Unlike Diablo/smace, Grim Promotes Global Ubiquitination and Specific Degradation of X Chromosome-linked Inhibitor of Apoptosis (XIAP) and Neither Cause Apoptosis*

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Grim is a Drosophila inhibitor of apoptosis (IAP) antagonist that directly interferes with inhibition of caspases by IAPs. Expression of Grim, or removal of DIAP1, is sufficient to activate apoptosis in fly cells. Transient expression of Grim in mammalian cells induces apoptosis, arguing for the conservation of apoptotic pathways, but cytoplasmic expression of the mammalian IAP antagonist Diablo/smace does not. To understand why, we compared Grim and Diablo. Although they have the same IAP binding specificity, only Grim promoted XIAP ubiquitination and degradation. Grim also synergized with XIAP to promote an increase in total cellular ubiquitination, whereas Diablo antagonized this activity. Surprisingly, Grim-induced ubiquitination of XIAP did not require the IAP RING finger. Analysis of a Grim mutant that promoted XIAP degradation, but was not cytotoxic, suggests that Grim killing in transient assays is due to a combination of IAP depletion, blocking of IAP-mediated caspase inhibition, and at least one other unidentified function. Unlike transiently transfected cells, inducible mammalian cell lines can sustain continuous expression of Grim and selective degradation of XIAP without undergoing apoptosis, demonstrating that down-regulation and antagonism of IAPs is not sufficient to cause apoptosis of mammalian cells.

The apoptotic program has been conserved in mammals and flies, with some interesting variations. The core molecules required for apoptosis, i.e. the adaptors required to activate caspases, the caspases themselves, and the inhibitors of apoptosis (IAPs),1 are found in both organisms. However, although the adaptor molecule Apaf-1 (mammals) and DARK (Drosophila) are both implicated in developmental cell death, Apaf-1 requires activation by cytochrome c whereas DARK does not (1, 2). Similarly, caspase inhibitory IAPs have been identified in both organisms. Although in Drosophila, removal of Drosophila inhibitor of apoptosis (DIAP1) has profound effects resulting in large numbers of ectopic cell deaths (3, 4), in mice, removal of X chromosome-linked inhibitor of apoptosis (XIAP) has no developmental or phenotypic consequence (5).

Proteins that bind to and antagonize IAPs have been identified in both flies and mammals. Five IAP antagonists have been identified in Drosophila: Hid, Grim, Reaper, Sickle, and Jafrec. Grim was described as a protein present in the genomic interval at 75C1 (6), a region that had previously been described to be required for programmed cell death in the fly (7). When ectopically expressed, Grim was able to induce cell death in flies and insect cells in tissue culture (6). It was subsequently shown to bind to and antagonize IAPs (8). Because depletion of DIAP1 by RNAi is sufficient to induce cell death in insect cell lines, these activities of Grim would also be sufficient to cause apoptosis (1, 9).

The mammalian protein XIAP contains three BIR domains that interact with both caspases and IAP antagonists, as well as a RING finger domain. The RING finger in XIAP has been shown to function as a ubiquitin ligase (10–12), as has that of DIAP1 (13, 14). The RING E3 ligase activity can be directed toward the IAP itself, allowing IAPs to regulate their own levels (10, 14). It may also promote ubiquitination of caspases, contributing to their inhibition (12, 14), and may target IAP antagonists for degradation (15).

Ubiquitination and degradation of XIAP can be stimulated by Reaper (16), just as ubiquitination and degradation of Hid is stimulated by DIAP1 (17), in each case in a RING-dependent manner. Because XIAP is specifically degraded in thymocytes following an apoptotic stimulus (10), it seems likely that there is a mammalian IAP antagonist that promotes IAP ubiquitination and degradation in an analogous way.

It has been reported that ectopic expression of Grim causes apoptosis when expressed in mammalian cell lines (18, 19). This is consistent with the notion that antagonism of IAPs in mammalian cells is also sufficient to initiate apoptosis. However, ectopic expression of the mammalian IAP antagonist Diablo/smace is not sufficient to cause apoptosis, even when the mature form is targeted to the cytoplasm (20). To investigate this discrepancy between fly and mammalian IAP antagonists we investigated whether Grim has other pro-apoptotic functions that are distinct from those of Diablo, and are in addition to IAP antagonism.

