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Direct inhibition of caspase 3 is dispensable for the anti-apoptotic activity of XIAP

John Silke[1,2], Paul G.Ekert[1], Catherine L.Day[3], Christine J.Hawkins[4], Manuel Baca[1], Joanne Chew[1], Miha Pakusch[1], Anne M.Verhagen[1] and David L.Vaux[1]

[1]The Walter and Eliza Hall Institute of Medical Research, Post Office Royal Melbourne Hospital, VIC 3050, [4]Department of Haematology and Oncology, Royal Children's Hospital, Flemington Road, Parkville 3052, Australia and [3]Institute of Molecular BioSciences, Massey University, Private Bag 11222, Palmerston North, New Zealand

[2]Corresponding author
e-mail: silke@wehi.edu.au

XIAP is a mammalian inhibitor of apoptosis protein (IAP). To determine residues within the second baculoviral IAP repeat (BIR2) required for inhibition of caspase 3, we screened a library of BIR2 mutants for loss of the ability to inhibit caspase 3 toxicity in the yeast *Schizosaccharomyces pombe*. Four of the mutations, not predicted to affect the structure of the BIR fold, clustered together on the N-terminal region that flanks BIR2, suggesting that this is a site of interaction with caspase 3. Introduction of these mutations into full-length XIAP reduced caspase 3 inhibitory activity up to 500-fold, but did not affect its ability to inhibit caspase 9 or interact with the IAP antagonist DIABLO. Furthermore, these mutants retained full ability to inhibit apoptosis in transfected cells, demonstrating that although XIAP is able to inhibit caspase 3, this activity is dispensable for inhibition of apoptosis by XIAP *in vivo*.

Keywords: BIR/caspase 3/caspase 9/XIAP/yeast

Introduction

Apoptosis is a physiological process of cell death common to metazoans (Vaux and Korsmeyer, 1999). Caspases, the key effector proteases of apoptosis, exist in healthy cells as inactive precursor molecules and their activation is, in large part, regulated by proteolytic processing between the p20 and p10 subunits. Autoprocessing of 'upstream' or 'initiator' caspases is facilitated by adaptor molecules such as FADD (Boldin *et al*., 1996; Muzio *et al*., 1996) and Apaf-1 (Li *et al*., 1997; Rodriguez and Lazebnik, 1999). 'Downstream' or 'effector' caspases can be activated following proteolytic processing by initiator caspases (Nicholson and Thornberry, 1997). Nevertheless, in some cases, proteolytic processing might not be required for proteolytic activity (Stennicke *et al*., 1999), and effector caspases such as caspase 3 can feed back to process upstream caspases such as caspase 8 and caspase 9 (Woo *et al*., 1999).

Inhibitor of apoptosis (IAP) proteins can inhibit apoptosis in both insect and mammalian organisms (Crook *et al*., 1993; Clem and Miller, 1994; Duckett *et al*., 1996; Hawkins *et al*., 1996; Liston *et al*., 1996). In particular, mutations in the *Drosophila* IAP locus, *thread*, result in increased programmed cell death and lethality early in development, demonstrating a fundamental role for DIAP-1 in regulating developmental apoptosis in the fly (Hay *et al*., 1995; Wang *et al*., 1999).

All IAPs bear baculoviral inhibitory repeats (BIRs), zinc-binding folds of ~70 amino acids. XIAP/hILP/MIHA, c-iap1/MIHB and c-iap2/MIHC each bear three BIRs followed by a C-terminal RING finger. Certain IAPs interact with either death (Rothe *et al*., 1995) or BMP receptors (Yamaguchi *et al*., 1999) in a BIR-dependent fashion, but it is their interaction with caspases that has generated the most interest. XIAP can inhibit caspase 3 *in vitro* with a K_i of 0.7 nM (Deveraux *et al*., 1997), and does so predominantly via its second BIR domain (BIR2) (Takahashi *et al*., 1998). In contrast, c-iap1 and c-iap2 have inhibitory activity for caspases 3 and 7 that is 100- to 1000-fold lower, indicating that they are unlikely to target these caspases *in vivo* (Roy *et al*., 1997).

The C-terminal fragment of XIAP, containing the BIR3 and RING finger, has also been reported to bind to the initiator caspase 9, although one report suggested an exclusive interaction with procaspase 9 (Deveraux *et al*., 1999), whereas another described inhibition of active caspase 9 with an IC_{50} of 10 nM (Sun *et al*., 2000).

Analysis of caspases in mammalian cells is complicated by the presence of many different caspases and caspase regulatory molecules. In contrast, the yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* do not encode any caspases or caspase inhibitors and thus provide a naïve system in which to test heterologous proteins. Furthermore, because expression of mammalian caspases in yeast is often lethal, they provide a convenient system for analysing caspases and their regulators, allowing libraries to be screened for novel inhibitors, inhibitor mutants or caspase mutants (Ekert *et al*., 1999; Hawkins *et al*., 1999; Ryser *et al*., 1999; Wang *et al*., 1999; Wright *et al*., 1999).

We performed a functional screen in the yeast *S.pombe* to identify mutations in the BIR2 of XIAP that prevented inhibition of caspase 3. Full-length XIAP, or a fragment encoding the BIR2 and flanking regions (Takahashi *et al*., 1998), was able to inhibit caspase 3-mediated death of *S.pombe* and *S.cerevisiae*. In a library of random BIR2 domain mutants we identified several mutants that no longer protected, either on their own or in the context of full-length XIAP. However, these XIAP mutants retained the ability to inhibit caspase 9 and to inhibit mammalian cell death induced by UV irradiation. Importantly, the mutants also retained the ability to bind to the mammalian

IAP antagonist DIABLO/smac, thus excluding the possibility that the mutant XIAPs inhibited cell death because they had lost the ability to be antagonized by DIABLO/smac (Du *et al*., 2000; Verhagen *et al*., 2000). Our results suggest that the primary point of action of XIAP is probably upstream of effector caspases, because abolition of the ability of XIAP to inhibit caspase 3 did not prevent it from protecting cells as efficiently as wild-type protein.

Results

Autoactivating, but not wild-type caspase 3 is toxic when expressed in S.pombe

In order to express active caspase 3 in *S.pombe*, we inserted the cDNA into the non-integrating plasmid pNeu, a pREP derivative that controls expression of the inserted gene by the full-strength *nmt* promoter (Maundrell, 1993). This allows caspase 3 expression to be induced by removal of thiamine from the media. While wild-type human caspase 3 does not kill *S.pombe* significantly because it fails to become processed, caspase 3 variants engineered to autoactivate are lethal (Ekert *et al*., 1999).

Wild-type caspase 3 was not toxic when its expression was induced in *S.pombe* (Figure 1A, compare C3 with C3mut). However, a caspase 3–β-Gal fusion protein autoactivated to a greater extent, probably due to multimer formation mediated by the β-galactosidase moiety, and was toxic to the yeast (Figure 1A and B). Toxicity required the catalytic activity of the caspase because the catalytic site mutant (QAGRG) caspase 3–β-Gal fusion protein was not toxic, and did not autoactivate (Figure 1A and B). This autoactivating caspase displays the same pH dependence as the unmodified enzyme in DEVD-AMC cleavage assays (data not shown), and in other respects behaves similarly to the unmodified enzyme, e.g. it can be inhibited by XIAP (see below).

