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Reaper Is Regulated by IAP-mediated Ubiquitination*

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In most cases, apoptotic cell death culminates in the activation of the caspase family of cysteine proteases, leading to the orderly dismantling and elimination of the cell. The IAPs (inhibitors of apoptosis) comprise a family of proteins that oppose caspases and thus act to raise the apoptotic threshold. Disruption of IAP-mediated caspase inhibition has been shown to be an important activity for pro-apoptotic proteins in *Drosophila* (Reaper, HID, and Grim) and in mammalian cells (Smac/DIABLO and Omi/HtrA2). In addition, in the case of the fly, these proteins are able to stimulate the ubiquitination and degradation of IAPs by a mechanism involving the ubiquitin ligase activity of the IAP itself. In this report, we show that the *Drosophila* RHG proteins (Reaper, HID, and Grim) are themselves substrates for IAP-mediated ubiquitination. This ubiquitination of Reaper requires IAP ubiquitin-ligase activity and a stable interaction between Reaper and the IAP. Additionally, degradation of Reaper can be blocked by mutating its potential ubiquitination sites. Most importantly, we also show that regulation of Reaper by ubiquitination is a significant factor in determining its biological activity. These data demonstrate a novel function for IAPs and suggest that IAPs and Reaper-like proteins mutually control each other's abundance.

Apoptosis is a regulated form of cell death that can be triggered by a variety of intracellular and extracellular signals. Although apoptosis research has revealed a plethora of signaling pathways that can contribute to the decision of a cell to die, the ultimate responsibility for completing the cell death program (in most cases) resides with the cysteine proteases known as caspases (reviewed in Refs. 1–3). Caspase-mediated cleavage of cellular substrates underlies many of the ordered processes that occur as the dying cell shrinks, degrades its DNA, and packages the remains for subsequent phagocytosis. As such, regulation of caspase activation is a key control point for the apoptotic machinery.

Acting in opposition to the caspases are the IAPs (inhibitors of apoptosis). These proteins function, at least in part, by directly binding to and inhibiting active caspases (Refs. 4–6; reviewed in Refs. 7 and 8). Many of the IAPs also contain a RING finger domain, and like other RING finger proteins, these IAPs can function as ubiquitin ligases (9, 10). Ubiquitin ligases work in conjunction with ubiquitin-activating and ubiquitin-conjugating enzymes to covalently link ubiquitin to lysines present in the target protein. Sequential linkage of multiple ubiquitin moieties (polyubiquitination) then results in targeting of the ubiquitinated protein for destruction by the proteasome (reviewed in Ref. 11). Although it is well established that IAPs can inhibit caspases through physical binding, the significance of IAP ubiquitin-ligase activity for the anti-apoptotic function of IAPs is not yet fully understood. For example, IAPs have been reported to ubiquitinate and promote the degradation of caspases, which could clearly favor cell survival (9, 12). Conversely, IAPs can auto-ubiquitinate and thereby promote their own destruction, which might be expected to favor cell death (9, 10, 12–17).

In *Drosophila*, the caspase-inhibiting function of IAPs is antagonized by the pro-apoptotic proteins Reaper, HID, and Grim (the RHG[1] proteins). The genes encoding these proteins are closely linked, and their combined deletion results in a generalized failure of apoptosis during development of the *Drosophila* embryo (18). Expression of these proteins is tightly controlled at the level of transcription, and ectopic expression of the RHG proteins in either insect or vertebrate cells can initiate apoptosis (19–26). Although Reaper, HID, and Grim do not share overall homology, they do share a short region at their extreme N termini that is responsible for binding IAPs (the so-called RHG motif). The interaction between the RHG motif and the IAP has been suggested to preclude the IAP/caspase interaction, thereby alleviating caspase inhibition (27–30). The N-terminal RHG motif is also present in several vertebrate apoptotic regulators, including Smac/Diablo and Omi/HtrA2 (18, 19, 22, 28, 29, 31–34). As a consequence, these proteins can directly bind and inhibit IAPs in a manner similar to that of Reaper, HID, and Grim.

Recently, we and others have reported that Reaper, HID, and Grim can also promote apoptosis by stimulating IAP ubiquitination and degradation (12–17). Both IAP ubiquitin-ligase activity and the RHG motif are required for this particular interaction, but it is not yet clear whether other regions of the RHG protein or the IAP will also be required. Moreover, although Smac and Omi bind IAPs, there have been no reports that they also stimulate IAP degradation.

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[1] The abbreviations used are: RHG, Reaper/HID/Grim; LLnL and ALLN, *N*-acetyl-Leu-Leu-Nle-CHO; Rpr, Reaper; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone; GST, glutathione *S*-transferase; HID, head involution defective, XIAP, X-linked IAP; DIAP, *Drosophila* IAP; GFP, green fluorescent protein; FACS, fluorescence activated cell sorting.

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In this report, we provide evidence that the interaction between the IAPs and the RHG proteins is a two-way street with regard to ubiquitination and proteasome-mediated degradation; that is, not only do the RHG proteins stimulate the ubiquitination and degradation of IAPs, but the IAPs also stimulate the ubiquitination and degradation of the RHG proteins. Our data demonstrate that the RHG motif is required for IAP-mediated degradation of Reaper, which suggests that a stable interaction between Reaper and the IAP is required for this form of regulation. Moreover, Reaper degradation can be blocked by inhibiting the proteasome, and when Reaper is stabilized by mutating potential ubiquitination sites, it becomes a markedly more potent inducer of apoptosis. Collectively, these data indicate that IAP regulators such as Reaper are targeted for degradation by IAP ubiquitin-ligase activity, and that this regulation is a significant factor in determining their biological activity.

