Apoptosis Induced by Drosophila Reaper and Grim in a Human System

ATTENUATION BY INHIBITOR OF APOPTOSIS PROTEINS (cIAPs)*

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Previous genetic studies have established Reaper and Grim as central regulators of apoptosis in Drosophila melanogaster. Reaper and Grim induce extensive apoptosis in Drosophila, yet share no homology to known vertebrate proteins. In this study, we show for the first time that ectopic expression of Reaper or Grim induced substantial apoptosis in mammalian cells. Reaper- or Grim-induced apoptosis was inhibited by a broad range of caspase inhibitors and by human inhibitor of apoptosis proteins cIAP1 and cIAP2. Additionally, in vivo binding studies demonstrated that both Reaper and Grim physically interacted with human IAPs through a homologous 15-amino acid N-terminal segment. Deletion of this segment from either Reaper or Grim abolished binding to cIAPs. In vitro binding experiments indicated that Reaper and Grim bound specifically to the BIR domain-containing region of cIAPs as deletion of this region resulted in loss of binding. The physical interaction was further confirmed by immunolocalization. When co-expressed, Reaper or Grim co-localized with cIAP1. However, deletion of the N-terminal 15 amino acids of Reaper or Grim abolished co-localization with cIAP1, suggesting that this homologous region can serve as a protein-protein interacting domain in regulating cell death. Moreover, by virtue of this interaction, we demonstrate that cIAPs can regulate Reaper and Grim by abrogating their ability to activate caspases and thereby inhibit apoptosis. This is the first function attributed to this 15-amino acid N-terminal domain that is the only region having significant homology between these Drosophila death inducers.

It is apparent that the underlying events in apoptosis are evolutionary conserved (1). First, there is similarity between the genetic, biochemical, and morphological events associated with apoptosis that are highly conserved between species (2, 3). Second, several of the key signaling and effector molecules have highly conserved counterparts that are functionally interchangeable between heterologous species (4–6). Finally, an increasing number of viral proteins that inhibit apoptosis exhibit anti-apoptotic activity in both vertebrates and invertebrates (7–12).

Three Drosophila melanogaster genes, reaper (rpr), grim, and hid, have been identified as key regulators of apoptosis during Drosophila embryogenesis (13–15). Deletion of a chromosomal segment encoding rpr, grim, and hid results in the loss of apoptotic cell death during fly development (16, 17). Loss of rpr alone inhibits nearly all apoptosis during Drosophila embryogenesis and attenuates cell death in response to several external inducers. Overexpression of rpr as a transgene in Drosophila eyes or by transfection in a Drosophila cell line results in apoptosis (10, 13). Apoptosis induced by Reaper, a surprisingly small protein of 65 amino acid residues, involves activation of caspases because death is inhibited by specific caspase inhibitors (13, 18, 19). Grim is predicted to encode a 138-amino acid polypeptide and, like rpr, expression of grim RNA coincides with the onset of apoptosis during embryonic development, and its overexpression induces extensive apoptosis in transgenic flies and cell lines (15). Reaper and Grim appear to function independently of each other, as cell death induced by Grim does not require expression of reaper (15). Both proteins do, however, share a highly homologous N-terminal motif.

The inhibitor of apoptosis proteins (IAP)† are a family of highly conserved anti-apoptotic proteins first identified in baculovirus by their ability to substitute functionally for the cell death inhibitor p35 (8, 20, 21). Baculovirus IAPs, namely Op-IAP and Cp-IAP, also block apoptosis in insect SF-21 cells induced by rpr expression (19) and actinomycin D (8). Additionally, Cp-IAP partially inhibits Reaper-induced cell killing in the Drosophila developing eye (10), and Op-IAP can significantly inhibit HeLa cell killing induced by mammalian caspase-1 (22) and the mammalian receptor-associated death adaptor molecule FADD (23). Cellular homologs of IAPs have also been described in humans (24) and Drosophila (10) that, like the baculovirus IAPs, block apoptosis in response to different stimuli.

