Mitochondrial Localization of Reaper to Promote Inhibitors of Apoptosis Protein Degradation Conferred by GH3 Domain-Lipid Interactions*

Christopher D. Freel1,2, D. Ashley Richardson1,3, Michael J. Thonemius, Eugene C. Gan, Sarah R. Horn, Michael R. Olson, and Sally Kornbluth1

From the Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina 27710

Morphological hallmarks of apoptosis result from activation of the caspase family of cysteine proteases, which are opposed by a pro-survival family of inhibitors of apoptosis proteins (IAPs). In Drosophila, disruption of IAP function by Reaper, Hid, and Grim (RHG) proteins is sufficient to induce cell death. RHG proteins have been reported to localize to mitochondria, which, in the case of both Reaper and Grim proteins, is mediated by an amphipathic helical domain known as the GH3. Through direct binding, Reaper can bring the Drosophila IAP (DIAP1) to mitochondria, concomitantly promoting IAP auto-ubiquitination and destruction. Whether this localization is sufficient to induce DIAP1 auto-ubiquitination has not been reported. In this study we characterize the interaction between Reaper and the mitochondria using both Xenopus and Drosophila systems. We find that Reaper concentrates on the outer surface of mitochondria in a nonperipheral manner largely mediated by GH3-lipid interactions. Importantly, we show that mitochondrial targeting of DIAP1 alone is not sufficient for degradation and requires Reaper binding. Conversely, Reaper able to bind IAPs, but lacking a mitochondrial targeting GH3 domain (ΔGH3 Reaper), can induce DIAP1 turnover only if DIAP1 is otherwise targeted to membranes. Surprisingly, targeting DIAP1 to the endoplasmic reticulum instead of mitochondria is partially effective in allowing ΔGH3 Reaper to promote DIAP1 degradation, suggesting that co-localization of DIAP and Reaper at a membrane surface is critical for the induction of DIAP degradation. Collectively, these data provide a specific function for the GH3 domain in conferring protein-lipid interactions, demonstrate that both Reaper binding and mitochondrial localization are required for accelerated IAP degradation, and suggest that membrane localization per se contributes to DIAP1 auto-ubiquitination and degradation.

Regulation of programmed cell death in Drosophila melanogaster is governed by a group of three related apoptotic inducers on chromosome 3: Reaper, Hid, and Grim. A chromosomal deletion that removes reaper, hid, and grim (H99 deletion) abrogates developmental apoptosis and can block cell death induced by cytotoxic agents such as ionizing radiation (1). In addition, ectopic expression of each individual gene product induces cell death in vivo and when expressed in cultured fly cells (2–4). Reaper, Hid, and Grim (RHG)5 also induce apoptosis in vertebrate model systems, raising the possibility that Drosophila proteins engage evolutionarily conserved proapoptotic pathways (5–8).

Both vertebrate and Drosophila signaling pathways that regulate apoptosis converge on the caspases, a conserved family of aspartate-directed cysteine proteases. Caspases reside within healthy cells as inactive zymogens; however, in response to proapoptotic stimuli, caspases are activated and cleave multiple intracellular substrates to produce the morphologic hallmarks of apoptosis, including cell shrinkage, nuclear condensation, and membrane blebbing (reviewed in Ref. 9). Although caspase activation is conserved, central control points that govern cellular commitment to apoptosis are distinct in fly and vertebrate models. Drosophila and vertebrate cells contain inhibitor of apoptosis proteins (IAP) that oppose caspase activity by directly binding and inhibiting active caspases. Yet only in Drosophila has loss of IAP-mediated caspase inhibition been shown to be sufficient to provoke cell death, implying that fly caspases are constitutively active, and this activity must be opposed by an IAP pool to ensure cell survival (10). A body of genetic and biochemical evidence indicates that RHG proteins can competitively bind IAPs through a homologous amino-terminal domain known as the IBM, or IAP-binding motif. These IBM-containing proteins thereby dislodge active caspases from the IAPs to induce apoptosis in the fly (11, 12).

In addition to caspase displacement, Reaper, Hid, and Grim can also oppose IAP function by promoting the proteosome-mediated degradation of IAP proteins. A subset of IAPs contain

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# To whom correspondence should be addressed: Dept. of Pharmacology and Cancer Biology, Duke University Medical Center, Box 3813, Durham, NC 27710. Tel: 919-613-8624; Fax: 919-681-1005; E-mail: kornb001@mc.duke.edu.

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5The abbreviations used are: RHG, Reaper, Hid, and Grim; IAP, inhibitors of apoptosis protein; DIAP, Drosophila IAP; ER, endoplasmic reticulum; GFP, green fluorescent protein; WT, wild type; HA, hemagglutinin; Z, benzyl-oxycarbonyl; fmk, fluoromethyl ketone; SFM, serum-free media; OMV, outer membrane vesicle; IBM, IAP-binding motif; MOM, mitochondrial outer membrane; TEM, transmission electron microscopy; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; UAS, upstream activating sequence.
RING domains and can act as E3 ubiquitin ligases. RHG proteins have been reported to promote auto-ubiquitination of DIAP1 (*Drosophila* inhibitor of apoptosis) in *vivo* and within cultured fly cells (13, 14). The ability to stimulate IAP auto-ubiquitination appears to be conserved, as Reaper also stimulates turnover of human XIAP (y-linked IAP) when expressed heterologously within cultured human cells as well as turnover of the *Xenopus* XIAP homolog, XLX, upon expression in *Xenopus* egg extracts (15). Significantly, the RING activity of DIAP1 is necessary for maximal antiapoptotic activity in *vivo* and within cultured fly cells (16, 17).