EXPERIMENTAL PROCEDURES

Immunofluoresence and in Situ Hybridization—The following genetic crosses were used to generate the imaginal discs in Fig. 1: EnG4 × UAS-P35 (*panels A* and *D*), EnG4 × UAS-HID, UAS-P35 (*panels B*, *E*, and *G*), and EnG4 × UAS-Rpr, UAS-P35 (*panels C*, *F*, *H*, and *I*). DIAP1 and HID proteins were detected by indirect antibody fluorescence using the appropriate antibodies and standard techniques. HID and Reaper RNAs were detected by hybridization of digoxigenin-labeled probes, followed by dye staining.

Preparation of Recombinant Drosophila Proteins—DIAP1D20E was prepared as described previously (43) from GST-TEV-DIAP1D20E followed by TEV cleavage. pET23a-Reaper-GST was expressed in BL21(DE3)pLysS by 0.4 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h and purified using glutathione-Sepharose 4B (Amersham Biosciences) per the manufacturer's instructions. HID-His$_6$ and Grim-His$_6$ were purified as described previously (17). All of the proteins for *in vitro* ubiquitination assays were dialyzed against the buffer UD (20 mM Tris, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, 10% glycerol) before use.

In Vitro Ubiquitination—*Drosophila* embryo extract was made as follows. 0–5-h-old embryos were collected and aged for 6 h at 25 °C. The embryos were dechorionated with 50% bleach, rinsed, suspended in equal volumes of buffer EX (20 mM Tris, pH 7.5, 100 mM NaCl, 5 mM ATP, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.25 M sucrose) and homogenized. The supernatant was collected after centrifugation at 12,000 × *g*. The concentration of the extract was ~10 μg/μl. The ubiquitination assay was carried out as follows. Hid-His$_6$, Reaper-GST, or Grim-His$_6$ protein (100 ng each) was preincubated with 1 μl of embryo extract at room temperature for 10 min. Subsequently, DIAP1D20E (400 ng) and His-ubiquitin (Calbiochem; 3 μg total) were added in buffer UR (25 mM Tris, pH 7.5, 0.5 mM dithiothreitol, 2 mM ATP, 5 mM $MgCl_2$). The reaction was incubated at 37 °C for 40 min and stopped by adding SDS sample buffer. The ubiquitination of each protein was visualized after separation of proteins by SDS-PAGE, transfer to polyvinylidene difluoride, and immunoblotting with the appropriate antibody.

Generation of Reaper Antibody—Anti-Reaper serum was obtained by standard immunization techniques using New Zealand White rabbits and a synthetic Reaper C-terminal peptide conjugated to keyhole limpet hemocyanin (Research Genetics). The sequence used for peptide synthesis was CHPKTGRKSGKYRKPSQ.

Generation of ReaperKR—Reaper was cloned into pcDNA3 by standard techniques. Further work required removal of a vector *Msc*I site; thus Reaper/pc3 was digested with *Pvu*II and recircularized. The oligonucleotides (GATCCATGGCAGTGGCATTCTACATACCCGATCAG-GCGACTCTGTTGCGGGAGGCGGAGCAGAGGGAGCAGCAGATTCT-CCGCTTGCGGGAGTCACAGTGGAGATTCCTGG; CCAGGAATCTC-CACTGTGACTCCCGCAAGCGGAGAATCTGCTGCTCCCTCTGCTC-CGCCTCCCGCAACAGAGTCGCCTGATCGGGTATGTAGAATGCCA-CTGCCATG; CCACCGTCGTCCTGGAAACCCTGCGCCAGTACACTT-CATGTCATCCGAGGACCGGAAGAAGGTCCGGCAGATATCGCAGG-CCATCGCAAT; and CTAGATTGCGATGGCCTGCGATATCTGCCGG-ACCTTCTTCCGGTCCTCGGATGACATGAAGTGTACTGGCGCAGG-GTTTCCAGGACGACGGTGG) were hybridized and cloned separately into Reaper-pcDNA3Δ*Pvu*II to generate clones A (N-terminal mutant) and B (C-terminal tetra-mutant). ReaperKR was generating by splicing the *Bam*HI/*Msc*I fragment from clone A into clone B above, and this insert was subcloned into the various vectors indicated.

In Vitro Translation—A variant of pcDNA3 was generated in which a c-Myc epitope tag was cloned downstream of the MCS *Xba*I site. The Reaper open reading frame minus its stop codon was cloned in frame with the Myc tag of pcDNA3-myc using standard techniques to generate Reaper-Myc. A variant of pSP64T, an *in vitro* SP6 expression vector with flanking 5′ and 3′ β-globin untranslated region and a polyadenosine tail was generated called pSP64BN. The *Bgl*II cloning site of pSP64T was replaced with an oligonucleotide encoding the multiple cloning site of pEBB, including unique *Bam*HI and *Not*I sites. ReaperKR-FLAG, Reaper-FLAG, Reaper, and ReaperΔ1–15 were subcloned into pSP64BN by standard techniques. To produce radioactive protein for half-life assays, Reaper-myc, Reaper, ReaperΔ1–15, Reaper-FLAG, ReaperKR-FLAG, Cdc25, and Grp94 templates were added at 20 ng μl^{-1} to rabbit reticulocyte lysate (Stratagene) containing 1 $\mu Ci\ \mu l^{-1}$ of S-35 Translabel (ICN), 1× ((−)-cysteine, (−)-methionine) amino acid mix and other components per manufacturer's protocol. Translated proteins were resolved by SDS-PAGE, soaked in 1 M salicylic acid for 1 h, dried, and exposed to Biomax MR film (Kodak). To assay protein stability, 100 $\mu g\ ml^{-1}$ cycloheximide was added to translated proteins, which were then incubated at 30 °C for an additional 30 and 60 min, boiled in SDS sample buffer, and processed as above.