The first discovered human IAP, the neuronal apoptosis inhibitory protein (NAIP), was identified based on its role in the neurodegenerative disorder spinal muscular atrophy (SMA) (25). Recently, four other human homologs, c-IAP1, c-IAP2, X-IAP, and survivin, have been identified, and all demonstrate anti-apoptotic activity (23, 24, 26–28). An important structural feature of all IAPs is an N-terminal motif termed the baculovirus IAP repeat (BIR). Human c-IAPs were originally identified as proteins that were recruited to the tumor necrosis factor receptor type 2 (TNFR2) signaling complex through an association involving the BIR-containing region and the receptor-associated adapter molecule TRAF2 (24). More recently, it has

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‡ The abbreviations used are: IAP, inhibitor of apoptosis proteins; rpr, reaper; SMA, spinal muscular atrophy; TNFR2, tumor necrosis factor receptor type 2; BIR, baculovirus IAP repeat; X-gal, 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside; DAPI, 4,6-diamidino-2-phenylindole; GST, glutathione S-transferase; PBS, phosphate-buffered saline; mAb, monoclonal antibody; FADD, Fas/APO-1-associated death domain protein.
FIG. 1. *Drosophila* Reaper and Grim induce apoptosis in mammalian cells. A, ectopic expression of Reaper or Grim kills MCF7 cells by inducing caspase activation and apoptosis. Cells were transiently transfected with plasmids encoding the indicated proteins and pCMV-β-galactosidase. Following staining with X-gal, transfected cells were examined by phase contrast microscopy. B, quantitation of apoptotic cell death in MCF7 cells. Cells were transiently transfected with the indicated expression constructs and pCMV-β-galactosidase. The broad range caspase inhibitor z-VAD-fmk was used at a concentration of 20 μM, whereas other cell death inhibitors were cotransfected at a 4-fold molar excess with total transfected DNA being kept constant. Cells were stained with X-gal and examined by phase contrast microscopy. The data (mean ± S.E.) are the percentage of X-gal-stained cells that possessed apoptotic morphology. C, human MCF7 cells were co-transfected with an expression construct encoding p35 and the indicated expression constructs. Twenty h post-transfection, cells were harvested, lysed, and immunoblotted with a polyclonal antibody raised against the large catalytic subunit of caspase-7 (left panel). Poly(ADP-ribose) polymerase (PARP) was detected in urea/SDS-solubilized nuclei by immunoblotting with a polyclonal antibody raised against PARP (right panel). D, human MCF7 cells were
been demonstrated that mammalian IAPs, X-IAP, and both cIAPs function to inhibit specific caspases, again an activity attributable to the BIR domain (29, 30). Inhibition of downstream caspases likely is not the sole mechanism of action of IAPs as in vivo binding studies demonstrate that both baculoviral and Drosophila IAPs can physically interact with Reaper (through the BIR domain), potentially inhibiting its ability to activate caspases (19). Although Reaper and Grim are key components of the death pathway in Drosophila, no mammalian homologs have been identified. However, we find that Drosophila Reaper and Grim can induce apoptosis in human cells. Furthermore, this is attenuated by several caspase inhibitors and, importantly, by human cIAPs. Reaper and Grim were found to bind the BIR domain-containing region of human cIAPs through an N-terminal 15-amino acid domain. Through this interaction, cIAPs can regulate Reaper and Grim by altering their subcellular distribution and blocking their access to downstream caspases and thereby inhibiting apoptosis. These results are the first evidence that the cell death machinery engaged by Reaper and Grim is functionally conserved in mammalian cells.

