

Identification of mammalian mitochondrial proteins that interact with IAPs via N-terminal IAP binding motifs

AM Verhagen^{*1}, TK Kratina¹, CJ Hawkins^{2,3}, J Silke^{1,3}, PG Eker^{1,2} and DL Vaux^{1,3}

Direct IAP binding protein with low pI/second mitochondrial activator of caspases, HtrA2/Omi and GstPT/eRF3 are mammalian proteins that bind via N-terminal inhibitor of apoptosis protein (IAP) binding motifs (IBMs) to the baculoviral IAP repeat (BIR) domains of IAPs. These interactions can prevent IAPs from inhibiting caspases, or displace active caspases, thereby promoting cell death. We have identified several additional potential IAP antagonists, including glutamate dehydrogenase (GdH), Nipsnap 3 and 4, CLPX, leucine-rich pentatricopeptide repeat motif-containing protein and 3-hydroxyisobutyrate dehydrogenase. All are mitochondrial proteins from which N-terminal import sequences are removed generating N-terminal IBMs. Whereas most of these proteins have alanine at the N-terminal position, as observed for previously described antagonists, GdH has an N-terminal serine residue that is essential for X-linked IAP (XIAP) interaction. These newly described IAP binding proteins interact with XIAP mainly via BIR2, with binding eliminated or significantly reduced by a single point mutation (D214S) within this domain. Through this interaction, many are able to antagonise XIAP inhibition of caspase 3 *in vitro*.

Cell Death and Differentiation (2007) 14, 348–357. doi:10.1038/sj.cdd.4402001; published online 23 June 2006

Inhibitor of apoptosis proteins (IAPs) suppress apoptosis by binding to and inhibiting active caspases, cysteine proteases that otherwise cleave proteins, resulting in disintegration of the cell. The IAPs X-linked IAP (XIAP), cIAP1, cIAP2 and ML-IAP bear one to three baculoviral IAP repeats (BIRs) and a C-terminal RING domain that has ubiquitin E3 ligase activity.^{1–3}

XIAP, the most potent inhibitor of caspases, is also the most well characterised of the mammalian IAPs.^{1,2} Its interaction with the processed caspase 9 and the activated forms of effector caspases 3 and 7 has been extensively characterised by mutagenesis, NMR and crystallographic studies. The key interactions with caspase 9 involve the BIR3 domain, whereas the linker region preceding the BIR2 domain and BIR2 itself mediate interaction with caspases 3 and 7.

Proapoptotic IAP antagonists can bind to the BIRs of IAPs and thereby release active caspases. They bind to the BIRs via a conserved N-terminal IAP binding motif (IBM).^{4,5} Whereas XIAP is a strong inhibitor of caspases, other IAPs such as ML-IAP, ILP2 and also the baculoviral IAP, OpiAP, have minimal or no ability to inhibit caspases directly, but are nevertheless able to inhibit cell death.^{6–8} These IAPs may be able to act indirectly by sequestering IAP antagonists, just as XIAP mutants with no caspase inhibitory activity could still suppress cell death as long they retained the ability to bind to IAP antagonists.⁹

Direct IAP binding protein with low pI (Diablo)/second mitochondrial activator of caspases (Smac), HtrA2/Omi and GstPT/eRF3 are mammalian proteins that interact with IAPs via N-terminal IBMs.^{10–16} All are expressed constitutively in a wide range of cell types, although some transcriptional regulation of HtrA2 has also been demonstrated.¹⁷ Diablo and HtrA2 are imported into the mitochondria as precursor proteins from which the N-terminal mitochondrial import sequence is removed to produce the mature IAP-interacting form.¹⁸ In healthy cells, they reside within the mitochondrial intermembrane space, and come in contact with IAPs only after apoptotic stress when they are released into the cytosol.

The IAP binding form of GstPT/eRF3 is a proteolytically processed isoform of an endoplasmic reticulum-associated protein whose normal role is to act during translation as a polypeptide chain release factor.¹⁶ It is likely that proteolytic removal of the N-terminus of GstPT by an unknown protease produces the IAP binding form of GstP1 and releases it into the cytoplasm.

In previous screens for IAP binding proteins, we used 293T cells transiently expressing the bait protein, Flag-epitope-tagged mouse XIAP (F-mXIAP).^{10,15} Large-scale immunoprecipitates of F-mXIAP were separated by 2D IEF/SDS-PAGE and co-immunoprecipitating proteins were identified by

¹The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, Victoria 3050, Australia and ²The Murdoch Children's Research Institute, Parkville, Victoria, 3052, Australia

*Corresponding author: AM Verhagen, The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, Victoria 3050, Australia. Tel: 61 3 9345 2555; Fax: 61 3 9347 0852; E-mail: verhagen@wehi.edu.au

³Current address: Department of Biochemistry, La Trobe University, Bundoora Victoria, 3086, Australia

Keywords: XIAP; IAP antagonist; Diablo; Smac; mitochondria; apoptosis; glutamate dehydrogenase; Nipsnap; CLPX; L-rich PPR motif containing protein; 3-hydroxyisobutyrate dehydrogenase; IBM

Abbreviations: IAP, inhibitor of apoptosis; BIR, baculoviral IAP repeat; Diablo, direct IAP binding protein with low pI; Smac, second mitochondrial activator of caspases; GdH, glutamate dehydrogenase; LRPPR, leucine-rich pentatricopeptide repeat motif-containing protein; 3HB, 3 hydroxyisobutyrate dehydrogenase; Nsp, Nipsnap; IBM, IAP binding motif

Received 30.3.06; revised 10.5.06; accepted 22.5.06; Edited by G Melino; published online 23.6.06

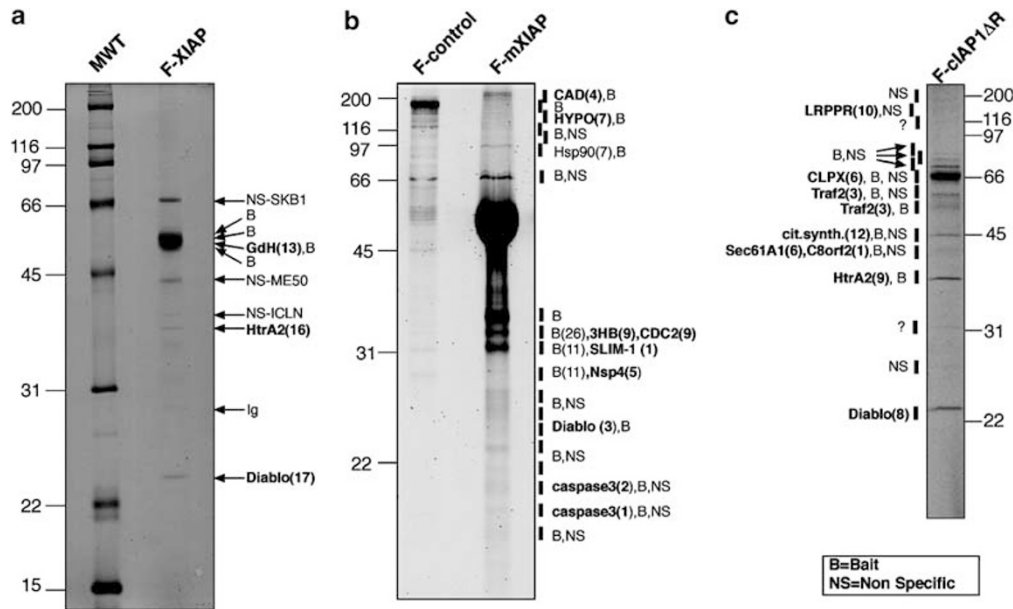


Figure 1 Proteomic screens for IAP interacting proteins (a) 293 cells stably expressing XIAP under a tetracycline-inducible promoter were treated with different apoptotic stimuli (UV, doxyrubicine, staurosporine, etoposide) and cytosolic extracts prepared at various time points post-treatment using a digitonin lysis procedure. The lysates, cleared of nuclear and cellular debris, were diluted with a more stringent Triton X-100-based lysis buffer. F-XIAP and interacting proteins were then isolated on Flag antibody columns, the pooled eluate concentrated, proteins separated by SDS-PAGE and visualised by sypro ruby gel staining. Gel slices were removed and mass spectrometry used to identify proteins. The number of peptides for which sequence was obtained is indicated in brackets. (b) F-mXIAP and interacting proteins were isolated from a combined pool of cytosolic extract from 293T cells transiently expressing F-mXIAP and cytosolic extract from NT2 cells harvested between 2 and 6 h following UV treatment. The immunoprecipitate was analysed as above. (c) F-clAP1 Δ R was immunoprecipitated from total cell lysates of transiently transfected 293T cells and analysed as described above

mass spectrometry. This approach allowed identification of Diablo/Smac and HtrA2/Omi.

