

The anti-apoptotic activity of XIAP is retained upon mutation of both the caspase 3- and caspase 9-interacting sites

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The X-linked mammalian inhibitor of apoptosis protein (XIAP) has been shown to bind several partners. These partners include caspase 3, caspase 9, DIABLO/Smac, HtrA2/Omi, TAB1, the bone morphogenetic protein receptor, and a presumptive E2 ubiquitin-conjugating enzyme. In addition, we show here that XIAP can bind to itself. To determine which of these interactions are required for it to inhibit apoptosis, we generated point mutant XIAP proteins and correlated their ability to bind other proteins with their ability to inhibit apoptosis. Δ RING point mutants

of XIAP were as competent as their full-length counterparts in inhibiting apoptosis, although impaired in their ability to oligomerize with full-length XIAP. Triple point mutants, unable to bind caspase 9, caspase 3, and DIABLO/HtrA2/Omi, were completely ineffectual in inhibiting apoptosis. However, point mutants that had lost the ability to inhibit caspase 9 and caspase 3 but retained the ability to inhibit DIABLO were still able to inhibit apoptosis, demonstrating that IAP antagonism is required for apoptosis to proceed following UV irradiation.

Introduction

All IAPs* bear baculoviral IAP repeats (BIRs), zinc-binding folds of ~70 amino acids (Deveraux and Reed, 1999; Silke and Vaux, 2001). X-linked mammalian inhibitor of apoptosis protein (XIAP)/hILP/MIHA, *c-iap1*/MIHB, and *c-iap2*/MIHC each bear three BIRs followed by a C-terminal RING finger. The BIRs and the regions that flank them are required for interaction of IAPs with tumor necrosis factor (TNF) receptor-associated factors (TRAFs) 1 and 2, TAB1, processed caspase 3, processed caspase 9, and processed DIABLO/smac (Rothe et al., 1995; Deveraux et al., 1997;

Yamaguchi et al., 1999; Chai et al., 2000; Ekert et al., 2001).

On the other hand, the RING finger domain of XIAP has been shown to bind the bone morphogenetic protein receptor 1A (Yamaguchi et al., 1999). The RING finger of XIAP and *c-iap1* has also been shown to function as an E3 ligase, promoting IAP ubiquitination (Yang et al., 2000). Therefore, the RING finger presumably binds to an as yet unidentified E2 ubiquitin-conjugating enzyme. The RING finger has been described to play an essential role in preventing apoptosis because XIAP Δ RING mutants were less effective at blocking Fas-induced cell death (Suzuki et al., 2001).

Whereas mammalian IAPs have been shown to be able to potently inhibit caspases, it remained possible that other protein interactions were important for its ability to prevent apoptosis, with the anti-caspase activity being redundant. For example, the very similar OpIAP is incapable of inhibiting viral-induced apoptosis when expressed without its RING finger. Moreover, the Δ RING protein appears to dominantly interfere with full-length OpIAPs anti-apoptotic

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*Abbreviations used in this paper: IAP, inhibitor of apoptosis protein; BIR, baculoviral IAP repeat; TNF, tumor necrosis factor; TRAF, TNF receptor associated factors; XIAP, X-linked mammalian inhibitor of apoptosis protein.

Key words: XIAP; caspase 9; caspase 3; DIABLO/smac; UV irradiation

function even though it contains the BIRs that might mediate caspase inhibition (Hozak et al., 2000). Other studies have linked XIAP's ability to inhibit cell death to jnk1 activation (Sanna et al., 1998).

Many previous studies using IAPs have used mutants that grossly disrupt structure of the BIR domains or the RING finger, or alternatively utilized domains to assess the functions of IAPs (Takahashi et al., 1998; Deveraux et al., 1999). Data obtained from mutants that interfere with gross structure have to be interpreted with care, and analyses with domains have obvious drawbacks. Apart from the fact that normally domains function within the whole protein and maybe regulated allosterically, it is difficult to compare results between domains because of varying expression levels. Also, domains cannot be assumed to fold correctly, and experiments with domains sometimes give counter-intuitive results. For example, the BIR3 RING domain of XIAP antagonises Fas-induced cell death, and when fused to GST the BIR3 RING domain binds the zymogen pro-caspase 9 rather than the active processed caspase 9 (Deveraux et al., 1999).

Although there is now a wealth of structural information on the BIR domains (Sun et al., 1999, 2000; Liu et al., 2000; Wu et al., 2000; Chai et al., 2001; Huang et al., 2001; Riedl et al., 2001), IAP point mutants have not been rigorously tested in their ability to inhibit apoptosis (Silke et al., 2001; Suzuki et al., 2001). Using structurally sound mutants, it is possible to test in a very precise manner whether the ability of XIAP to inhibit apoptosis resides in its ability to inhibit processed caspases or whether it also has another anti-apoptotic activity distinct from its ability to inhibit caspases.

Therefore, we constructed a panel of single point mutants of XIAP that no longer bind to caspase 3 or caspase 9 or DIABLO, but maintain the structural integrity of XIAP and its ability to oligomerize. To assess the importance of the RING finger we also expressed some of the key point mutants without the RING finger. The C-terminal end chosen for the ∂ RING constructs gives rise to a correctly folded BIR3 domain (Sun et al., 2000); therefore, these particular deletion mutants should be correctly folded.

Using two different death stimuli, we show that XIAP's ability to inhibit cell death is unimpaired if either the caspase 3 or caspase 9 binding activity is lost. Simultaneous disruption of XIAP's ability to bind caspase 3 and caspase 9 and DIABLO completely abrogated its ability to inhibit apoptosis, therefore XIAP requires only these three activities to inhibit UV/etoposide-induced cell death. ∂ RING mutants which were severely impaired in their ability to oligomerize with full-length XIAP were as competent as full-length XIAP mutants in inhibiting cell death, indicating that neither the RING, nor oligomerization, are essential to XIAP's function. Interestingly, mutants that had lost the ability to inhibit caspase 9 and caspase 3 yet maintained their ability to bind DIABLO/smac were able to inhibit cell death. These results indicate that endogenous XIAP is sufficient to prevent apoptosis provided it is not antagonized by an IAP antagonist such as DIABLO/smac, revealing a requirement for IAP antagonism in this model death system.

Results

In order to analyze the requirement for different protein interactions for XIAP to inhibit cell death, we generated a large number of single point mutants of XIAP. These point mutant proteins have been examined by nuclear magnetic resonance and do not affect the folding of the BIR domains (Sun et al., 1999, 2000), and therefore should leave the structure of the full length molecule intact (Fig. 1, top). As the point mutations were designed to disrupt particular interactions and were themselves based on domain analyses, they have rarely been tested against other partners but are assumed to specifically affect only the target partner (Fig. 1, top). Previously published *in vitro* data for binding and inhibition of most of the mutants used in this study are tabulated with details described in the figure legend.

XIAP interaction with processed caspase 3

We and others have previously described analysis of single point mutations within the linker region between the BIR1 and BIR2 of XIAP including T143A, V146A, and D148A (Sun et al., 1999; Silke et al., 2001) (Fig. 1, top). Full-length XIAP bearing the D148A mutation to the linker N-terminal of BIR2 cannot bind processed caspase 3 in coimmunoprecipitation analysis *in vivo* and its ability to inhibit caspase 3 *in vitro* is reduced >500-fold (Silke et al., 2001).