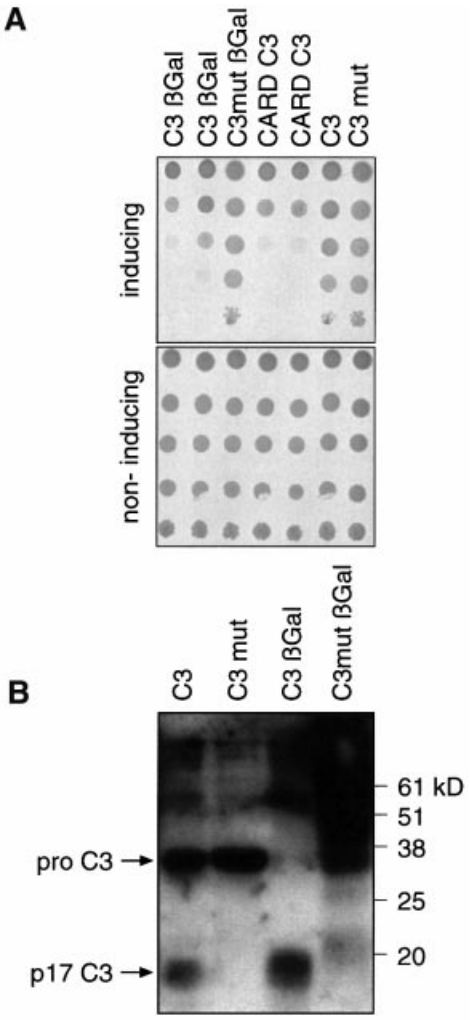


Fig. 1. Autoactivating caspase 3 kills *S.pombe*. (**A**) Yeast expressing a caspase 3–β-Gal fusion (C3 βGal), a caspase 3 catalytic mutant–β-Gal fusion (C3mut βGal), caspase 3 fused at the N-terminus with the CARD of caspase 2 (CARD C3) (Colussi *et al*., 1998), caspase 3 (C3) and caspase 3 catalytic mutant (C3mut) under the inducible *nmt* promoter were plated in serial 10-fold dilutions on solid inducing media. (**B**) Caspase 3 processing requires the catalytic cysteine and occurs in both caspase 3- and caspase 3–β-Gal fusion-expressing yeast. Yeast were induced in minimal media without thiamine, and proteins were harvested and run on an SDS–polyacrylamide gel, transferred and blotted with anti-caspase 3.

Full-length MIHA, or BIR2 plus flanking regions, can inhibit caspase 3-mediated death of S.pombe

To test which IAPs were able to inhibit caspase 3-mediated killing of *S.pombe*, we inserted cDNAs for XIAP, its murine homologue MIHA, c-iap1/MIHB, c-iap2/MIHC and survivin/MIHD into the non-integrating yeast vector pURAS K, which drives expression using the constitutive ADH promoter (Losson and Lacroute, 1983), and pREP, which drives expression from the inducible *nmt* promoter. Expression of XIAP and MIHA from both the pURAS and pREP vectors was able to suppress caspase 3 toxicity (Figure 2A and data not shown), but neither a construct expressing XIAP BIR1+3, nor any of the other IAPs, were able to do so. Expression of c-iap1, c-iap2, XIAP, XIAP BIR1+3 and XIAP BIR2 was confirmed by western blotting (Figure 2B).

To confirm further that protection by XIAP was not due to inhibition of caspase activation by the β-galactosidase moiety, we also tested the ability of XIAP to inhibit another construct that uses the caspase recruitment domain (CARD) of caspase 2 to autoactivate caspase 3 (Colussi *et al*., 1998). XIAP and MIHA were both able to inhibit death mediated by this CARD–caspase 3 construct (Figure 2A). The portion of XIAP including the BIR2 and flanking regions (Takahashi *et al*., 1998) was also able to inhibit caspase 3-mediated killing of yeast, but a construct without BIR2, BIR1+3, was not able to do so (Figure 2A), confirming that the region of XIAP containing BIR2 and its flanking regions is both necessary and sufficient for inhibition of caspase 3.

XIAP BIR2s with mutations to conserved BIR residues, or the N-terminal flanking region, fail to inhibit caspase 3

The BIR2 fragment of XIAP has a K_i against caspase 3 similar to that of the full-length protein (Deveraux *et al*., 1997; Takahashi *et al*., 1998), and can inhibit caspase 3-mediated death of yeast (Figure 2A). We therefore generated mutations in this region to identify residues necessary for caspase inhibition. Error-prone PCR with limiting nucleotides was used to generate a library of BIR2

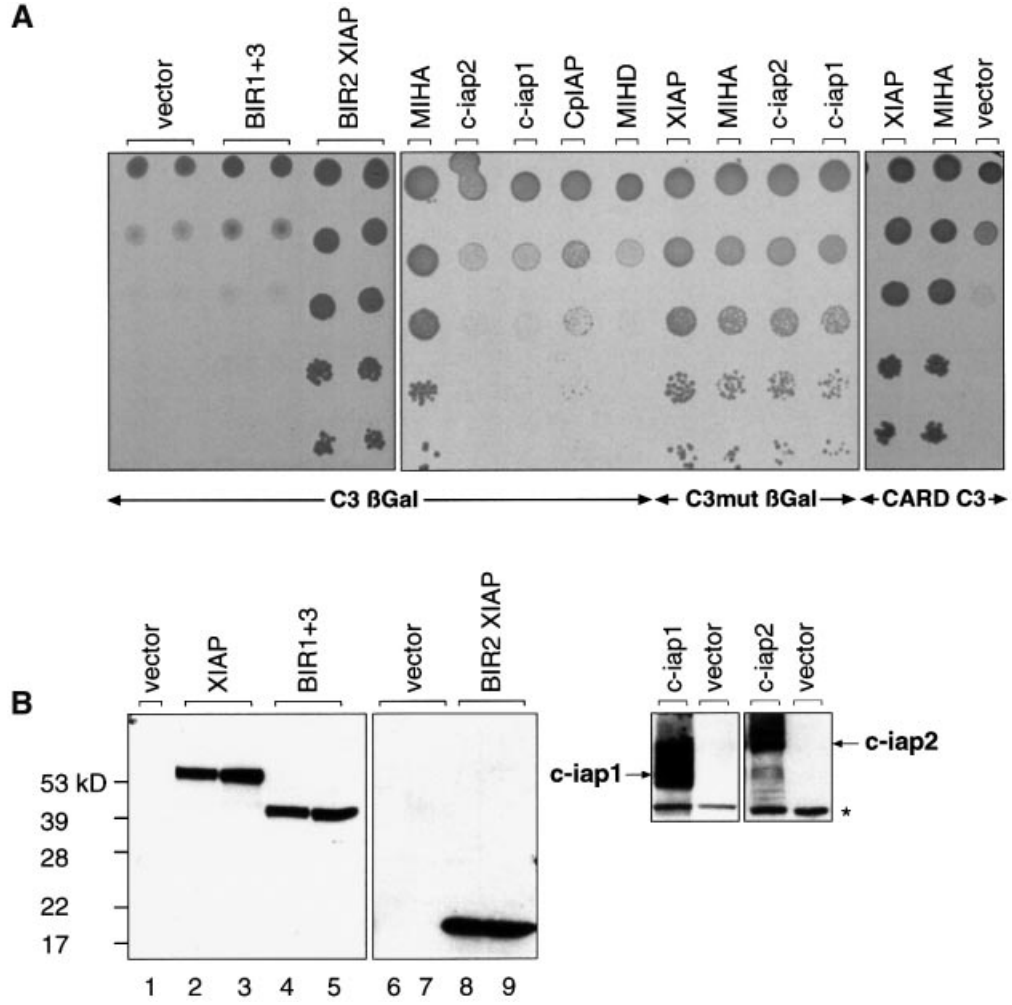


Fig. 2. The BIR2 and full-length XIAP inhibit caspase 3 toxicity in yeast. (**A**) Yeast expressing either a caspase 3–β-Gal fusion (C3 βGal), a caspase 3 catalytic mutant–β-Gal fusion (C3mut βGal) or CARD caspase (CARD C3) under the inducible *nmt* promoter and co-expressing the IAP indicated on a constitutive promoter were plated in serial 10-fold dilutions on solid inducing media. (**B**) Expression of the IAPs. Yeast were grown in minimal media, and the proteins were extracted, run on SDS–polyacrylamide gels and transferred to nitrocellulose. pURAS vector (lane 1), XIAP (lanes 2 and 3) and XIAP BIR1+3 (lanes 4 and 5) were probed with anti-XIAP, and pURAS vector (lanes 6 and 7) and XIAP BIR2 (lanes 8 and 9) were probed with anti-tetraHis. Likewise, c-iap1 and c-iap2 were probed with anti-c-iap1 and c-iap2, respectively.

genes with random point mutations. Sequence analysis of individual clones from the library revealed that ~50% contained a single mutation and 5–10% contained double point mutations. Yeast expressing an inducible caspase 3–β-Gal fusion were transformed with the library and grown on non-inducing selective media.