In the present study, protein immunoprecipitates were separated by extended 1D SDS-PAGE, so that proteins with high molecular weight or extreme p/s that would normally be excluded by IEF/SDS-PAGE could be identified. In addition, lysates from both healthy and apoptotic cells were utilised to include proteins that may only be expressed following apoptotic stress.

Several new mammalian IAP binding proteins were identified that bind via N-terminal IBMs to conserved residues within the BIR2 domain of XIAP. Through this interaction, several of these proteins are able to antagonise XIAP inhibition of caspase 3 *in vitro*, and may serve to promote or accelerate apoptosis.

Results

Identification of novel IAP interacting proteins. For the first proteomic screen, 293 cells were used in which a single copy of a cDNA encoding C-terminally Flag-tagged human XIAP (F-XIAP) had been stably integrated under a tetracycline-inducible promoter (see Materials and Methods). These cells were exposed to a variety of apoptotic stimuli and were harvested at variable times post-treatment, and cytosolic extracts prepared using a digitonin lysis procedure. After clearing of nuclear and cellular debris, the lysates were then diluted with a more stringent Triton X-100-based lysis buffer and F-XIAP and co-immunoprecipitating proteins isolated by passing the lysate

through a column of Flag M2 antibody coupled to agarose beads. The eluted proteins were then separated by 1D SDS-PAGE electrophoresis and visualised by sypro ruby and Coomassie blue staining (Figure 1a).

In addition to Diablo and HtrA2, another mitochondrial protein, GdH, was detected. GdH comigrated with XIAP, and was not observed in immunoprecipitates of unrelated Flag-tagged proteins (data not shown). Although GdH is a mitochondrial matrix protein, it has previously been observed to be released following tBid treatment of liver mitochondria, suggesting either that some GdH may reside within the intermembrane space¹⁹ or that at late stages of apoptosis matrix proteins are also released.

In an additional screen, cytosolic extracts from NT2 cells were prepared between 2 and 6 h after treatment with UV. This extract was mixed with cytosolic extract from transiently transfected 293T cells expressing high levels of C-terminally F-mXIAP. As described above, F-mXIAP and coimmunoprecipitating proteins were isolated on Flag antibody columns and the proteins analysed by SDS-PAGE. In addition to Diablo and caspase 3, several additional proteins were identified that were not seen in control immunoprecipitates (Figure 1b).

A further screen was conducted for proteins that bind to cIAP1. In this experiment, 293T cells were transiently transfected with a construct that expressed a Flag-tagged form of cIAP1 lacking its RING domain (F-clAP1 Δ R), because this protein is much more stable than the full-length protein. F-clAP1 Δ R and co-immunoprecipitating proteins were isolated from total cell lysates and analysed as before (Figure 1c). Co-immunoprecipitating with cIAP1 were Diablo and HtrA2, as found previously.^{10–16} Another known cIAP1 interacting

wild-type XIAP or XIAP point mutants in which conserved residues within the BIR2 or BIR3 domain necessary for binding DIABLO and HtrA2 were mutated. As shown in Figure 3a, both GdH and Nsp4 retained interaction with the BIR3 domain mutant (E314S), whereas interaction was eliminated by mutation of the BIR2 domain (D214S). Furthermore, GdH was found to interact with XIAP fragments possessing only the BIR2 domain, but could not interact with the BIR3 domain on its own, even though well expressed (Figure 3b). The interaction of Nsp4 with fragments of XIAP was difficult to detect, although weak interaction with well-expressed fragments encompassing the BIR2 domain could be detected (Figure 3b).

Another mitochondrial protein, 3HB, was also found to interact specifically with XIAP, although this interaction appeared to be weaker than with the other proteins. In addition, whereas human 3HB could interact with F-XIAP and F-mXIAP, we could not demonstrate interaction of either protein with mouse 3HB (data not shown). Like GdH and Nsp4, human 3HB also appeared to interact predominately via

the BIR2 domain because the XIAP D214S mutant had bound very small amounts of 3HB (Figure 3c).

Interaction of GdH and Nsp4 with XIAP is mediated via N-terminal IBMs. Diablo, HtrA2 and processed caspase 9, like Grim, HID, Reaper and other IAP antagonists from *Drosophila*, interact with the BIR domains of IAPs via exposed N-terminal IBMs. The interaction of Nsp4 and GdH with XIAP involves residues within the XIAP BIR2 domain that are required for interaction of the XIAP BIR2 domain with Diablo and HtrA2. Diablo and HtrA2, however, also interact via the BIR3 domain of XIAP. To examine whether an N-terminal IBM was also required for Nsp4 and GdH, the N-terminal amino acid of the mature proteins was mutated to a glycine residue. For both of these proteins, this mutation eliminated interaction with XIAP (Figure 4a).

Nsp4 is highly related to another protein Nsp3, and the N-terminal 15 residues of Nsp3 and Nsp4 are identical. As expected, although Nsp3 was less well expressed than Nsp4, it was also able to interact with XIAP (Figure 4b).