Therefore, to confirm that all D148A mutants described here had lost the ability to inhibit caspase 3, we performed immunoprecipitation analysis with FLAG-tagged XIAP in 293T cells transfected with an autoactivating caspase 3-LacZ construct that is toxic to 293 cells. The autoactivating caspase 3 LacZ is a convenient approach to obtain active processed caspase 3, the partner of XIAP. All XIAPs used in this study that carried the D148A mutation accumulated less caspase 3 because of the poor survival of cells containing unbound and uninhibited caspase 3. All the other mutants without D148A were able to bind active caspase 3 and accumulated more active caspase 3. These observations are consistent with the idea that if active caspase 3 is not inhibited, small amounts are sufficient to kill the cell, leaving little time for processed caspase 3 to accumulate. Note the similar lack of processed caspase 3 in the cells transfected with TAB1.

Interestingly, although structural studies (Riedl et al., 2001) have shown that the processed N-terminus of the p10 subunit of caspase 3 can make contact with BIR2 the D214S mutation to this region did not affect the ability of caspase 3 to bind XIAP in cells.

XIAP interaction with processed caspase 9

E314 and W310 are residues that contribute to the formation of a groove in the BIR3 of XIAP that is involved in binding to the N terminus of DIABLO and processed caspase 9, and neither E314S nor W310A mutants bind or inhibit processed caspase 9 in the context of the BIR3 alone (Fig. 1, top) (Sun et al., 2000). H343A mutants also do not inhibit caspase 9 in the context of the BIR3 but as H343 is not present in the groove, this residue must contact another important surface of active caspase 9, perhaps acting in a manner analogous to the D148 residue in caspase 3 inhibition. To determine whether E314S, W310A, or H343A affects

the ability of full-length XIAP to inhibit caspase 9, we transiently transfected 293T cells with constructs expressing XIAP mutants together with full-length caspase 9. Ectopic expression of caspase 9 in 293T cells results in autoprocessing of caspase 9, presumably because the large over expression drives dimerization and activation, and lysates contain both unprocessed and processed caspase 9. This active caspase 9 is toxic to 293T cells in a similar manner to the auto-activating caspase 3.

After transfection, XIAP mutants were immunoprecipitated and the presence of coimmunoprecipitated caspase 9 was determined by Western blot analysis using anti-caspase 9 antibodies. As observed previously (Ekert et al., 2001), wild-type XIAP bound only to processed caspase 9, and,

consistent with the results observed for the BIR3 domain alone (Sun et al., 2000), the E314S or W310A single point mutants had greatly reduced ability to bind processed caspase 9 (Fig. 2). Whenever E314S, W310A, or H343A were combined with another mutant affecting either DIABLO binding or caspase 3 inhibition, there was a significant reduction in the amount of processed caspase 9 accumulating because of the inability of the mutant XIAPs to prevent caspase 9 induced toxicity which, as expected, requires caspase 3. Importantly, the mutant combinations that had unmutated W310, E314, or H343 were able to accumulate and bind processed caspase 9.

The analogous residue to G306S (G269S) has been described in the *Drosophila* DIAP1 to create a gain of function

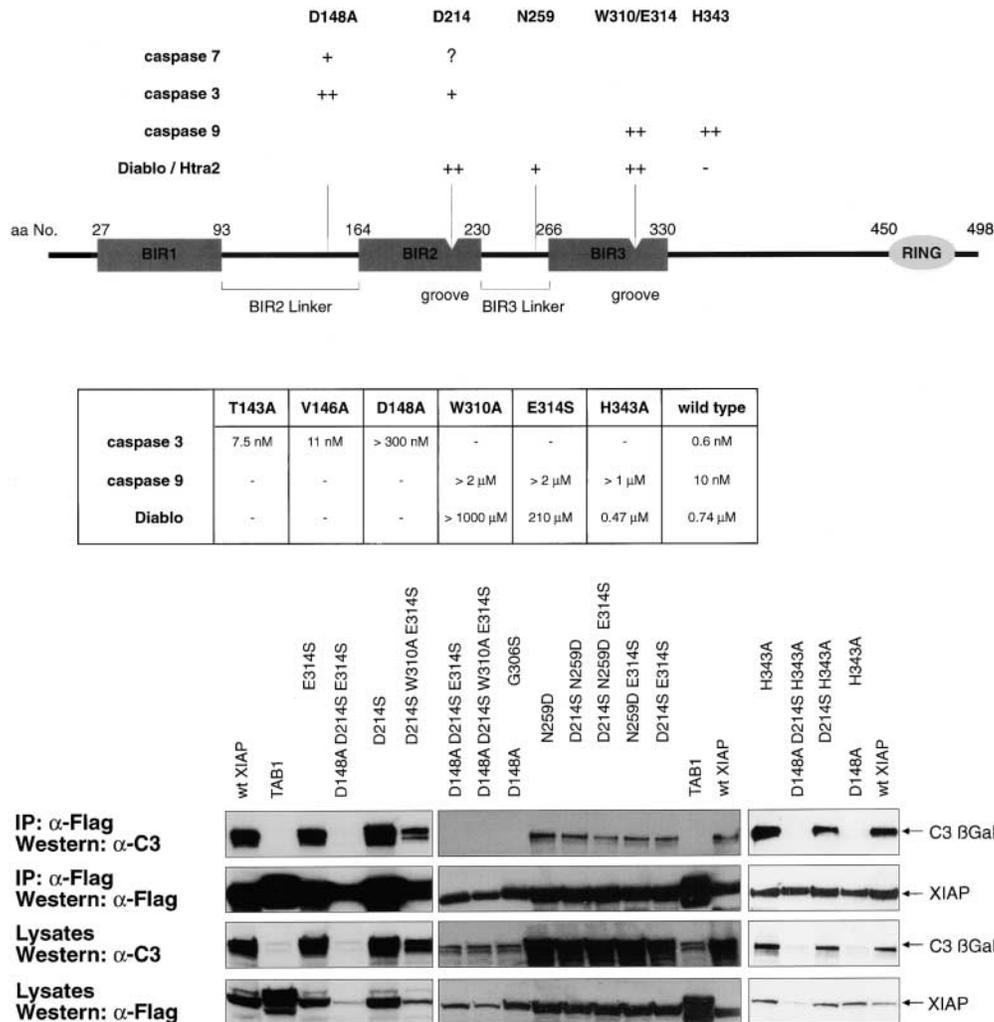


Figure 1. **The D148A mutation abolishes the ability of full length XIAP to interact with caspase 3 in cells.** (top) ++, Schematic of XIAP showing the location of key residues and their role in binding to partner proteins; +, Strong contribution of residue to binding; -, Weak contribution of residue to binding; -, No contribution; ?, No interaction in crystal structure. An empty space indicates that this residue is not presumed to interact but has never been tested. The roles of particular residues in binding have only been tested in the context of domains of XIAP, the importance of these residues in the context of the full-length molecule, with the exception of D148A has not been looked at previously. The mutants described in this paper have been tested in vitro and K_i s and K_D s have been established and the relevant data is tabulated below the schematic. Caspase 3 inhibition is K_i data for mutant in the context of full-length XIAP (Silke et al., 2001), caspase 9 inhibition is IC_{50} data for mutant in the context of the BIR3 (Sun et al., 2000), DIABLO is K_D data for a peptide of the NH_2 -terminal of DIABLO binding to mutant in the context of the BIR3 domain of XIAP (Liu et al., 2000). (bottom) 293T cells were transiently transfected with plasmids expressing FLAG XIAP, FLAG XIAP mutants, or FLAG TAB1 and caspase 3 LacZ. Cell lysates were immunoprecipitated with anti-FLAG beads, separated on SDS/PAGE gels, transferred to PVDF membranes, and immunoblotted with anti-caspase 3 and anti-FLAG.