EXPERIMENTAL PROCEDURES

Cell Death Assays—Human MCF7 breast carcinoma cells were transiently transfected as described previously (31, 32). Briefly, 2.5 × 10⁵ MCF7 cells were transfected with 0.1 μg of the reporter plasmid pCMV β-galactosidase plus 1 μg of test plasmid in 6-well tissue culture dishes using LipofectAMINE (Life Technologies, Inc.) as per the manufacturer’s instructions. Thirty h post-transfection, the cells were fixed with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) for 3–4 h. Cells were visualized by phase contrast microscopy. Approximately 300–400 β-galactosidase-positive cells were assayed for each transfection (n = 3) from three randomly selected fields, and the mean of these was used to calculate percentage apoptosis. Viable or apoptotic cells were distinguished based on morphological alterations typical of adherent cells undergoing apoptosis including becoming rounded, condensed, and detached from the dish (2).

Expression Vectors—cDNA encoding C-terminal FLAG-tagged Reaper (Rpr-Flag) and Grim (Grim-Flag) were generated by standard polymerase chain reaction techniques using custom-designed primers and subcloned into the mammalian expression vector pcDNA3 (Invitrogen). Truncated versions of Rpr (ΔRpr-15)-Flag and Grim (ΔGrim-2-15)-Flag were generated by deletional polymerase chain reaction mutagenesis. Reaper and Grim were expressed as GST fusion proteins in the pGEX-6P vector (Amersham Pharmacia Biotech). Co-immunoprecipitation and Western Blot Analysis—Reaper or Grim could induce apoptosis in mammalian cells. Ecotropic expression of Reaper or Grim in human MCF7 breast carcinoma cells induced rapid apoptosis (Fig. 1). Morphologically, both Reaper- and Grim-transfected cells displayed alterations characteristic of cells undergoing apoptosis, becoming rounded, shrunken, and detached from the culture dish (Fig. 1A). We next determined whether known suppressors of apoptosis inhibited Reaper- or Grim-induced mammalian cell death. Consistent with studies in Drosophila (13, 18, 19), ecotopic cell death induced by overexpression of Reaper and Grim in mammalian cells was blocked by various caspase inhibitors, including zVAD-fmk, p35, and CrmA (Fig. 1B). In addition, mammalian cIAP1 and cIAP2 abrogated both Reaper- and Grim-induced cell killing (Fig. 1, A and B), further suggesting that Reaper and Grim function upstream of caspases.

To confirm this, the activation of one of the distal caspases, caspase-7, was examined. Activation of caspase family members occurs following the proteolytic processing of a single polypeptide-inactive zymogen to an active dimeric species consisting of large and small subunits. Given this, the activation of caspase-7 was monitored by the emergence of the active enzyme products in cells transfected with Grim or Reaper. As shown in Fig. 1C, in vector transfected cells (first lane), caspase-7 is present in its zymogen form, whereas in cells overexpressing the p55 tumor necrosis factor death receptor (second lane), this caspase is converted to the active processed form as evidenced by the appearance of the large catalytic subunit. Because both Grim and Reaper overexpression also resulted in the conversion of caspase-7 to its active form (third and fourth lanes, respectively), cell death induced by these Drosophila death proteins must involve the activation of mammalian apoptotic caspases. However, we were unable to detect direct association between these death proteins and caspases, transfected as described above. Cells were transfected with equimolar amounts of death inducers and a 4-fold molar excess of cell death inhibitors. The data (mean ± S.E.) are the percentage of X-gal-stained cells that possessed apoptotic morphology.

RESULTS AND DISCUSSION

Drosophila Reaper and Grim Induce Apoptosis in Mammalian Cells—Key inducers and regulators of apoptosis, including caspases and mammalian Bcl-2 family homologues, are highly conserved between species, suggesting that the basic mechanism of apoptosis is similar. Given this, we asked if Drosophila Reaper or Grim could induce apoptosis in mammalian cells. Electropic expression of Reaper or Grim in human MCF7 breast carcinoma cells induced rapid apoptosis (Fig. 1). Morphologically, both Reaper- and Grim-transfected cells displayed alterations characteristic of cells undergoing apoptosis, becoming rounded, shrunken, and detached from the culture dish (Fig. 1A). We next determined whether known suppressors of apoptosis inhibited Reaper- or Grim-induced mammalian cell death. Consistent with studies in Drosophila (13, 18, 19), ecotopic cell death induced by overexpression of Reaper and Grim in mammalian cells was blocked by various caspase inhibitors, including zVAD-fmk, p35, and CrmA (Fig. 1B). In addition, mammalian cIAP1 and cIAP2 abrogated both Reaper- and Grim-induced cell killing (Fig. 1, A and B), further suggesting that Reaper and Grim function upstream of caspases.