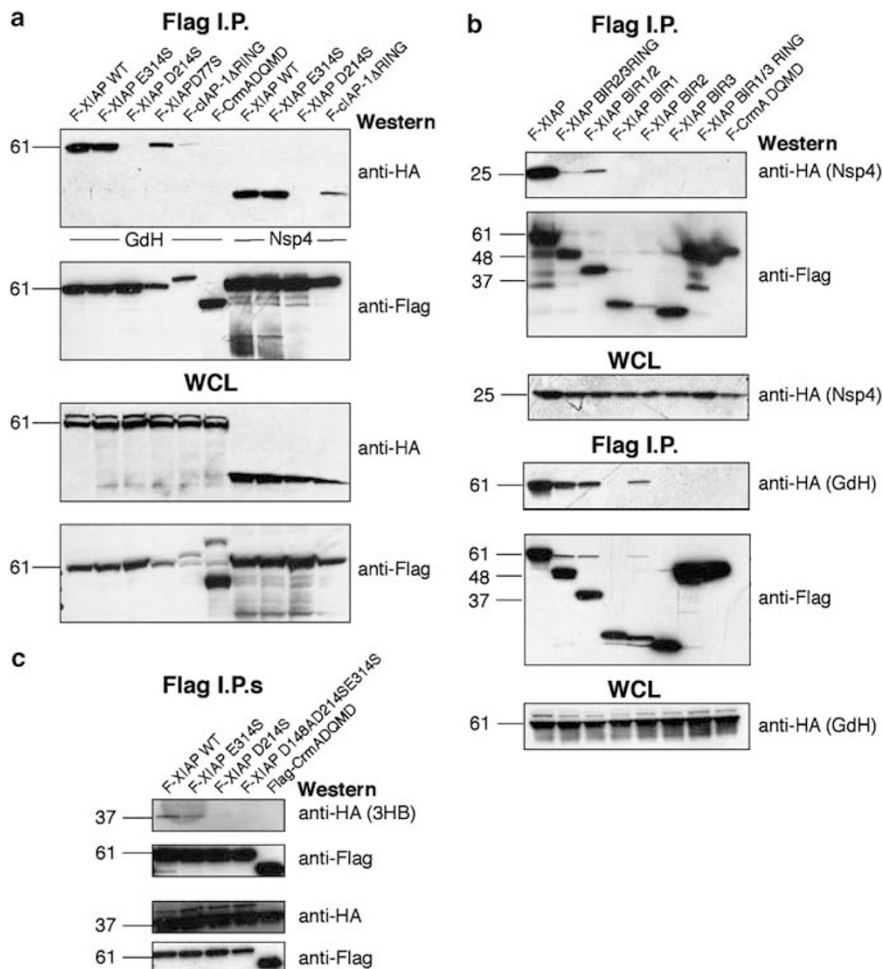


Figure 3 GdH, Nsp4 and 3HB interact with the XIAP BIR2 domain. (a) F-XIAP WT, F-XIAP BIR1 domain mutant (D77S), BIR2 domain mutant (D214S) or BIR3 domain mutant (E314S), F-cIAP1ΔR or F-DQMD, were immunoprecipitated from the lysate of transiently transfected 293T cells and the immunoprecipitate examined for co-immunoprecipitating HA-GdH or HA-Nsp4 by Western blot analysis. (b) F-XIAP WT and Flag-tagged XIAP domains were examined for the ability to interact with HA-GdH or HA-Nsp4. (c) Flag-tagged XIAP mutants and F-DQMD were examined for interaction with 3HB. The XIAP mutant D148AD214SE314S has a mutation of the caspase 3/7 interaction site within the BIR1/2 linker (D148) as well as mutations of both the BIR2 and BIR3 domains

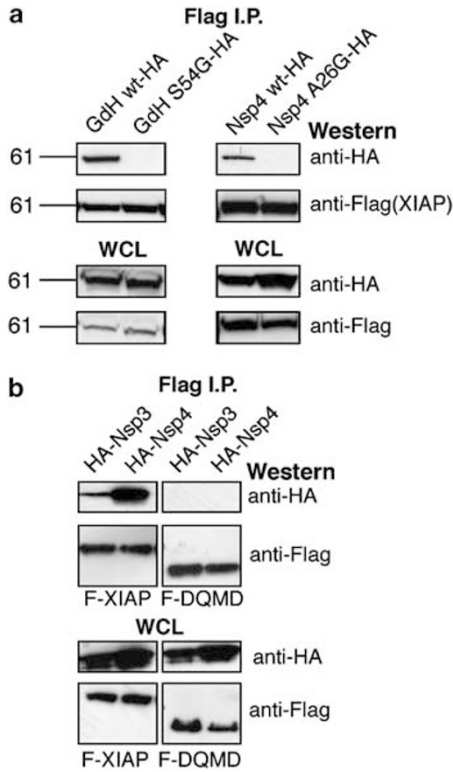


Figure 4 GdH and Nsp4 interact with XIAP via N-terminal IBMs. (a) F-XIAP was examined for interaction with wild-type GdH and Nsp4 proteins alongside GdH and Nsp4 mutants in which the N-terminal amino acid within the IBM of the mature protein has been mutated. (b) Nsp3, a highly related protein to Nsp4, was examined for its ability to interact with F-XIAP or control F-DQMD

CLPX and LRPPR interact with the XIAP BIR2 domain. Another two mitochondrial proteins, CLPX and LRPPR, identified in the screen for cIAP interacting proteins, were also able to interact with XIAP (Figure 2). As with Nsp4 and GdH, CLPX and LRPPR interacted with key residues in the BIR2 domain of XIAP, and mutation of residues within the XIAP BIR3 domain did not affect interaction (Figure 5a and b).

CLPX and LRPPR interact via N-terminal IBMs with IAPs. CLPX, a regulatory component of mitochondrial Clp protease (CLPP),²³ and LRPPR²⁴ are produced as precursor proteins from which a mitochondrial import sequence is removed. Cleavage is thought to occur after amino acid 64 in CLPX and 59 in LRPPR. For both of these proteins, cleavage of the import sequence would result in an alanine at the N-terminus. Mutation of this alanine within LRPPR to a glycine residue dramatically reduced its binding to both XIAP and cIAP1 to the level typically observed as nonspecific background binding for this protein in interaction with an unrelated protein (F-DQMD) (Figure 5c).

When CLPX was transiently expressed in 293T cells, both unprocessed and processed forms were readily detected (Figure 5c, third panel, lanes 3, 7 and 11). Mutation of the alanine of mature CLPX eliminated interaction of the processed form with both XIAP and cIAP1 (Figure 5c, top panel, lanes 4 and 8). Curiously, the apparently unprocessed

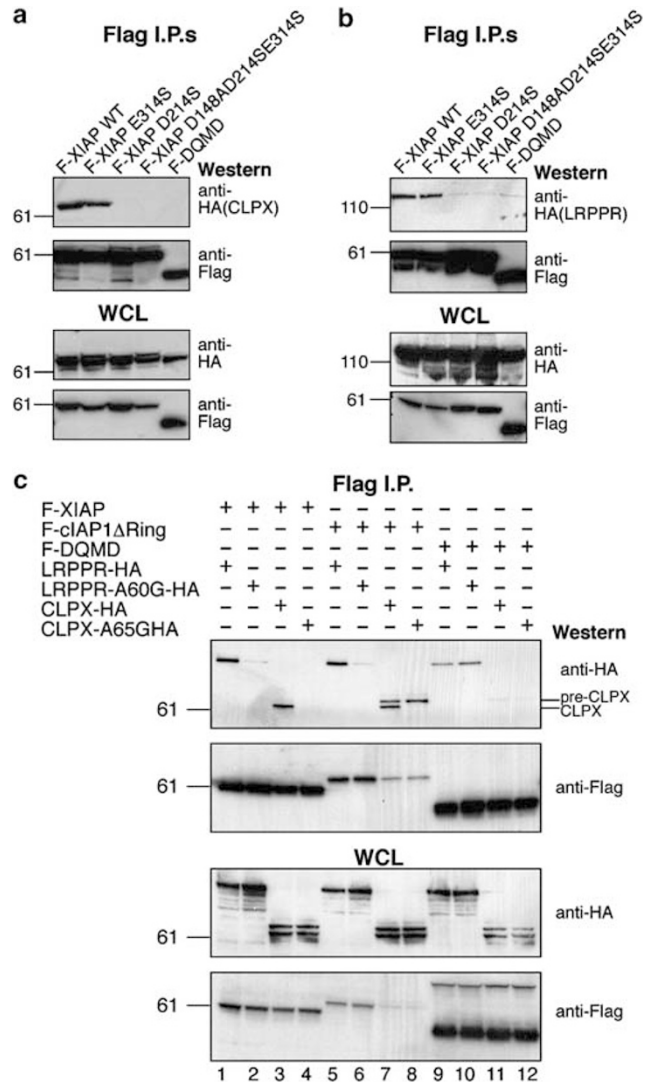


Figure 5 CLPX and LRPPR interact with XIAP via the XIAP BIR2 domain. F-XIAP WT and BIR domain mutants were examined for interaction with (a) C-terminally HA-tagged CLPX or (b) HA-LRPPR. (c) CLPX and LRPPR IBM point mutants were compared with their wild-type counterparts for interaction with F-XIAP, F-clAP1 Δ Ring and control F-DQMD

form of CLPX also appeared to be able to bind to cIAP1, but not with XIAP (Figure 5c, top panel, lane 3 versus lane 7), and this form retained interaction even when the N-terminal alanine of mature CLPX was mutated (Figure 5c, top panel, lane 8). The nature of this interaction is unclear, although a weaker nonspecific interaction of this unprocessed form was also noted with the control protein F-DQMD (Figure 5c, top panel, lane 11). Indeed, we have sometimes noticed nonspecific binding of other incompletely processed proteins, including Diablo (see Figure 2) with the control protein. It is possible that when the mitochondria are overloaded and unable to process rapidly all imported proteins, as may occur in transient overexpression, aggregates of incompletely processed forms occurs.

Slim1 was another protein that was co-immunoprecipitated with XIAP, although some weak binding to the control protein was also observed. Slim 1 is a nuclear protein that has a Lim

domain reported to confer E3 ligase activity.²⁵ Unlike the other proteins investigated, its interaction was not affected by mutation of either the BIR2 or BIR3 domain and did not resemble that of an antagonist (data not shown).

