Akt and its protease activity was not inhibited (Fig. 4C), which suggests that Ser^{196} is the predominant Akt phosphorylation site. In contrast, the Casp9(S183A) protein was still phosphorylated by Akt and its protease activity was reduced.

Mass spectrometry (MS) analysis of in vitro phosphorylated Casp9 was also undertaken as an alternative to site-directed mutagenesis, using a modified form mass spectroscopy: surface-enhanced laser desorption/ionization (SELDI) (*21, 22*). SELDI analysis of V8 protease digests of recombinant Casp9 after treatment with GST-Akt (but not GST control) revealed the presence of one 80-dalton mass-shifted peptide (*4*), which is consistent with the presence of a single Akt phosphorylation site in Casp9. The relevant phosphopeptide corresponded to amino acids 187 to 200 of Casp9 (as deduced from the V8 protease map of C9 and the MS profile), which contains Ser^{196} but not Ser^{183}. To confirm that Akt could phosphorylate this site, a peptide representing the deduced V8 fragment KLRRRFSSLHFMVE (*3*) was synthesized and used as a substrate for in vitro kinase assays employing GST-Akt. This Ser^{196}-containing peptide was phosphoryl-

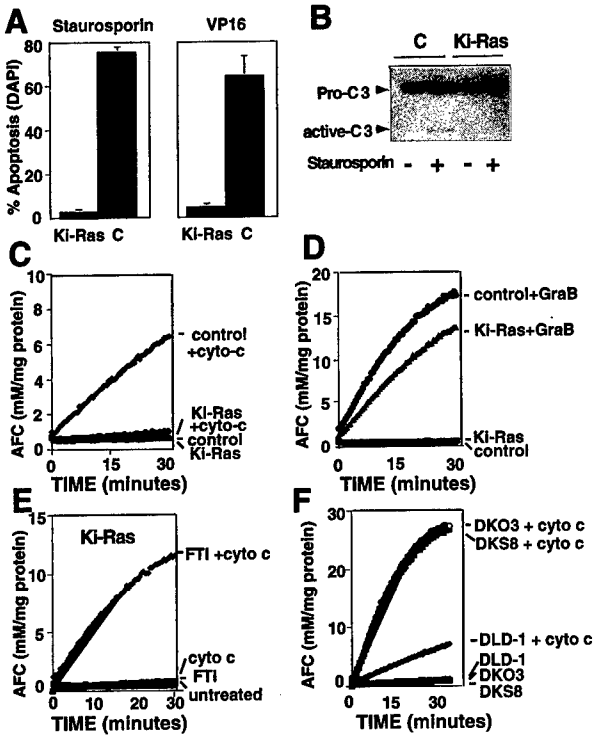

Fig. 1. Cytosolic extracts from Ras(V12)-expressing cells are refractory to cyto c–induced caspase activation. 267 and 267-Ki-Ras(V12) cells (*28*) were cultured with (+) or without (−) 1 μM staurosporin for 5 hours or 10 μM VP16 for ~18 hours before (**A**) determination of the percent of apoptotic cells by staining with 4′,6′-diamidino-2-phenylidole (DAPI) (Sigma) (mean ± SE; $n = 3$) (*4*) or (**B**) preparation of lysates for SDS-PAGE and immunoblot analysis (50 μg per lane) using antiserum to Casp3 with enhanced chemiluminescence (ECL) detection (*29*). Untreated cells were >95% nonapoptotic. In (**C**) through (**F**), cytosolic extracts were prepared (*30*) from 267 (control) and 267-Ki-Ras cells, with or without prior culture with 2 μM FTIase inhibitor [*N*-(amino-3-mercaptopropylamino-3-methyl-butyl)-Phe-Met-OH (Alexis, San Diego, California)] for 48 hours, or from DLD-1 cells, which contain an endogenous Ki-Ras(V12) allele and two clones, DK03 and DKS8, in which the Ki-Ras(V12) allele was interrupted by homologous gene recombination (*6*). Extracts were normalized for protein concentration and incubated at 30°C with or without 10 μM cyto c (and 1 mM dATP) or with 10 μM GraB (*5*). Ac-DEVD-AFC hydrolysis was measured with continuous-reading instruments (*31*).