In contrast to *Drosophila* apoptotic regulation, mitochondria play a central role upstream of caspase activation in vertebrates. During vertebrate apoptosis, cytochrome *c* is a necessary cofactor for caspase activation (reviewed in Ref. 18), and its release from the mitochondrial intermembrane space is tightly controlled. In vertebrate cells, Bcl2 family members regulate mitochondrial permeability (reviewed in Ref. 9). In particular, Bax and Bak serve as a gateway to mitochondrial permeability, and their oligomerization in the mitochondrial outer membrane (MOM) is necessary and sufficient to release cytochrome *c* (reviewed in Ref. 18). Once cytosolic, cytochrome *c* promotes the assembly of the apoptosome to activate caspase 9, leading to the eventual destruction of the cell. The role of mitochondria during *Drosophila* apoptosis, however, remains uncertain. Although the role of cytochrome *c* in fly apoptosis has been controversial, cytochrome *c* has been shown to change conformation and expose previously masked epitopes upon receipt of an apoptotic stimulus (20). Moreover, cytochrome *c* has been implicated in the caspase-dependent process of spermatid individuation in flies (21). Unlike Apaf1, the fly homolog DARK does not require cytochrome *c* to recruit and activate the apical caspase Dronc (22). Moreover, knockdown of cytochrome *c* by RNA interference does not impair Reaper-induced apoptosis in cultured fly cells (23).

Although cytochrome *c* has not been implicated in fly apoptosis, several lines of evidence suggest that *Drosophila* mitochondria participate in programmed cell death. Previous studies have shown that Reaper and Grim localize to mitochondria in cultured *Drosophila* cells (24, 25). In these studies, the Reaper-mitochondrion interaction promoted DIAP1 auto-ubiquitination and was necessary for maximal apoptosis. Recent work by White and co-workers (26) Goyal *et al.* (27) has demonstrated the ability of Reaper to permeabilize mitochondria and release cytochrome *c* in *Drosophila* S2 cells. Reaper localization was also found to impact mitochondrial ultrastructure dependent on the dynamin-related protein, Drp1.

Mutational analysis of Reaper and Grim has identified a region outside the IBM, termed the GH3 domain, as necessary and sufficient for mitochondrial localization (24, 25); however, the molecular basis for this association has not been known. In this study, we used both the biochemically tractable *Xenopus* egg extract system and cultured *Drosophila* cells to characterize the Reaper-mitochondrion interaction and clarify the role of mitochondria in DIAP1 destabilization. We demonstrate that the GH3 domain promotes interactions of Reaper with the outer mitochondrial membrane and that Reaper-outer membrane interactions depend primarily on GH3-lipid interactions. We also show that forcible localization of DIAP1 to the outer mitochondrial membrane, although alone not sufficient for DIAP1 degradation, can compensate for deletion of the Reaper GH3 domain in Reaper-induced DIAP1 degradation, suggesting that Reaper binding and mitochondrial binding provide distinct inputs required for the induction of DIAP1 degradation. We also report the surprising finding that localization of DIAP1 to the cytoplasmic face of the endoplasmic reticulum, rather than the mitochondria, partially restores the ability of Reaper lacking a GH3 domain to induce DIAP1 degradation. These findings suggest that concentration at a membrane surface facilitates DIAP auto-ubiquitination and degradation and that this mitochondrial function in IAP degradation can be served by the endoplasmic reticulum.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Recombinant Proteins**—The pIE3-HA-DIAP1-wt plasmid was a generous gift from Dr. Kristin White (Harvard, Massachusetts General Hospital). The pIE3-HA-DIAP1-xL plasmid was generated by PCR using the following primers: 5′-AAACGCC-CAATTGGCCCAAACCGGCATG and 5′-TATGGATCTCTCATTTTCCAGTACGAGTGGAGCCCAGCAGCCGCCACATCAGTCCAGCTGATCCCATGTATAGGCCGCGCGCATCACATCGGT. The pIE3-HA-DIAP1-Ch5 plasmid was generated by PCR using the following primers: 5′-ACCGATGTGATGCGCGTATATTTCTCATTGGGTTTCCGACTGAAGAGTGAGCCCAGCAGAACCAGCGCGCGCACAGTCATGCCCGTCAGGAACCAAGAAAAATATAATGAGTCGGCGCATACATACCTGATCGGT. These PCR products were digested with MfeI and BamHI and then digested with BamHI and NotI for subcloning into pENTR. We also show that forcible localization of DIAP1 to the outer mitochondrial membrane, although alone not sufficient for DIAP1 degradation, can compensate for deletion of the Reaper GH3 domain in Reaper-induced DIAP1 degradation, suggesting that Reaper binding and mitochondrial binding provide distinct inputs required for the induction of DIAP1 degradation. We also report the surprising finding that localization of DIAP1 to the cytoplasmic face of the endoplasmic reticulum, rather than the mitochondria, partially restores the ability of Reaper lacking a GH3 domain to induce DIAP1 degradation. These findings suggest that concentration at a membrane surface facilitates DIAP auto-ubiquitination and degradation and that this mitochondrial function in IAP degradation can be served by the endoplasmic reticulum.