A total of 2200 colonies were picked and replica plated onto solid media with (non-inducing) or without (inducing) thiamine. Colonies that only grew on the non-inducing plates, indicating loss of the ability to counter caspase 3 toxicity, were isolated and the plasmids recovered.

Fifteen of the plasmids contained a single point mutation in the BIR2, four had two point mutations, and nine had a single nucleotide deletion (Figure 3A). All single point mutants in non-structural residues were expressed to approximately the same levels as the wild-type protein in yeast, demonstrating that loss of caspase 3 inhibition was not due to lower expression levels (data not shown).

All deletion mutants had lost the highly conserved BIR2 structure but retained some of the N-terminal amino acids. One of the mutants, F228L, had a frameshift immediately after the last Zn co-ordinating cysteine (Figure 3A), indicating that the whole BIR2 is required for caspase 3 inhibition.

Three of the single point mutations (C200R, H220Y and C203R) were in three of the four amino acids responsible for co-ordinating the Zn ion, emphasizing the requirement for a correctly folded BIR domain for caspase 3 inhibition. One of the mutations, R166G, was to the arginine residue conserved in all BIRs. Three of the mutations (T143A, M160T and C203R) were to residues that chemically shift when incubated with active caspase 3 and which are presumed to interact directly with caspase 3 (Figure 3B and C; and Sun *et al*., 1999). A major group of four single point mutations occurred in the linker region between BIR1 and BIR2 of XIAP, a region that has been shown to be important for inhibition of caspase 3 by the BIR2 domain (Sun *et al*., 1999).

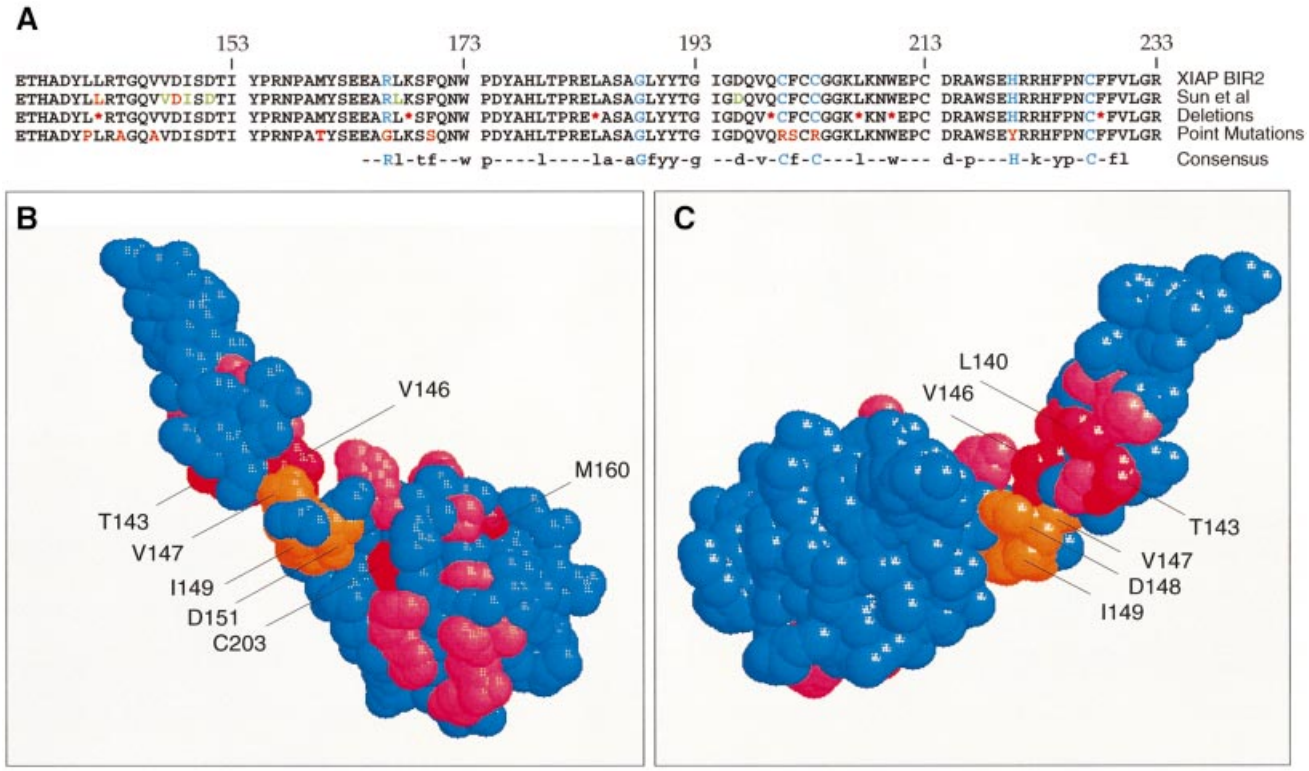


Fig. 3. Mutations in the BIR2 domain that attenuate its inhibitory function against caspase 3. (**A**) Sequence alignment of the wild-type BIR domain and those of the mutants mapped onto a linear sequence; the numbering indicates the amino acid position according to full-length XIAP. The BIR2 domain mutants reported in Sun *et al*. (1999) are shown. Residues that when mutated attenuated BIR2's inhibitory activity against caspase 3 by >200-fold, i.e. L141 and D148, are highlighted in red, and mutants V147, I149 and D151, whose activity was attenuated 11.5-, 10- and 4.3-fold, respectively, are highlighted in green. The single nucleotide deletions that resulted in truncation of the BIR2 domain in yeast and loss of caspase 3 inhibitory potential are indicated with a red asterisk. Point mutations, the point mutants in the BIR2 domain that resulted in loss of caspase 3 inhibition in yeast; Consensus, residues conserved in all BIRs identified to date are in upper case and highlighted in blue, residues conserved in most BIRs are in lower case. (**B**) Residues that chemically shift when the BIR2 domain is incubated with caspase 3 (Sun *et al*., 1999). D148A, V147, I149 and D151 are highlighted in orange, mutations identified in this screen are shown in red and indicated with text, and other residues that interact with caspase 3 are shown in pink. (**C**) The opposing face of BIR2 with the same colour scheme as in (B). Prepared using data given to us by Stephen Fesik with the program RasMol (Sayle and Milner-White, 1995).