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Fig. 3. Reaper and Grim interact with human cIAP1. A, Grim and Reaper interact with human cIAP1. 293 cells were transiently transfected with expression constructs encoding Grim-Flag or Reaper-Flag and cIAP1-Myc. Thirty-six h post-transfection, extracts were prepared and immunoprecipitated (IP) with a control monclonal antibody (mAb) (designated C) or a mAb to the specified epitope tag. Co-precipitating cIAP1-Myc was detected by immunoblotting with anti-Myc monoclonal antibody. B, cIAP1 interacts through its BIR domains with Reaper and Grim. In vitro translated 35S-labeled cIAP1 (left panel), cIAP1-BIR, and cIAP1-RING (right panel) were incubated with GST alone or GST fusions of Reaper and Grim immobilized onto glutathione-Sepharose. Bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. Lower panels confirm equivalency of loading.

Fig. 4. The N-terminal 15 amino acids of Reaper or Grim mediate association with cIAP1. A, cIAP1 interacts with Rpr but not with ΔRpr2–15. 293 cells were transiently co-transfected with the indicated expression constructs for cIAP1-Myc and Rpr-Flag or ΔRpr2–15-Flag. Co-precipitation of cIAP1-Myc with Rpr-Flag was detected by immunoblotting (Blot) with an anti-Myc monoclonal antibody (top panel). Expression of Rpr-Flag and ΔRpr2–15-Flag are shown in lower insets. B, cIAP1 interacts with Grim but not with ΔGrim2–15. Cells were transfected and lysates immunoprecipitated as in panel A. Coprecipitation of cIAP1-Myc was detected by immunoblotting with anti-Myc. Expression of Grim-Flag, ΔGrim2–15-Flag, and cIAP1-Myc are shown in the lower insets. C, quantitation of apoptotic cell death induced by ΔRpr2–15 and ΔGrim2–15 in MCF7 cells. Cells were transiently transfected with the indicated expression constructs and pCMV-β-galactosidase. The broad range caspase inhibitor z-VAD-fmk was used at a concentration of 20 μM, whereas other cell death inhibitors were cotransfected at a 4-fold molar excess with total transfected DNA being kept constant. Cells were stained with X-gal and examined by phase contrast microscopy. The data (mean ± S.E.) are the percentage of X-gal-stained cells that possessed apoptotic morphology.
suggesting an indirect mechanism of caspase activation (data not shown). Furthermore, we analyzed the state of the endogenous apoptotic substrate, PARP. In nonapoptotic cells, PARP is observed in the uncleaved form (116 kDa), whereas in cells transfected with Grim or Reaper, which are undergoing apoptosis, PARP is characteristically cleaved to an indicator apoptotic fragment (85 kDa) (Fig. 1C). This provides additional confirmation that Grim and Reaper induce apoptosis by activating mammalian caspases.

To determine where exactly in the apoptotic pathway Grim or Reaper were functioning, we examined whether two recently described inhibitors of death receptor (CD-95 and TNFR1) mediated apoptosis-inhibited cell death induced by Reaper or Grim. The *Molluscum contagiosum* viral protein MC159 and
the equine herpesvirus 2 protein E8 specifically inhibit recruitment of caspase-8 to the adapter molecule FADD, thereby disrupting assembly of the death receptor signaling complex and abrogating cell death (12, 36). Overexpression of either MC159 or E8 significantly inhibited FADD-induced cell killing, but did not inhibit Reaper- or Grim-induced killing (Fig. 1D), suggesting that Grim and Reaper must function downstream of caspase-8 recruitment to FADD. Therefore, Grim and Reaper do not appear to engage components of the death pathway involved in proximal death receptor signaling.