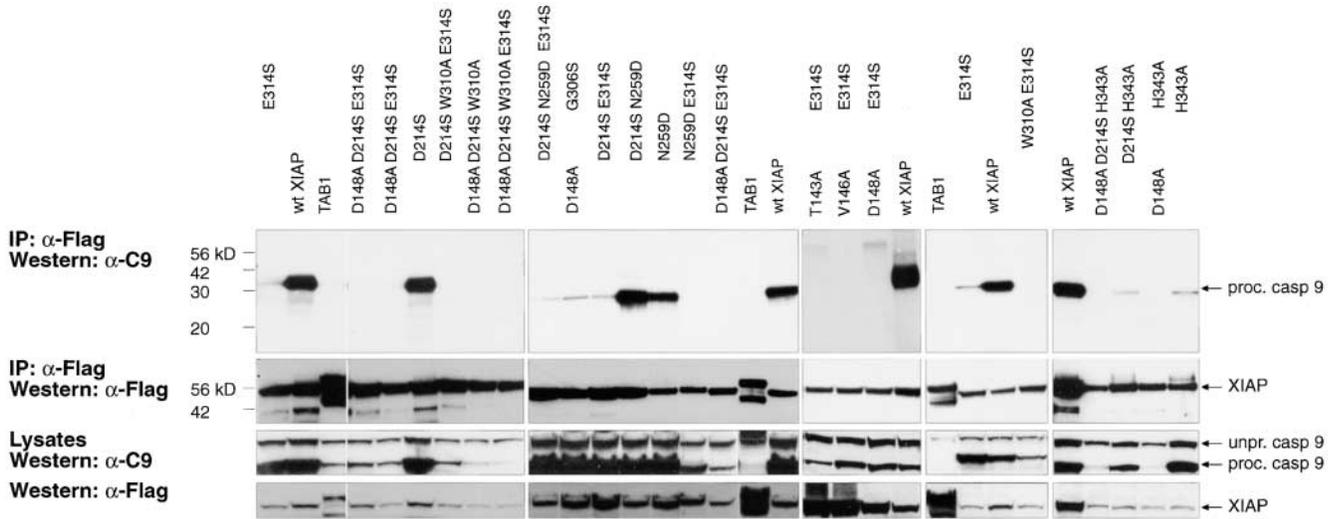


Figure 2. The E314S, W310A, and G306S mutations significantly impair the ability of full length XIAP to interact with and inhibit processed caspase 9. 293T cells were transiently transfected with plasmids expressing FLAG-tagged XIAP, FLAG XIAP mutants, or FLAG TAB1 and caspase 9. Cell lysates were immunoprecipitated with anti-FLAG beads, separated on SDS/PAGE gels, transferred to PVDF membranes, and immunoblotted with anti-caspase 9 and anti-FLAG.

mutant (Goyal et al., 2000) that is a strong suppressor of Grim and Reaper-induced cell death. Mammalian XIAP D148A G306S binds processed caspase 9 as weakly as an E314S mutant does in this immunoprecipitation assay. A BIR3 domain containing an H343A mutation in the context of the BIR3 alone fails to inhibit caspase 9, with an IC₅₀ of >1 μM (Fig. 1, top) (Sun et al., 2000). In the context of the full-length protein, H343A XIAP still appears to be able to bind caspase 9, if due account is taken for the levels of XIAP protein in the immunoprecipitation (Fig. 2). However, on three independent occasions we have observed that none of the H343A mutants accumulate to the same levels as wild-type XIAP when coexpressed with caspase 9. Coex-

pression of caspase 3 or DIABLO does not affect levels of H343A mutants, indicating that the reduced levels are most likely due to the inability of these XIAP mutants to inhibit caspase 9 (Figs. 1 B and 3).

XIAP interaction with DIABLO/smac

Three regions of DIABLO have been implicated in interactions with XIAP. DIABLO has been shown to bind XIAP with a preference for the BIR3 (Chai et al., 2000; Srinivasula et al., 2000). The processed N-terminal finger of DIABLO contacts the same residues of the BIR3 that the processed end of the p10 of caspase 9 does, namely E314 and W310 (Liu et al., 2000; Wu et al., 2000). The middle portion of

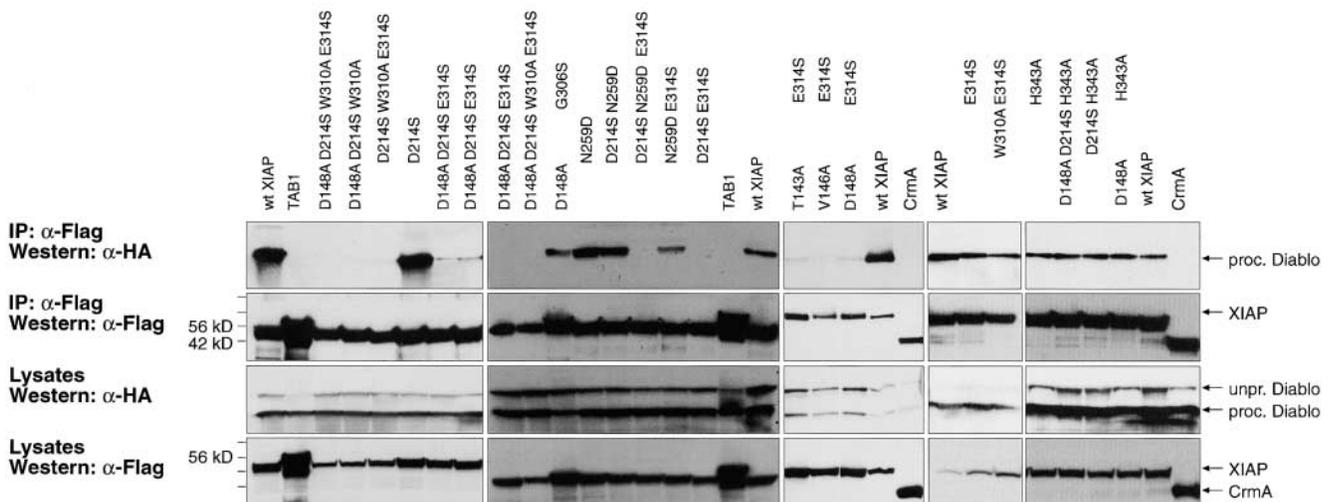


Figure 3. The E314S, W310A mutations impair, and the D214S E314S combination abolishes the ability of full-length XIAP to interact with processed DIABLO. 293T cells were transiently transfected with plasmids expressing FLAG XIAP, FLAG XIAP mutants, FLAG TAB1, or FLAG CrmA and HA-tagged DIABLO. Cell lysates were immunoprecipitated with anti-FLAG beads, separated on SDS/PAGE gels, transferred to PVDF membranes, and then immunoblotted with anti-HA and anti-FLAG.