Most IBMs possess an N-terminal alanine residue, although serine can be tolerated in interaction with BIR2. CLPX, LRPPR, GdH, Nsp4 and 3HB interact with the BIR2 domain of XIAP via their processed N-termini. An alignment of the N-termini of these IAP binding proteins with previously described IAP antagonists and caspases is shown in Figure 6. All previously described IAP antagonists have an N-terminal alanine residue, and this was also observed with CLPX, LRPPR, Nsp4 and 3HB. Unlike them, GdH has an N-terminal serine residue; however, an N-terminal serine residue has also been demonstrated in one IAP binding form of caspase 7 and in interaction of peptides with the BIR2 domain (see Discussion).^{26,27}

GdH, LRPPR and Nsp4 antagonise XIAP inhibition of caspase 3 *in vitro*. The IAP binding proteins identified above all interact with the XIAP BIR2 domain. Therefore, we were interested in examining whether through this interaction these proteins were able to antagonise XIAP inhibition of caspase 3. As shown in Figure 7a, GdH and LRPPR were able to antagonise XIAP inhibition of caspase 3 *in vitro*. Caspase activity in the presence of Nsp4 was also slightly greater than in the presence of XIAP alone (Figure 7a), observed in four independent experiments (data not shown). For GdH, we were successful in purifying high levels of material, which enabled us to test this protein at a range of concentrations. As shown in Figure 7b, GdH antagonised

XIAP inhibition of caspase 3 in a dose-dependent manner. Furthermore, the purified mammalian GdH S/G mutant only increased caspase 3 activity slightly, indicating that the effect of GdH was dependent on the N-terminal serine residue through which it binds to XIAP. The small effect of the mutant at high concentrations might have been due to some wild-type GdH in multimeric complexes, as it was isolated from transiently transfected 293T cells that contain endogenous GdH.

In our previous studies, Diablo and HtrA2 were found to antagonise XIAP inhibition of UV-induced cell death. We therefore sought to test whether these newly identified IAP binding proteins could promote apoptosis in this assay. For these experiments, an NT2 cell line was used that is resistant to UV-induced apoptosis because of stable expression of an XIAP construct. Consistent with our previous studies,

Caspase 9	A	T	P	F	Q	E	G	L	R
Diablo/Smac	A	V	P	I	A	Q	K	S	E
HtrA2/Omi	A	V	P	S	P	P	P	A	S
GSPT/eRF3	A	K	P	F	V	P	N	V	H
Reaper	A	V	A	F	Y	I	P	D	Q
Grim	A	I	A	Y	F	I	P	D	Q
Hid	A	V	P	F	Y	L	P	E	G
Sickle	A	I	P	F	F	E	E	E	H
Jafrac2	A	K	P	E	D	N	E	S	C
Nsp4	A	T	G	P	R	Q	Y	D	G
GdH	S	E	A	V	A	D	R	E	D
Caspase 7	S	G	P	I	N	D	T	D	A
CLPX	A	S	K	D	G	I	S	K	D
LRPPR	A	I	A	A	K	E	K	D	I
3HB	A	S	K	T	P	V	G	F	I

Figure 6 Comparison of the N-termini of mammalian and *Drosophila* IAP binding proteins. Identical amino acids are given a black background, whereas similar residues have a dark grey background. Frequently observed alternative amino acids in the second and third position have been given a light grey background. Although most proteins have an N-terminal alanine residue, serine residues can be tolerated in interaction with the BIR2 domain as noted for one processed form of caspase 7 and for GdH. Valine in the second position and proline in the third position is evident in several IAP antagonists. Alternatively, three IAP binding proteins Grim, Reaper and LRPPR have an isoleucine residue in the second position and four proteins, Reaper, Grim, GdH and LRPPR, have an alanine residue in the third position

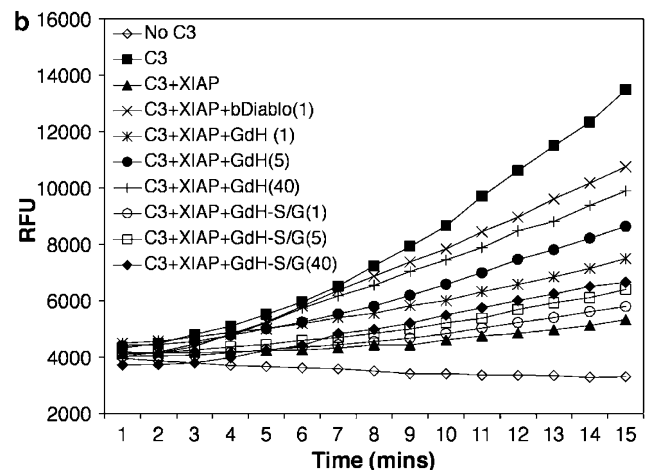
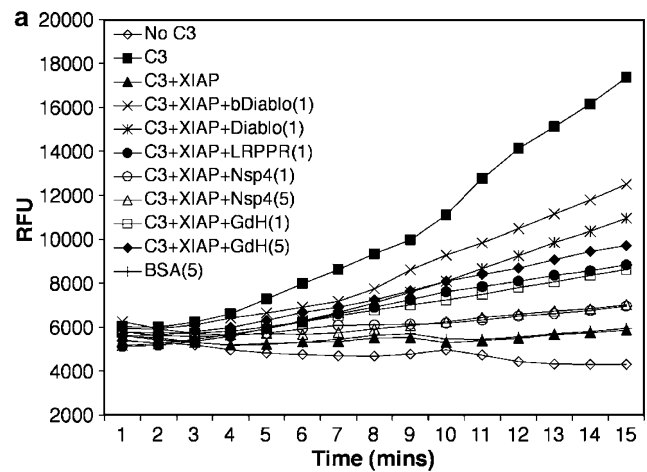


Figure 7 GdH, LRPPR and Nsp4 can antagonise XIAP inhibition of caspase 3 *in vitro*. (a) Recombinant active caspase 3 (approximately 15 ng) was mixed with XIAP alone (500 ng) or with XIAP premixed with BSA (5 μ g), bacterially expressed Diablo (bDiablo-1 μ g) or mammalian prepared Diablo (1 μ g), GdH (1 or 5 μ g), Nsp4 (1 or 5 μ g) or LRPPR (1 μ g). Caspase activity (relative fluorescent units – RFU) was measured at 37°C by cleavage of the fluorogenic substrate DEVD-AMC. (b) Mammalian GdH and its IBM mutant GdH S/G were compared at different concentrations (1, 5 and 40 μ g) for the ability to antagonise XIAP inhibition of caspase 3. Caspase assays were performed as described above

transiently expressed Diablo and HtrA2 were able to promote apoptosis of these cells following treatment with UV above that of control cells transiently transfected with a β -galactosidase expression construct. However, no increase in cell death was observed with the other IAP binding proteins GdH, Nsp4, CLPX, LRPPR and 3HB, despite reasonable expression for all proteins (Figure 8a and b). It is likely that because these proteins do not interact with the BIR3 domain, they are unable to antagonise XIAP inhibition of caspase 9, a necessary requirement for UV-induced cell death to proceed. They may, however, act at a later stage of cell death by antagonising XIAP inhibition of caspase 3. It is also possible that they have a role in promoting apoptosis through death receptor pathways, in the form of an amplification loop or in responses to granzyme B, where XIAP may have a role in inhibiting caspase 3 or caspase 7.^{28,29}