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**Antibodies**—Generation of the Reaper antibody was described previously (15). This antibody was affinity-purified using a biotin-tagged epitope peptide bound to streptavidin beads (GE Healthcare). Affinity-purified Reaper antibody was...
0.3 mg/ml and used at a dilution of 1:1000 for immunoblot. Antibodies directed against human Bcl-xL and Bid were purchased from Cell Signaling Technologies (Danver, MA). Calnexin and ribophorin A antibodies were obtained from AbCam (Cambridge, UK). Antibody directed against ATP synthase was from Molecular Probes (Invitrogen), and our VDAC antibody was purchased from EMD Biosciences (San Diego). Goat antirabbit Alexa Fluor 680 (Molecular Probes) and goat anti-mouse IRDye800CW (Rockland Immunochemicals Inc., Philadelphia) secondary antibodies were used for detection using an Odyssey Infrared Imager (Licor Inc., Lincoln, NE).

Apoptosis Assay in the Developing Fly Retina—The pGMR vector was a generous gift from Dr. Bruce Hay (California Institute of Technology, Pasadena). pGMR-RPR transgenic flies were obtained from the Bloomington Stock Center (Bloomington, IN). Flies expressing mutants of RPR in the GMR vector were generated using standard practices by the Duke University Model Systems Genomics Facility (Durham, NC) in a W1118 background. Images shown are representative of multiple different transgenic lines.

Xenopus Egg Extract Preparation and Fractionation—Crude interphase Xenopus egg extracts were prepared as described previously (8) and supplemented with 50 μM Z-VAD-fmk (BioMol, Plymouth Meeting, MA). To fractionate the crude extracts into cytosol and membranous components, crude extract was further centrifuged at 200,000 × g for 70 min in a Beckman TLS-55 rotor. The cytosolic fraction was removed and recentrifuged for an additional 25 min at 200,000 × g. The endoplasmic reticulum (ER)-enriched light membrane fraction was diluted into 1.5 ml of egg lysis buffer (ELB: 250 mM sucrose, 2.5 mM MgCl₂, 50 mM KCl, 10 mM HEPES, pH 7.7, and 1 mM dithiothreitol) and pelleted through a 0.5 mM sucrose cushion by centrifugation at 20,000 rpm for 20 min in a Beckman TLS-55 rotor. The heavy membrane fraction enriched in mitochondria was further purified on a discontinuous Percoll gradient composed of 42, 37, 30, and 25% Percoll prepared in mitochondrial isolation buffer (MIB: 840 mM mannitol, 240 mM sucrose, 40 mM KCl, 1 mM succinate, 0.5 mM EDTA, 0.5 mM EGTA) (29). Percoll gradients were centrifuged at 25,000 rpm for 25 min in a Beckman TLS-55 rotor with the brake off. Purified mitochondria were further washed with 5 volumes of MIB before use. To test the relative purity of our mitochondria preparations, and to rule out ER contamination, NADPH cytochrome c reductase enzymatic assays (Sigma) were performed for each extract compartment.

Isolation of Mitochondria from Drosophila Embryos—Mitochondria were isolated from W1118 Drosophila embryos based on a protocol previously described by Schwarze et al. (30). In short, 1 g of embryos (collected after 24 h of laying) was dechorionated with 50% bleach, resuspended in 1 ml of cold MIB, and homogenized in a Dounce tissue homogenizer (50 strokes on ice; Wheaton Science Products, Millville, NJ). Samples were spun twice at 1,000 × g for 5 min at 4 °C to remove unlysed cells and debris, followed by a single spin at 13,000 × g for 5 min at 4 °C. The resulting pellet was washed twice in MIB and pelleted through a 0.5 M sucrose cushion as described above. The mitochondria-enriched pellet was either suspended in 2 volumes of SDS sample buffer for SDS-PAGE and Western blotting or processed for transmission electron microscopy.

Egg Extract Localization—Synthetic Reaper peptides were added to crude interphase extract at 4 μM. An equal amount of dimethyl sulfoxide (Me₂SO) vehicle and 0.5 μM recombinant Bcl-xL were included as controls. After Reaper peptide and Bcl-xL addition, the extract was rotated at 4 °C for 1 h and then fractionated as described above. Aliquots of each fraction were resuspended in 2 volumes of sample buffer, resolved on a 10–20% gradient SDS-PAGE, and immunoblotted with Reaper or Bcl-xL antibody.

Mitochondrial Subfractionation—Fractionation of Xenopus mitochondria was based on the protocol of Pedersen et al. (31) with the following modifications. Two ml of crude egg extract was incubated with 4 μM WT Reaper, 0.5 μM recombinant Bcl-xL, or Me₂SO for 1 h at 4 °C with agitation in the presence of 50 μM Z-VAD-fmk (Biomol). Crude extract was fractionated as described above, and mitochondria were purified over a Percoll gradient. Note that 2 ml of crude extract yielded ~50 mg of purified mitochondria per sample. These mitochondria were then subfractionated as described (31). Reaper peptides and recombinant proteins were then detected by Western blot.