**Drosophila Reaper and Grim Display Significant Homology to Mammalian IAPs**—It has previously been shown that the amino-terminal end of Reaper and Grim share significant homology (15). Ten of the first 14 residues are identical in both proteins, whereas three of the remainder are conserved substitutions. Using this domain, data bases were searched for proteins showing significant homology. A surprising similarity emerged between this N-terminal segment and the BIR domains of IAPs (Fig. 2). Analysis of the individual BIR domains of cIAP1 and cIAP2 revealed that the second BIR domain (amino acid 207–222 or 192–207, respectively) shared highest homology with the amino terminus of both Reaper and Grim (Fig. 2).

**Reaper and Grim Interact with Mammalian cIAP1**—Previous studies have shown that both baculovirus and *Drosophila* IAPs physically interact with and inhibit Reaper-induced killing in *Drosophila* cells (19). Because of the observed sequence similarity between the N-terminal domain of Grim and Reaper with the BIR domain of cIAPs, we determined if Grim and Reaper could interact with the cIAPs. Human 293 cells were transiently transfected with expression constructs that directed the synthesis of Flag epitope-tagged Grim or Reaper and Myc epitope-tagged cIAP1. Immunoprecipitation of both Grim-Flag and Reaper-Flag quantitatively co-precipitated cIAP1-Myc (Fig. 3A). Previous studies have indicated that the BIR domain-containing region of cIAP1 and cIAP2 acts as a protein-protein interaction motif, mediating binding to the TRAF family of receptor-associated adapter proteins (24) and, more recently, to specific caspases (29, 30). To determine whether the BIR domain-containing region of cIAPs was indeed mediating binding, Reaper and Grim were expressed as GST fusion proteins in bacteria and tested for interaction with *in vitro* translated cIAP1, cIAP1-BIR (deleted C-terminal Ring finger domain) and cIAP1-Ring (deleted N-terminal BIR-containing domain). As anticipated, full-length cIAP1 bound to both GST-Reaper and GST-Grim (Fig. 3B, left panel). cIAP1-BIR convincingly bound to both GST-Reaper and GST-Grim but cIAP1-Ring did not, suggesting that the interaction between Reaper and Grim and the human cIAPs was mediated by the BIR-containing domain.

**The Interaction between Reaper or Grim and cIAP1 Is Mediated through Their N-terminal 15 Amino Acids**—A characteristic of a number of components of the cell death pathway is the existence of discrete protein-protein interaction segments. These domains recruit and assemble components of the apoptotic pathway through homophilic interactions and allow for the transduction of apoptotic signals. To determine whether the homologous domain shared between the *Drosophila* death proteins and the cIAPs is one such interaction domain, truncated versions of Reaper (ΔRpr2–15-Flag) and Grim (ΔGrim2–15-Flag) were generated in which amino acids 2–15 were deleted from the N terminus of both proteins. Co-immunoprecipitation analysis demonstrated that the ability of Reaper and Grim to bind cIAP1 was abolished when N-terminal residues 2–15 were deleted (Fig. 4, A and B). To further investigate the significance of this interaction, we determined whether the truncated derivatives of Reaper and Grim, ΔRpr2–15-Flag and ΔGrim2–15-Flag, could still induce apoptosis, and if so, could the cIAPs inhibit apoptosis under these circumstances. Ectopic expression of ΔRpr2–15-Flag or ΔGrim2–15-Flag in human MCF7 breast carcinoma cells induced apoptosis at levels comparable with full-length Reaper and Grim (Figs. 4C and 1B). Surprisingly, apoptosis induced by ΔRpr2–15 and ΔGrim2–15 was still blocked by mammalian cIAP1 and cIAP2 (Fig. 4C). Recent studies have shown that human IAPs function as inhibitors of downstream active caspases. This suggests that cIAPs may be blocking apoptosis induced by ΔRpr2–15 and ΔGrim2–15 at such a downstream site.