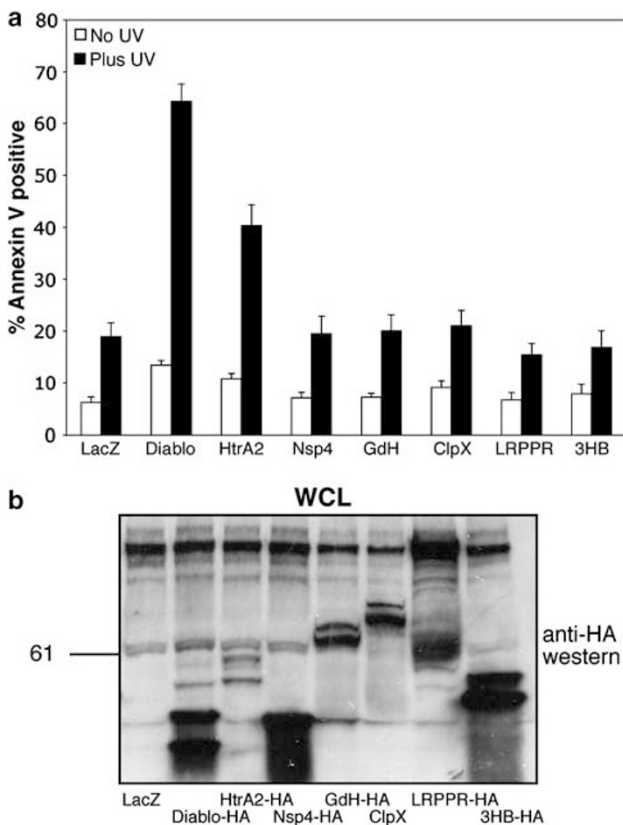


Figure 8 Unlike Diablo and HtrA2, other IAP binding proteins GdH, Nsp4, LRPPR, CLPX and 3HB do not antagonise XIAP inhibition of UV-induced cell death. (a) IAP binding proteins were examined for their ability to antagonise XIAP inhibition of UV-induced cell death of UV-resistant XIAP stably expressed NT2 cells. Cells were transiently transfected with mammalian expression constructs encoding HA-tagged proteins together with $\frac{1}{10}$ th concentration of GFP-encoding vector. After 48 h post-transfection, the cells were untreated or UV irradiated (50 J/M^2), returned to the incubator for 6 h and then harvested. The cells were then incubated with annexin V biotin followed by a tricolor-streptavidin conjugate and GFP-expressing cells, representing the transiently transfected population were selectively analysed for annexin V binding to indicate apoptotic cells. The results represent the mean plus standard error for six independent experiments, except for LRPPR and 3HB, which were tested in three experiments. (b) Expression of the IAP binding proteins in the transfected cells above (a) was examined by Western blot

Discussion

We have identified several additional mammalian proteins that, like Diablo and HtrA2, can bind to the IAPs XIAP and cIAP1. Strikingly, all of these proteins normally reside in the mitochondria, and bear an N-terminal IBM that is generated by proteolytic removal of a mitochondrial import sequence.

The proteins identified in this study all interact preferentially with the XIAP BIR2 domain. Whereas the requirements for interaction of Diablo with the XIAP BIR3 domain have been extensively characterised, much less is known about interaction with the XIAP BIR2 domain. For XIAP-BIR3-Diablo interaction, only the first four amino acids of Diablo are critical, these residues fitting snugly within the surface groove of BIR3.^{5,30} There appears to be additional requirement for interaction of Diablo with XIAP BIR2 fragments and monomeric mutants of Diablo, normally a dimer, do not interact. It is possible that additional amino acids are important for interaction with XIAP BIR2 similarly to interaction of *Drosophila* IAP antagonists Grim and Hid with DIAP1 BIR2.³¹ It is likely, however, that the first 3–4 residues in the IBM are critical for BIR2 interaction and that the more C-terminal residues contribute to the strength of interaction, but cannot in the absence of acceptable residues in the first positions allow binding.

Like all previously described IAP antagonists, the mature forms of Nsp4, CLPX, LRPPR and 3HB each have an amino-terminal alanine residue; however, mature GdH has an amino-terminal serine residue. An N-terminal serine residue is so far the only known exception to the requirement for an N-terminal alanine and occurs in caspase 8 processed caspase 7 allowing caspase 7 binding to the BIR2 of XIAP.²⁶

The occurrence of serine containing IBMs binding to the BIR2 of XIAP is also consistent with a study using phage display that showed that 1% of phage display peptides capable of binding XIAP BIR2 bore N-terminal serine residues,²⁷ whereas alanine was the only N-terminal amino acid observed in peptides that bound to the BIR3 domain of XIAP. Interestingly, the favoured amino acids for the second, third and fourth positions in XIAP BIR2 interactions of phage display peptides were E (45%), A (51%) and V (65%), which are identical to the second, third and fourth amino acids of mature GdH, perhaps, implying that GdH's interaction with the BIR2 of XIAP is likely to be relatively strong and has been selected for. Furthermore, A and D in the fifth and sixth positions were observed in 9.2 and 8.2% of phage display peptides.²⁷

The analysis of XIAP BIR2 interacting peptides by Franklin *et al.*²⁷ gives a valuable insight into requirements for protein interaction with this domain. The N-terminal three amino acids of LRPPR, (AIA), are identical to those of the *Drosophila* IAP antagonist Grim, and were also commonly seen among phage display peptides binding to XIAP BIR2,²⁷ with frequencies of 98, 8.5 and 51%, respectively. The next three amino acids of the LRPPR IBM, AKE were observed with frequencies of 1.4, 0 and 13%, respectively. Although no lysine residues were observed by phage display in the fifth position, the high frequencies for the other positions promote a strong interaction of LRPPR with XIAP BIR2. Although the three N-terminal residues of Nsp4 (and Nsp3) (ATG) were observed in

BIR2-binding phage display peptides with frequencies of 98, 12, 15%, the next three residues were not observed and therefore perhaps not critical for interaction, but do not favour such a strong interaction as for LRPPR.²⁷ Consistent with these observations, LRPPR was more effective at antagonising XIAP inhibition of caspase 3 *in vitro* than Nsp4, suggesting a higher affinity interaction.

The three N-terminal residues of CLPX and 3HB (ASK) were observed with frequencies of 98, 0.7 and 3.1% in XIAP BIR2-interacting peptides,²⁷ with low frequencies in the second and third position likely to negatively impact on interaction of these proteins with XIAP. This is probably compensated for in the case of CLPX by a favourable amino acid in position 5 (G) observed in 12% of peptides, and in 3HB by amino acids in positions 5 and 6 (PV) observed in 13 and 5.2% of cases.

Whereas an N-terminal IBM is essential for IAP interaction, it seems likely that the multimeric nature of several of the IAP binding proteins we have identified also enhances their capacity to interact with IAPs, as noted for Diablo, a dimer, and HtrA2, a hexamer.

Whether these IAP binding proteins have a role in promoting apoptosis is unclear. Their selective binding to the BIR2 domain and not the BIR3 domain suggests that they would not be able to antagonise inhibition of caspase 9 by XIAP. Indeed, none of these proteins overcame XIAP inhibition of UV-induced cell death in a model where XIAP acts by binding to caspase 9 via BIR3. In contrast, Diablo and HtrA2, proteins that bind to both the BIR3 and BIR2 domains of XIAP, were able to antagonise XIAP inhibition of UV-induced cell death.

Selective binding of IAP antagonists to distinct BIRs has been observed in *Drosophila*,³² with Hid binding selectively to the second BIR of DIAP1. The significance of this selective BIR binding is still unclear, but appears to influence the ability of DIAP1 mutants to prevent cell death induced by over-expression of these IAP antagonists in the *Drosophila* eye.^{33,34}

The N-termini of the proteins described in this study enable them to bind specifically to the XIAP BIR2 and, through this interaction, GdH, LRPPR and, to a lesser extent, Nsp4 were able to antagonise XIAP inhibition of caspase 3 *in vitro*. Although there may be some biological role of this interaction in intrinsic cell death pathways, it would probably occur late during apoptosis, and may serve only to accelerate the cell death process. The IAP binding proteins we have described may, however, be more likely to assist in amplification of death receptor pathways or responses to granzyme B, as these pathways do not involve initiator caspases inhibitable by IAPs but do nevertheless require IAP inhibitable effector caspases 3 and 7.^{28,29}

The C-terminal RING domain of IAPs possesses E3 ligase activity, an activity exploited by IAPs to regulate their own stability as well as that of other interacting proteins, including other IAPs.^{3,35} IAP antagonists not only compete for caspase interaction, in several circumstances they have been shown to promote IAP ubiquitination and degradation, an effect that is largely dependent on the C-terminal IAP RING domain.² It will be interesting to examine whether cytoplasmically expressed forms of the IAP binding proteins described in

the present study also have the potential to regulate IAP stability.