As recently described for Reaper and Hid (16, 17), we show here that in addition to IAP antagonism, Grim promotes XIAP ubiquitination and destruction. However, in marked contrast to Reaper or Hid, Grim is able to promote ubiquitination of an XIAP lacking its RING domain. As well as targeting XIAP for degradation, Grim also promotes a massive increase in total cellular ubiquitination, whereas Diablo does neither. In addition to promoting an increase in general protein ubiq-
expression can be regulated by tetracycline we confirm that Grim must interact with XIAP for Grim to induce global ubiquitination. In stable cell lines in which Tet can regulate Grim, we show that stably expressed Grim is able to promote degradation of endogenous XIAP. Surprisingly XIAP is specifically targeted by Grim, and levels of both ML-IAP remain constant. Thus it is still possible that mammalian cells like Drosophila cells are unable to tolerate reduction in all IAPs, but consistent with the XIAP knock-out we show they can tolerate expression of an IAP antagonist and reduction in XIAP.

**EXPERIMENTAL PROCEDURES**

**Transfections and Constructs**—pEF expression constructs encoding C-terminal FLAG-tagged XIAP, XIAP mutants, N-terminally-FLAG-tagged CrmA DMQD, pEF caspase-9, and pEF cytoplasmic Diablo have been previously described (20–23). pcDNA3 FLAG Skp2 was described in Ref. 24. pEF N-terminal FLAG tag SOCS1 and pEF FLAG Jak2 were kind gifts from Tracy Wilson. UehH5 was a kind gift from Martin Scheffner and was cloned with PCR from pET 3a UehH5 into pEF with primers 2675’-ggaggatccgctgagttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
Grim Depletes XIAP but Does Not Induce Apoptosis

Fig. 1. Grim and Diablo bind to the same residues in XIAP. XIAP mutants that are unable to bind Diablo are also significantly impaired in their ability to bind Grim. There is an inverse correlation between the extent of Grim interaction and the amount of XIAP in the lysates. 293T cells were transiently transfected with plasmids expressing FLAG-XIAP and mutants thereof, and C-terminal HA-tagged Grim. Cell lysates were immunoprecipitated with anti-FLAG beads, separated on SDS-PAGE gels, transferred to PVDF membranes, and immuno-blotted with anti-HA (to reveal Grim) and anti-FLAG (to reveal XIAP mutants).

We performed co-transfection experiments with XIAP and a range of other proteins to determine whether Grim specifically targeted XIAP for ubiquitination and degradation, and whether the increase in total cellular ubiquitination was a specific effect. Total cellular ubiquitination was very uniform regardless of which protein was transfected, even when the other protein was either an unrelated E3 ligase such as SOCS1, or an E2 ubiquitin conjugating enzyme such as UbcH5.

Cytoplasmic Diablo (Fig. 2B, lane 6) and Grim (Fig. 2B, lane 5) make two notable exceptions. When cytoplasmic Diablo was co-transfected with XIAP there was a general suppression of total cellular ubiquitination, even when the other protein was either an unrelated E3 ligase such as SOCS1, or an E2 ubiquitin conjugating enzyme such as UbcH5.

The total amount of XIAP was also very uniform except when Grim was transfected, causing a dramatic decrease in the amount of XIAP. As XIAP is a ubiquitin ligase, it is expected to have a certain basal level of ubiquitination, and, provided other elements within the ubiquitin/proteasome system remain constant, it is expected that the ratio of ubiquitinated to non-ubiquitinated XIAP should remain constant. If the amount of ubiquitinated XIAP does not have an obvious role in the ubiquitin/proteasome pathway of Jak2 and paracaspase (lanes 3 and 4). Co-expression of proteins that do play a role in the ubiquitin/proteasome pathway, such as SOCS1 and UbcH5, does affect the ratio of ubiquitinated to non-ubiquitinated XIAP, but at least in the case of SOCS1 this is unlikely to be due to a specific effect on XIAP but rather a general nonspecific effect. Even when such perturbations on the ubiquitin/proteasome pathway are taken into account, Grim and Diablo produce the most profound disruption in the ratio of ubiquitinated to non-ubiquitinated XIAP. When cytoplasmic Diablo is transfected there is a large amount of XIAP but no ubiquitinated XIAP, and when Grim is transfected there is a greatly reduced level of XIAP but a very large amount of ubiquitinated XIAP. Due to the transient nature of the assay and over-expression of the proteins, an increased ratio of ubiquitinated to non-ubiquitinated XIAP does not always give rise to an unambiguous reduction in XIAP; however, prolonged expression in stable cell lines of proteins that alter this ratio, such as Grim, would be expected to, and does, result in such a reduction (see Fig. 6A).