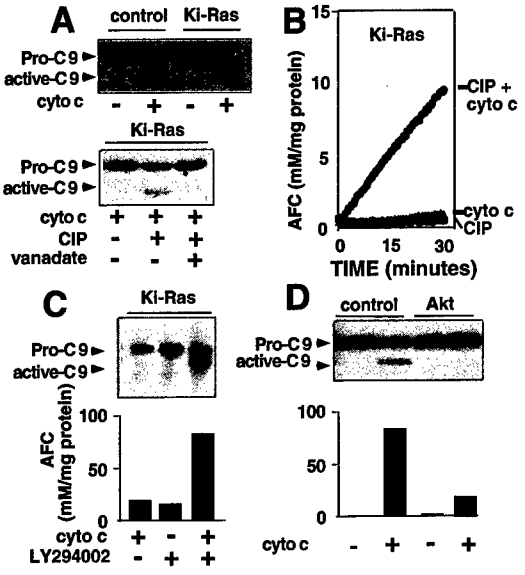

Fig. 2. Inhibition of cyto c–induced processing of pro-Casp9 in cytosolic extracts from Ras(V12)- or v-akt-expressing cells. (**A**) Cytosolic extracts from 267B or 267B-Ki-Ras(V12) cells were normalized for total protein content, then pretreated at 37°C for 1 hour with (+) or without (−) 1 U of PBS-exchanged calf intestinal alkaline phosphatase (CIP) (Boehringer-Mannheim) or with CIP and 2 mM Na-orthovanadate. In vitro translated (IVT) [^{35}S]pro-Casp9 in reticulocyte lysates (10% v:v) was added, and the extracts were treated with (+) or without (−) 10 μM cyto c and 1 mM dATP at 30°C for 0.5 hour before analysis by SDS-PAGE and autoradiography. (**B**) Cytosolic extracts (normalized for total protein) from 267B-Ki-Ras cells were pretreated at 37°C for 1 hour with nothing or with 1 U of CIP, then incubated at 30°C in the presence of 10 μM Ac-DEVD-AFC with (+) or without (−) 10 μM cyto c and 1 mM dATP. Substrate cleavage was monitored by AFC fluorescence. (**C** and **D**) Cytosolic extracts were prepared from either (C) Ki-Ras 267 cells that had been cultured with or without 10 μM Ly294002 for 1 hour or (D) NIH-3T3 cells that had been stably infected with control or v-akt–encoding retroviruses (*10*). IVT [^{35}S]pro-Casp9 was added (top panels) or Ac-DEVD-AFC cleavage was monitored (bottom panels) for 0.5 hour as above.

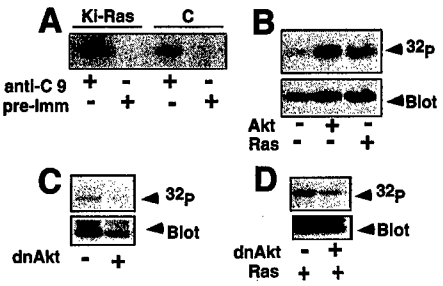

Fig. 3. Ki-Ras(V12) and Akt induce phosphorylation of pro-Casp9. (**A**) 267B and 267B-Ki-Ras(V12) cells were cultured in $^{32}PO_4$-containing medium (*11*). Immunoprecipitations were performed with either antibody to Casp9 or preimmune serum (*12*). Immune complexes were analyzed by SDS-PAGE and autoradiography. Immunoblotting confirmed equivalent amounts of endogenous pro-Casp9 in 267B and 267B-Ki-Ras(V12) cell lysates (*4*). (**B** through **D**) 293T cells were transfected with plasmids encoding FLAG-pro-Casp9 (Cys287Ala) alone or together with pCMV6-Akt(E40K), pCMV-dn-Akt (*15*), pZipNeo-Ha-Ras(V12) (gift of C. Hauser), or combinations of these plasmids. Cells were ^{32}P-labeled (*11*) and pro-Casp9 was immunoprecipitated with an antibody to FLAG (Kodak). Immune complexes were subjected to SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes for autoradiography (top) or anti-FLAG immunoblot (bottom) analysis using ECL-based detection to verify immunoprecipitation of equivalent amounts of FLAG–pro-Casp9.

ated in vitro by Akt (Fig. 4D), as judged by a characteristic phosphatase-sensitive 80-dalton shift in peptide mass.