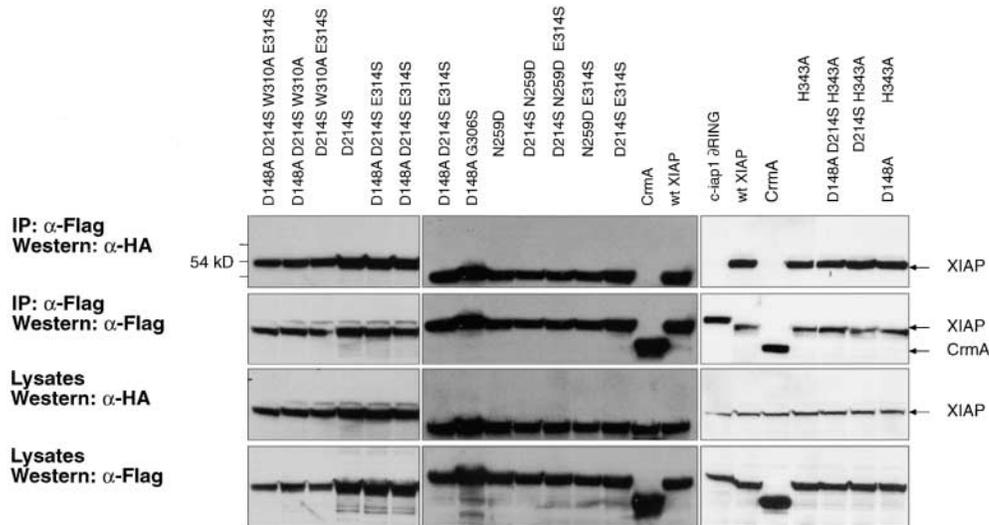


Figure 4. **XIAP is able to oligomerize in cells, and neither a D148A, D214S, N259D, W310, E314S, or H343A mutation, nor any combination thereof affects the ability of full-length XIAP to interact with itself.** 293T cells were transiently transfected with plasmids expressing FLAG XIAP, FLAG XIAP mutants, FLAG *c-iap1* Δ RING, or FLAG CrmA, and HA-tagged XIAP wild-type. Cell lysates were immunoprecipitated with anti-FLAG beads, separated on SDS/PAGE gels, transferred to PVDF membranes, and immunoblotted with anti-HA and anti-FLAG.

helices H2 and H3 in DIABLO also make contact with residues N259, S261, and R258 in the linker region immediately N-terminal to the BIR3 of XIAP (Wu et al., 2000). Finally, it has been proposed that the finger of DIABLO may also contact D214 in the groove of BIR2, in an analogous fashion to its interactions with E314 in the groove of BIR3. To determine the importance of each of these contact points in full-length XIAP we performed cotransfection analysis with the XIAP mutants and DIABLO followed by coimmunoprecipitation and Western blotting (Fig. 3). Mutation of either N259 or D214 alone did not significantly impair the ability of XIAP to bind processed DIABLO, confirming that the groove on BIR3 is the most important site. The N259D E314S double mutant was impaired in its interaction with DIABLO, but this would appear to be attributable to the presence of the E314S mutation as similar impairment was observed for the E314S, W310A E314S mutants and for BIR2 linker E314S double mutants, V146A E314S and D148A E314S (Fig. 3). Mutation of the BIR2 linker region alone has been previously shown to have no effect on DIABLO interaction (Silke et al., 2001). Although mutation of E314 in XIAP significantly impairs interaction with DIABLO, it does not eliminate interaction, and the amount of DIABLO immunoprecipitated by the E314S mutant varied between 5–50% depending upon the amount of processed DIABLO in the lysate. The residual interaction is presumably because DIABLO is also able to interact with the BIR2 domain. Simultaneous mutation of the predicted BIR2 binding site (D214S) and BIR3 binding site, E314S, completely eliminates XIAP interaction with DIABLO (Fig. 3). These results indicate that the most important interaction of DIABLO with XIAP is via the groove in BIR3, but there is also a significant interaction with the analogous groove of BIR2 and that the combination of D214S E314S eliminates interaction of XIAP with DIABLO. Similar data have been obtained for HtrA2/Omi (Verhagen et al., 2001).

XIAP oligomerization

As OpiAP has been described to form oligomers (Hozak et al., 2000) we wanted to determine whether XIAP exists as dimers or higher order oligomers within cells. In a transient transfection and immunoprecipitation analysis, wild-type XIAP is able to bind to itself (Fig. 4). We also determined whether any of the mutations affected the ability of the mutant XIAPs to bind to wild-type XIAP. Consistent with the fact that none of the point mutations should affect the structure of XIAP, all of the mutants tested behaved indistinguishably from wild-type XIAP in their ability to bind wild-type XIAP (Fig. 4). This interaction is likely to be specific and not an overexpression artifact, as Δ RING *c-iap1* did not interact with XIAP even though expressed to higher levels than the full-length XIAP. Although it is possible that the observed XIAP self-interaction is an indirect result of aggregation in the apoptosome, the inability of the mutant D148A D214S W310A E314S to bind caspase 3 (Fig. 1 B) and caspase 9 (Fig. 2) suggests that the interaction between different XIAP molecules is direct.

Ability of XIAP mutants to prevent apoptosis

The purpose of testing mutants in immunoprecipitation analyses was to confirm within mammalian cells that the constructs performed consistently. Nearly all of the mutations have been described and rigorously tested *in vitro* (Fig. 1 A), and thus in most cases the assays were not designed to test interaction but control that the construct had the mutation in question. This was also the purpose behind including several mutants that should behave analogously, thus the triple mutant D148A D214S E314S should perform the same as either D148A D214S W310A or D148A D214S W310A E314S as all target exactly the same interactions. Therefore, the three mutants can be considered to belong to a single class, and in Table I the mutants are grouped into classes so that easy comparison of the results from the mutants is pos-

Table 1. Summary of the properties of XIAP mutants

Class of mutant	Average UV death	Average Etoposide death	DIABLO binding	Casp3 Inhibition	Casp9 Inhibition	XIAP binding
	%	%				
Vector	79	50	—	—	—	—
NT2 cells	69	52	—	—	—	—
Wild-type						
XIAP	11	5	10	10	10	10
XIAP N259D	10	3	10	10	10	10
Caspase 9 only						
XIAP H343A	11	—	10	10	0	10
Part DIABLO						
XIAP D214S	9	3	9	10	10	10
XIAP D214S N259D	11	3	9	10	10	10
Caspase 9 and part DIABLO						
XIAP E314S	13	6	5	10	0	10
XIAP N259D E314S	14	5	5	10	0	10
XIAP W310A E314S	16	6	4	10	0	—
XIAP D214S H343A	11	—	8	10	0	10
Caspase 9 and DIABLO						
XIAP D214S E314S	23	10	0	10	0	10
XIAP D214S N259D E314S	24	9	0	10	0	10
XIAP D214S W310A E314S	33	18	0	10	0	10
Caspase 3, caspase 9, and part DIABLO						
XIAP T143A E314S	29	24	5	5	0	—
XIAP V146A E314S	32	26	5	2	0	—
XIAP D148A E314S	50	46	5	0	0	—
XIAP D148A W310A E314S	49	56	4	0	0	—
XIAP D148A D214S H343A	15	—	9	0	0	10
XIAP D148A G306S	13	5	8	0	0	10
Caspase 3, caspase 9, and DIABLO						
XIAP D148A D214S E314S	67	52	0	0	0	10
XIAP D148A D214S W310A	65	50	0	0	0	10
XIAP D148A D214S W310A E314S	70	49	0	0	0	10
Caspase 3 and caspase 9						
XIAP	9	—	10	0	0	10

Mutants are grouped into classes to allow easy comparison of data between particular sets of mutants. N259D has been allocated as wild-type because it had no apparent effect on DIABLO binding. Arbitrary values for XIAP binding or inhibition have been allocated based on consideration of previously unpublished *in vitro* inhibition data and binding data presented in this paper, with 10 representing 100%, and 0 representing 0%, relative to wild-type XIAP. The mean apoptosis is the data presented in bar graph format in Fig. 5, A and B.

sible. In all cases, the mutants performed consistently and we were thus in a position to assay these mutants in a physiological apoptosis setting.