Another possibility is that, rather than these new proteins being antagonists of IAPs, these interactions allow IAPs to target mislocalised mitochondrial proteins for ubiquitination and disposal by the proteasome. In this regard, it is interesting that all of the mammalian IAP binding proteins described to date, with the exception of Diablo, appear to have other essential functions in the cell. HtrA2/Omi is a serine protease and chaperone protein required for mitochondrial maintenance and, whereas HtrA2-deficient mice have many gross abnormalities associated with mitochondrial dysfunction, none suggest a defect in cell death pathways.³⁶ GstPT/eRF3 is a proteolytically processed isoform of an endoplasmic reticulum-associated protein, with a role in translation as a polypeptide chain release factor.¹⁶ GdH and 3HB are mitochondrial metabolic enzymes. CLPX is a hexameric protein that regulates the activity of CLPP, another serine protease with chaperone activity.²³ The function of Nsp4 or other Nsp proteins is not clear and although it has been suggested that these proteins have a role in vesicular transport and may be associated with lipid rafts,^{21,22} our results for Nsp4 suggest it is a mitochondrial protein because it bears a classic mitochondrial import sequence that is removed to generate the mature protein. LRPPR is thought to be an RNA binding protein involved in mitochondrial RNA transcript processing. Mutations of the LRPPR gene have been observed in Leigh syndrome, French-Canadian type, a disease characterised by cytochrome *c* oxidase deficiency.²⁴

The fact that a subset of processed mitochondrial proteins described here such as GdH have ideal IBMs for binding the BIR2 of XIAP, and do bind XIAP, suggests that the interaction has been selected for and is important. However, it is impossible to be sure that this interaction is physiologically important for regulating developmental apoptosis. For example, mice deficient for Diablo/Smac, the most convincing mammalian IAP antagonist with the highest affinity IAP interactions, and with no other known function, appears to be normal.³⁷ This is in direct contrast to the profound developmental and apoptotic defects observed when *Drosophila* IAP antagonists are deleted. These data suggest that other IAP binding proteins, such as HtrA2, GstPT and the mitochondrial IAP binding proteins described in the present study, can compensate for the loss of Diablo, and it is important therefore to recognise the numbers of potential IAP antagonists secreted within the mitochondria. The observation that cells from mice doubly deficient for Diablo/Smac and HtrA2/Omi do not show increased resistance to cell death may suggest compensation from IAP antagonists other than HtrA2.³⁸ Alternatively, it is possible that IAPs and their antagonists do not have a significant role in mammalian developmental cell death. IAPs also have a so far poorly characterised nonapoptotic role interfacing with the TNF and TGF receptor superfamilies and thus a third possibility remains that the role of IAP antagonist proteins is to regulate IAP interactions at these points. Ultimately, therefore, a clear understanding of the importance of IAP antagonism in mammals will require a more thorough understanding of the exact role of IAPs themselves.

Materials and Methods

Constructs. Mammalian expression constructs (pEF BOS) encoding C- and N-terminally F-XIAP, C-Flag-tagged XIAP mutants, N-terminally Flag-tagged CrmA^{DQMD} variant (F-DQMD), C-terminally Flag-tagged MIH Δ Ring (F-clAP Δ R) and C-terminally HA-tagged mouse Htra2 as well as a pcDNA3 expression construct encoding mouse C-terminally HA-tagged Diablo have been described previously.^{9,10,15} For all new candidate IAP binding proteins, cDNA fragments with Kozak initiation sites were amplified either from human kidney marathron cDNA (Clontech, CA, USA) or from ATCC clones and were subcloned into pcDNA3.HA1 or pcDNA3Flag1 vectors as indicated (cloning vectors kindly provided by Dr David Huang). Human CLPX cDNA and LRPPR cDNA were amplified from ATCC clones 7313841 (Genbank BM800613) and 9125880 (BC050311), respectively. cDNAs encoding the C-terminally HA-tagged proteins were also removed from pcDNA3HA1 and subcloned into pEF Bos mammalian expression vectors. Overlap PCR technology was used to generate pcDNA3 or pEF expression vectors encoding C-HA-tagged Nipsnap 4 (Nsp4) A26G mutant, glutamate dehydrogenase (GdH) S54G mutant, CLPX A65G mutant and LRPPR A60G mutant.

Bacterial expression constructs pGEX-6P-3 XIAP, encoding GST-XIAP and pET24b Diablo, encoding Delta N (mature) Diablo with a C-terminal Hex-His tag have been described previously.¹⁵

Cell lines. The HEK293T, human Flp-InTM T-TRExTM 293 cells (Invitrogen) and the human teratocarcinoma cell line NT2 were grown in DME media supplemented with 10% fetal calf serum (FCS), 1% penicillin–streptomycin and 2% glutamine. NT2 cell lines stably transfected with vectors encoding human C-Flag XIAP have been described previously.^{10,15,39} Flp-InTM T-TRExTM 293 cells with a single integrated copy of a cDNA encoding C-terminally Flag-tagged XIAP under the control of tetracycline-inducible promoter have been described previously.⁴⁰ Expression of Flag-XIAP was induced by the overnight addition of doxycycline.

Transfections and cell death assays. 293T cells were transiently transfected with various constructs using EffecteneTM (Qiagen, Germany) according to the manufacturer's instructions. Typically, 1–2 μ g of DNA was used per 10 cm plate of cells. For the transfection of NT2 cells, six-well plates were seeded with 3×10^5 NT2 cells/well. Several hours later, each well of cells was transiently transfected with 1 μ g pEF expression constructs plus 100 ng pEGFP using Fugene (Qiagen) at a 3:1 Fugene:DNA ratio (see manufacturer's instructions). Forty-eight hours post-transfection, the cells were left untreated or UV-irradiated (50 J/M²) and then returned to the incubator for 6 h before cell viability was assessed. Harvested cells were incubated with biotinylated annexin V (Molecular Probes) followed by incubation with Streptavidin-Tricolor (Caltag). GFP-positive cells were analysed by flow cytometry for annexin V positivity as a measure of dead cells.

Immunoprecipitations and Western blot analysis. For total cell extracts, 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, and the nuclear and cellular debris cleared by centrifugation. Immunoprecipitations were performed using Flag-specific monoclonal antibody (mAb) M2 covalently coupled agarose beads (Sigma, Australia). The immunoprecipitates were washed five times in lysis buffer and proteins eluted with 100 μ M glycine (pH 3) and then neutralised with $\frac{1}{10}$ th volume of 1 M Tris (pH 8). Proteins were separated by SDS-PAGE. Immunoprecipitates were examined by Western blot analysis following transfer of proteins to nitrocellulose membranes (Hybond C-Extra, Amersham, UK). mAbs used for Western blots were anti-Flag M2 (Sigma) and anti-HA High-Affinity 3F10 (Boehringer Mannheim, Mannheim, Germany) Proteins were visualised by ECL (Amersham, UK) following incubation of membranes with HRP-coupled secondary antibodies.

Proteomic screens. For the isolation of XIAP interacting proteins, the 293 XIAP-inducible line was initially incubated with doxycycline overnight to induce F-XIAP. Cells were then treated with etoposide (10–25 μ g/ml), staurosporine (10 nM–10 μ M), doxorubicin (0.4–2 μ M) or UV-irradiation (25–100 J/M²). Between 5 and 12 h later, the cells were harvested, and cells lysed for 15 min in a digitonin lysis buffer (0.025% digitonin in 250 mM sucrose, 20 mM HEPES (pH 7.4), 5 mM MgCl₂, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 10 mM Tris (pH 7.4), 10 μ g/ml aprotinin, 10 μ g/ml leupeptin and 1 mM PMSF). A brief centrifugation (2 min, 5000 \times g) was performed and the supernatant retained (cytosolic fraction). The extract was then

diluted 1:4 with 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, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin and 1 mM PMSF) and passed through columns of Flag-M2 antibody coupled to agarose beads. Following extensive washing, F-XIAP and co-immunoprecipitating proteins were eluted from the column using acidic glycine (100 μ M, pH 3), and then neutralised with a final concentration of 100 mM Tris (pH 8). The elutions from five separate column procedures, using lysates from a total of 128 \times 15 cm plates of cells, were pooled and the sample concentrated by ultrafiltration using an Amicon ultrafiltration unit (molecular weight cutoff 10 kDa, Amicon). The sample was then separated by SDS-PAGE on 12% long (22 cm) gels (Hoefer). The proteins were initially visualised following staining with sypro ruby (Molecular Probes, Invitrogen), according to the manufacturer's instruction, followed by Coomassie blue staining with Phast-Blue Coomassie (Pharmacia).