Full-length mutant XIAPs are no longer able to inhibit caspase 3

We then tested whether mutations to BIR2 affected inhibition of caspase 3 by the full-length XIAP protein in *S.cerevisiae*. In addition to our mutants, we also analysed an XIAP mutant, D148A, described by Sun *et al*. (1999). The mutants were tested against the caspase 3–β-Gal fusion protein expressed in *S.cerevisiae* under a glucose-suppressable promoter. Wild-type MIHA, XIAP and the baculoviral p35 all inhibited yeast death caused by caspase 3, and, consistent with our previous result, all the BIR2 mutants had reduced caspase 3 inhibitory activity, even in the context of the full-length protein (Figure 4A). While mutants L140P and V146A retained a small amount of activity in this assay, C200R (a Zn co-ordinating mutant) and the D148A mutant displayed no detectable activity.

To quantitate the changes to the inhibitory constant (K_i) caused by the mutations, we expressed full-length wild-type XIAP and the mutants in *Escherichia coli*, and partially purified the recombinant proteins (Figure 4C). Analysis of the proteins by size exclusion chromatography indicated that they all existed as high molecular weight complexes (data not shown). These proteins were then characterized for their ability to inhibit caspase 3 in an *in vitro* DEVD-AMC cleavage assay. In accordance with previously published results (Deveraux *et al*., 1997), we determined the K_i for wild-type XIAP against caspase 3 to be 0.6 ± 0.1 nM (Figure 4B). Consistent with the results obtained in yeast, XIAP mutants L140P, T143A and V146A had a 10- to 20-fold reduction in their activity, and the D148A mutant had a >500-fold reduced ability to inhibit caspase 3 (Figure 4B). It was not possible to produce recombinant mutants C200R, R166G, M160T or F170S. Wild-type XIAP is itself difficult to produce *in vitro* and we suspect that mutations that even slightly affect the structure of XIAP affect its stability *in vitro*.

Processed caspase 3 interacts with XIAP (Sun *et al*., 1999) and to evaluate the effect of the mutants we immunoprecipitated the transiently transfected mutants with the autoactivating caspase 3–β-Gal from mammalian cells. As expected, all mutations interfered with the ability of XIAP to bind caspase 3. Consistent with the *in vitro* inhibition data, mutants L140P and V146A retained a small amount of caspase 3 binding activity, whereas D148A, M160T, F170S C200R and R166G had significantly lost the ability to bind caspase 3 in this assay. Mutant T143A retained some caspase 3 binding, indicating that

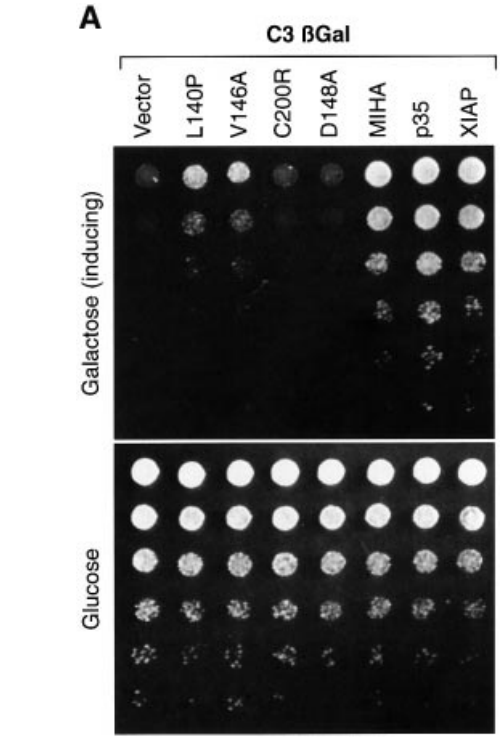
the lack of inhibition of caspase 3 is not due to its inability to bind.

Full-length mutant XIAPs retain the ability to inhibit caspase 9 and to bind to caspase 9 and DIABLO

The ability of the full-length XIAP mutants to inhibit caspase 9 was tested in the *S.cerevisiae* system. Apaf-1 lacking its WD40 repeats and wild-type procaspases 3 and 9 were all co-expressed together with full-length wild-type or mutant XIAP. In this system, caspase 3 does not autoactivate significantly, but requires processing by Apaf-1-activated caspase 9 for activation and death of the yeast (Hawkins *et al*., 2001). Death of the yeast in this system is dependent on both caspase 9 and caspase 3, but inhibition of caspase 9 is sufficient to prevent cell death because a BIR3-only construct was able to protect the yeast fully (Figure 5A). Mutants L140P, V146A and T143A protected the yeast cells as well as wild-type XIAP, and the D148A mutant retained significant activity (Figure 5A), whereas C200R, R166G, F170S and M160T were not able to block this caspase 9-mediated death.

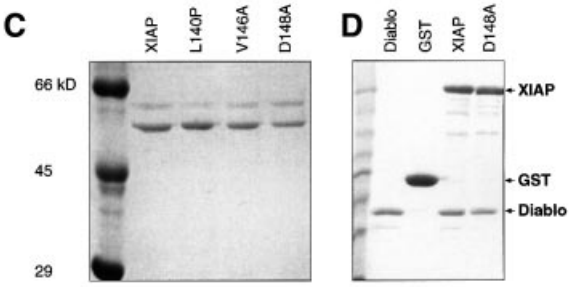
To confirm that mutations in the linker region did not affect interaction with caspase 9, we immunoprecipitated the XIAP mutants from 293T cells co-transfected with full-length caspase 9. In this system, caspase 9 auto-activates, and both processed and unprocessed forms of caspase 9 are present in the lysates (Figure 5B). All XIAP mutants in the BIR2 linker were able to immunoprecipitate processed caspase 9. Mutant D148A showed a slight reduction in its ability to bind caspase 9 (Figure 5B), but the reduction was not as dramatic as for mutant C200R. Mutants M160T, F170S, R166G and C200R all showed reduced binding to processed caspase 9. However, the amount of processed caspase 9 present in the lysates was less, and this was most probably due to increased cell death among cells expressing the mutants (or the negative control TAB1), so that less caspase 9 accumulated. This finding corroborates our observation in yeast that these mutants are less effective at blocking caspase 9.

We determined the IC_{50} of XIAP against caspase 9 in an *in vitro* LEHD-AMC cleavage assay (Figure 4B). Surprisingly, the IC_{50}s for the full-length protein were higher than for the BIR3 domain alone (Sun *et al*., 2000). To exclude the possibility that only the BIR2 domain was folded correctly in the bacterially produced XIAP, we performed *in vitro* precipitations with bacterially produced DIABLO (Figure 4D). Because DIABLO binding is determined to a large extent by the BIR3 of XIAP (Chai *et al*., 2000), and the bacterially produced XIAP and XIAP D148A bound DIABLO to the same extent, it seems likely



B

	K_i vs caspase 3	Fold reduction	IC50 vs caspase 9
XIAP	0.6 ± 0.1 nM (0.7 nM)		291 nM ± 20
L140P	12.9 ± 2.4 nM	21.5	284 nM
T143A	11.1 ± 3.3 nM	17.9	
V146A	7.5 ± 2 nM	12.5	314 nM
D148A	> 300 nM	>500	340 nM ± 40
DEVD-CHO	0.7 nM (0.3 nM)		