Cell Culture, Transfections, Immunoblotting, Pulse-Chase Analysis, and Apoptosis Assay—All of the cell culture reagents were obtained from Invitrogen unless otherwise specified. Details of cell culture, vector constructs, immunoblotting, affinity precipitations, and pulse-chase analysis were as previously described (14), with the following exceptions. HEK 293T cells were plated at a density of 1×10^6 cells/10-cm dish for immunoblotting experiments and 200,000 cells/well in 6-well dishes for pulse-chase analysis and apoptosis assay. The cells were transfected 24 h after plating using a standard protocol of calcium phosphate and HEPES-buffered saline. 10-cm dishes were transfected with a total of 10 μg of DNA, and 6-well plates were transfected with a total of 1.6 μg of DNA/well. Where indicated, the proteasome inhibitor LLnL (ALLN, Calbiochem) was added to a final concentration of 20 μM for 45 min prior to harvesting cells. When appropriate, the cells were harvested by rinsing once with phosphate-buffered saline and lysing the cells on ice with buffer IP (50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 2.5 mM $MgCl_2$, and 1% Nonidet P-40, plus 1× Complete protease inhibitor (Roche Molecular)). Bead-bound material following affinity precipitation was washed three times with buffer IP prior to analysis by SDS-PAGE and immunoblotting or autoradiography. The apoptosis-inducing ability of Reaper and ReaperKR was assayed by co-transfecting pEGFP-C1 (Clontech) with the vectors indicated. After 48 h, live cells (as determined by forward and side scatter) were analyzed for GFP fluorescence by flow cytometry. Each transfection was performed in duplicate, with and without 50 μM zVAD-fmk (Biomol) to inhibit caspase activation, for a total of four transfections/construct. The percentage of live GFP+ cells for each construct in the absence of zVAD-fmk was normalized to the percentage of live GFP+ cells in the presence of zVAD-fmk such that the results shown indicate caspase-dependent loss of GFP+ cells while correcting for any differences in transfection efficiency between constructs.

SL2 Cell Culture—All of the cell culture reagents were obtained from Invitrogen unless otherwise specified. SL2 cells were obtained from the ATCC via the Duke Cell Culture Facility and were maintained in Schneider's *Drosophila* medium supplemented with 10% heat-inactivated fetal bovine serum (HyClone). For transfection, 3×10^6 cells (at 5×10^5 cells/ml) were seeded in T-25 flasks. 24 h later, DNA was prepared for transfection by mixing 20–30 μg of appropriate constructs with 62 μl of 2 M $CaCl_2$ and 438 μl of sterile water. 500 μl of 2× HEPES buffered saline was bubbled in to each sample over 1 min. DNA mixtures were allowed to sit for 30 min at room temperature and were then added to cells for 16–24 h, after which cells were pelleted and resuspended in fresh medium.

SL2 Killing Assay—Enhanced GFP was subcloned into the *Eco*RI and *Xba*I sites of pCasper3, downstream of the ubiquitin promoter, using standard techniques. Wild type Reaper and ReaperKR were cloned into the *Eco*RI and *Bam*HI sites of pMT, downstream of the metallothionine promoter, using standard techniques. SL2 cells were co-transfected with 2 μg of GFP and 20 μg of pMT vector, Reaper, or ReaperKR. After 24 h, the cells were pelleted at 1,000 × *g* for 5 min and then resuspended in fresh medium. Following an additional 24 h, Reaper or ReaperKR was induced with 70 nM $CuSO_4$, and the cells were incubated at 25 °C for 3 days. FACS analysis was then performed. Transfection efficiency was controlled for by normalizing each transfection to its percentage of GFP (+) in the presence of zVAD-fmk. Each transformation was analyzed in triplicate (for a total of 18 samples: nine with zVAD-fmk and nine without). Forward and side scatter were used to gate viable cells, with the same gate settings used for all

samples. 100,000 gated cells/sample were counted and then analyzed for GFP fluorescence using FACS ANALYZER (BD). Cells with $>10^2$ GFP signal were taken as positive. The percentage of survival for sample A was calculated as $[\%GFP(+)_A - zVAD\text{-}fmk]/[\%GFP(+)_A + zVAD\text{-}fmk]$. The average percentage of GFP(+) was calculated, and the standard deviations for each sample were used to determine error.

Δ1–15 versus Wild Type Stability Assay—Full-length or Δ1–15 reaper-GFP fusions were generated by overlap PCR using the following oligonucleotides: 5′ reaper, GAAGGAGGATCCATGGCAGTGGCATTC-TACATACCC; 5′ overlap, TATCGCAAGCCATCGCAAAGATCTATGG-TGAGCAAGGGCGAG; 3′ overlap, CTCGCCCTTGCTCACCATAGATC-TTTGCGATGGCTTGCGATA; and 3′ GFP, CCTCCGGATCCCTACTT-GTACAGCTCGTCCATGCCGAG. Fusion open reading frames were subcloned into the *Bam*HI and *Eco*RI sites of pMT, downstream of the metallothionine promoter, using standard techniques. pIE3-DIAP-HA was a gift from Kristin White (Harvard/MGH). 10 μg of DIAP and 20 μg of full-length or Δ1–15 reaper-GFP were transfected into SL2 cells as described above. 24 h after resuspension in fresh medium, GFP fusions were induced with 700 nM $CuSO_4$ and placed into 50 μM zVAD-fmk to prevent cell death. After 16 h of induction, the cells were pelleted, washed with fresh medium, and resuspended in medium lacking copper but supplemented with 50 μM zVAD-fmk. The cells were immediately subjected to FACS analysis as above, with additional analyses at 16 and 24 h. Each sample was analyzed in triplicate as above. The averages were calculated for each sample, and the standard deviations were used to determine error. The percentage of Reaper remaining in the presence of DIAP1 = [+D]/[no D] where [+D] = [% GFP(+) with DIAP at time *T*]/[% GFP(+) with DIAP at time 0], and [no D] = [% GFP(+) without DIAP at time T]/[% GFP(+) without DIAP at time 0].