Evidence that Ser^{196} is phosphorylated by Akt in vivo was also obtained by mass spectroscopy and ^{32}P metabolic labeling comparisons of pro-Casp9 and pro-Casp9(S196A) recovered by immunoprecipitation from transfected 293 HEK cells (*23, 24*) (Fig. 4, E and F). For these experiments, however, it was necessary to mutate the active site cysteine (Cys^{287}) of Casp9 and Casp9(S196A) to avoid inducing apoptosis. MS analysis of V8 digests of pro-Casp9 immunoprecipitated from cells that had been transfected with active Akt revealed an 80-dalton mass-shifted form of the peptide fragment containing Ser^{196}. In contrast, the corresponding peptide fragment from pro-Casp9(S196A) recovered from Akt-transfected cells was not phosphorylated (Fig. 4E). Similarly, when coexpressed with active Akt in ^{32}P-labeled 293 cells and then recovered by immunoprecipitation (*11*), less ^{32}P incorporation into the pro-Casp9(S196A) protein was observed than in the wild-type pro-Casp9 (Fig. 4F).

Overexpressing pro-Casp9 in 293T HEK cells induces apoptosis (*24*). Thus, Akt should suppress apoptosis induced by pro-Casp9 but not by the pro-Casp9(S196A) mutant that lacks the identified Akt phosphorylation site. Transfection of plasmids encoding pro-Casp9 or pro-Casp9(S196A) into 293T HEK cells resulted in apoptosis of most of the successfully transfected cells (Fig. 4G) (*4*). Coexpression of active Akt with wild-type pro-Casp9 rescued ~40% of the cells from apoptosis. In contrast, Akt rescued approximately half the cells expressing pro-Casp9(S196A), which is consistent with the failure of the Ser^{196} mutant to serve as a substrate for Akt in vitro and in vivo.

Akt has been implicated in signal transduction pathways for apoptosis suppression induced by Ras, growth factor receptors, neurotrophin receptors, and some oncoproteins (*9*). The data presented here suggest that phosphorylation and inactivation of Casp9 may be one of several mechanisms used by Akt to promote cell survival (*25*). Pro-Casp9 activation by Apaf-1/cyto c oligomeric complexes reportedly involves both autocatalytic self-processing and trans-processing of inactive pro-Casp9 molecules by active Casp9 molecules held within the same complex (*26*). Occupation of binding sites on Apaf-1 by endogenous phosphorylated Casp9 molecules, therefore, presumably explains why cyto c–induced processing of pro-Casp9 was inhibited in extracts from cells with elevated Akt activity. Ser^{196} in Casp9 is predicted to be distal from the substrate binding pocket, based on the three-dimensional structure of the homolog Casp3 (*27*). Thus, inhibition by phosphorylation may involve an allosteric mechanism that affects subunit dimerization or that alters the catalytic machinery of the substrate cleft through conformational changes. Though further studies are required to delineate the enzymological and structural details of how Akt-mediated phosphorylation of Ser^{196} in Casp9 inhibits its proteolytic activity, the findings reported here elucidate a mechanism for regulating caspases.