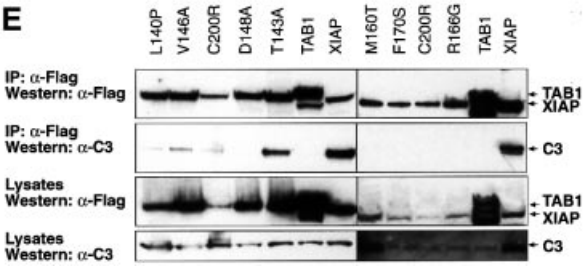


Fig. 4. Full-length XIAPs with mutations in the BIR1–BIR2 linker are attenuated in their ability to inhibit caspase 3. (**A**) Yeast expressing a caspase 3–β-Gal fusion from the pGALL-inducible vector were co-transformed with full-length XIAP mutants, the baculoviral p35 (p35), wild-type MIHA or XIAP and plated in serial 10-fold dilutions on solid inducing (galactose) and non-inducing (glucose) media. (**B**) K_is for full-length XIAP, XIAP mutants and the tetrapeptide aldehyde DEVD-CHO against caspase 3, and IC_{50}s for full-length XIAP against caspase 9. (**C**) Purified XIAP and XIAP mutants separated on a 12% SDS–polyacrylamide gel and stained with Coomassie Blue. (**D**) XIAP D148A interacts with DIABLO. Purified XIAP and XIAP D148A were used to co-immunoprecipitate bacterially produced DIABLO, which were separated on a 12% SDS–polyacrylamide gel and stained with Coomassie Blue. (**E**) XIAP mutants are impaired in their ability to interact with caspase 3 *in vivo*. 293T cells were transiently transfected with plasmids expressing Flag-tagged XIAP, XIAP mutants or TAB1 and caspase 3–β-Gal (C3 βGal). Cell lysates were immunoprecipitated with anti-Flag beads and immunoblotted with anti-caspase 3.

that the bacterially produced XIAPs have a correctly folded BIR3.

We recently have identified a novel mammalian antagonist of IAPs called DIABLO/smac (Du *et al*., 2000; Verhagen *et al*., 2000). DIABLO was identified originally due to its ability to bind to XIAP. The XIAP mutants were therefore tested for their ability to bind to DIABLO *in vivo*, and, consistent with data from the bacterially produced proteins, all linker mutants behaved indistinguishably from wild-type XIAP in an immunoprecipitation assay (Figure 5C). Both the caspase 9 and DIABLO binding assays demonstrate that the linker mutations generated have not greatly interfered with the structure or other capabilities of XIAP.

XIAP mutants that do not inhibit caspase 3, but still inhibit caspase 9 and interact with DIABLO, block UV-induced apoptosis as well as wild-type XIAP

To test whether loss of caspase 3 inhibitory activity affected the ability of XIAP to inhibit apoptosis of mammalian cells, we expressed the mutants in NT2 teratocarcinoma cells and exposed them to UV radiation (Figure 6A). Some of the mutants were able to protect against UV-induced apoptosis as efficently as wild-type XIAP, while others were unable to protect, even though all except for mutant C200R were expressed equivalently (Figure 6B). Mutants R166G, F170S and C200R, which contain mutations that probably affect the BIR2 fold, could not inhibit UV-induced death. However, mutants L140P, V146A, T143A and D148A, which no longer inhibit caspase 3, still retained full activity against UV-induced apoptosis. These four independent mutants demonstrate that caspase 3 inhibitory activity is independent of caspase 9 inhibitory activity, and is not required for XIAP to inhibit UV-induced cell death.

Discussion

If caspases are the major effectors of the apoptotic programme, and IAPs function to block caspases, IAPs are in a pivotal position to determine whether a cell undergoes apoptosis or not. Consistent with a key role in the decision process, mutants of the *diap-1* locus, *thread*, in *Drosophila*, do not develop due to massive ectopic cell death. However, a similar drastic phenotype for mammalian IAP knock-outs has yet to be reported.

XIAP can inhibit caspase 3 with a K_i of 0.7 nM (Deveraux *et al*., 1997). The BIR2 of XIAP plus flanking regions of 60 amino acids has been shown to account for nearly all this caspase 3 inhibitory activity (Takahashi *et al*., 1998). Further studies have shown that the small region upstream of the conserved BIR2 is required for caspase 3 inhibition (Sun *et al*., 1999) and, although our studies involved a non-directional approach, they are in accord with this data. First, a structurally intact BIR is required for caspase 3 inhibition by the BIR2 fragment

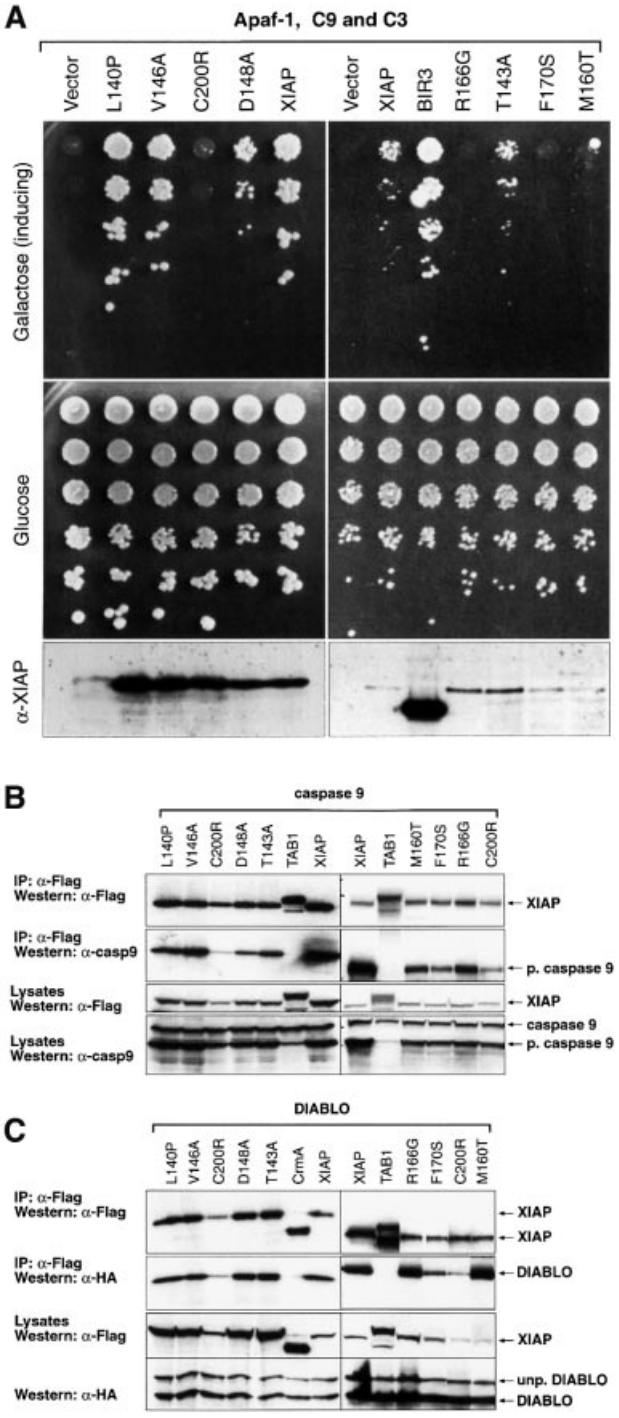


Fig. 5. Full-length XIAPs with mutations in the BIR1–BIR2 linker retain their ability to inhibit caspase 9 and interact with DIABLO. (**A**) Yeast expressing Apaf-1 –WD40, caspase 9 and caspase 3 were co-transformed with full-length XIAP mutants or a BIR3–eGFP fusion construct, and plated in serial 10-fold dilutions on solid inducing (galactose) and non-inducing (glucose) media. Western blots of the mutants were performed with anti-XIAP. (**B**) Co-immunoprecipitation of XIAP mutants and caspase 9 from cell lysates. 293T cells were transiently transfected with plasmids expressing Flag-tagged XIAP, XIAP mutants or TAB1 and caspase 9. Cell lysates were immunoprecipitated with anti-Flag beads and immunoblotted with anti-caspase 9 and anti-Flag antibodies. p. caspase 9 = processed caspase 9; caspase 9 = full-length caspase 9. (**C**) Co-immunoprecipitation of XIAP mutants and DIABLO from cell lysates. 293T cells were transiently transfected with plasmids expressing Flag-tagged XIAP, XIAP mutants, CrmA or TAB1 and DIABLO-HA tag. Cell lysates were immunoprecipitated with anti-Flag beads and immunoblotted with anti-HA and anti-Flag. unp. DIABLO = unprocessed DIABLO; DIABLO = full-length processed DIABLO (Verhagen *et al*., 2000).

because mutants that had lost any part of the BIR2 were no longer able to inhibit caspase 3. Secondly, the N-terminal linker region is required for caspase 3 inhibition because single point mutants in this region were unable to inhibit caspase 3. Strikingly two of these mutants, V146A and T143A, represented very subtle changes to the primary sequence, yet the effect on the ability of the full-length XIAP to inhibit caspase 3 was marked, with a loss of inhibitory activity of >12- to 18-fold. It seems unlikely that such modest changes would have any effect on the structure of the full-length protein. The fact that the mutant XIAPs retained the ability to inhibit caspase 9 and bind DIABLO corroborates this assumption.