Carbonate Treatment of Xenopus Mitochondria—Crude Xenopus egg extract was prepared as above and supplemented with 50 μM Z-VAD-fmk, Me₂SO, 4 μM WT Reaper, 0.5 μM Bcl-xL, and 100 μl in vitro translated hexokinase I added to 2-ml aliquots of crude extract. After incubation at 4 °C for 1 h, samples were fractionated and the mitochondria purified by centrifugation over a Percoll gradient. Purified mitochondria were resuspended in 1 volume of MIB, and 10 μl of each suspension was further divided in half for carbonate treatment. Five microliters of mitochondria suspension was treated with 20 μl of 0.1 M sodium carbonate, pH 11.5, whereas the other 5-μl mitochondrial suspension was mock-treated with 20 μl of water for 30 min at 4 °C. Supernatants were retained after treatment and resuspended in an equal volume of 2× SDS-PAGE sample buffer. Mitochondrial pellets were washed in MIB and resuspended in 2 volumes of 5× SDS-PAGE sample buffer.

Protease Protection Assay—Crude egg extract was prepared and supplemented with 50 μM Z-VAD-fmk, Me₂SO, 4 μM WT Reaper, or 0.5 μM Bcl-xL added to 2-ml aliquots of crude extract. Binding reactions were incubated at 4 °C for 1 h and then fractionated. Mitochondria were washed with MIB, and divided into 5 equal aliquots for proteinase K treatment. Aliquots of mitochondria were treated with 0, 0.1, 0.5, 1, or 10 μg/ml proteinase K in a total volume of 200 μl of MIB. Proteinase K digestion reactions were incubated at 4 °C for 10 min with agitation. After digestion, proteinase K was quenched by addition of 1× Complete Protease Inhibitor (Roche Applied Science) followed by incubation at 4 °C for 15 min. Digested mitochondria were washed in MIB and resuspended in 2 volumes of 5× SDS-PAGE sample buffer.

Mitochondrial Pre-digestion with Proteinase K—Crude egg extract was fractionated, and the mitochondria were purified. Fifty milligrams of mitochondria were resuspended in 200 μl of MIB for proteinase K treatment. Mitochondria were treated with 0, 1, 5, and 10 μg/ml proteinase K for 10 min at 4 °C with agitation. After digestion, proteinase K was quenched, the
mitochondria were washed in MIB and resuspended in 100 μl of *Xenopus* cytosol supplemented with 100 μM Z-VAD-fmk. This mitochondrial suspension was treated with Me2SO, a 1:10 dilution of *in vitro* translated hexokinase I, 0.5 μM Bcl-xL, or 4 μM WT Reaper for 1 h at 4 °C with agitation. Mitochondria were then pelleted, washed with MIB, and resuspended in 2 volumes of 5× SDS-PAGE sample buffer. Recombinant proteins and endogenous VDAC were detected by immunoblotting with their respective antibodies. Hexokinase I was detected by autoradiography.

**Localization in Drosophila S2 Cell Lysate—** S2 cells adapted for growth in serum-free media (SFM) were purchased from Invitrogen and subcultured according to manufacturer protocols. A 2.5-liter culture of S2 cells was inoculated at 2 × 10⁶ cells per ml and harvested when the culture reached a density of 15.3 × 10⁶ cells per ml. The cells were washed once in 5 volumes of ice-cold PBS and once more with 5 volumes of hypotonic lysis buffer and allowed to swell on ice for 1 h. Cells were resuspended in 1 volume of ice-cold PBS and once more with 5 volumes of hypotonic lysis buffer and loaded onto a discontinuous Percoll gradient containing 0, 25, 30, 37, and 42% Percoll in 1× lysis buffer. This lysate was further spun at 100,000 g for 1 h with rotation. Liposomes and mitochondria were separated by first diluting the binding reaction 1:5 with ELB containing no sucrose. This dilution was subsequently centrifuged through ELB containing 0.5 M sucrose to separate mitochondria from liposomes.

**In Vitro Caspase Assay—** Crude egg extract was supplemented with an energy-regenerating system composed of 2 mM ATP, 5 mg/ml creatine kinase, and 20 mM phosphocreatine. Reaper peptides were diluted in Me2SO to 3.2 or 1.6 mM. The peptides were then rapidly diluted 1:100 into crude extract. As a control caspase-8 cleaved recombinant human Bid was added to crude extract at a final concentration of 25 nM. After addition of Reaper peptide or recombinant proteins, the DEVD cleavage assay was performed as described (15).

**Xenopus Extract XLX Stability Assay—** The XLX stability assay was performed as described (15) with the following modifications. In the indicated samples purified mitochondria were diluted 1:4 in MIB and then added 1:10 to the final 100-μl reaction. OMVs or mitochondrial liposomes prepared as described above were added 1:10 in the final reaction. Radiolabeled XLX was produced *in vitro* using the rabbit reticulocyte lysate TN(T kit (Promega, Madison, WI), resolved by 12% SDS-PAGE, and quantified using a STORM 840 Phospho-Imager (GE Healthcare).