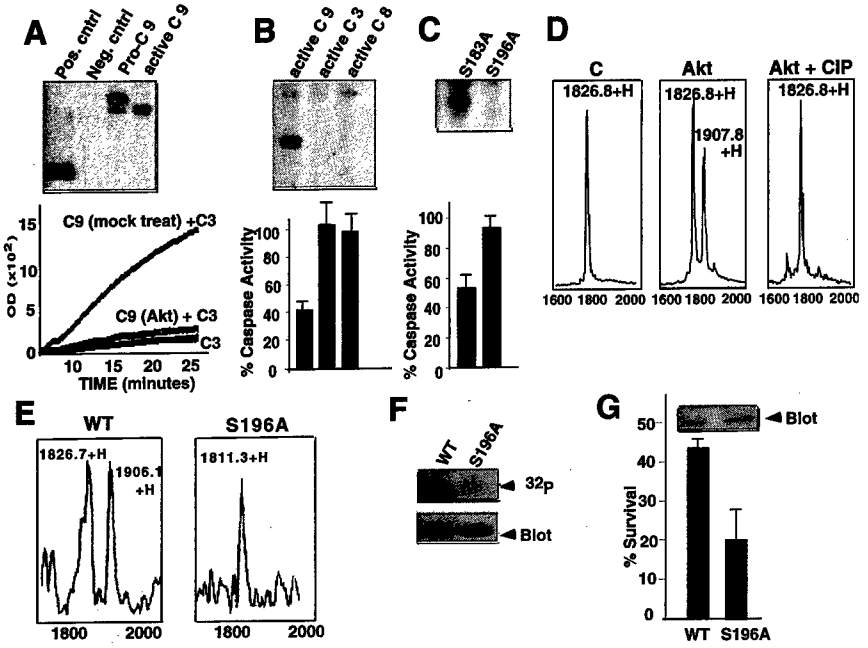

Fig. 4. Akt phosphorylates and inactivates Casp9. (**A**) Akt-containing immune complexes (*15*) were used to phosphorylate histone 2B as a positive control (Pos. cntrl), calmodulin as a negative control (Neg. cntrl), or recombinant unprocessed pro-Casp9(C287A) and active processed Casp9 in vitro in the presence of [γ^{32}P]ATP (*17*), followed by SDS-PAGE and autoradiography analysis (upper panel). Alternatively, active Casp9 was treated with Akt or control immune complexes using unlabeled ATP, and Casp9 activity was measured on the basis of its ability to activate pro-Casp3, which then cleaves the colorimetric substrate AC-DEVD-pNA (lower panel) (*19*). (**B** and **C**) Purified GST-Akt (*18*) was incubated with recombinant active Casp3, Casp8, Casp9, Casp9(S183A), or Casp9(S196A) (*20*) in the presence of [γ-^{32}P]ATP for SDS-PAGE and autoradiography analysis (upper panel) (*17*) or unlabeled ATP for caspase activity assays (lower panel) (mean ± SE; n = 3) (*19*). (**D**) A synthetic peptide KLRRRFSSLHFMVE (*3*) corresponding to residues 187 to 200 of pro-Casp9 was used for in vitro kinase reactions with either control protein (left) or GST-Akt (middle). A portion of the Akt-kinased peptide (50 pmol) was subsequently treated with 0.2 U of CIP for 4 hours at 37°C (right). Peptides were analyzed by MS. (**E**) V8 digests of FLAG-Casp9 (WT) and FLAG-Casp9(S196A) immunoprecipitated from 293 cells and analyzed by SELDI (*21, 22*). The V8-produced peptide corresponding to Casp9(187–200) is shown, demonstrating the ~80-dalton mass-shifted species in Casp9 but not Casp9(S196A). (**F**) 293T cells were transfected with pCMV6myrAkt and FLAG-tagged C287A mutants of Casp9 (WT) or Casp9 (S196A). The next day, cells were labeled with ^{32}P, and pro-Casp9 proteins were recovered by anti-FLAG immunoprecipitation, subjected to SDS-PAGE, and transferred to PVDF membranes for analysis by autoradiography (top panel) and anti-Casp9 immunoblot (ECL) (bottom panel) analysis. (**G**) 293T cells were transfected with 3 μg of pcDNA3-FLAG plasmids encoding pro-Casp9, or pro-Casp9(S196A), together with either 5 μg of control plasmid or pCMV6-myrAkt-HA and 1 μg of pEGFP. Sixteen hours after transfection, cells were deprived of serum and adherent and floating cells were collected 5 hours later and stained with DAPI. The percent of green fluorescent protein–positive cells with nonapoptotic nuclei was determined (mean ± SE; n = 3) (*30*). Lysates were analyzed by anti-FLAG immunoblotting to confirm expression of equivalent amounts of the wild-type and S196A FLAG-Casp9 proteins (inset).