RESULTS

IAPs Can Ubiquitinate Reaper, HID, and Grim—Our previous work and that of others have shown that the interaction between IAPs and Reaper (and Grim and HID) lowers cellular IAP levels by stimulating ubiquitin-mediated degradation of the IAPs (12–17). In the course of these experiments involving Reaper, HID, and DIAP1, we noticed that overexpression of Reaper in *Drosophila* imaginal discs led not only to lower DIAP1 levels but also to elevated levels of HID as detected by immunofluorescence (Fig. 1, *A–F*). The reciprocal experiment examining Reaper protein levels in the presence of HID overexpression was uninformative because of the inability of our Reaper antibodies to detect Reaper *in situ*. To eliminate the possibility that Reaper expression was affecting HID transcription (Fig. 1*F*), we analyzed the amount of HID mRNA by *in situ* hybridization and found that there was no increase in the amount of HID message (Fig. 1*H*). These results implied that the regulation of Reaper and HID levels were somehow linked at a post-transcriptional step. This seemed particularly significant in light of the fact that the Reaper, HID, and Grim proteins all play a key role in developmental and radiation-induced apoptosis in *Drosophila*, but there have been no published reports on post-transcriptional mechanisms controlling the abundances of these proteins.

Although an effect on translational regulation remained a possible explanation for the elevated HID levels, this seemed unlikely because Reaper has been shown to suppress rather than enhance translation (14, 17). However, we had also noted that Reaper immunoprecipitates from cells transfected with both Reaper and a human IAP (XIAP) contained a prominent 18-kDa species recognized by anti-Reaper immunoblotting. Given the established link between the RHG proteins, IAPs, and ubiquitin-mediated degradation, we strongly suspected that this species was monoubiquitinated Reaper. Indeed, the 18-kDa band evident in the Reaper immunoblot of the anti-Reaper immunoprecipitate was also recognized by anti-ubiquitin antibody (Fig. 2*A*). Given that *Drosophila* IAP-1 (DIAP1) is the physiologically relevant IAP with regard to Reaper, we repeated these experiments with Reaper and DIAP1 with similar results (Fig. 2*A*).

We then transfected cells with Reaper and either XIAP or

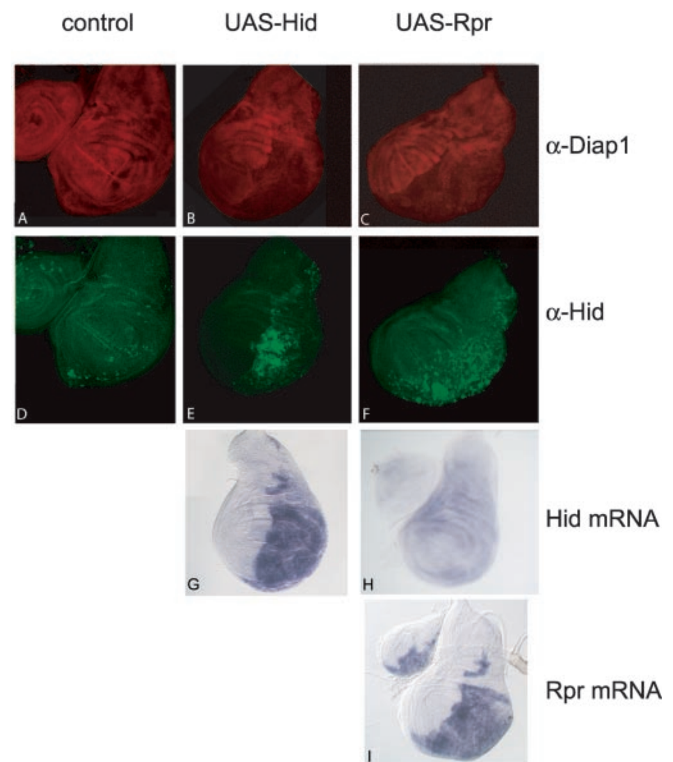

FIG. 1. **Reaper expression lowers DIAP1 levels and post-transcriptionally increases HID levels.** DIAP1 and HID were detected by immunofluorescence of imaginal discs from *Drosophila* embryos overexpressing p35 (*A* and *D*), HID + p35 (*B* and *E*), and Reaper + p35 (*C* and *F*). Expression of the caspase inhibitor p35 prevented Reaper- and HID-induced apoptosis. *in situ* hybridization was used to detect mRNA expression of HID (*G* and *H*) and Reaper (*I*).

DIAP1 and looked for Reaper-ubiquitin conjugates in the IAP immunoprecipitate. We detected multiple ubiquitinated species at 9-kDa intervals above the 9-kDa nonubiquitinated Reaper (Fig. 2*B*, *black arrowheads*). Importantly, these protein species were absent when cells were transfected with Reaper and an XIAP RING finger point mutant (H467A) that lacks ubiquitin ligase activity. Note also that only the 18- and 27-kDa species could be detected in the anti-Reaper immunoblot because the Reaper antibody was prepared against an extreme C-terminal peptide from Reaper, where four of five ubiquitin-modifiable lysines are located (see Fig. 5*A*). As such, it is likely that multiubiquitination of Reaper was interfering with antibody binding.

The presence of ubiquitinated Reaper species in the IAP co-precipitate suggested that Reaper might be a substrate of IAP ubiquitin-ligase activity. Additionally, we suspected that the RHG protein HID might also serve as a substrate for IAP-mediated ubiquitination because our initial experiment showed that HID levels increased as DIAP1 levels were lowered by Reaper (Fig. 1). To prove that Reaper and HID could in fact serve as substrates for IAP-mediated ubiquitination, we performed *in vitro* ubiquitination reactions with recombinant forms of these proteins. These results show clearly that Reaper and HID are substrates for DIAP1-stimulated ubiquitination (Fig. 2*C*). Furthermore, we found that Grim is also ubiquitinated *in vitro* (Fig. 2*C*), suggesting that all three of these *Drosophila* RHG proteins may be regulated at the level of protein stability.