It has been proposed that the BIR1–BIR2 linker region of XIAP binds to the active site of caspase 3 and that D148 acts as the P_4 residue (Sun *et al*., 1999). However, if the linker acts as a pseudosubstrate, then mutation of the P_1 would be expected to be as disruptive, if not more so, as mutation of P_4. When the proposed P_1 position was mutated, however, a very minor (4-fold) loss of activity was observed (Sun *et al*., 1999). When combined with the data of Sun *et al*. (1999) it seems more likely that if caspase 3 does bind a tetrapeptide in XIAP, it is 145QVVD148, rather than 148DISD151. Consistent with this interpretation, the conservative V to A substitution at position 146, being the P_3 position in the revised scheme, has a disproportionately large influence on the ability to inhibit caspase 3 that would not be expected at the P_7 position. A similar argument can be made for the V147A mutant (Sun *et al*., 1999), which has lost 11-fold of its activity and would now be considered as the P_2 residue. Intriguingly, caspase 7 prefers valine to alanine at the P_3 position, and both caspases 3 and 7 have a preference for valine at the P_2 position (Thornberry *et al*., 1997). Likewise, although DIAP-1 has been shown to inhibit mammalian caspase 3, drICE and DCP1 (Kaiser *et al*., 1998; Hawkins *et al*., 1999; Wang *et al*., 1999), all of which can be presumed to have the optimal DXXD specificity, there is no DXXD motif in the linker of BIR2 but rather a QATGD motif (Figure 7). Intriguingly, DIAP-2 contains an SVVD tetrapeptide in the BIR2 linker (Figure 7). Finally, in contrast to the baculoviral p35, XIAP is not cleaved by its target caspase at this site, and if an optimal tetrapeptide were present in the linker this might result in conversion of XIAP to a substrate rather than an inhibitor (Ekert *et al*., 1999). Therefore, it is not surprising that QVVD is not an optimal tetrapeptide cleavage site for caspase 3 (DEVD).

That the linker region of the BIR2 is required for the ability of the whole molecule to inhibit caspase 3 was demonstrated by placing the mutations in the context of the full-length protein. The linker mutants retained the ability to bind and inhibit caspase 9, demonstrating that the ability to inhibit caspase 3 can be separated from the caspase 9 inhibitory activity. These results corroborate Deveraux *et al*. (1999) who showed that a fragment containing the BIR3 of XIAP contained caspase 9 inhibitory activity. However, in the context of the whole molecule, there is clearly interplay between inhibition of apoptosis and the structure of the BIR2, because mutants R166G, F170S and C200R had lost the ability to inhibit UV-induced death even though their BIR3 and RING domains were intact, and also showed reduced processed caspase 9 binding. Possibly, caspase 3 and caspase 9 bind XIAP simultaneously and contribute to each other's binding, because D148A, the linker mutation that does not affect the structure of XIAP, affected caspase 3

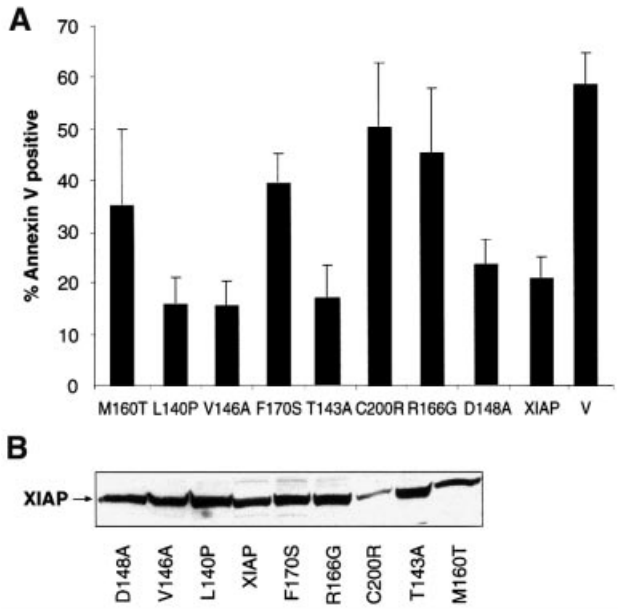


Fig. 6. Full-length XIAPs that retain caspase 9 inhibitory potential inhibit UV-induced cell death. (**A**) The XIAP mutants (M160T, etc), empty vector (V) and wild-type XIAP (XIAP) were cloned into a pEF vector, and transiently transfected into NT2 cells with a GFP marker plasmid. Cells subsequently were induced to undergo cell death with UV irradiation, and stained with annexin V. The fractions of cells that were positive for GFP and annexin V over GFP-positive cells were expressed as the percentage of annexin V-positive cells. Error bars are two standard errors of the mean for three independent experiments. (**B**) Extracts of transiently transfected NT2 cells were made, separated on SDS–polyacrylamide gels, transferred to nitrocellulose and probed with anti-Flag antibody.

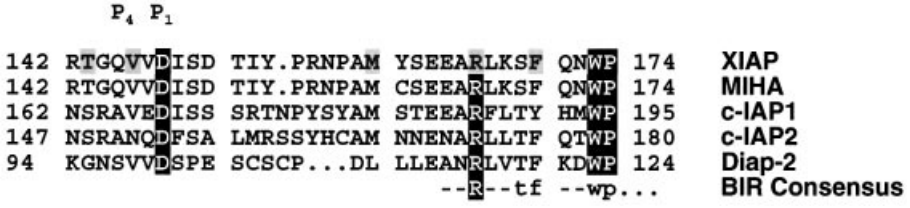


Fig. 7. A tetrapeptide that is not optimal for caspase 3 is present in many BIR linkers. Mutations described in this study are indicated with grey shading and black text, residues conserved in all BIR linkers are indicated with black shading and white text. The N-terminal part of the BIR consensus is shown underneath.

binding the most, and also subtly but reproducibly reduced the ability of XIAP to bind to and inhibit caspase 9.

UV-induced cell death has been shown, at least in mouse embryonic fibroblasts (MEFs) and, in some cases, embryonic stem cells, to require Apaf-1 (Yoshida *et al*., 1998), caspase 9 (Hakem *et al*., 1998) and caspase 3 (Woo *et al*., 1998). These results provide the strongest evidence that the UV-induced death pathway in MEFs proceeds through Apaf-1-mediated activation of caspase 9 and caspase 3, and we have shown that stably expressed XIAP is able to block UV-induced cell death completely with a potency similar to Bcl-2 (Verhagen *et al*., 2000).