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12. A rabbit antiserum to Casp9 was raised against purified recombinant His_6-active Casp9 and verified to be specific for Casp9 by immunoblotting experiments using a panel of recombinant caspases, including Casp3, Casp6, Casp7, Casp8, and Casp10.
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15. 293T cells (10^7) were transiently transfected with 25 μg of pCMV6-myrAkt-HA or pcDNA (control) DNA. The activated form of Akt was generated by adding the NH_2-terminal Src myristoylation sequence to a pre-existing construct expressing Akt-HA in pCMV-6 (*19*). Cells were lysed 1 day later in 1.5 ml of 20 mM tris-HCl (pH 7.4), 140 mM NaCl, 1% NP-40, 10 mM NaF, 1 mM Na_3VaO_4, 1 mM EDTA, and protease inhibitors. After normalizing for protein concentration, lysates were precleared with protein G–Sepharose and preimmune serum for 1 hour and incubated at 4°C with 0.5 μg of rat high-affinity mAb to hemagglutinin (HA) (Boehringer-Mannheim), followed by addition of 10 μl of protein G–Sepharose (Pharmacia) for 1 hour. Alternatively, endogenous Akt was immunoprecipitated from 267 or 267-Ki-Ras cells with antibody to Akt (Santa Cruz Biotech), producing similar results (*4*). Immunoprecipitates were washed three times in lysis solution and two times in kinase solution [20 mM Hepes (pH 7.2), 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT, and 3 μM ATP].
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20. Pro-Casp9 and Pro-Casp9(C287A) cDNAs, as well as S183A and S196A mutants of these, were expressed with NH_2-terminal His_6-tags from pET23b in BL21 cells for production of processed Casp9 and unprocessed Casp9, respectively (*16*). Expression was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside at $OD_{600} \cong 0.6$ to 0.8 and ~25°C for 4 hours for the S183A mutant and for 1 hour for the S196A mutant. Proteins were affinity purified by Ni-chelate Sepharose (Pharmacia).
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23. Casp9 mutants were generated by site-directed polymerase chain reaction (PCR) mutagenesis from a human pro-Casp9 cDNA (V. Dixit) and subcloned into pcDNA3-FLAG, pCMV2-FLAG, or pET23b plasmids. The primer pairs used to generate the S183A and S196A mutants were 5′-CCGCACCCGCACTGGCGCGAACATCGACTGTGAG-3′ plus 5′-CTCACAGTCGATGTTCGCGCCAGTGCGGGTGCGG-3′; and 5′-CGGCGTCGCTTCTCCGCGCTGCATTTCCTGGTGG-3′ plus 5′-CCACCATGAAATGCAGCGCGGAGAAGCGACGCCG-3′, respectively. PCR was performed for 16 cycles at 95°C for 30 s, 55°C for 1 min, and 68°C for 12 min. Twenty microliters of the reactions was digested with Dpn I (10 U) for subsequent subcloning into plasmids.
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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;

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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).

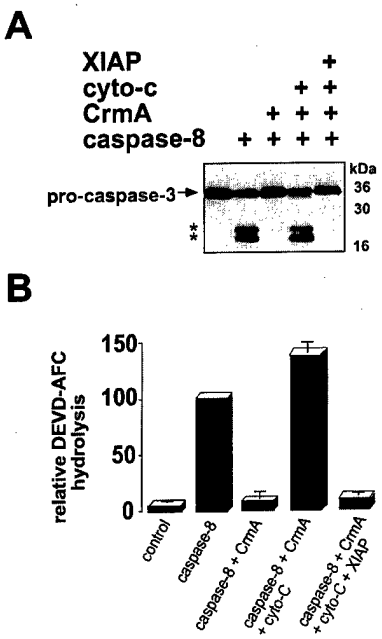

Fig. 1. CrmA and XIAP inhibition of caspase-8 and cytochrome *c*-induced processing and activation of pro-caspase-3 in cytosolic extracts. Recombinant purified and active caspase-8 (0.1 μM) was added to cytoplasmic extracts from 293 cells in the absence or presence of various combinations of CrmA (0.5 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,