Reaper Is Stabilized by Inhibiting the Proteasome—To further elucidate the machinery involved with this phenomenon, we focused on the regulation of Reaper stability and asked whether or not the proteasome was involved in the degradation

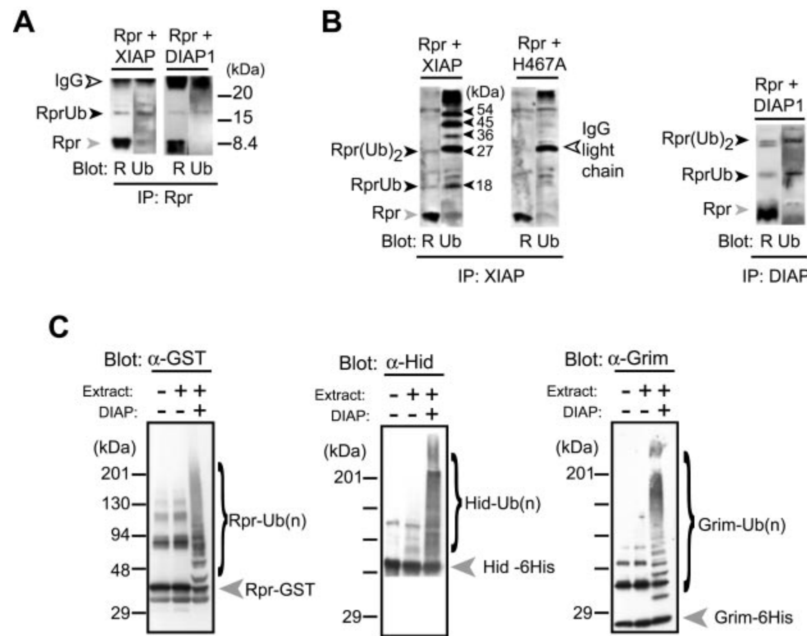

FIG. 2. **Reaper, Hid, and Grim ubiquitination.** *A*, Rpr and FLAG-tagged XIAP or Reaper and GST-tagged DIAP1 were transfected into HEK 293T cells. Immunoprecipitations were performed using anti-Reaper serum coupled to protein A (*IP: Rpr*). The precipitates were resolved by SDS-PAGE and blotted using anti-Reaper serum (*R*) or anti-ubiquitin antibody (*Ub*). The Reaper parent species is indicated (*gray arrowhead*), as is the 18-kDa monoubiquitinated species (*black arrowhead*). *B*, Rpr and FLAG-XIAP, Rpr and FLAG-H467A XIAP ligase mutant (H467A), or Rpr and GST-DIAP1 were transfected into HEK 293T cells. Affinity precipitations were performed using anti-FLAG antibody coupled to protein G (*IP: XIAP*) or glutathione-Sepharose (*IP: DIAP*). The precipitates were resolved by SDS-PAGE and blotted using anti-Reaper serum (*R*) or anti-ubiquitin antibody (*Ub*). Reaper parent species is indicated (*gray arrowhead*), as are the mono- and polyubiquitinated species (*black arrowheads*). *C*, *left panel*, recombinant Reaper-GST. *Middle panel*, Hid-His_6. *Right panel*, Grim-His_6 were mixed with *Drosophila* embryo extract for 10 min. Subsequently, recombinant DIAP1 and His-ubiquitin were added, and the mixture shifted to 37 °C for 40 min. The samples were resolved by SDS-PAGE and blotted using indicated antibodies. Note parent species (*gray arrows*) and polyubiquitinated species (*brackets*).

of ubiquitinated Reaper. When reticulocyte lysates were used to transcribe and translate Reaper, the addition of LLnL (also known as ALLN) to inhibit proteasomal degradation resulted in much higher levels of Reaper production (Fig. 3*A*). Transcription and translation of two unrelated control proteins (cdc25 and Grp94) demonstrated that the effect of LLnL was not a nonspecific increase in protein production (Fig. 3*A*). Extending these results to the more complex milieu of transfected 293T cells, we found that the addition of LLnL for 45 min significantly increased the amount of Reaper detected by immunoprecipitation and immunoblotting (Fig. 3*B*). Both experiments suggest that Reaper is targeted for proteasomal degradation.

The Reaper N Terminus Is Required for IAP-mediated Degradation—Given the ability of the RHG proteins to interact physically with IAPs, we hypothesized that this direct binding would be necessary for IAPs to promote Reaper, HID, and Grim ubiquitination and degradation. To verify this, we tested a deletion mutant of Reaper that lacked the first 15 amino acids and was therefore missing the canonical RHG IAP-binding motif (Reaper Δ1–15). In a previous report, Reaper Δ1–15 failed to co-precipitate with cellular IAP (24), and we have shown that Reaper Δ1–15 was unable to stimulate XIAP degradation (14). As expected, Reaper Δ1–15 failed to bind DIAP1 (Fig. 4*A*), whereas full-length Reaper co-precipitated with DIAP1 quite well (Fig. 2*B*). Both wild type and Reaper Δ1–15 were then transcribed and translated in rabbit reticulocyte lysates, and the results were analyzed by autoradiography. Our results showed that the Reaper Δ1–15 protein was considerably more abundant than the wild type, consistent with the mutant being more stable (Fig. 4*B*). When cycloheximide was added to the reticulocyte lysates to stop translation, the Reaper Δ1–15 protein was markedly more stable than wild type Reaper over a 60-min time course (Fig. 4*C*).