To test whether loss of caspase 3, caspase 9, or DIABLO binding affected the ability of XIAP to inhibit apoptosis in mammalian cells, we expressed the mutants in NT2 teratocarcinoma cells and exposed them to UV radiation or etoposide (Fig. 5). After 8 or 24 h (in the case of etoposide), all cells were collected and stained with Annexin V and analysed by flow cytometry, with transfected (GFP positive) cells classed as apoptotic or viable based on their annexin binding. A representative sample of the data obtained is shown in Fig. 5 A. These experiments were repeated on three independent occasions and the mean and standard deviation calculated for each mutant (Fig. 5, B and C).

We have previously shown that loss of caspase 3 inhibition does not affect XIAPs ability to prevent UV-induced cell death (Silke et al., 2001) and in this study the double mutant D148A D214S is also unimpaired. Likewise, loss of the ability to inhibit caspase 9 alone did not affect the ability of

the mutants to inhibit apoptosis, because neither E314S XIAP, H343A XIAP, nor the W310A E314S double mutant were impaired in their ability to protect the cells. Loss of ability to bind DIABLO/caspase 9 did affect XIAP function in inhibiting cell death because the D214S E314S double and the D214S W310A E314S, D214S N259D E314S triple mutants while still able to inhibit cell death, were not as competent as the wild-type protein.

Any XIAP mutant that contained the combination of the D148A and E314S (or W310A) mutations, and therefore had lost the ability to inhibit both caspase 3 and caspase 9, was impaired in its ability to inhibit cell death. Mutations within the BIR2 linker region that are not as compromised in their ability to inhibit caspase 3 as D148A, i.e., V146A and T143A (Silke et al., 2001), when combined with E314S, also reduced the ability of XIAP to inhibit cell death (Fig. 5, A and B). These double mutants provide a type of dose-response and confirm in an independent fashion that simultaneous loss of the ability to inhibit caspase 3 and caspase 9 markedly reduces the potential of XIAP to inhibit cell

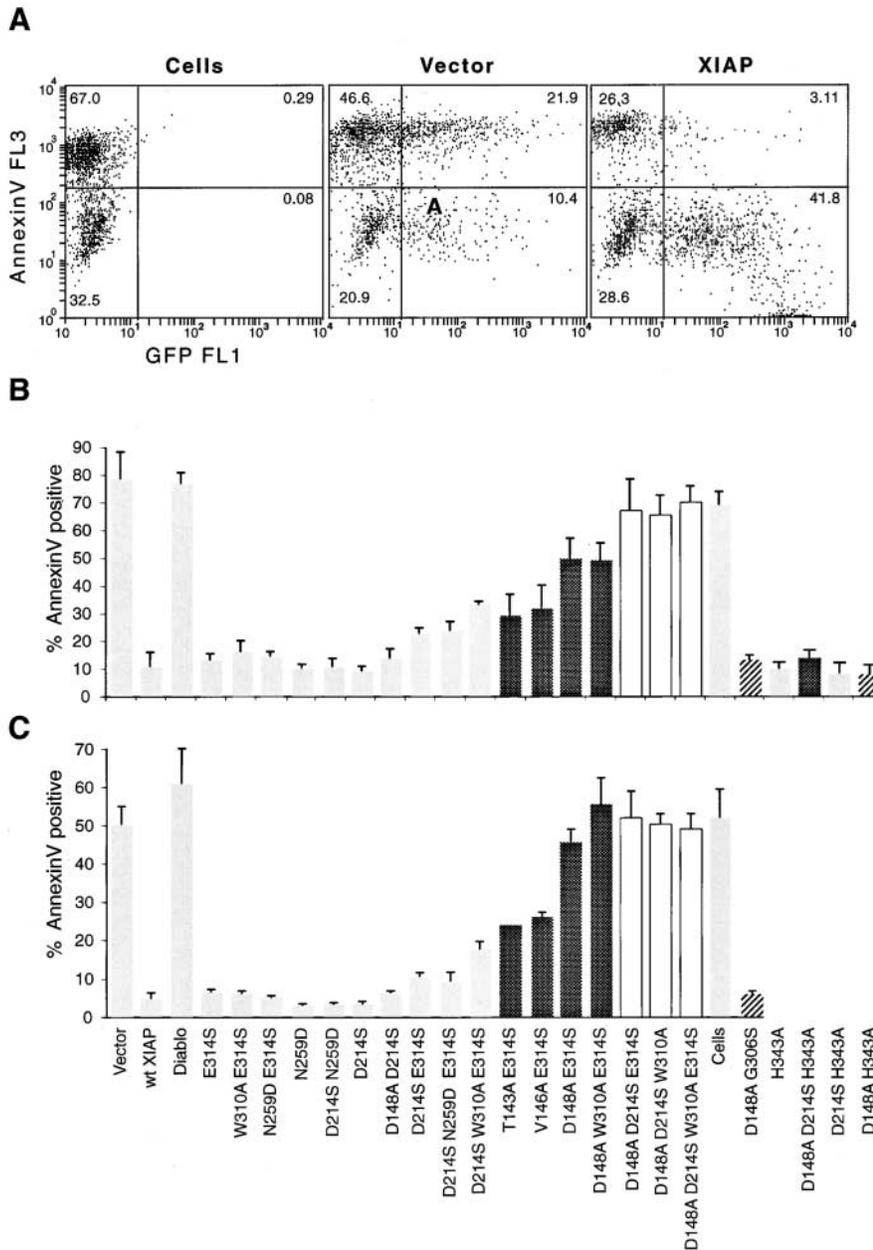


Figure 5. XIAP that is competent to bind DIABLO is able to inhibit cell death. (A) FACS analysis of transiently transfected NT2 cells stained with Annexin V 8 h after UV irradiation. (B) The ability of XIAP mutants to protect against UV induced death. NT2 cells were transiently transfected with FLAG XIAP (wt XIAP), FLAG XIAP mutants, empty vector (vector), DIABLO/smac (DIABLO), or were untransfected (cells). Cells were subsequently irradiated with UV, and stained with Annexin V 7–8 h later. The fraction of cells that were positive for GFP and Annexin V over total number of GFP positive cells were expressed as the percent of Annexin V positive cells, except in the case of untransfected cells where the percent of Annexin V positive cells over the total number of cells were expressed as the percent of Annexin V positive cells. Error bars are standard deviation for three independent experiments. Different classes of mutants are indicated by different shading to enable easy comparison. (unfilled bars) Mutants that do not inhibit caspase 3, caspase 9 or DIABLO. (dark shaded bars) Mutants that affect caspase 3, caspase 9, and also affect DIABLO binding. (hatched bars) Mutants that affect caspase 3 and caspase 9 only. (C) The ability of XIAP mutants to protect against etoposide induced death. As above for UV-induced death except NT2 cells were rather incubated with etoposide for 20–24 h and then stained with Annexin V.

death. However, it is significant that the E314S/W310A mutant also interferes with XIAPs ability to bind DIABLO/smac, although it does not abrogate it entirely. Therefore, these mutants are grouped in the loss of caspase 3, caspase 9 and part loss of DIABLO binding class in Table I.

Triple mutants that had completely lost their ability to bind not only caspase 3 and caspase 9 but also DIABLO/smac were completely ineffective in blocking either UV or etoposide induced apoptosis, confirming that the essential points of XIAP inhibition of cell death are controlled through these three interactions.

Two types of mutants that have lost the ability to inhibit caspase 9 but retain the ability to bind DIABLO (the D148 G306S double mutant and the H343A double and triple mutants) were notable because they were still able to inhibit apoptosis as effectively as wild type XIAP.