**S2 Cell Culture and DIAP1 Degradation Assay—** S2 cells were maintained in *Drosophila* SFM and subcultured as described (24). S2 cells were transfected with Cellfectin (Invitrogen) as directed by the manufacturer. For the HA-DIAP1 degradation assay, 1 × 10⁶ S2 cells were seeded in each well of a 12-well tissue culture plate (Corning Life Sciences, Lowell, MA) and allowed to attach overnight. In each well, 1 μg of pIE3-HA-DIAP1 WT, pIE3-HA-DIAP1-xL, or pIE3-HA-DIAP1-Cb5 was co-transfected with 6 μg of pMT-WTRPr, pMT-ΔGH3BPr, or empty vector. The HA-DIAP1 plasmids were allowed to express for 15 h in the presence of 50 μM Z-VAD-fmk. Reaper expression was induced by incubating cells in SFM, including 50 μM Z-VAD-fmk and 1 mM copper sulfate for 6 h. Cells were washed in PBS and lysed in lysis buffer (50 mM HEPES-KOH, pH 7.5, 100 mM KC1, 0.5% Triton X-100, 10 mM 2-mercaptoethanol, and 1× Complete protease inhibitor (Roche Applied Science)). Crude lysate was clarified by centrifugation at 15,000 × g for 5 min, resolved by SDS-PAGE, and immunoblotted for the presence of HA-DIAP1, Reaper, and actin. After Western blot, membranes were scanned using an Odyssey infrared imager, and the intensity of actin and HA-DIAP1 bands was quantified using the Odyssey software. The fraction of HA-DIAP1 remaining was calculated by taking the ratio of the HA-DIAP1/Reaper co-transfected samples to their respective actin controls. These normalized values were averaged and reported ± S.E. using StatView statistical software (version 5.0.1, SAS Institute Inc., Cary, NC).
Confocal Microscopy of S2 Cells—The amino termini of the HA-DIAP1-WT, HA-DIAP1-xL, and HA-DIAP1-Cb5 open reading frames were fused to GFP by recombination into the pAGW vector (Drosophila Genomics Resource Center). 1 μg of each pAGW DIAP1 plasmid was transfected into S2 cells. After a 16-h incubation, cells were transferred to glass-bottomed chamber slides and allowed to reattach for 2 h. Cells were subsequently stained with 5 nM MitoTracker Red (Invitrogen) for 30 min in SFM. Cells were washed twice in PBS and imaged using fluorescence confocal microscopy.

Electron Microscopy Sample Preparation and Analysis—Isolated Xenopus and Drosophila embryo mitochondria were chemically fixed in suspension with 2.5% glutaraldehyde, 2% paraformaldehyde, and 1% tannic acid in 0.1 M sodium cacodylate buffer, pH 7.2, for 12–18 h. Following fixation, the mitochondria were washed with 0.1 M sodium cacodylate buffer and treated for 1 h with cold 0.5% osmium tetroxide. Samples were then washed once with water and once with 50% ethanol, stained in 2% uranyl acetate in the dark for 30 min, and dehydrated through a graded ethanol series. Fixed mitochondria were infiltrated and embedded in an epoxy resin (EMbed-812, Electron Microscopy Sciences, Ft. Washington, PA) and mounted for ultramicrotome sectioning. Mesas were raised in the regions of interest, and 70 nm thin sections were cut with a diamond knife (Diatome, Hatfield, PA), collected on copper grids, and stained with uranyl acetate and lead citrate. The specimens were examined using the FEI Philips Tecnai 12 TEM (FEI Co., Hillsboro, OR) at 80 kv, and images were captured using the Gatan 794 digital camera (Gatan Inc., Pleasanton, CA) and Digital Micrograph software (version 3.4, Gatan Inc.).

RESULTS

The GH3 Domain Is Necessary for Reaper-induced Apoptosis in Vivo and in Xenopus Egg Extract—We previously demonstrated that the GH3 domain is necessary for maximal cell killing by Reaper when expressed in cultured Drosophila S2 cells (24); however, the role of the GH3 domain during Reaper-induced apoptosis in vivo has not been reported. The GH3 domain of Grim is necessary for its maximum activity when expressed in the fly eye, notum, and wing (25). To formally test the role of the Reaper GH3 domain during fly apoptosis in vivo, we expressed wild type Reaper and a mutant protein lacking the GH3 domain (ΔGH3 Reaper) in fly eyes under the control of the UAS promoter and the eye-specific GMR enhancer. As shown in Fig. 1A, expression of ΔGH3 Reaper fails to induce apoptosis within the fly eye, whereas ectopic expression of wild type Reaper ablates the eye. A Reaper molecule bearing point mutations within the GH3 domain (L35Q, A36R, and T37S) attenuates Reaper activity resulting in a less severe rough eye phenotype (LAT). Therefore, the GH3 domain is necessary for maximal Reaper activity in the context of the whole fly, as we had observed in cultured S2 cells.

Reaper Co-fractionates with Mitochondria in a GH3 Domain-dependent Manner—Using Reaper-GFP to monitor Reaper localization, we reported previously that Reaper localized to mitochondria in a GH3 domain-dependent manner, both in S2 and cultured mammalian cells (24). Moreover, as reported by Claveria et al. (25) using immunofluorescence techniques, the GH3 domain found in Grim also confers mitochondrial localization on both Grim and heterologous proteins to which it is fused. Although the GH3 domain appeared to serve as a mitochondrial targeting sequence, its mechanism of action and the type of mitochondrial association it conferred remained unclear. Therefore, to biochemically characterize the mechanism of Reaper targeting to mitochondria, we took advantage of both cultured Drosophila cells and the Xenopus egg extract system, where Reaper promotes caspase activation.