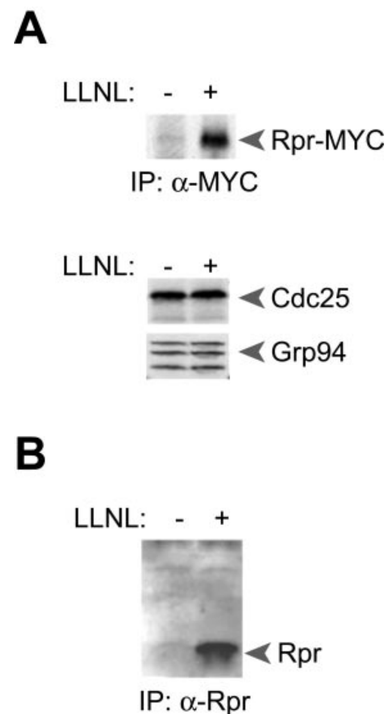

FIG. 3. **Proteasome-dependent degradation of Reaper.** *A*, Myc-tagged Reaper (Rpr-myc), Cdc25, or Grp94 were translated in the presence of 20 μM LLnL or Me_2SO carrier in rabbit reticulocyte lysate. For Reaper, equal amounts of lysate were immunoprecipitated using the 9E10 anti-Myc antibody. For controls, equal volumes of lysate were loaded for analysis. The samples were resolved by SDS-PAGE and exposed to film. *B*, untagged Reaper was transfected into HEK 293T cells that were treated for 45 min prior to harvesting with 20 μM LLnL or Me_2SO carrier. The cells were harvested and subjected to immunoprecipitation and immunoblotting using anti-Reaper serum.

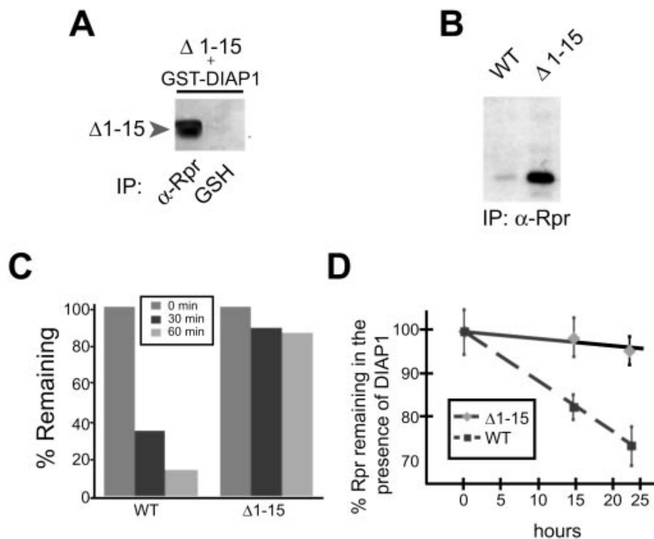

FIG. 4. **Reaper Δ1–15 is not an IAP substrate.** *A*, HEK 293T cells were co-transfected with GST-DIAP1 and Δ1–15 Reaper (Δ1–15). Co-precipitations were performed using either anti-Reaper serum (α-Rpr) or glutathione-Sepharose (*GSH*). The samples were resolved by SDS-PAGE and blotted using anti-Reaper serum. Note that Fig. 2*B* demonstrates co-immunoprecipitation of full-length Reaper and DIAP1. *B*, untagged Reaper and Reaper Δ1–15 were translated in rabbit reticulocyte lysate. Equal amounts of lysate were subject to immunoprecipitation using anti-Reaper serum. The samples were resolved by SDS-PAGE and exposed to film. *C*, reaper or Reaper Δ1–15 were produced in reticulocyte lysates, cycloheximide was added, and the proteins were incubated for a further 30 or 60 min at 30 °C. Equal volumes of reticulocyte lysate were immunoprecipitated with anti-Reaper serum and processed as above. The precipitates were resolved by SDS-PAGE and exposed to film. The results were quantified by ImageJ application (NIH). *D*, *Drosophila* SL2 cells were transfected with either copper-inducible Reaper-GFP or Δ1–15 Reaper-GFP in the presence or absence of constitutively expressed DIAP1. At time 0, copper was removed, and the cells were harvested at 0, 16, and 24 h and subjected to FACS analysis to determine the percentage of GFP-positive cells. The percentage of Reaper-GFP in the presence of DIAP1 was determined by comparing the percentage of GFP to that at time 0 in cells containing DIAP1 relative to the percentage of GFP in cells lacking DIAP1. The samples were processed and analyzed in triplicate, and the standard deviations were used to determine error. *IP*, immunoprecipitation; *WT*, wild type.

To demonstrate the biological significance of the Reaper-IAP interaction in modulating Reaper protein levels, we examined the relative stability of Reaper and Reaper Δ1–15 in *Drosophila* SL2 cells. Because SL2 cells have a low transfection efficiency, it was difficult to follow Reaper protein levels by immunoblotting or radiolabeling. Therefore, we generated Reaper-GFP and Reaper Δ1–15-GFP constructs under the control of a metallothionine promoter to perform a fluorescence-based protein stability assay. Each Reaper construct was transfected into SL2 cells, with or without DIAP1 that was driven by the constitutive baculovirus IE1 promoter. After 16 h of induction with copper, the copper containing medium was replaced with fresh (copper-free) medium, thereby inactivating the Reaper promoter. SL2 cells were then immediately analyzed for GFP fluorescence (and then analyzed again at 16 and 24 h). The results of this experiment confirmed our hypothesis that wild type Reaper was subject to DIAP1-stimulated degradation, but Reaper Δ1–15 was not (Fig. 4*D*). An identical experiment in which cycloheximide was used in place of copper-removal gave similar results (data not shown). It is worth noting that Reaper Δ1–15 retains all five of the lysines in Reaper (Fig. 5*A*), so the enhanced stability of the Reaper deletion mutant most likely stems from its inability to interact with IAPs and not from a lack of potential ubiquitin conjugation sites.