RING finger function

To assess the function of the RING finger in XIAPs ability to inhibit UV-induced apoptosis we created XIAP point mutants without the RING finger. The ∂ RING point mutants were assayed at the same time as the full-length XIAP mutants, and comparison of Figs. 5 B and 6 A shows that the full-length mutants gave the same levels of protection as before. Altogether, the ∂ RING mutants performed similarly to the full-length protein with single mutants protecting as well as wild-type XIAP, and the triple D148A D214S E314S mutant having lost the ability to inhibit UV-induced apoptosis (Fig. 6 A). Interestingly, the D148A E314S ∂ RING protected better than the full-length protein. This was most likely due to the increased stability/expression of the ∂ RING mutants in NT2 cells (Fig. 6 B).

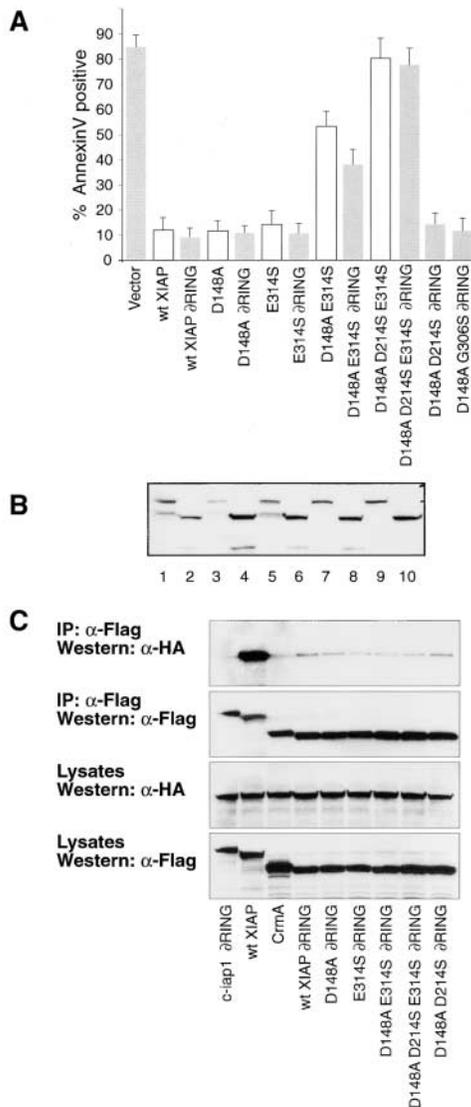


Figure 6. Δ RING XIAP is as competent as full-length XIAP to inhibit cell death, but is impaired in its ability to oligomerize. (A) FACS analysis of transiently transfected NT2 cells stained with Annexin V 8 h after UV irradiation. The full-length protein is indicated with unfilled bars and the Δ RING mutants with gray bars. (B) Western blot of transiently transfected NT2 cell extracts separated on SDS/PAGE gels and probed with anti-FLAG, samples are loaded as in the UV assay above, the lower molecular weight band detected by the flag antibody is present in both full-length XIAP and Δ RING mutants and might represent a cleavage product of XIAP although the cells were not treated with any apoptotic stimulus. (C) 293T cells were transiently transfected with plasmids expressing FLAG XIAP Δ RING, FLAG XIAP Δ RING mutants, FLAG c-iap1 Δ RING, or FLAG CrmA and HA-tagged XIAP wild-type. Cell lysates were immunoprecipitated with anti-FLAG beads, separated on SDS/PAGE gels, transferred to PVDF membranes, and immunoblotted with anti-HA.

To study oligomerization with wild-type XIAP we performed coimmunoprecipitation analyses as before. Significantly, the Δ RING mutants were impaired in their ability to dimerize, even though their expression levels were if anything slightly above those of the full-length protein. Thus, it appears that the RING finger promotes XIAP oligomerization but is not required for XIAP to inhibit cell death.

Discussion

An array of single point mutations that should not affect the structure of XIAP (Sun et al., 1999, 2000) and do not affect its ability to oligomerize (Fig. 4), were used to analyze many of the distinct functions of XIAP in isolation. These results show that an XIAP mutant that cannot inhibit caspase 3, caspase 9, and DIABLO is no longer able to inhibit UV or etoposide induced cell death.

Mutation of D148 in the N-terminal flank of BIR2 prevents XIAP interacting with processed caspase 3 (Sun et al., 1999), but leaves intact binding to processed caspase 9, DIABLO, (Silke et al., 2001), XIAP (Fig. 4), and TAB1 (unpublished data). The structure of the BIR2 of XIAP bound to caspase 3 (Riedl et al., 2001) indicates that, aside from D148, residues in the D214 groove in the core of BIR2 make contact with the processed N-terminus of the p10 of caspase 3. However, in this case the processed p10 of caspase 3 would contact a BIR2 in a different XIAP molecule to the BIR2 that binds via its N-terminal flank to the active site of caspase 3. In other words, the structure implies that XIAP exists as a multimer when it is bound to processed caspase 3. At least in the context of the full-length molecule, mutation of D214 does not drastically affect the ability of XIAP to inhibit caspase 3 because a D214S E314S XIAP double point mutant (in which the grooves in both BIR2 and BIR3 are mutated) is able to inhibit cell death much better than a D148A E314S double mutant (Fig. 6, A and B). This result is in accord with an analysis of the BIR2 alone, in which mutation of D214 did not affect the ability of the BIR2 to inhibit caspase 3 (Sun et al., 1999).

The crystal structure of DIABLO bound to the BIR3 of XIAP has been solved and shows that in addition to E314 and W310 other residues also make contact with DIABLO (Wu et al., 2000; Srinivasula et al., 2001). In particular, the middle portion of helices H2 and H3 in DIABLO makes contacts via amino acids in its helix domain to residues N259, S261, and R258 of the BIR3 of XIAP. Although this interface consists of seven hydrogen bonds and two patches of van der Waals interactions, with $>2,000 \text{ \AA}^2$ of buried surface area, mutation of N259 alone makes little difference to the ability of XIAP to bind DIABLO. Furthermore, the N259D D214S and N259D E314S double mutants still bind DIABLO, indicating that the contribution of N259 in the full-length molecule is not as significant as either D214 or E314. In contrast, the D214S E314S double mutant has completely lost the ability to bind DIABLO, indicating that the grooves in BIR2 and BIR3 make the most critical contacts to DIABLO. We have also recently shown that the D214S E314S double mutant is incapable of binding another IAP antagonist HtrA2/Omi (Verhagen et al., 2001). Although simultaneous mutation of D214 and E314 abolishes DIABLO, HtrA2/Omi binding (Verhagen et al., 2001) and simultaneously caspase 9 binding, it nevertheless only slightly affects the ability of the overexpressed XIAP to inhibit cell death (Fig. 5, B and C). Thus, overexpressed XIAP can partially inhibit cell death by blocking caspase 3 alone, which is consistent with the idea that there is a feedback loop where caspase 3 can process caspase 9.

Both DIABLO and caspase 9 contact E314 in the groove of BIR3 (Wu et al., 2000). However, E314 is clearly more criti-

cal for caspase 9 binding because the E314S mutation alone completely abolishes the ability of XIAP to immunoprecipitate processed caspase 9, whereas it leaves some DIABLO binding intact (compare Figs. 2 and 3). G306 is also present in the BIR3 groove and makes contacts to the fourth residue (Ile57) of the processed N-terminal of DIABLO (Liu et al., 2000; Wu et al., 2000). Mutation of Ile57 impairs DIABLO N-terminal peptide binding to the BIR3 of XIAP, but is >100-fold less disruptive than mutation of the first residue of processed DIABLO (Ala54). E314 and W310 of XIAP contact Ala54 of DIABLO, whereas G306 contacts Ile57, thus it is likely that the G306S XIAP binds DIABLO better than the E314S or W310A mutants (Liu et al., 2000). Furthermore, the G306S is likely to be more disruptive of the bulky Phe in the fourth position of processed caspase 9 (Srinivasula et al., 2001) than the Ile of DIABLO or the Ala of HtrA2. H343A does not affect binding of the N-terminal peptide of DIABLO to the BIR3 of XIAP (Liu et al., 2000).