When the XIAP mutants were tested in a transient transfection assay, two classes of mutants became apparent: a class that could still inhibit cell death as efficiently as wild-type XIAP, and a class that was ineffective at preventing UV-induced cell death. Because the mutants that were still able to inhibit caspase 9 were able to block UV-induced cell death, we propose that XIAP, when overexpressed, can block cell death at the level of processed caspase 9, although we cannot exclude the possibility that XIAP inhibits other caspases or processes that are responsible for caspase 9 activation. At least in this overexpression system, therefore, XIAP mutants that are crippled in their ability to inhibit caspase 3 can still block cell death.

XIAP appears to be a highly complex molecule with interactions described for caspase 3, caspase 9 and DIABLO. While we have shown that inhibition of caspase 3 by XIAP is dispensable for the ability of XIAP to inhibit cell death, XIAP is nevertheless clearly able to block caspase 3. In a similar way, DIAP-1 is able to inhibit both a caspase 9 homologue (DRONC) (Meier *et al*., 2000) and at least two downstream effector type caspases (or caspase 3 homologues), DCP1 and drICE (Hawkins *et al*., 1999; Wang *et al*., 1999), arguing that this dual role has been preserved throughout evolution. How this dual activity functions in a cell death situation remains to be determined, but the results described here show that XIAP can block cell death prior to effector caspase activation, presumably at the level of caspase 9.

Materials and methods

Plasmids

The plasmid pURAS is a pFL20 derivative containing a pBR322 backbone, a *URA3* gene for selection and *S.pombe* ars1 and stb elements (Losson and Lacroute, 1983). Transcription is driven from a constitutive ADH promoter. The plasmid pNeu is a pREP derivative (Maundrell, 1993) and contains a pUC backbone and a LEU2 selectable marker. Transcription is driven from a *nmt*1 full-strength promoter. pURAS was modified by insertion of an oligonucleotide containing a consensus Kozak and *Asc*I site and recreating a unique *Bam*HI site to create pURAS K. The same Kozak (K) oligonucleotide was introduced into pNeu and downstream of this was introduced a multiple cloning site oligonucleotide, adaptor (A): Kozak (K), 5′-gatcgccaccatggcgcgccg-3′, 5′-cggtggtaccgcgcggcctag-3′; adaptor (A), 5′-gatcccatatgctagcgtcgacaaggcgcgccattaatctagatagt-3′, 5′-tcgaactatctagattaatggcgcgccttgtcgacgctagccatatgg-3′ to create pNeu KA. pNeu KA β-Gal was created by *Pfu*I PCR and inserting a β-Gal cassette into *Nhe*I–*Sal*I sites, and the β-Gal gene was tested for functionality in yeast. Caspase 3 was *Pfu*I PCR amplified with primers 1.12 (5′-cgggatcctcatggagaacactgaaaactca-3′) and 1.17 (5′-cgcgctagcgtgataaaaatagagttcttttg-3′) and inserted into pNeu KA β-Gal via *Bam*HI–*Nhe*I. The caspase 3 mutant was generated with *Pfu*I PCR using 1.12, 1.17, 1.18 (5′-cggatgctcttccggccgtggtacagaactg-3′) and 1.19 (5′-cggatgctcttcggccggcctgaataatgaaaagt-3′). The caspase 3 cassette was placed into pEF KA β-Gal using *Bam*HI–*Nhe*I. The CARD caspase 3 construct was made by amplifying the plasmid pGFP-N1 CARD caspase 3 [pro C2 caspase 3–green fluorescent protein (GFP); a kind gift of S.Kumar (Colussi *et al*., 1998)] with the primers 1.17 and 1.22 (5′-cgggatcctcatggcggcgccgagcggg-3′) and inserted into pNeu KA with *Bam*HI–*Nhe*I.

The BIR2 was amplified with the primers 7.28 (5′-cgccagatcttgagagatcattttgccttagaca-3′) and 7.30 (5′-gccgcagctgttacaggatccttggatttcttggaagatttgttga-3′) cloned into pURAS K with *Bam*HI–*Pvu*II for mutagenesis screening. To reclone the mutants obtained in the yeast screen, the product of the *Pfu*I PCR with primers 7.43 (5′-cgggatccaccatgcgtgatcattttgccttagacag-3′) and 7.44 (5′-cgcgctagctggattcctaggaagatttgttgaatttggg-3′) was cloned into pURAS K His6 with *Bam*HI–*Nhe*I. These plasmids were digested with *Bam*HI–*Xba*I and the fragments cloned into a bacterial expression pET 15b variant created with the oligos 2.05 (5′-catgggatcccatatggctagccaataacccggga-3′) and 2.06 (5′-gatctcccgggttattggctagccatatgggatcc-3′) via *Bam*HI–*Nhe*I. To create the full-length mutants of XIAP, XIAP was *Pfu*I amplified with primers 7.15 (5′-cgggatcctgatgacttttaacagttttgaaggatct-3′) and 7.45 (5′-ccgccctagggcaaaaagatctctgcttcccagatagtt-3′) to create a BIR1 fragment, and 7.47 (5′-ccgccctaggaatccatccatggcagattat-3′), 7.48 (5′-tcacttgaggttctggttgcggatctagtgaatgctcagaaag-3′) and 7.49 (5′-cgtctagattaagacataaaaatttttttgcttgaa-3′) to remove the *Bgl*II site silently from XIAP. These two fragments were then cloned into pURAS K Flag via *Bgl*II–*Nhe*I to create pURAS XIAP B1C1. pURAS XIAP B1C1 was digested with *Bgl*II–*Avr*II and the BIR2 mutants were inserted with *Bcl*I–*Avr*II ends to create pURAS XIAP B1A1C1.

For the *S.cerevisiae* plasmids, caspase 3 was amplified from CPP32α (Fernandes *et al*., 1994) with the oligonucleotides 5′-cgggatccatggagaacactgaaaactcagtgg-3′ and 5′-gctctagattagtgataaaaatagagttcttttgtgagc-3′, digested with *Bam*HI and *Xba*I, and cloned into pGALL-(URA), which had been generated by swapping the *Pvu*I fragment of pGALL-(TRP1) (Hawkins *et al*., 1999) containing the *TRP-1* gene with that of pRS316 containing the *URA* selection gene. pYX 143 KAS caspase 3–β-Gal was made by digesting pNeu KAS caspase 3–β-Gal with *Bam*HI–*Xba*I into pYX 143 KAS Flag *Bam*HI–*Nhe*I. pYX 143 KAS Flag was made by digesting pYX 143 with *Nco*I–*Nhe*I and inserting KAS Flag *Nco*I–*Xba*I from pNeu KAS Flag. The caspase 9 and constitutively active Apaf-1 expression constructs have been described previously (Hawkins *et al*., 1999). Full-length wild-type XIAP and mutants were excised from the pURAS vectors using *Sac*I and *Sal*I and cloned into pADH TRP1 (Wang *et al*., 1999).

To create the mammalian expression vectors, pEF kozak was digested with *Bam*HI–*Nhe*I and the full-length XIAP mutants from pURAS XIAP B1A1C1 with *Bam*HI–*Xba*I. All constructs were verified by digest and sequencing. pFlag TAB1 was a kind gift from Kuni Matsumoto (Shibuya *et al*., 1996); pEF Flag CrmA and pcDNA3 DIABLO HA tag have been described (Ekert *et al*., 1999; Verhagen *et al*., 2000).