Ubiquitination-resistant Reaper Is Not Destabilized by IAPs—To fully address the biological significance of this novel mechanism for regulating Reaper levels, we wanted to ask whether or not the ubiquitination and degradation of Reaper *per se* affects its abundance and biological activity. This question precluded the use of Reaper Δ1–15 because that mutant lacks the IAP interaction domain and is therefore unable to inhibit IAP activity or stimulate IAP degradation. We therefore chose to construct an additional Reaper mutant that would interact normally with the IAPs but would itself be impervious to ubiquitination. Accordingly, we mutated all of the lysines in Reaper to arginines (ReaperKR) to inhibit ubiquitin conjugation (Fig. 5*A*). As expected, ReaperKR still interacted quite stably with DIAP1 (Fig. 5*B*). When ReaperKR was produced *in vitro* using reticulocyte lysates, we observed that much more of the mutant was made than wild type Reaper, suggesting that the lysine mutations were in fact stabilizing the protein (Fig. 5*C*). We then expressed Reaper and ReaperKR by transient transfection of 293T cells and observed that the ReaperKR produced to much higher steady state levels than wild type Reaper (Fig. 5*D*). Furthermore, although inhibition of the proteasome with LLnL increased Reaper levels, it had no effect on ReaperKR levels (Fig. 5*D*). We then performed a pulse-chase assay in 293T cells to directly examine the intrinsic half-lives of Reaper *versus* ReaperKR. As expected, the lysine mutant had a markedly increased half-life relative to wild type Reaper (Fig. 5*E*). These experiments supported our hypothesis that the ubiquitin-proteasome pathway is important for the stability of the Reaper protein and that mutation of the lysines in Reaper makes it resistant to degradation.

IAP Ligase Activity Contributes to Reaper Instability—If IAPs were in fact mediating the degradation of Reaper, we reasoned that ReaperKR should be resistant to the effects of IAP overproduction. We therefore performed pulse-chase analysis on Reaper and ReaperKR in 293T cells that had also been transfected with XIAP. The results from this assay confirmed that ReaperKR was significantly more stable than the wild type protein (Fig. 5*F*). In contrast, when the pulse-chase analysis was repeated using the XIAP H467A ubiquitin ligase mutant, wild type Reaper was stable, implying that the destabilization of Reaper was in fact specific to IAP ubiquitin-ligase activity (Fig. 5*F*).

Regulation of Reaper Stability Affects Its Ability to Induce Apoptosis—Finally, because Reaper is a potent pro-apoptotic protein, we wanted to assay the killing ability of the degradation-resistant ReaperKR with respect to wild type Reaper. We reasoned that if the regulation of Reaper levels by IAP-stimulated ubiquitination was biologically significant, then the degradation-resistant ReaperKR would be an even more potent killer than wild type Reaper. To test this hypothesis, we first compared the killing activities of Reaper and ReaperKR in transfected human cells. As shown in Fig. 6*A*, ReaperKR was a substantially better inducer of apoptosis than Reaper. Finally, to assay the biological function of Reaper in the context of its native species, we examined the relative killing activities of Reaper and ReaperKR in *Drosophila* SL2 cells. Once again, the degradation-resistant ReaperKR was a much better inducer of caspase-dependent cell death than wild type Reaper (Fig. 6*B*). Collectively, these experiments demonstrate that regulation of Reaper by IAP-mediated ubiquitination and degradation has a significant impact on the ability of Reaper to initiate apoptosis.

DISCUSSION

Our results implicate the ubiquitin-proteasome pathway in the regulation of Reaper stability and biological activity. *In vitro* ubiquitination assays coupled with overexpression and mutant studies strongly suggest that IAPs such as XIAP and DIAP1 can serve as ubiquitin ligases for Reaper, HID, and Grim. Furthermore, the regulation of Reaper stability has a

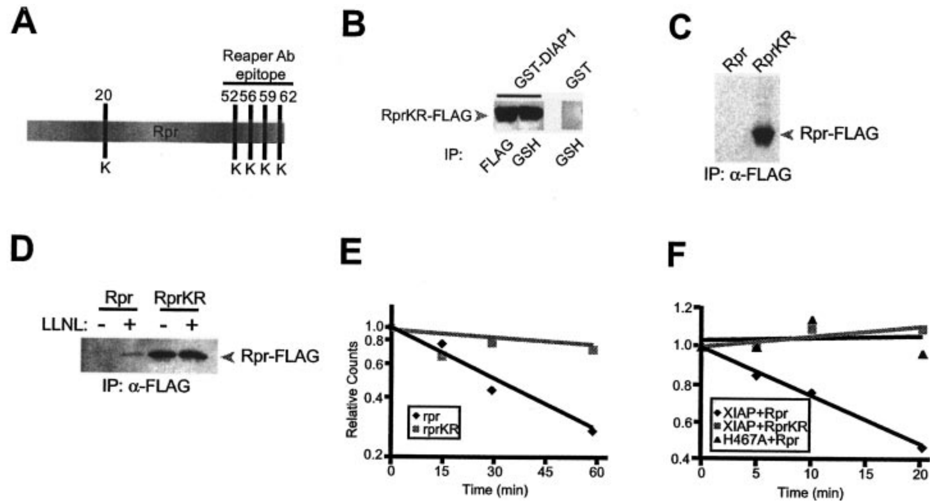

FIG. 5. **Lysine-deficient Reaper exhibits increased stability.** *A*, schematic of the Reaper protein sequence, showing the number and relative distribution of lysines. The amino acid positions are indicated. *B*, HEK 293T cells were co-transfected with GST or GST-DIAP1 and lysine-deficient Reaper (ReaperKR-FLAG). Co-precipitations were performed using either anti-FLAG antibody or glutathione-Sepharose (*GSH*). The samples were resolved by SDS-PAGE and blotted using anti-FLAG antibody. *C*, FLAG-tagged Rpr and lysine-deficient Reaper (*RprKR*) were translated in rabbit reticulocyte lysate. Equal volumes of lysate were immunoprecipitated with anti-FLAG antibody. The samples were resolved by SDS-PAGE and exposed to film. *D*, HEK 293T cells were transfected with FLAG-tagged Rpr and RprKR. 45 min prior to harvest, the cells were treated with 20 μM LLnL (or not). The cells were harvested, and the Reapers were precipitated using anti-FLAG antibody. The samples were resolved by SDS-PAGE and blotted using anti-FLAG antibody. *E*, HEK 293T cells were transfected with Rpr or RprKR. At the indicated times, the cell lysates were subjected to immunoprecipitation using anti-FLAG antibody to precipitate Rpr and RprKR. The samples were resolved by SDS-PAGE and exposed to film, and the results were quantified using the ImageJ application (NIH). *F*, HEK 293T cells were transfected with Rpr or RprKR and either GST-XIAP or GST-XIAP H467A. The samples were processed as in *E*.

significant effect on the ability of Reaper to initiate apoptosis. As such, the work reported here ascribes a new anti-apoptotic function to the IAP RING finger domain in that it promotes the degradation of RHG family members.