E314S and W310A mutations both simultaneously interfere with DIABLO and caspase 9 binding. In the UV death assay, both the D148A E314S and D148A W310A E314S mutants still protect partially even though they have lost caspase 3 and caspase 9 binding and have reduced DIABLO binding. The protection the D148A E314S mutant affords can be further increased by deleting the RING finger of XIAP (Fig. 6 A) which increases the levels of XIAP in NT2 cells (Fig. 6 B). This indicates that even residual DIABLO binding is sufficient to partially block apoptosis. The mutants G306S and H343A further confirm that DIABLO binding is sufficient for exogenous XIAP to block cell death. In these cases, either the double mutants D148A G306S, D148A H343A or the triple mutant D148A D214S H343A, which retain significant DIABLO binding, still protect NT2 cells as well as wild-type XIAP against UV-induced cell death.

Deletion of the RING finger has little effect on the ability to inhibit UV-induced cell death. As with fly DIAP1 (Hay et al., 1995), removal of the RING finger makes XIAP a marginally more potent inhibitor of cell death, presumably due to its increased abundance. Contrary to OpIAP (Hozak et al., 2000), we find that the RING finger makes a strong contribution to XIAPs ability to oligomerize and Δ RING c-iap1 does not interact at all. These results mean that oligomerization of the full-length protein is likely to be physiological, and at least in the context of this assay, that the ability to oligomerize is not required for XIAP to inhibit cell death.

Because an IAP that has lost the ability to bind caspase 3, caspase 9, DIABLO and HtrA2/Omi is ineffective at blocking UV cell death, all of XIAPs anti-apoptotic activity must reside in these functions. By selectively removing the ability to inhibit caspase 3 and caspase 9, we have created several XIAP mutants that can only bind IAP antagonists, and these mutants still inhibit cell death as well as wild-type XIAP. The most likely mechanism for this inhibition is that exogenous expression of these mutants is sufficient to bind IAP antagonists that are released after a death stimulus such as DIABLO/smac and HtrA2/Omi, thereby allowing endogenous IAPs to block caspases without being antagonized. As there are several other IAP antagonists in addition to DIABLO and HtrA2/Omi (unpublished data), it might be dif-

ficult to prove the requirement for IAP antagonism by knock-out strategies alone. Therefore, these results demonstrate a requirement for IAP antagonism in cell death and indicate that the XIAP mutants described here will be useful tools in other similar analyses.

Materials and methods

Transfections and constructs

pEF expression constructs encoding C-terminal FLAG-tagged XIAP, N-terminally FLAG-tagged CrmA DQMD, caspase 3 LacZ, caspase 9, and pFLAG TAB1 have been previously described (Ekert et al., 1999; Verhagen et al., 2000; Silke et al., 2001). pEF TAB1 HA Tag was constructed by digesting the PCR product of primers 226 TAB1 5' cgggatccaccatggcggcagaggaggag 3' and 227 TAB1 5' cgcgctagccgggtcgtcaccacgctctgct 3' with *Bam*H I, *Nhe* I and cloning into pEF HA Tag digested with *Bam*H I, *Nhe* I. pEF constructs encoding FLAG-tagged XIAP mutants were generated using overlap PCR technology. The D148A construct has already been described (Silke et al., 2001). The other mutants were generated with combinations of the primers 759 *Bst*B I 5' tgctcttgggtttggggcggga 3', 760 E314S 5' aactgattggaagcccagttccgacccttgggaacaacatgct 3', 766 W310A 5' tggaggaggcctaactgatgtagcgaagcccagtggaagacc 3', 770 *Eco*R V 5' ctgtcttctgagcattcactagat 3', 771 D214S 5' tgggaacctgtctcagagcctgtcagaacacag 3', and 772 N259D 5' tcaacaactctctcagagatccatccatggcagatta 3', and inserted into pEF FLAG XIAP digested with *Bst*B I and *Eco*R V. FLAG-tagged XIAP domain constructs were generated using PfuI polymerase PCR, and the primers 743 BIR2 5' cgggatccaccatcgctgatcttttgccttagacag 3', 744 BIR2 5' cgcgctagctggattcctaggaagatttggtaattggg 3', 771 BIR1 5' cgggatccaccatgacttttaacagttttgaaggatca 3', 745 BIR1 5' cgcctcaggggcaaaaagatctctgcttccagatagt 3', 747 BIR3 5' cgcctcagggaatccatccatggcagattat 3', 750 XIAP 5' cgtctagacataaaatgtttgctttaa 3', 761 BIR3 5' cggaaatcggatccaccatgctgatgctgtgagttc 3', and 762 BIR3 5' gcgttagctagcggagatctgagtgttctaccagacact 3' and cloned into the vector pEF c-term FLAG using sites compatible with *Bam*H I and *Nhe* I. pEF XIAP Δ RING constructs were made using PCR primers 746 5' cgggatccaccatgacttttaacagttttgaaggatca 3' and 762 and cloned *Bam*H I *Nhe* I into pEF C-terminal FLAG digested with *Bam*H I *Xba* I. All constructs were verified by digest and sequencing.

Immunoprecipitations and Western blot analysis

Cells were lysed in a Triton X-100 based lysis buffer (1% Triton X-100, 10% glycerol, 150 mM NaCl, 20 mM Tris, pH 7.5, 2 mM EDTA, 1 mM PMSF, 10 μ g/ml aprotinin and 10 μ g/ml leupeptin) for 1 h at 4°C and the debris cleared by centrifugation. Immunoprecipitations were performed using FLAG specific mAb M2 covalently coupled agarose beads (Sigma-Aldrich). The immunoprecipitates were washed five times in lysis buffer and proteins eluted with 100 mM glycine, pH 3, and then neutralized with 1/10th vol of 1 M Tris, pH 8. Proteins were separated by SDS PAGE and immunoprecipitates were examined by Western blot analysis after transfer of proteins to Immobilon™-P membranes (Millipore). Antibodies used for Western blots were anti-FLAG M2 (Sigma-Aldrich), anti-HA High Affinity 3F10 (Boehringer Mannheim), anti-caspase 9, a gift of Y. Lazebnik, and anti-caspase 3 (Pharmingen). Proteins were visualized by ECL (Amersham Pharmacia Biotech) after incubation of membranes with HRP-coupled secondary antibodies.

Apoptosis assays

The cell death assay is described in detail in Silke et al. (2001). In brief, NT2 cells were transfected with equal amounts of the mutant constructs and 1:20 (wt/wt) of pEGFP for 24 h. The cells were washed and induced to undergo apoptosis by irradiating with 25 J/m² of UV and harvested 6–7 h later, or treating with etoposide (100 μ g/ml) and harvesting 20–24 h later. All cells, both adherent and nonadherent, were recovered and stained with recombinant Annexin V-Biotin and streptavidin Tricolor (CalTag Laboratories) with appropriate washing steps. The stained cells were analysed with a FACS scan for FL1 fluorescence (EGFP positive) and FL3 fluorescence (Annexin V positive) and the proportion of cells that were EGFP and Annexin V positive (transfected and apoptotic), to the total of EGFP positive (transfected) cells was determined.