Grim, Not Apoptosis, Promotes Ubiquitination and Degradation of XIAP—One possible confounding effect in the above analyses was that Grim but not Diablo promotes apoptosis when transiently transfected. Therefore it was possible that it was the induction of apoptosis that indirectly induced XIAP ubiquitination, rather than Grim itself. To test this we performed a similar co-transfection experiment, with two additional controls. First, caspase-9 auto-processes and causes apoptosis when over-expressed in cells (25, 26), and as processed caspase-9, like Grim, binds to XIAP in a BIR-dependent manner, we reasoned that this was an excellent control to determine whether apoptosis induced XIAP ubiquitination. Second, we identified a double point mutant of Grim (L88A/L89A) that did not promote apoptosis when co-transfected in mammalian cells and that was expressed to the same levels as the wild type protein. A similar point mutant (L89E) has recently been described that destroys Grim’s ability to induce cell death in Drosophila cells (27). A quantitation of the cell death induced by these constructs in 293T cells shows that Grim (L88A/L89A) is as non-toxic as Diablo (Fig. 3A).

From Fig. 3B it is apparent that apoptosis caused by caspase-9 over-expression does not result in XIAP ubiquitination (see lanes 1–3, α-Flag IP and α-HA panels). Furthermore there was no evidence for ubiquitination of caspase-9 itself by XIAP. The control blots show that equal levels of protein were loaded (lanes 1–3, α-Hsp70 lysate panel), that caspase-9 was processed (lanes 1–3, α-caspase 9 lysate panel), and that wild type XIAP was able to immunoprecipitate processed caspase-9, but that the mutant XIAP D214S E314S was not (cf. lanes 1 and 3, IP: α-Flag α-caspase-9 panel), as expected from previous results (23, 28). Consistent with the previous results, wild type Grim promoted both XIAP degradation and a global increase in ubiquitination when expressed with wild type XIAP, but caused significantly less degradation and global ubiquitination when expressed with a mutant XIAP (D214S/E314S) that is significantly impaired in its ability to bind Grim (cf. lanes 7 and 9, α-HA and α-Flag panels). As in Fig. 4, the ratio of ubiquitinated to non-ubiquitinated XIAP has been changed dramatically by Grim co-transfection, resulting in reduced levels of XIAP, but the ratio of ubiquitinated to non-ubiquitinated XIAP in a mutant that cannot interact with Grim remains the same, regardless of whether Grim is transfected or not.

The reduction in XIAP levels was not as pronounced in the samples transfected with Grim L88A/L89A, nevertheless comparison of the levels of XIAP in the lysates shows a significant reduction, and there is also an increase in global ubiquitination when
XIAP and Grim L88A/L89A are expressed. As in the case of wild type Grim, this required XIAP interaction because the mutant D214S/E314S XIAP was not degraded (Fig. 3B, lanes 6 and 9).

**Grim Promotion of XIAP Ubiquitination Requires Grim XIAP Interaction**—The obvious mechanism by which Grim might promote XIAP ubiquitination is by activating the RING finger of XIAP that has previously been shown to function as an E3 ligase. To determine whether the RING finger was required, and to confirm that direct Grim-XIAP interaction was necessary for XIAP ubiquitination, co-transfection analyses were performed with different XIAP mutants, C-terminally myc-tagged Grim and HA-tagged ubiquitin.

XIAPs co-transfected with an empty myc vector have an almost constant ratio of ubiquitinated to non-ubiquitinated protein regardless of the mutation. The one exception to this being the structurally catastrophic H467A mutant, which is clearly unstable. The δRING XIAPs have a greatly reduced level of ubiquitination consistent with the idea that the RING is responsible for IAP auto-ubiquitination.

Wild type XIAP co-transfected with Grim shows a significant increase in ubiquitination and an equally significant decrease in protein when compared with the vector control (cf. lanes 1 and 9). However a mutant XIAP that is unable to bind Grim (D214S/W310A/E314S) maintains the same ratio of ubiquitinated to non-ubiquitinated in both the Grim and vector control transfections (Fig. 4, cf. lanes 4 and 12). This confirms that...
XIAP interaction with Grim is required for XIAP ubiquitination and degradation.

Surprisingly, although removal of the RING finger impaired XIAP ubiquitination by Grim, it did not abolish it (Fig. 4, lane 2). The most marked difference in ubiquitination of XIAP was among XIAP mutants that are unable to bind Grim, and this effect was striking; in either the full-length molecule or in the 8RING molecules (Fig. 4, cf. lanes 2 and 3). These results show that promotion of XIAP ubiquitination by Grim requires XIAP binding, but contrary to expectation the RING finger was not required for Grim-induced XIAP or cellular ubiquitination. Mutations just N-terminal to the RING finger in a region conserved among different mammalian IAPs (E438A/E439A and R442A/R443A) did not impair Grim-induced ubiquitination and degradation of XIAP and appeared to enhance it, presumably because they actually enhanced binding (cf. lanes 1, 7, and 8). Interestingly both these double mutations make XIAP more similar to DIAP1, probably accounting for the increased affinity of XIAP to Grim. Conversely a mutation in the RING finger itself (K451S/I452A) decreases the affinity of XIAP for Grim and as expected renders the mutant protein more resistant to Grim-induced ubiquitination and degradation.