The findings reported here suggest that IAP proteins can ubiquitinate Reaper and its relatives and that this requires a stable interaction between the RHG protein and the IAP. We and several other groups recently reported that the interaction between Reaper, HID, and Grim and the IAPs can stimulate IAP auto-ubiquitination and degradation, thereby facilitating caspase activation and cell death (12–17). Given the ability of Reaper to stimulate IAP degradation and vice versa, it is not entirely clear how the outcome of the Reaper-IAP battle is determined. Because Reaper is also transcriptionally regulated (35–39), the balance between Reaper-mediated death and IAP-mediated survival may be partially determined by the strength of Reaper induction following a particular apoptotic stimulus. Similarly, it is likely that cells with different levels and types of IAPs will differ in their susceptibility to Reaper. Almost certainly, other modulatory factors also help to determine the outcome of the Reaper-IAP interaction. One such candidate factor is Morgue, a newly identified protein related to variant ubiquitin-conjugases that was isolated in a screen for modifiers of Reaper and Grim cell death (13, 16). This protein may assist Reaper- and Grim-mediated IAP degradation in some way, helping to shift the balance toward death when Morgue is present. Also, the ability of Reaper to suppress translation may assist in shifting the IAP-Reaper balance toward cell death.

Our data demonstrating that Reaper, HID, and Grim are all subject to IAP-stimulated ubiquitination may help to explain previous reports that have noted a cooperative apoptosis-inducing effect when more than one RHG protein is present (40, 41). Although it has been thought that this effect might be due to slightly different biological functions, the data presented here suggest that these proteins may cooperate *in vivo* by indirectly modulating each other's abundance; that is, as the RHG proteins successfully stimulate ubiquitin-mediated destruction of the IAPs, their own half-lives are extended, and they are able

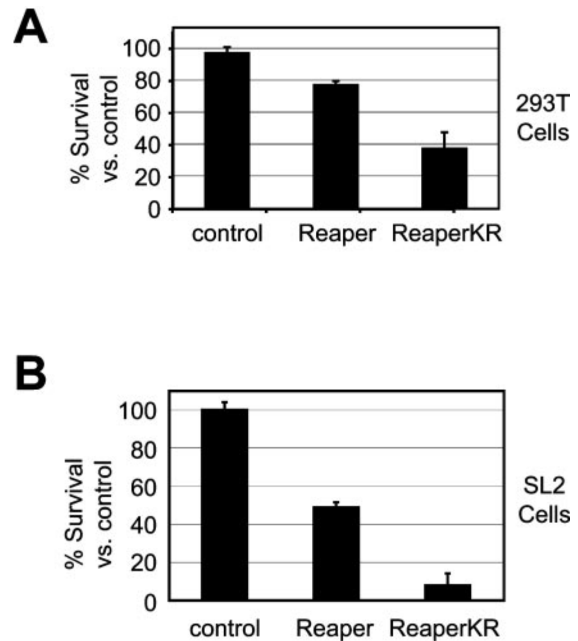

FIG. 6. **Lysine-deficient Reaper is a more potent inducer of apoptosis.** *A*, HEK 293T cells were co-transfected with GFP and vector alone (*control*), Reaper, or lysine-deficient ReaperKR in the presence or absence of the irreversible caspase inhibitor zVAD-fmk. After 48 h, the cells were harvested and subjected to FACS analysis to determine the percentage of GFP-positive cells. The percentage of survival was calculated by the percentage of GFP-positive cells without zVAD-fmk relative to the percentage of GFP-positive cells with zVAD-fmk. The samples were processed in duplicate, and the standard deviations were used to determine error. *B*, *Drosophila* SL2 cells were co-transfected with constitutively expressed GFP and metallothionine-driven Reaper, ReaperKR, or vector control. Reaper was induced 16 h after transfection (to allow for GFP expression), and the cells were harvested after a further 48 h of incubation. The cells were subjected to FACS analysis as above. The percentage of survival was calculated by dividing the percentage of GFP-positive induced cells by the percentage of GFP-positive induced cells treated with zVAD-fmk. The samples were processed in triplicate, and the standard deviations were used to determine error.

to accumulate to higher levels. This would explain the rise in HID levels following Reaper overexpression (Fig. 1).

Interestingly, the vertebrate IAP antagonist Smac is also a substrate for IAP-mediated ubiquitination, suggesting that ubiquitination of IAP-binding partners may be widespread (42). In this regard, it would be interesting to determine whether the stability of Omi is regulated by IAP proteins as well. Conversely, the weakly pro-apoptotic proteins Smac and Omi have not been reported to stimulate IAP degradation. If Smac and Omi do not, in fact, have this activity, their interaction with the IAP ubiquitin-ligase would be unidirectional, with the IAP targeting Smac and Omi for destruction, while the IAP itself remained stable. This may be the case if Smac and Omi do not engage the IAPs in precisely the same way as Reaper, HID, and Grim or if a domain in addition to the RHG motif is also required to stimulate IAP auto-ubiquitination.

Finally, the interplay that we have described between Reaper and the IAPs illustrates that the decision to undergo apoptosis (or not) is an active struggle within the cell. In this particular struggle, the outcome can be tipped one way or the other by regulating the protein stability of these antagonistic apoptotic regulators.

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