Software

An EPSON scanner was used to scan all autoradiographs and the scanned images were imported directly into FreeHand9 and cropped with Freehand9.

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References

- Chai, J., C. Du, J.W. Wu, S. Kyin, X. Wang, and Y. Shi. 2000. Structural and biochemical basis of apoptotic activation by Smac/DIABLO. *Nature*. 406:855–862.
- Chai, J., E. Shiozaki, S.M. Srinivasula, Q. Wu, P. Dataa, E.S. Alnemri, and Y. Shi. 2001. Structural basis of caspase-7 inhibition by XIAP. *Cell*. 104:769–780.
- Deveraux, Q.L., E. Leo, H.R. Stennicke, K. Welsh, G.S. Salvesen, and J.C. Reed. 1999. Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. *EMBO J.* 18:5242–5251.
- Deveraux, Q.L., and J.C. Reed. 1999. IAP family proteins—suppressors of apoptosis. *Genes Dev.* 13:239–252.
- Deveraux, Q.L., R. Takahashi, G.S. Salvesen, and J.C. Reed. 1997. X-linked IAP is a direct inhibitor of cell-death proteases. *Nature*. 388:300–304.
- Ekert, P.G., J. Silke, and D.L. Vaux. 1999. Inhibition of apoptosis and clonogenic survival of cells expressing crmA variants: optimal caspase substrates are not necessarily optimal inhibitors. *EMBO J.* 18:330–338.
- Ekert, P.G., J. Silke, C.J. Hawkins, A.M. Verhagen, and D.L. Vaux. 2001. DIABLO promotes apoptosis by removing MIHA/XIAP from processed caspase 9. *J. Cell Biol.* 152:483–490.
- Goyal, L., K. McCall, J. Agapite, E. Hartwig, and H. Steller. 2000. Induction of apoptosis by *Drosophila* Reaper, Hid and Grim through inhibition of IAP function. *EMBO J.* 19:589–597.
- Hay, B.A., D.A. Wassarman, and G.M. Rubin. 1995. *Drosophila* homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. *Cell*. 83:1253–1262.
- Hozak, R.R., G.A. Manji, and P.D. Friesen. 2000. The BIR motifs mediate dominant interference and oligomerization of inhibitor of apoptosis Op-IAP. *Mol. Cell Biol.* 20:1877–1885.
- Huang, Y., Y.C. Park, R.L. Rich, D. Segal, D.G. Myszka, and H. Wu. 2001. Structural basis of caspase inhibition by XIAP. Differential roles of the linker versus the BIR domain. *Cell*. 104:781–790.
- Liu, Z.H., C.H. Sun, E.T. Olejniczak, R.P. Meadows, S.F. Betz, T. Oost, J. Herrmann, J.C. Wu, and S.W. Fesik. 2000. Structural basis for binding of Smac/DIABLO to the XIAP BIR3 domain. *Nature*. 408:1004–1008.
- Riedl, S.J., M. Ratus, R. Schwarzenbacher, Q. Zhou, C. Sun, S.W. Fesik, R.C. Liddington, and G.S. Salvesen. 2001. Structural basis for the inhibition of caspase-3 by XIAP. *Cell*. 104:791–800.
- Rothe, M., M.G. Pan, W.J. Henzel, T.M. Ayres, and D.V. Goeddel. 1995. The TNF-R2-TRAF signaling complex contains two novel proteins related to baculoviral-inhibitor of apoptosis proteins. *Cell*. 83:1243–1252.
- Sanna, M.G., C.S. Duckett, B. Richter, C.B. Thompson, and R.J. Ulevitch. 1998. Selective activation of jnk1 is necessary for the anti-apoptotic activity of hI κ B. *Proc. Natl. Acad. Sci. USA*. 95:6015–6020.
- Silke, J., and D.L. Vaux. 2001. Two kinds of BIR-containing protein-inhibitors of apoptosis, or required for mitosis. *J. Cell Sci.* 114:1821–1827.
- Silke, J., P.G. Ekert, C.L. Day, C.J. Hawkins, M. Baca, J. Chew, M. Pakusch, A.M. Verhagen, and D.L. Vaux. 2001. Direct inhibition of caspase 3 is dispensable for the anti-apoptotic activity of XIAP. *EMBO J.* 20:3114–3123.
- Srinivasula, S.M., P. Datta, X.J. Fan, T. Fernandes-Alnemri, Z. Huang, and E.S. Alnemri. 2000. Molecular determinants of the caspase-promoting activity of Smac/DIABLO and its role in the death receptor pathway. *J. Biol. Chem.* 275:36152–36157.
- Srinivasula, S.M., R. Hegde, A. Saleh, P. Datta, E. Shiozaki, J. Chai, R.A. Lee, P.D. Robbins, T. Fernandes-Alnemri, Y. Shi, and E.S. Alnemri. 2001. A conserved XIAP-interaction motif in caspase-9 and Smac/DIABLO regulates caspase activity and apoptosis. *Nature*. 410:112–116.
- Sun, C., M. Cai, A.H. Gunasekera, R.P. Meadows, H. Wang, J. Chen, H. Zhang, W. Wu, N. Xu, S.C. Ng, and S.W. Fesik. 1999. NMR structure and mutagenesis of the inhibitor-of-apoptosis protein XIAP. *Nature*. 401:818–821.
- Sun, C., M. Cai, R.P. Meadows, N. Xu, A.H. Gunasekera, J. Herrmann, J.C. Wu, and S.W. Fesik. 2000. NMR structure and mutagenesis of the third BIR domain of the inhibitor of apoptosis protein XIAP. *J. Biol. Chem.* 275:33777–33781.
- Suzuki, Y., Y. Nakabayashi, and R. Takahashi. 2001. Ubiquitin-protein ligase activity of X-linked inhibitor of apoptosis protein promotes proteasomal degradation of caspase-3 and enhances its anti-apoptotic effect in Fas-induced cell death. *Proc. Natl. Acad. Sci. USA*. 98:8662–8667.
- Takahashi, R., Q. Deveraux, I. Tamm, K. Welsh, N. Assamunt, G.S. Salvesen, and J.C. Reed. 1998. A single BIR domain of XIAP sufficient for inhibiting caspases. *J. Biol. Chem.* 273:7787–7790.
- Verhagen, A.M., P.G. Ekert, M. Pakusch, J. Silke, L.M. Connolly, G.E. Reid, R.L. Moritz, R.J. Simpson, and D.L. Vaux. 2000. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell*. 102:42–53.
- Verhagen, A.M., J. Silke, P.G. Ekert, M. Pakusch, H. Kaufmann, L.M. Connolly, C.L. Day, A. Tikoo, R. Burke, C. Wrobel, et al. 2001. HtrA2 promotes cell death through its serine protease activity and its ability to antagonize inhibitor of apoptosis proteins. *J. Biol. Chem.* 277:445–454.
- Wu, G., J.J. Chai, T.L. Suber, J.W. Wu, C.Y. Du, X.D. Wang, and Y.G. Shi. 2000. Structural basis of IAP recognition by Smac/DIABLO. *Nature*. 408:1008–1012.
- Yamaguchi, K., S. Nagai, J. Ninomiya-Tsuji, M. Nishita, K. Tamai, K. Irie, N. Ueno, E. Nishida, H. Shibuya, and K. Matsumoto. 1999. XIAP, a cellular member of the inhibitor of apoptosis protein family, links the receptors to TAB1-TAK1 in the bone morphogenetic protein signaling pathway. *EMBO J.* 18:179–187.
- Yang, Y., S. Fang, J.P. Jensen, A.M. Weissman, and J.D. Ashwell. 2000. Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. *Science*. 288:874–877.