Previous publications have described an H467A XIAP mutant that was unable to auto-ubiquitinate (10, 12, 16). However even in transient transfection experiments this structurally catastrophic mutant was considerably less stable than wild type XIAP (Fig. 4, lanes 6 and 14, α-HA and α-Flag panels), and in isogenic stable cell lines this mutant was almost undetectable (data not shown), and therefore it is difficult to draw conclusions about its ability to auto-ubiquitinate in the presence of Grim.

Grim Promotes Ubiquitination in the Presence of Stably Expressed XIAP—Although Grim was able to promote XIAP degradation when both were transiently transfected, it remained possible that this was merely an artifact of the transfection process. To address this issue we created isogenic stable cell lines bearing tetracycline regulatable constructs. Using the Flp-In™ T-REX™ 293 cell line (Invitrogen) we created FLAG XIAP and FLAG XIAP mutant cell lines. The isogenic nature of these lines is shown by the equal levels of expression of XIAP obtained from different cell lines (Fig. 5, cf. lanes 1, 5, and 7).

The stable cell lines were transiently transfected with FLAG Grim and HA-ubiquitin and treated with or without tetracycline. Presumably because of the low transfection efficiencies (≈15%) and the lack of T-antigen driven replication of the transfected plasmid in this 293 cell line, it was not possible to see a reduction in the amount of stable XIAP in these assays. Consistent with the transient transfection analyses, however, Grim induced an increase in ubiquitination of wild type XIAP in two different cell lines, but not of a mutant XIAP (D214S/E314S) that is unable to bind Grim (Fig. 5, cf. lanes 2 or 7 with lane 6). In contrast, Diablo did not induce an increase in XIAP

FIG. 3. Grim, but not apoptosis, increases XIAP ubiquitination. A, transiently transfected Grim L88A/L89A does not kill 293T cells. 293T cells were transiently transfected with various constructs expressing Grim and Grim L88A/L89A from different promoters or caspase-9.

GFP was used as a marker to indicate transfected cells that were subsequently scored for viability by morphology. The mean ± S.E. of 3 independent transfections are shown. B, Grim and Grim L88A/L89A promote a general increase in ubiquitination and increase in ubiquitination of XIAP. As for Fig. 2A, CrmA is included as a control for the specificity of XIAP degradation by Grim. cl. casp9, processed caspase-9 p37 subunit; cl. XIAP, XIAP processed by caspase-3 between the BIR2 and BIR3; pro casp9, zymogen full-length caspase-9. 293T cells were transiently transfected with caspase-9, FLAG-Grim, or FLAG-Grim L88A/L89A. Cell lysates were immunoprecipitated with anti-FLAG beads, separated on SDS-PAGE gels, transferred to PVDF membranes, and immunoblotted with anti-HA (to reveal ubiquitination), anti-FLAG (to reveal XIAP and Grim), anti-Diablo, anti-caspase-9, and anti-Hsp70 (to control loading of lysates).

2 Unpublished observations.
ubiquitination. The slight increase in ubiquitination caused by transient transfection of Grim apparent in lanes 4, 6, and 9 is probably due to activation of the E3 ligase activity of endogenous XIAP, because we were not able to show Grim promoting ubiquitination of either ML-IAP or ciap-1 in transient transfection assays (see Supplemental Data).

Expression of Grim in Stable Lines Promotes Degradation of XIAP—To investigate the ability of Grim to promote degradation of XIAP in stable lines, we used the Flp-InTM T-RExTM system to create cells that inducibly express either Grim or the Grim LL88AA mutant. Consistent with the transient transfection experiments, Grim promoted ubiquitination and degradation of transiently transfected wild type XIAP and /H11128 RING XIAP (data not shown).

Immunoprecipitations of lysates of the stable lines were performed to look at the ability of Grim to bind to endogenous XIAP and promote its degradation, but initially we were not able to immunoprecipitate endogenous XIAP with FLAG-Grim from lysates made from these cell lines, even though both FLAG-Grim and XIAP were present (Fig. 6). Because the lysis buffer used released endogenous IAP antagonists, such as Diablo and HtrA2/Omi from the mitochondria, it was possible that these were competing with Grim for binding to XIAP post lysis. We therefore induced expression of Grim in the stable cell lines for 48 h and then fractionated the cells with digitonin into membrane and cytoplasmic fractions.