To examine the localization of native, untagged full-length Reaper, we synthesized the full 65-amino acid Reaper protein as
A GH3-lipid Interaction Is Required for Reaper Mitochondrial Localization

A synthetic peptide. As reported previously (15), this peptide exhibited potent pro-apoptotic activity when supplemented into egg extracts (Fig. 1B). Moreover, deletion of the GH3 domain abrogated this activity. Following crude fractionation of the Xenopus egg extract into cytosolic, light membrane, and heavy membrane fractions, we further purified the mitochondrial fraction by Percoll gradient centrifugation of the heavy membrane. As shown in Fig. 2A, Reaper co-fractionated with the mitochondria, and co-localizes in the gradients with the mitochondrial marker, VDAC, which lies embedded in the mitochondrial outer membrane. Importantly, co-fractionation of Reaper with mitochondria depended upon the GH3 domain, as similar peptides lacking the GH3 domain were almost entirely cytosolic (Fig. 2A, ΔGH3 rpr). Because Drosophila mitochondria could, when supplemented into Xenopus egg cytosol, support Reaper-induced caspase activation,6 we suspected that the ability of Reaper to associate with native fly mitochondria would be similar to that we observed for Xenopus mitochondria. To test this formally, we added the Reaper peptide to crude hypotonic Drosophila S2 cell lysates. This S2 lysate was fractionated as described under “Experimental Procedures,” and the Reaper peptide was localized by immunoblotting with Reaper antibody. As demonstrated by co-localization with the mitochondrial inner membrane protein ATP synthase, Reaper localized to Drosophila mitochondria as we had observed in Xenopus extract (Fig. 2B, upper); moreover, mitochondrial localization in S2 cell lysates was entirely dependent upon the GH3 domain. These fractionation results support our previously reported characterization of Reaper-GFP transfected into S2 cells where we observed mitochondrial localization by fluorescence microscopy (24). Thus in both frog and fly systems, Reaper localization to fractions enriched in mitochondria is GH3-dependent. Note that we previously reported that a fraction of Reaper can associate with ribosomes; the ribosome-containing fraction (not shown here) is largely pelleted during our fractionation procedure, along with large amounts of glycogen and intermediate filaments (33).

The biochemical localization of endogenous Reaper protein has previously proven challenging because of its restricted expression (during development or in response to cytotoxic stress) and its potency in promoting rapid cell death (and thus loss of mitochondrial integrity). However, using lysates created from large numbers of early developing fly embryos, we were able to fractionate and detect endogenous Reaper by immunoblotting in a mitochondrial enriched compartment (Fig. 2B, lower).

Reaper Associates with the Mitochondrial Outer Membrane—Although various imaging techniques had been used to localize Reaper to mitochondria, the resolution in these experiments was not sufficient to determine whether Reaper bound to the outer mitochondrial membrane or translocated into mitochondria...
A GH3-lipid Interaction Is Required for Reaper Mitochondrial Localization

FIGURE 3. The Reaper-mitochondria association is not peripheral and is independent of intact mitochondrial surface proteins. A, Reaper resists carbonate extraction. Mitochondria purified from crude Xenopus egg extract supplemented with WT Reaper (WT rpr), recombinant Bcl-xL, or radiolabeled hexokinase I (HK I) were washed with 0.1 M sodium carbonate, pH 11.5. The supernatant (S) and pellet (P) fractions were analyzed by immunoblotting to detect Reaper peptides and Bcl-xL. Hexokinase I was visualized by autoradiography. B, Reaper is protected from protease digestion when associated with mitochondria. Mitochondria were purified from egg extracts supplemented with WT Reaper or Bcl-xL and treated with increasing amounts of proteinase K. Endogenous VDAC and cytochrome c (cyto c) immunoblot monitor mitochondrial integrity during proteinase K digestion. C, intact mitochondrial surface proteins are not necessary for Reaper association. Purified Xenopus mitochondria were digested with increasing amounts of proteinase K. After protease inactivation, WT Reaper, Bcl-xL, or radiolabeled HK I were incubated with digested mitochondria resuspended in Xenopus cytosol. D, Reaper-bound Xenopus mitochondria (and nontreated controls) were incubated in buffer (lane 1), buffer with 5 μg/ml proteinase K (ProK) (lane 2), or buffer with 5 μg/ml proteinase K and 0.6% digitonin (lane 3) for 15 min at 4 °C with agitation. Only following permeabilization of the MOM with digitonin could WT Reaper be digested by proteinase K, as with VDAC.

Reaper Is Not Peripheraly Associated with the MOM—To further characterize the nature of the Reaper-mitochondria association, we wished to determine whether Reaper behaves as a peripheral or integral membrane protein. Toward this end, crude Xenopus extract was supplemented with wild type Reaper and the mitochondria purified over a Percoll gradient. Subsequent extraction of isolated mitochondria with alkaline carbonate buffer failed to disassociate Reaper (Fig. 3A), whereas hexokinase I, a peripherally associated MOM protein (36), was effectively solubilized by carbonate extraction. In contrast, endogenous XenopusVDAC remained stably embedded in the mitochondrial outer membrane after carbonate treatment, as did recombinant human Bcl-xL. Thus, Reaper resists carbonate extraction in a manner similar to integral MOM proteins, suggesting that Reaper may be embedded within the MOM.