Generation and screening of a mutant library

PCR-based mutagenesis was performed using *Taq* polymerase with limiting nucleotides (four separate mixes of 4 μM of the three dNTPs and 0.8 μM of each individual nucleotide). The PCR was performed for eight cycles, $MnCl_2$ (100 μM) was added and a further 28 cycles were performed. A total of 120 clones were picked that were viable on the non-inducing plates but died on the inducing plates, indicating loss of the ability of the BIR2 mutant to inhibit caspase 3 toxicity. DNAs were purified successfully from 41 of these colonies, retransformed into bacteria and three minipreps were made from each clone. All three minipreps were digested with a diagnostic *Nhe*I to reveal those that contained a correctly sized BIR2 insert. In all cases, all three plasmids had the same restriction pattern, indicating that the BIR2 plasmids were replicated faithfully in the yeast and did not undergo any gross recombination events. Twenty eight plasmids contained the correct restriction fragment, and one miniprep from each of these 28 sets was sequenced in both orientations. A complete overlapping sequence was obtained for each clone.

Yeast transformation

Transformation was performed with a standard LiAc protocol and plating on selective media; see 'http://www.bio.uva.nl/pombe/handbook'. All clones were always maintained on selective media.

Yeast growth assays

Yeast transformants were inoculated from a selective plate overnight in non-inducing Edinburgh minimal medium (EMM) at 30°C to an OD_{600} of 2–3. The OD_{600} was then quantitated using a spectrophotometer and

10-fold serial dilutions made in sterile tissue culture plates. Yeast dilutions were plated on selective non-inducing and inducing agar plates and grown for 3–4 days at 30°C. *Saccharomyces cerevisiae* transformants were processed for survival assays as described (Hawkins *et al*., 2000).

Western blotting and immunoprecipitation analysis

Protein samples from *S.pombe* were obtained with a trichloroacetic acid (TCA) precipitation protocol and run on 4–20% gradient gels (Gradipore). Protein extracts from mammalian cells were obtained using DISC lysis buffer (150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 20 mM Tris pH 7.5; Muzio *et al*., 1996). Western blots were carried out using standard protocols and the antibodies anti-caspase 3 (Pharmingen), anti-Flag (Kodak), anti-XIAP (MBL; Abacus), tetra-His (Qiagen), anti-HA (3F10) (Boehringer Mannheim), anti-caspase 9 (Yuri Lazebnik), anti-c-IAP1 (R&D Systems) and anti-c-IAP2 (R&D Systems). Proteins were visualized by ECL (Amersham, UK) following incubation of membranes with horseradish peroxidase (HRP)-coupled secondary antibodies. The *S.cerevisiae* protein extracts were isolated for SDS–PAGE and immunoblotting analysis as previously described (Hawkins *et al*., 2000). Immunoprecipitations were performed using Flag-specific monoclonal antibody M2 covalently coupled to agarose beads (Sigma). The immunoprecipitates were washed five times in DISC lysis buffer and proteins eluted with 100 mM glycine (pH 3).

Transfections

Transfections were performed with Effectene™ (Qiagen). A 0.5 μg aliquot of the XIAP DNA and 25 ng of pEGFP (Clontech) were co-transfected onto a 2 cm plate of 25–40% confluent cells. The effectene mix was plated onto the cells and removed 12 h after transfection. Transfection efficiencies were ~20–30% as judged by fluorescence-activated cell sorting (FACS) analysis of green cells over the total population. For co-immunoprecipitation experiments, 10 cm plates of 30% confluent 293 cells were transfected with 0.5 μg of XIAP mutants and 0.5 μg of the partner (DIABLO or caspase 3-βGal) and harvested 36 h later.

Cell viability and annexin V staining

NT2 cells were transfected with equal amounts of the mutant constructs and 1:20 (w/w) of pEGFP and allowed to proliferate for a day. The cells were washed and induced to undergo apoptosis by irradiating 2 cm plates with 25 J/m^2 of UV radiation and harvested 6–7 h later by recovering the media (containing non-adherent and apoptotic cells), trypsinizing the adherent cells for 5 min, inhibiting trypsin with an equal volume of fetal calf serum (FCS) and combining both adherent and non-adherent cell fractions. The cells were washed with 3 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 mM $CaCl_2$ and 5% FCS. Recombinant annexin V was produced in house and conjugated with biotin. Cells were incubated in 100–200 μl of DMEM supplemented with 10 mM $CaCl_2$ and biotinylated annexin V at 1:100 dilution at room temperature for 20 min. Cells were washed with 2 × 3 ml of DMEM and resuspended in 1–200 μl of DMEM supplemented with 10 mM $CaCl_2$, 5% FCS and streptavidin Tricolor (CalTag Laboratories) at 1:100 dilution on ice for 15 min. The 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 annexin V positive, and hence apoptotic, determined.

Preparation of recombinant full-length XIAP proteins

The cDNAs for full-length human XIAP (residues 1–497) and the mutants V146A, L140P, C200R and D148A (Sun *et al*., 1999) were amplified by PCR and then cloned into the expression vector pGEX-6P-3 (Pharmacia) using *Eco*RI and *Bam*HI. The sequence of each construct was confirmed by DNA sequencing. Recombinant proteins were expressed in *E.coli* strain SG13009 (Qiagen) at 25°C overnight after induction with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were pelleted and sonicated in 1× phosphate-buffered saline (PBS; 11.8 mM sodium phosphate buffer pH 7.3, 2.7 mM KCl, 140 mM NaCl), the supernatant was recovered and GST fusion proteins were purified by affinity chromatography on glutathione–Sepharose (Pharmacia). The GST fusion proteins were then digested with PreScission protease (Pharmacia) in 1× PBS containing 1 mM dithiothreitol (DTT) while still bound to the resin. Soluble XIAP was recovered from the resin and the purity was confirmed by SDS–PAGE. Following cleavage, five additional N-terminal vector-derived residues (GPLGS) remained.

Caspase cleavage assay and determination of K_is for XIAP mutants

Caspase 3 was produced from 500 ml of liquid culture of *S.pombe* and native extracts were made using glass beads and breaking in TEEG buffer (50 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 0.1% CHAPS) supplemented with the protease inhibitors phenylmethylsulfonyl fluoride (PMSF), leupeptin and aprotinin. Glycerol was added to 10% and the extracts were stored at –20°C. Caspase 9 was obtained from BIOMOL Research Laboratories, Inc. Inhibition assays were performed at pH 7.5 in 0.1 M HEPES, 10% sucrose, 0.1% CHAPS and 10 mM DTT buffer. Caspases 3 or 9 were pre-incubated with varying concentrations of inhibitor for 10 min at 37°C. Aliquots (50 μl) of enzyme inhibitor were then mixed in a 96-well plate with an equal volume of 50 μM DEVD-AMC (caspase 3) or 50 μM LEHD-AMC (caspase 9, Bachem), and fluorescence was measured in a fluorimeter instrument (TECAN) at 37°C for 40 min, with individual readings made every 45 s (excitation filter 360 nm; emission filter 465 nm). Substrate cleavage rates were calculated from the slopes of the initial rates, and inhibition data were analysed by using Dixon plots (Dixon, 1953). Inhibitor dissociation constants (K_i) were calculated from the derived IC_{50} values by use of the expression $K_i = IC_{50}/(1 + [S]/K_m)$, where $[S] = 50$ μM and $K_m = 10$ μM as previously determined for caspase 3-mediated cleavage of DEVD-AMC under identical assay conditions (Garcia-Calvo *et al*., 1999). Three independent experiments were performed for each inhibitor and the mean and standard deviation were calculated. To validate the assay system, we determined the K_i for the peptide aldehyde DEVD-CHO (Bachem), and found it to be 0.6 nM (Figure 4B), which is in close agreement with the value of 0.3 nM determined by Nicholson *et al*. (1995). Because the IC_{50} obtained for XIAP against caspase 9 was higher than expected from data with the BIR3 alone, inhibition assays were performed simultaneously with caspase 3 inhibition assays to verify that the concentrations used gave the expected results for caspase 3 inhibition. Similar values were obtained for IC_{50} regardless of whether the inhibition assay was performed at pH 6.5 or 7.5.

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