The fractionations were performed successfully as both VDAC/porin and cytochrome c were present only in the membrane fractions (Fig. 6A). Both XIAP and Grim localized to both membrane and cytoplasmic fractions, with XIAP being more abundant in the cytoplasm. The /XIAP panel in Fig. 6A shows that the level of endogenous XIAP was specifically reduced in both cytoplasmic and membrane fractions of the Grim stable cell line, because none of the other proteins examined (e.g. Hsp70, cytC, VDAC/porin, caspase-9, or caspase-3) were reduced. Although only a comparison with a stably expressing paracaspase cell line is shown as a control, levels of endogenous XIAP in this cell line were identical with other stable cell lines tested (data not shown). Importantly, FLAG-Grim immunoprecipitation from the cytoplasmic fraction, which contained Grim but did not contain any mitochondrial proteins, was able to pull down endogenous XIAP (Fig. 6B). Although the Grim L88A/L89A mutant was not able to immunoprecipitate endogenous XIAP from the cytoplasmic fraction, it is clear from an examination of the lysates and immunoprecipitations that there was less Grim LL88AA than wild type Grim in the cytoplasmic fraction, and it is therefore not possible to make a conclusion about the ability of Grim LL88AA to bind to XIAP. Nevertheless there was significantly less XIAP in the membrane fraction from the Grim LL88AA stable cell line, which is consistent with our previous observations that Grim L88A/L89A was able to promote XIAP ubiquitination, albeit with a slightly reduced activity, and consistent with the idea that XIAP can still bind Grim LL88AA.

Induction of Grim in Stable Cell Lines Does Not Induce Apoptosis and Does Not Greatly Sensitize Cells to Apoptotic
Insults—As expected, the viability of all the stable cell lines used in this study was approximately equal in the absence of tetracycline (data not shown). Surprisingly, however, in the presence of tetracycline, all stable cell lines tested were equally viable, with the Grim lines showing more or less the same viability as cell lines over-expressing XIAP (Fig. 7). This was true whether the cells were exposed to a short pulse of tetracycline (Fig. 7) or prolonged induction with tetracycline over a month of tetracycline induction, all isogenic lines, including Grim, maintained expression of their respective transgene (data not shown). Thus continuous expression of Grim, despite reducing levels of endogenous XIAP, had little effect on normal cell viability.

To ascertain whether reduced levels of XIAP might sensitize cells to apoptotic stimuli we determined cellular viability following low doses of etoposide treatment (Fig. 7). Although cells over-expressing wild type XIAP showed decreased sensitivity toward etoposide, cells expressing Grim were only slightly more sensitive than isogenic cell lines expressing an inactive XIAP mutant D148A/D214S/E314S or GFP (Fig. 7).

Six recent publications reported the ability of Reaper, Grim and Hid to regulate DIAP1 levels in Drosophila (13, 14, 16, 17, 29, 30). Hay and co-workers (17) reported that Hid caused a RING-dependent degradation of DIAP1, and that in contrast Reaper and Grim down-regulated DIAP1 levels in a less specific manner by interfering with general translation (17). The finding of RING-dependent degradation by Hid was consistent with other observations that a DIAP1 RING finger mutant was able to suppress Hid, but not Reaper and Grim, induced cell death in the eye (4, 14, 31). In contrast, Holley et al. (16) and Steller and co-workers (15) reported that Reaper was able to greatly decrease IAP abundance by increasing IAP degradation, a function that required the IAP RING finger, but in addition Holley et al. (16) also reported that Reaper repressed total protein translation. Cagan and co-workers (20) similarly reported that Grim and Reaper, but not Hid, promoted degradation of DIAP1 in vivo. Overall, therefore, although there was a broad area of agreement between the six studies that some of the Drosophila IAP antagonists affect levels of DIAP1, they proposed different mechanisms and a consensus of how Drosophila IAP antagonists affect DIAP1 levels remains elusive.