As described previously, the GH3 domain is predicted to form an amphipathic α-helix with a hydrophobic face, raising the possibility that this domain may allow Reaper to insert into the MOM through a direct interaction with the outer mitochondrial lipid bilayer; if Reaper integrates into the outer mitochondrial surface, it might be, at least in part, shielded from the cytosol. To determine whether Reaper remained exposed to the cytosol after binding mitochondria, we allowed Reaper to bind mitochondria in crude Xenopus egg extract, purified the mitochondria, and treated the Reaper-mitochondrial complexes with increasing amounts of the serine endopeptidase proteinase K. As shown in Fig. 3B, Reaper was digested only by high concentrations of protease that also compromised mitochondrial integrity as shown by the cleavage of VDAC. In contrast, the exposed tail-anchored protein Bcl-xL was digested by low concentrations of protease. These data indicate that Reaper binds mitochondria in a protease-protected manner, possibly by inserting into the outer mitochondrial membrane.

Intact Mitochondrial Surface Proteins Are Not Required for Reaper Association—To determine whether mitochondrial surface proteins mediate Reaper binding, purified Xenopus mitochondria were treated with proteinase K to digest accessible regions of mitochondrial surface proteins. After proteinase K inactivation with complete protease inhibitor (Roche Applied Science), WT Reaper was added to the digested mitochondria and incubated in Xenopus cytosol. Despite digestion of surface proteins, Reaper associated with mitochondria (Fig. 3C). To verify that peripherally associated mitochondrial proteins could not associate with digested mitochondria, we...
assayed the ability of hexokinase I to associate with the mitochondrial surface following protease treatment. Taken together, these data suggest that the Reaper-mitochondria association is not peripheral, nor is the binding likely to be mediated by a protein-protein interaction that occurs on the mitochondrial outer membrane. To verify that Reaper itself was not incapable of digestion by protease K, mitochondrially localized WT Reaper was subjected to mixture of digitonin and protease. As can be seen in Fig. 3D, permeabilizing the MOM with digitonin permits the destruction of Reaper by protease K as well as endogenous VDAC.

To confirm that mitochondrial proteins are dispensable for Reaper binding, we assayed the ability of Reaper to bind protein-free liposomes composed of mitochondrial phospholipids. Phospholipid liposomes derived from purified Xenopus mitochondria were prepared using techniques developed by Kuwana et al. (32). The resulting unilamellar liposomes were composed entirely of mitochondrial lipids in their native molar ratios; however, we would expect endogenous lipid microdomains (e.g. cardiolipin-rich contact sites between the inner and outer mitochondrial membranes) to be scrambled. We found that WT Reaper bound mitochondrial liposomes in a GH3 domain-dependent manner when incubated in the presence of Xenopus cytosol despite the absence of mitochondrial surface proteins (Fig. 4A, upper). These data suggest that the GH3 domain can confer lipid association. Interestingly, the Bcl-2 family member tBid could not bind mitochondrial lipid vesicles. This observation agrees with published data demonstrating that tBid directly binds cardiolipin localized at mitochondrial contact sites (37, 38). These data strongly suggest that Reaper and tBid employ distinct mechanisms to bind mitochondria. Furthermore, mitochondrial proteins are not required for Reaper to bind the MOM. To test if Reaper specifically bound to liposomes of mitochondrial origin versus liposomes of a different composition, we repeated the above experiment using protein-free liposomes produced from Xenopus ER membrane. Interestingly, wild type Reaper was capable of binding the ER liposomes, whereas the GH3 mutant protein remained cytosolic (Fig. 4A, lower). The close resemblance in polar lipid composition between the two organelles may account for these observations (39, 40). However, the ability of Reaper to bind mitochondria, but not ER, in the full extracts and in intact cells suggests that there are membrane proteins or lipid microdomains in the intact mitochondria that confer binding specificity in the complex cellular environment, even though the GH3 domain can confer stable association with lipid vesicles derived from either membrane.

Given the affinity of Reaper for liposomes, we reasoned that these phospholipid vesicles might, if present at molar excess, be able to compete with mitochondria for Reaper association. To test this hypothesis, purified mitochondria were mixed with an excess of mitochondria-derived vesicles. After incubation with WT Reaper, the liposomes were separated from mitochondria, and both components were assayed for Reaper association by immunoblotting with a Reaper antibody. As shown in Fig. 4B, excess lipid vesicles can titrate Reaper away from the mitochondria. The purity of our liposome preparation is demonstrated in Fig. 4C, which compares the protein content of our mitochondria-localizing tail from Bcl-xL to Reaper lacking the GH3 domain, it remained possible that the Bcl-xL tail conferred a function on Reaper distinct from localization and shared with mitochondrial liposomes to that of vesicles formed of the MOM (outer mitochondrial vesicles; OMVs) by the silver staining of an SDS-polyacrylamide gel. Additionally, outer and inner mitochondrial membrane marker proteins (VDAC and ATP synthase, respectively) as well as cystolic (C) and mitochondria (M) compartments.