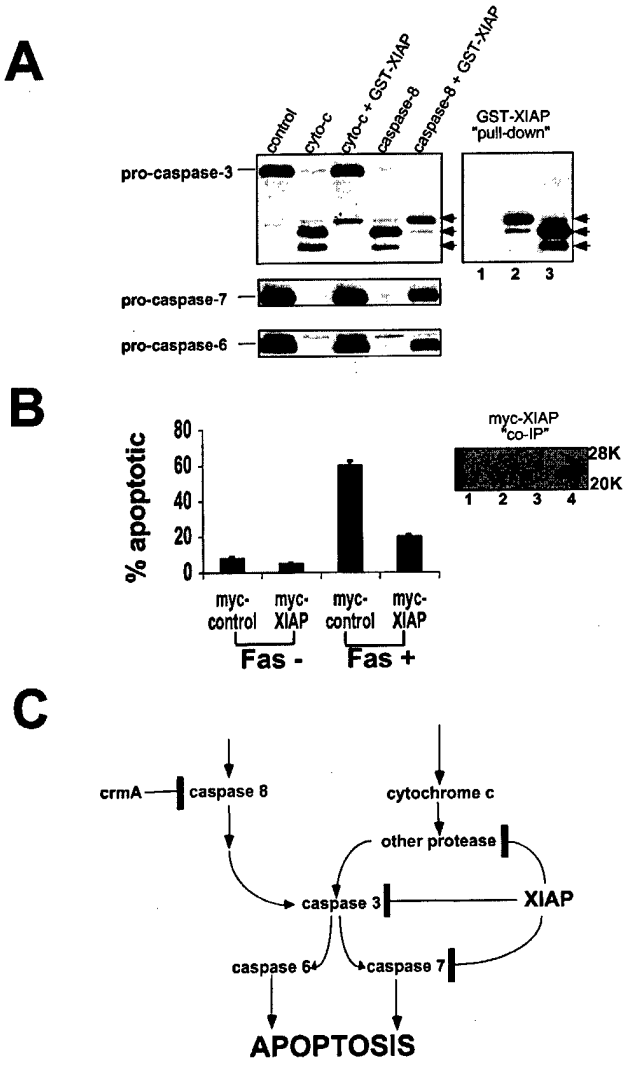

Fig. 2. XIAP-mediated inhibition of pro-caspases 3, -6 and -7 processing in cytochrome *c*- and caspase-8-treated extracts. (**A**) Cytochrome *c* (10 μM) together with dATP (1 mM) or active caspase-8 (0.1 μM) were added to cytosolic extracts from 293 cells with or without GST-XIAP (0.2 μM). Extracts 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.

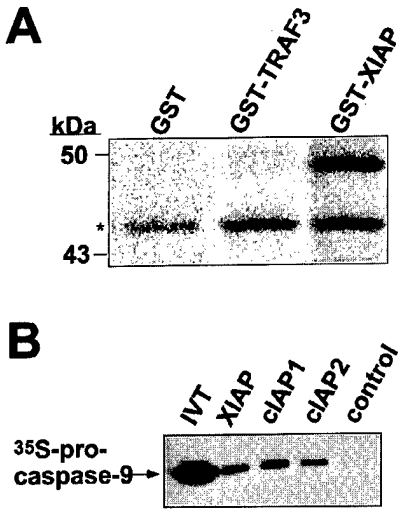

Fig. 3. Pro-caspase-9 binds to XIAP, cIAP1 and cIAP2. (**A**) GST-XIAP was incubated in lysates from U937 cells that had been cultured in [^{35}S]L-methionine-containing media. Lysates were incubated at 4°C for 1.5 h in GST, GST-TRAF-3 (1-357), or GST-XIAP. Proteins were separated on SDS–PAGE gels and 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).

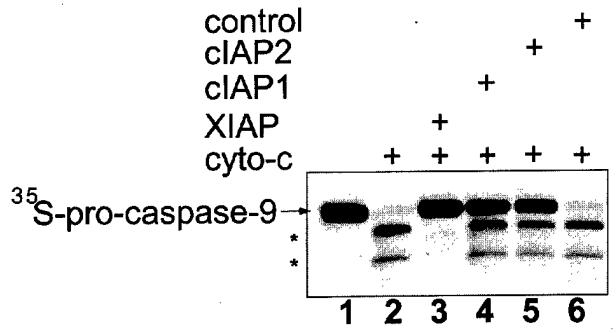

Fig. 4. Inhibition of cytochrome *c*-induced caspase-9 processing by XIAP, cIAP1 and cIAP2. *In vitro*-translated ^{35}S-labeled pro-caspase-9 was added to cytosolic extracts from 293 cells which were then incubated for 30 min at 30°C with (lanes 2–6) or without (lane 1) cytochrome *c* (10 μM) and dATP (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

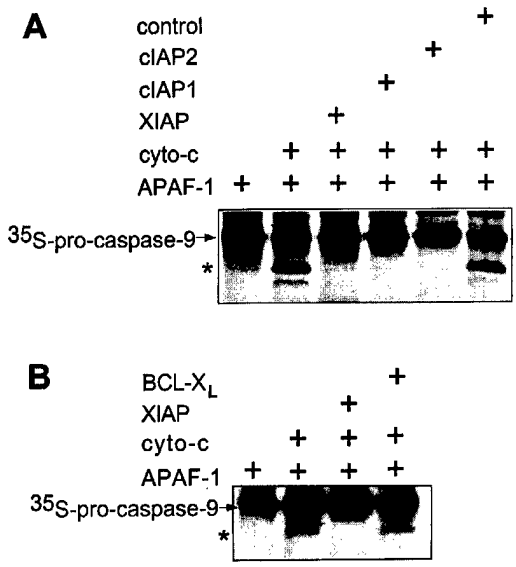

Fig. 5. Pro-caspase-9 processing requires Apaf-1 and cytochrome *c* and is inhibited by XIAP, cIAP1 and cIAP2. *In vitro*-translated ^{35}S-labeled pro-caspase-9 and Apaf-1 were incubated individually or together with cytochrome *c* (10 μM) and dATP (1 mM). Processing of pro-caspase-9 in the absence 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).