The observation that DIAP1 became more multi-ubiquitinated in an IAP antagonist-dependent fashion prompted us to investigate the ability of the best characterized mammalian IAP antagonist, Diablo/smacc, to promote degradation of mammalian IAPs. By using Grim as a positive control we have decisively shown that Diablo cannot promote ubiquitination of XIAP, and in fact actually has the opposite activity. However, our findings do show that a mechanism for “Grim-like” induction of IAP degradation exists in mammals. Consistent with observations for Reaper (16), we find that Grim induces a specific degradation of XIAP that occurs through an increase in XIAP ubiquitination. Consistent with several of the above studies we also see a decrease in total protein when Grim is transiently transfected into mammalian cells, which might result from a general inhibition of translation (16, 17, 30). This decrease in total protein is not caused by the cytotoxicity associated with Grim, as a non-toxic Grim mutant was as effective as wild type Grim in reducing total protein. An alternative explanation for the decrease in total protein would be that Grim induces an increase in protein turnover as evidenced by the increased ubiquitination present when Grim is transiently transfected. Possibly a combination of both these processes occur and Grim promotes the ubiquitination and degradation of an essential translational component. Whatever the hypothesis, it seems as though this effect is small when compared with the ability of Grim to promote ubiquitination and subsequent degradation of XIAP, because stably expressed Grim promoted destruction of endogenous XIAP, but did not noticeably affect the levels of any other proteins tested. Although our results are consistent with Holley et al. (16) they are not consistent with those from Hay and co-workers (17) who showed in vitro that Hid, but not Grim, was capable of inducing DIAP1 auto-ubiquitination. The two systems are obviously quite different and Hay and co-workers (17) cautioned that “lack of in vitro activity may well have represented adoption of unphysiological conformations of Grim”; however, it would be unusual if Grim could promote the ubiquitination of XIAP but not DIAP1.

Our findings also add to our understanding of the mechanism of Grim-induced IAP degradation. Firstly we find that XIAP RING becomes ubiquitinated and degraded when co-expressed with Grim. This finding is incompatible with the proposal that Grim promotes the E3 ligase activity of IAPs. It is possible that Grim activates the E3 ligase activity of the endogenous XIAP, which then oligomerizes with and trans-
ubiquitinates the delta RING construct; however, we do not favor this model for several reasons. First, δRING XIAP oligomerizes with full-length XIAP significantly less well than full-length XIAP with itself (23). Second, transiently transfected δRING XIAP became efficiently ubiquitinated even though amounts of this protein vastly exceed those of the endogenous full-length protein. Last, and most telling, δRING XIAP must be able to bind to Grim to become ubiquitinated. We therefore propose that Grim actually serves as a surrogate E3 ligase to directly recruit and activate the E2 ubiquitin conjugating enzyme. Holley et al. (16) found that Reaper was able to stimulate XIAP degradation but not that of the RING finger H467A mutant. As our data were obtained with Grim, it is possible that Grim and Reaper function differently in promoting IAP degradation. However, we show that the H467A mutant is extremely unstable, as might be expected from such a structurally catastrophic mutant, and we believe, therefore, that it is difficult to draw conclusions regarding this particular mutant.

Although the mechanisms regulating levels of IAPs appear to have been conserved between mammals and flies, an important difference exists. XIAP with a RING finger that has had a Zn co-ordinating ligand disrupted (e.g. H467A) is significantly less stable than the wild type protein. It appears therefore that although RING mediated degradation of XIAP is important in determining the level of XIAP in the cell, other factors such as structural integrity are in fact more important than RING mediated degradation alone. This is in contrast to the reports that show that DIAP1 with a Zn co-ordinating ligand disrupted (i.e. C412Y) is in fact more stable than wild type DIAP1 (14).

We also observed a significant increase in total cellular ubiquitination when Grim was either transfected or induced in stable cell lines. This increase in total cellular ubiquitination was further augmented when XIAP was co-expressed with Grim. It is noteworthy that cytoplasmic Diablo decreased the levels of total cellular ubiquitination, arguing that the increase in ubiquitination is specific to XIAP. The increase in ubiquitination due to Grim could result from a general activation of XIAP’s ubiquitinating activity, but might also occur if Grim activates other RING finger proteins, such as ciap-1 or ciap-2. We favor the hypothesis that the increase in total ubiquitination results predominantly from an activation of the E3 ligase activity of XIAP, because in transient transfection experiments we saw no significant increase in total cellular ubiquitination when Grim was co-transfected with ciap-1 or -2 (see Supplemental Figure). The large general increase in ubiquitination caused by XIAP indicates that there are a large number of XIAP ubiquitination substrates, and these substrates are un-

FIG. 6. Expression of Grim in stable lines results in degradation of endogenous XIAP. Stable Grim cell lines were generated using a commercially available Flp recombinase system to create isogenic stables that are induced by tetracycline. A, XIAP and Grim localize to the cytoplasmic and membrane fractions and endogenous XIAP is specifically reduced in the Grim and Grim L88A/L89A mutant cell lines. Cells were induced with tetracycline for 48 h and lysed using a digitonin fractionation protocol. Cytoplasmic and membrane fractions were separated on SDS-PAGE gels, transferred to PVDF membranes, and immunoblotted with anti-XIAP, anti-VDAC/Porin, anti-Hsp70, anti-FLAG, anti-cytochrome c, anti-caspase-3, and anti-caspase-9. B, Grim localized in the cytoplasmic fraction is able to immunoprecipitate endogenous XIAP present in the cytoplasmic fraction of FLAG Grim stable cell lines. Cells were induced with tetracycline for 48 h and lysed using a digitonin fractionation protocol. The cytoplasmic fraction was immunoprecipitated with anti-FLAG beads, separated on SDS-PAGE gels, transferred to PVDF membranes, and immunoblotted with anti-XIAP and anti-FLAG.
Grim Depletes XIAP but Does Not Induce Apoptosis