**cIAPs Alter Subcellular Distribution of Reaper and Grim via their N-terminal 15 Amino Acids**—It has previously been shown that Reaper co-localizes with both baculoviral and *Drosophila* IAPs in SF-21 insect cells (19). To confirm the above interactions between Reaper and Grim and the human cIAPs, 293 cells were transfected with epitope-tagged versions of Reaper and Grim2–15-Flag, when co-expressed with cIAP1-Myc, full-length Reaper and Grim displayed a punctate perinuclear localization (Fig. 5, A and B). However, in the presence of cIAP1-Myc, full-length Reaper and Grim displayed a punctate perinuclear localization (Fig. 5, D and J, respectively) that coincided with the subcellular localization of cIAP1-Myc (Fig. 5, E and K). This same perinuclear localization was observed for cIAP1-Myc when expressed alone (Fig. 5C). Interestingly, ΔRpr2–15-Flag or ΔGrim2–15-Flag, when co-expressed with cIAP1-Myc, maintained a diffuse cytoplasmic localization (Fig. 5, G and M), consistent with the binding studies demonstrating...
that an intact N terminus is required for the binding of Reaper or Grim to cIAPs.

cIAPs Regulate Reaper and Grim by Binding to their N-terminal 15 Amino Acids and Prevent Activation of Downstream Caspases—The physical association of cIAPs with Grim and Reaper and the altered subcellular localization mediated by this 15-amino acid region suggests a mechanism by which cIAPs may inhibit apoptosis. By associating with and altering the subcellular localization of Reaper and Grim, the cIAPs may prevent their access to downstream pro-apoptotic effectors, such as caspases. To determine whether such a scenario is indeed true, we examined the ability of Reaper and Grim (full-length and truncated versions) to activate downstream caspase-7 in the presence and absence of cIAP1. As shown in Fig. 6A, ectopic expression of Reaper or Grim in MCF7 cells can activate caspase-7, as evidenced by the appearance of the intermediate and large catalytic cleavage products. Similarly, both ΔRpr2–15 and ΔGrim2–15 activated caspase-7. However, in keeping with the above hypothesis, when co-expressed with cIAP1, Reaper and Grim were no longer able to activate caspase-7 (Fig. 6A), consistent with the notion that their association with cIAP1 prevented their access to the downstream caspase death machinery. In contrast, ΔRpr2–15 and ΔGrim2–15 activated caspase-7 at comparable levels in the presence of cIAP1 (Fig. 6A). In this instance, the cells still remained viable (Fig. 4C) as any active caspase-7 generated was presumably inhibited by cIAP1 functioning as a caspase inhibitor. These results support the hypothesis that the cIAPs initially inhibit Reaper and Grim by a sequestration mechanism involving their N-terminal 15 amino acids (Fig. 6B). However, if Reaper or Grim bypass this sequestration mechanism (as might occur by proteolytic removal of the N-terminal 15 amino acids), cIAPs are still able to attenuate death by inhibiting downstream active caspases (Fig. 6B).

In conclusion, we have demonstrated for the first time that Drosophila Reaper and Grim can interact with human cIAPs. Furthermore, we have mapped the interacting domains to the N-terminal 15 amino acids of Reaper and Grim and the BIR domain of cIAPs. Moreover, through this interaction cIAPs can sequester Reaper and Grim and abrogate their ability to activate caspases. This is the first demonstration of a regulatory function conferred by the homologous N-terminal 15-amino acid segment present in Reaper, Grim, and Hid. Prior to this study, the significance of the striking homology between these three death inducers was unknown. Taken together, these results and previously reported observations, it is likely that the cIAPs function at several levels in the apoptotic cascade, including direct inhibition of downstream caspases and sequestration of initiators of apoptosis such as Reaper and Grim.

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