In a second screen for XIAP interacting proteins, the cytosolic extract from 40 \times 15 cm plates of 293T cells transiently transfected with F-mXIAP was combined with the cytosolic extract from 120 plates of WT NT2 cells and 120 plates of the XIAP NT2 line prepared between 3 and 5 h following 50 J/M² UV. The pooled extract was diluted with a Triton X-100-based lysis buffer and F-XIAP and interacting proteins prepared on a column of Flag-antibody beads as described above.

For the identification of clAP1 interaction proteins, 30 \times 15 cm plates of 293T cells were transiently transfected with pEF Bos vector encoding F-clAP1 Δ R. Forty-eight hours post-transfection, total cell lysate was prepared using a Triton X-100-based lysis buffer (above) and F-clAP1 Δ R and interacting proteins immunoprecipitated using a Flag-antibody column as described above.

Protein analysis and mass spectrometry. Protein bands of interest were excised, digested *in situ* with trypsin and analysed by tandem mass (ms/ms) spectrometry as described previously.^{10,15} The mass spectrometry and peptide sequence analysis was performed at the Joint Protein Structure Facility (JPSL), Melbourne, Australia. The number of identifying peptides and coverage for the individual proteins was as follows: LRPPR – 10 peptides, 8% coverage; CLPX – 6 peptides, 12% coverage; Nsp4 – 5 peptides, 23% coverage; GdH – 13 peptides, 29% coverage; 3 HB – 11 peptides, 39% coverage; Slim 1 – 1 peptide, 6% coverage; citrate synthase – 12 peptides, 23% coverage. To obtain N-terminal sequence information for Nsp4, C-terminally Flag-tagged Nsp4 was immunoprecipitated from 293T cells transiently transfected with a vector encoding the full-length protein. The immunoprecipitate was separated by SDS-PAGE and transferred to PVDF membrane. Nsp4 protein was visualised by light staining with Coomassie blue and the band submitted to the JPSL where N-terminal sequence determination was performed.

Preparation of recombinant protein. Recombinant XIAP was produced as described previously.¹⁵ In brief, GST-XIAP protein was produced in *Escherichia coli* transformed with a vector encoding GST-XIAP (pGEX-6P-3 XIAP) and was affinity purified on glutathione sepharose. XIAP was released by incubation of the resin with PreScission protease (Pharmacia) in release buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.01% Triton X-100, 1 mM DTT, pH 7) overnight at 4°C. Recombinant mature Diablo was similarly generated in bacteria but with a C-terminal Hex-His tag. The protein was purified on Ni-NTA agarose beads (Qiagen) and released using Imidazole release buffer (50 mM Na₂HPO₄, 300 mM NaCl, 250 mM imidazole). IAP binding proteins Diablo, GdH, Nsp4 and LRPPR were prepared as C-terminally Flag-tagged proteins from transiently transfected 293T cells. They were purified from total cell lysates using anti-Flag M2 affinity gel and were eluted from the column with Flag peptide (Sigma). All proteins were dialysed against PBS and concentrated using Amicon Ultrafiltration Units (molecular weight cutoff 10 kDa). The purity of all proteins was assessed by SDS-PAGE followed by staining of the gel with Coomassie blue and protein concentrations estimated by comparison to proteins of known concentration in combination with measurement by Bradford assay (Biorad).

Caspase cleavage assay. Caspase 3 activity was detected by cleavage of the fluorogenic substrate DEVD-AMC (Bachem). Caspase 3 (approximately 15 ng) was incubated in 1 \times Caspase cleavage buffer (0.05 M HEPES (pH 7.5), 5% PEG4000, 0.05% CHAPS, 10 mM DTT) in the presence or absence of XIAP (500 ng), mature recombinant bacterially prepared Diablo (1 μ g), mammalian-expressed and purified IAP binding proteins Diablo, GdH, GdH S/G mutant Nsp4 and LRPPR at various concentrations as indicated or BSA (5 μ g) in a final volume of 50 μ l. Caspase 3 was added last to the mix after XIAP had been allowed to preincubate with IAP antagonists on ice for 30 min. The mixes were incubated for a

forther 5 min on ice after the addition of the caspase. The substrate DEVD-AMC was then added to a final concentration of 25 μ M and fluorescent emission, indicating substrate cleavage, was determined at 37°C with 1-min intervals on a fluorimeter (Tecan) using excitation and emission filters of 360 and 465 nm, respectively.

Cell death assays. The teratocarcinoma cell line NT2 was grown in DMEM supplemented with 10% FCS, 1% penicillin–streptomycin and 2% glutamine. Stable NT2 lines expressing F-XIAP have been described previously.^{10,39} Cells were transfected as described above. After 48 h post-transfection, the cells were left untreated or UV-irradiated (50 J/M²) and then returned to the incubator for 6 h before cell viability was assessed. Harvested cells were incubated with biotinylated annexin V (Molecular Probes) followed by incubation with Streptavidin-Tricolor (Caltag). GFP-positive cells were analysed by flow cytometry for annexin V binding to detect dead cells.

Acknowledgements. We acknowledge Lisa Connolly, Andrew Clippingdale and Robert Moritz from the Joint Protein Service Facility for their mass spectrometric analysis, Diep Chu for recombinant Diablo protein and David Huang for vectors provided. AMV is a recipient of an Australian Research Council (ARC) QEII fellowship. DLV is an National Health and Medical Research (NHMRC) Senior Principle Research Fellow. PGE, JS and CJH are all supported by NHMRC Career Development fellowships. This work was supported by NHMRC Program Grant (257502) and a Leukemia and Lymphoma Society Center Grant. CH's work is supported by grants from the ARC (#DP0343431) and NHMRC (#284513).