In the “intrinsinc” model of apoptosis cytchrome c released from mitochondria activates Apaf-1 that in turn activates caspase-9, which then activates caspase-3. Activated caspase-3 is able to feed back to process and activate caspase-9, amplifying the original signal. We have proposed that IAPs in mammalian cells function as a safety catch that prevents initiation of the caspase-9, caspase-3 amplification loop until IAP antagonists release them. Our data suggest that in mammalian cells activation of caspasers is tightly regulated, and that in most normal circumstances there is no accidental activation of either caspase-3 or caspase-9. These results are also consistent with the lack of phenotype of XIAP knock-out mice. If this model is correct, the challenge is to find situations where the existence of the IAP safety catch is critical.

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REFERENCES

1. Zimmermann, K. C., Ricci, J. E., Droin, N. M., and Green, D. R. (2002) J. Cell Biol. 156, 1077–1089
2. Bersten, L., Read, S., Cakouros, D., Huh, J. R., Hay, B. A., and Kumar, S. (2002) J. Cell Biol. 156, 1089–1098
3. Wang, S. L., Hawkins, C. J., Yu, H., Hay, B. A., and Kumar, S. (1999) Cell 98, 453–463
4. Goyal, L., McCall, K., Agapite, J., Hartwig, E., and Steller, H. (2000) EMBO J. 19, 589–597
5. Harlin, H., Ruffle, S. B., Duckett, C. S., Lindsten, T., and Thompson, C. B. (2001) Mol. Cell. Biol. 21, 3604–3608
6. Chen, P., Nordstrom, W., Gish, A., and Abrams, J. M. (1996) Genes Dev. 10, 1773–1782
7. White, K., Grether, M. E., Abrams, J. M., Young, L., Farrell, K., and Steller, H. (1994) Science 264, 677–683
8. Vucic, D., Kaiser, W. J., and Miller, L. K. (1998) Mol. Cell. Biol. 18, 3300–3309
9. Rodriguez, A., Chen, P., Oliver, H., and Abrams, J. M. (2002) EMBO J. 21, 2189–2197
10. Yang, Y., Fang, S., Jensen, J. P., Weissman, A. M., and Ashwell, J. D. (2000) Science 288, 874–877
11. Huang, H., Joaozeiro, C. A., Bonfoco, E., Kamada, S., Leveson, J. D., and Hunter, T. (2000) J. Biol. Chem. 275, 26661–26664
12. Suzuki, Y., Nakabayashi, Y., and Takashashi, R. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 8662–8667
13. Ryoo, H. D., Bergman, A., Gonen, H., Ciechanover, A., and Steller, H. (2002) Nat. Cell Biol. 4, 432–438
14. Wilson, R., Goyal, L., Ditta, M., Zacharias, A., Baker, D. A., Agapite, J., Steller, H., and Meier, P. (2002) Nat. Cell Biol. 4, 445–450
15. MacFarlane, M., Merrison, W., Britton, S. B., and Cohen, G. M. (2001) J. Cell Biol. 153, 3661–3661
16. Hollyer, C. L., Ohon, M. R., Colon-Ramos, D. A., and Kornbluth, S. (2002) Nat. Cell Biol. 4, 439–444
17. Yoo, S. J., Huh, J. R., Muro, I., Yu, H., Wang, L., Wang, S. L., Feldman, R. M., Clem, B. J., Muller, H. A., and Hay, B. A. (2002) Nat. Cell Biol. 4, 416–424
18. Claveria, C. A., and Torres, M. (1998) EMBO J. 17, 7199–7208
19. Mccarthy, J. V., and Dixit, V. M. (1998) J. Biol. Chem. 273, 24009–24015
20. Verhagen, A. M., Eckert, P. G., Pakusch, M., Silke, J., Connolly, L. M., Reid, G. E., Moriz, R. L., Simpson, R. J., and Vaux, D. L. (2000) Cell 102, 42–53
21. Eckert, P. G., Silke, J., and Vaux, D. L. (1999) EMBO J. 18, 319–328
22. Silke, J., Eckert, P. G., Day, C. L., Hawkins, C. J., Bace, M. C., Chow, J., Pakusch, M., Verhagen, A. M., and Vaux, D. L. (2001) EMBO J. 20, 5314–5323
23. Silke, J., Hawkins, C. J., Eckert, P. G., Chow, J., Day, C. L., Pakusch, M., Verhagen, A. M., and Vaux, D. L. (2001) EMBO J. 20, 5314–5323
24. Lissztwan, J., Marti, A., Sutterluty, H., Getaia, M., Wirbelauer, C., and Krek, W. (1998) EMBO J. 17, 368–373
25. Duax, H. J., Orth, K., Chanezard, A., Poirier, G. G., Frolich, C. J. H., Eckert, P. G., and Dixit, V. M. (1996) J. Biol. Chem. 271, 16720–16724
26. Eckert, P. G., Silke, J., Hawkins, C. J., Verhagen, A. M., and Vaux, D. L. (2001) J. Cell Biol. 153, 483–490
27. Claveria, C., Caminero, E., Martinez-A, C., Campuzano, S., and Torres, M. (2002) EMBO J. 21, 3327–3336
28. Sinivasan, S. M., Hegde, R., Saleh, A., Datta, P., Shnaike, E., Chai, J., Lee, C., Robbins, P. D., Fernandez-Almener, T., Shi, Y., and Almener, E. S. (2001) Nature 410, 112–116
29. Wing, J. P., Schreider, B. A., Yokokura, T., Wang, Y., Andrews, P. S., Huseinovic, N., Dong, C. K., Ordahl, J. L., Schwartz, L. M., White, K., and Nambu, J. R. (2002) Nat. Cell Biol. 4, 451–456
30. Nembhard, I. M., and Cagan, R. (2002) Nat. Cell Biol. 4, 425–431
31. Li, S., Mazon, I., and White, K. (2000) Genetics 154, 669–678