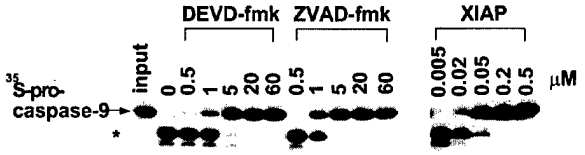

Fig. 6. Comparison of pro-caspase-9 inhibition by Ac-DEVD-fmk, zVAD-fmk and XIAP. *In vitro*-translated ^{35}S-labeled pro-caspase-9 was added to cytosolic extracts from 293 cells containing 10 mM cytochrome *c* and 1 mM dATP. Samples were incubated at 30°C for 30 min in the presence of the indicated 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

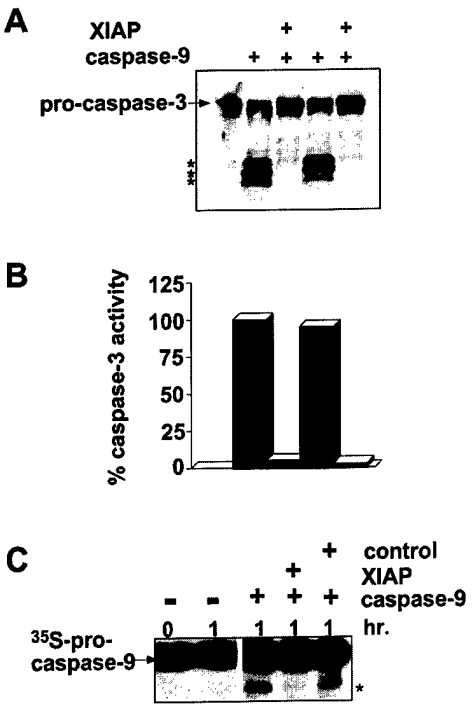

Fig. 7. Inhibition of purified active caspase-9 by XIAP. Active caspase-9 was produced in bacteria and purified as a His_6-tagged protein. Caspase-9 activity was measured by monitoring the processing (A) and activity (B) of the purified recombinant zymogen form of caspase-3 that was produced in bacteria. 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).

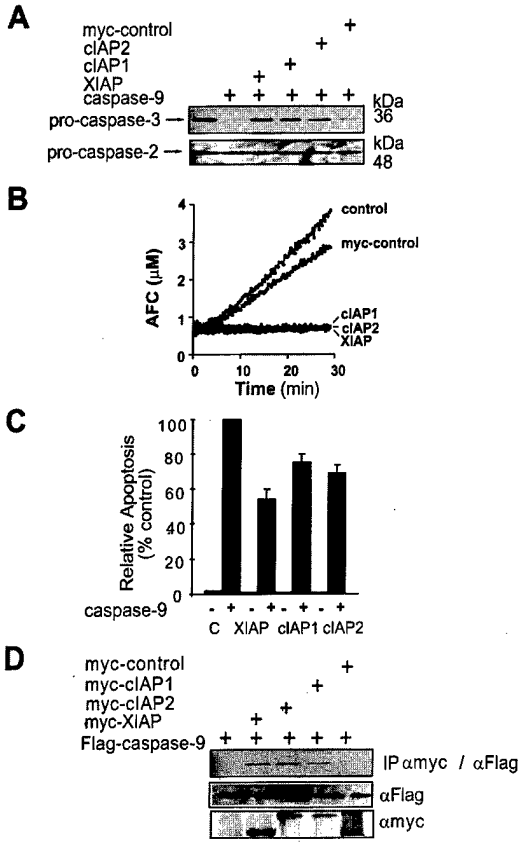

Fig. 8. XIAP, cIAP1 and cIAP2 bind pro-caspase-9 *in vivo* and inhibit caspase-9-induced processing of caspase-3. 293T cells were transfected with either FLAG-tagged pro-caspase-9 or pcDNA-myc-tag control plasmid DNA alone or in combination with myc-tagged XIAP, cIAP1, cIAP2 or a myc-tagged control protein. 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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