- Verhagen AM, Coulson EJ, Vaux DL. Inhibitor of apoptosis proteins and their relatives: IAPs and other BIRPs. *Genome Biol* 2001; **2**: 3009.1–3009.10.
- Gerl RE, Verhagen AM. IAPs, their antagonists and their role in neurological disease and cancer. *Curr Med Chem – Anti-Inflamm Anti-Allergy Agent* 2005; **4**: 393–406.
- Vaux DL, Silke J. IAPs, RINGs and ubiquitylation. *Nat Rev Mol Cell Biol* 2005; **6**: 287–297.
- Liu ZH, Sun CH, Olejniczak ET, Meadows RP, Betz SF, Oost T *et al*. Structural basis for binding of Smac/DIABLO to the XIAP BIR3 domain. *Nature* 2000; **408**: 1004–1008.
- Wu G, Chai JJ, Suber TL, Wu JW, Du CY, Wang XD *et al*. Structural basis of IAP recognition by Smac/DIABLO. *Nature* 2000; **408**: 1008–1012.
- Shin H, Renatus M, Eckelman BP, Nunes VA, Sampaio CA, Salvesen GS. The BIR domain of IAP-like protein 2 is conformationally unstable: implications for caspase inhibition. *Biochem J* 2005; **385**: 1–10.
- Vucic D, Franklin MC, Wallweber HJ, Das K, Eckelman BP, Shin H *et al*. Engineering ML-IAP to produce an extraordinarily potent caspase 9 inhibitor: implications for Smac-dependent anti-apoptotic activity of ML-IAP. *Biochem J* 2005; **385**: 11–20.
- Wilkinson JC, Wilkinson AS, Scott FL, Csomos RA, Salvesen GS, Duckett CS. Neutralization of Smac/Diablo by inhibitors of apoptosis (IAPs). A caspase-independent mechanism for apoptotic inhibition. *J Biol Chem* 2004; **279**: 51082–51090.
- Silke J, Hawkins CJ, Ekert PG, Chew J, Day CL, Pakusch M *et al*. The anti-apoptotic activity of XIAP is retained upon mutation of both the caspase 3- and caspase 9-interacting sites. *J Cell Biol* 2002; **157**: 115–124.
- Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE *et al*. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* 2000; **102**: 42–53.
- Du C, Fang M, Li Y, Li L, Wang X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* 2000; **102**: 33–42.
- Suzuki Y, Imai Y, Nakayama H, Takahashi K, Takio K, Takahashi R. A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. *Mol Cell* 2001; **8**: 613–621.
- Hegde R, Srinivasula SM, Zhang Z, Wassell R, Mukattash R, Cilenti L *et al*. Identification of Omi/HtrA2 as a mitochondrial apoptotic serine protease that disrupts inhibitor of apoptosis protein–caspase interaction. *J Biol Chem* 2001; **277**: 432–438.
- Martins LM, Iaccarino I, Tenev T, Gschmeissner S, Totty NF, Lemoine NR *et al*. The serine protease Omi/HtrA2 regulates apoptosis by binding XIAP through a reaper-like motif. *J Biol Chem* 2001; **277**: 439–444.
- Verhagen AM, Silke J, Ekert PG, Pakusch M, Kaufmann H, Connolly LM *et al*. HtrA2 promotes cell death through its serine protease activity and its ability to antagonize inhibitor of apoptosis proteins. *J Biol Chem* 2002; **277**: 445–454.
- Hegde R, Srinivasula SM, Datta P, Madesh M, Wassell R, Zhang Z *et al*. The polypeptide chain-releasing factor GSPT1/eRF3 is proteolytically processed into an IAP-binding protein. *J Biol Chem* 2003; **278**: 38699–38706.
- Jin S, Kalkum M, Overholzer M, Stoffel A, Chait BT, Levine AJ. CIAP1 and the serine protease HTRA2 are involved in a novel p53-dependent apoptosis pathway in mammals. *Genes Dev* 2003; **17**: 359–367.
- Burri L, Strahm Y, Hawkins CJ, Gentle IE, Puryer MA, Verhagen A *et al*. Mature DIABLO/Smac is produced by the IMP protease complex on the mitochondrial inner membrane. *Mol Biol Cell* 2005; **16**: 2926–2933.
- Van Loo G, Demol H, van Gurp M, Hoorelbeke B, Schotte P, Beyaert R *et al*. A matrix-assisted laser desorption/ionization post-source decay (MALDI-PSD) analysis of proteins released from isolated liver mitochondria treated with recombinant truncated Bid. *Cell Death Differ* 2002; **9**: 301–308.
- Rothe M, Pan MG, Henzel WJ, Ayres TM, Goeddel DV. The TNF-R2–TRAF signaling complex contains two novel proteins related to baculoviral-inhibitor of apoptosis proteins. *Cell* 1995; **83**: 1243–1252.
- Lee AH, Zareei MP, Daefler S. Identification of a NIPSNAP homologue as host cell target for *Salmonella* virulence protein SpiC. *Cell Microbiol* 2002; **4**: 739–750.
- Buechler C, Bodzioch M, Bared SM, Sigrüener A, Boettcher A, Lapicka-Bodzioch K *et al*. Expression pattern and raft association of NIPSNAP3 and NIPSNAP4, highly homologous proteins encoded by genes in close proximity to the ATP-binding cassette transporter A1. *Genomics* 2004; **83**: 1116–1124.
- Kang SG, Dimitrova MN, Ortega J, Ginsburg A, Maurizi MR. Human mitochondrial ClpP is a stable heptamer that assembles into a tetradecamer in the presence of ClpX. *J Biol Chem* 2005; **280**: 35424–35432.
- Mootha VK, Lepage P, Miller K, Kunkenborg J, Reich M, Hjerrild M *et al*. Identification of a gene causing human cytochrome c oxidase deficiency by integrative genomics. *Proc Natl Acad Sci USA* 2003; **100**: 605–610.
- Tanaka T, Soriano MA, Grusby MJ. SLIM is a nuclear ubiquitin E3 ligase that negatively regulates STAT signaling. *Immunity* 2005; **22**: 729–736.
- Scott FL, Denault JB, Riedl SJ, Shin H, Renatus M, Salvesen GS. XIAP inhibits caspase-3 and -7 using two binding sites: evolutionarily conserved mechanism of IAPs. *EMBO J* 2005; **24**: 645–655.
- Franklin MC, Kadkhodayan S, Ackerly H, Alexandru D, Distefano MD, Elliott LO *et al*. Structure and function analysis of peptide antagonists of melanoma inhibitor of apoptosis (ML-IAP). *Biochemistry* 2003; **42**: 8223–8231.
- Sutton VR, Wowk ME, Cancilla M, Trapani JA. Caspase activation by granzyme B is indirect, and caspase autoprocessing requires the release of proapoptotic mitochondrial factors. *Immunity* 2003; **18**: 319–329.
- Goping IS, Barry M, Liston P, Sawchuk T, Constantinescu G, Michalak KM *et al*. Granzyme B-induced apoptosis requires both direct caspase activation and relief of caspase inhibition. *Immunity* 2003; **18**: 355–365.
- Chai J, Du C, Wu JW, Kyin S, Wang X, Shi Y. Structural and biochemical basis of apoptotic activation by Smac/DIABLO. *Nature* 2000; **406**: 855–862.
- Wu JW, Cocina AE, Chai J, Hay BA, Shi Y. Structural analysis of a functional DIAP1 fragment bound to grim and hid peptides. *Mol Cell* 2001; **8**: 95–104.
- Zachariou A, Tenev T, Goyal L, Agapite J, Steller H, Meier P. IAP-antagonists exhibit non-redundant modes of action through differential DIAP1 binding. *EMBO J* 2003; **22**: 6642–6652.
- Lisi S, Mazzon I, White K. Diverse domains of THREAD/DIAP1 are required to inhibit apoptosis induced by REAPER and HID in *Drosophila*[in process citation]. *Genetics* 2000; **154**: 669–678.
- Goyal L, McCall K, Agapite J, Hartwig E, Steller H. Induction of apoptosis by *Drosophila* Reaper, Hid and Grim through inhibition of IAP function. *EMBO J* 2000; **19**: 589–597.
- Silke J, Kratina T, Chu D, Ekert PG, Day CL, Pakusch M *et al*. Determination of cell survival by RING-mediated regulation of inhibitor of apoptosis (IAP) protein abundance. *Proc Natl Acad Sci USA* 2005; **102**: 16182–16187.
- Jones JM, Datta P, Srinivasula SM, Ji W, Gupta S, Zhang Z *et al*. Loss of Omi mitochondrial protease activity causes the neuromuscular disorder of mnd2 mutant mice. *Nature* 2003; **425**: 721–727.
- Okada H, Suh W-K, Jin J, Woo M, Du C, Elia A *et al*. Generation and characterization of Smac/DIABLO-deficient mice. *Mol Cell Biol* 2002; **22**: 3509–3517.
- Martins LM, Morrison A, Klupsch K, Fedele V, Moiso N, Teismann P *et al*. Neuroprotective role of the Reaper-related serine protease HtrA2/Omi revealed by targeted deletion in mice. *Mol Cell Biol* 2004; **24**: 9848–9862.
- Ekert PG, Silke J, Hawkins CJ, Verhagen AM, Vaux DL. DIABLO promotes apoptosis by removing MIHA/XIAP from processed caspase 9. *J Cell Biol* 2001; **152**: 483–490.
- Silke J, Kratina T, Ekert PG, Pakusch M, Vaux DL. Unlike Diablo/smac, Grim promotes global ubiquitination and specific degradation of X chromosome-linked inhibitor of apoptosis (XIAP) and neither cause apoptosis. *J Biol Chem* 2004; **279**: 4313–4321.