FIG. 7. Induction of stably expressed Grim does not induce apoptosis. Stable Grim cell lines were generated using a commercially available Flp recombinase system to create isogenic stables that are induced by tetracycline. Two individual stable cell lines expressing Grim (A and G), XIAP (B and C), and XIAP mutant (A and D) were analyzed. A, cells were induced with 10 µg/ml tetracycline for 30 h, without further treatment (black bars) or in addition, etoposide at a final concentration of 2.5 µg/ml for the last 24 h of the 30 h tetracycline induction (unfilled bars). Viability was assessed by staining cells with propidium iodide and analyzing on a fluorescence-activated cell sorter scanner for sub G1 DNA content. Error bars represent standard deviation from either 3 (black bars) or 6 (unfilled bars) independent measurements.

likely to be only known XIAP interacting proteins because strikingly we saw no evidence for caspase-9 or Diablo ubiquitination by XIAP.

It has been previously reported (18, 19) that Grim is able to induce apoptosis in mammalian cells. We confirmed these observations and also identified a mutant of Grim that was unable to promote cell death in transient transfection, i.e. LL88AA. We tested this mutant because it is part of a partially conserved helix that is present in Hid, Grim, Reaper, and Drosophila. A point mutant Grim (L89E) was also analyzed, and consistent with our observations it was unable to promote cell death in Drosophila. Our results support the idea that Grim has a pro-apoptotic function distinct from IAP antagonism, and additionally indicate that this pro-apoptotic function is distinct from IAP degradation as this mutant was almost as effective as wild type in promoting XIAP degradation. However, our results indicate that mammalian cells are able to tolerate lower levels of this cytotoxic function, because cells with stably integrated Grim were almost as healthy as cells stably transfected with GFP. Most likely the partner for the cytotoxic activity of Grim is not as well conserved in mammals as the IAPs are.

Stable cell lines containing an inducible Grim construct continuously induced with tetracycline were as viable as any other isogenic cell line we analyzed. This was despite a significant reduction in the levels of XIAP. This reduction in levels of XIAP was very specific as none of VDAC, cytochrome C, Hsp70 caspase-9, or caspase-3 were reduced. Although not all XIAP was lost from these cells, reduction of DIAP1 mRNA to one quarter of the initial amount by double-stranded RNA treatment was sufficient to induce cell death in Drosophila SL2 cells (1). This ability of mammalian cells to withstand a reduction in XIAP implies a significantly different role for IAPs in mammalian compared with Drosophila cells. This ability is also consistent with the fact that mammalian cells tolerate high levels of ectopic expression of Diablo in the cytoplasm, and the lack of a phenotype when XIAP is knocked out in mice.
Unlike Diablo/smac, Grim Promotes Global Ubiquitination and Specific Degradation of X Chromosome-linked Inhibitor of Apoptosis (XIAP) and Neither Cause Apoptosis

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