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![FIGURE 4. Reaper binds protein-free liposomes derived from mitochondrial and ER phospholipids. A, WT Reaper (WT rpr), ΔGH3 Reaper (ΔGH3 rpr), recombinant human Bcl-xL, and recombinant caspase-8 cleaved Bid (tBid) were incubated in Xenopus cytosol with liposomes prepared from Xenopus mitochondria (upper) and ER (lower). Following binding, the supernatant from each reaction was recovered, and the liposome pellets were washed extensively with egg lysis buffer. The supernatant (S) and pellet (P) fractions for all binding reactions were resolved by SDS-PAGE followed by Western blotting. B, liposomes attenuate Reaper association with mitochondria. Purified mitochondria alone (1) or a mixture of purified mitochondria and mitochondria-derived liposomes were added to Xenopus cytosol previously supplemented with WT Reaper. After incubation, the mitochondria and liposomes were separated by centrifugation through a sucrose cushion. The liposome (2) and mitochondria (3) fractions were collected and analyzed for the presence of Reaper peptide by immunoblotting. C, left, silver-stained SDS-PAGE of mitochondrial liposome (L) preparation to verify that they contain no detectable protein as compared with OMV. Right, the purity of our OMV and mitochondrial liposome (L) preparations was evaluated by Western blotting for mitochondrial protein markers located in the outer and inner mitochondrial membranes (VDAC and ATP synthase, respectively) as well as cystolic (C) and mitochondria (M) compartments.**

Mitochondrial Association Is Required for Maximal Biological Activity of Reaper—Our previously reported work demonstrated that the Reaper GH3 domain was required both for mitochondrial localization and for the maximal apoptotic activity of Reaper (24). However, the conclusion that mitochondria are critical for the biological activity of Reaper was subject to the caveat that the absence of a GH3 domain might have produced an inherently defective protein. Although we were able to restore Reaper function by appending the mitochondria-localizing tail from Bcl-xL to Reaper lacking the GH3 domain, it remained possible that the Bcl-xL tail conferred a function on Reaper distinct from localization and shared with...
the GH3 domain. Because our findings indicated that mitochondrial lipids were sufficient for Reaper localization to mitochondria, we reasoned that addition of excess mitochondrial liposomes might interfere with Reaper-mediated apoptosis by providing additional Reaper-binding sites and therefore displacing intact, wild type Reaper from mitochondria. Therefore, to further validate the direct connection between Reaper-mitochondrion association and the ability of Reaper to promote apoptosis, we supplemented egg extract with an excess of mitochondrial lipid vesicles, and we examined the effect of these vesicles on caspase activation in crude Xenopus egg extract. As shown in Fig. 5A, Reaper-induced caspase activation in the egg extract was attenuated in the presence of mitochondrial liposomes. However, caspase activation induced by tBid, which does not bind appreciably to the liposomes (Fig. 4A), was not impaired. These data are fully consistent with the idea that Reaper must localize to mitochondria to exert its maximal proapoptotic activity.

Because Reaper-induced apoptosis in the egg extract relies upon mitochondrial cytochrome c release, it is perhaps not surprising that removal of Reaper from mitochondria impaired apoptosis in that system. As stated above, in S2 cells, we were able to restore full apoptotic potency to Reaper lacking the GH3 domain by appending the Bcl-xL tail. This suggested that the feature of the GH3 domain germane to its function was mitochondrial localization. However, to bolster these conclusions and to examine biochemically the effects of mitochondrion on a function of Reaper demonstrably important for maximally efficient apoptosis in the fly, the induction of IAP degradation, we refined our previously reported in vitro IAP degradation assay (24). In this assay, radiolabeled IAP protein (XLX, a Xenopus IAP) is mixed with Xenopus cytosol supplemented with Reaper. This assay represents a powerful platform to define more completely the biochemical components necessary for IAP degradation. Addition of full-length Reaper promoted IAP degradation as observed previously (15); however, the addition of mitochondria to the degradation reaction markedly accelerated Reaper-stimulated IAP degradation (Fig. 5B). This disappearance was not because of caspase-mediated IAP cleavage, as the pan-caspase inhibitor p35 was present in all reactions and did not hinder IAP degradation. Furthermore, addition of the 26 S proteasome inhibitor MG132 abrogated IAP loss (data not shown), strongly suggesting that any observed IAP turnover was because of Reaper-stimulated degradation. In agreement with this idea, when protein-free liposomes derived from mitochondrial lipids (which we demonstrated above could compete Reaper away from mitochondria) were included along with mitochondria in the IAP degradation reaction, Reaper no longer enhanced IAP degradation (Fig. 5B). The addition of OMVs to this assay also decreased the ability of Reaper to degrade XLX (Fig. 5C). These data suggest that the localization of Reaper to intact mitochondria is necessary for efficient degradation of the IAP as Reaper directed away from mitochondria by the presence of liposomes or bound to mitochondrial outer membrane vesicles increases IAP stability, even when WT Reaper containing a GH3 domain is used in the assay. Collectively, these data demonstrate that Reaper localization to the mito-

FIGURE 5. Mitochondrial association is required for maximal Reaper activity. A, liposomes derived from mitochondrial phospholipids were assayed for their ability to attenuate Reaper-induced caspase activation. WT Reaper peptide (WT rpr) or recombinant caspase-8 cleaved Bid (tBid) was added to crude Xenopus egg extract supplemented with lipid vesicles. The colorimetric caspase assay was performed as described as Fig. 1B. B and C, mitochondrial liposomes and OMVs were analyzed for their effect on Reaper-induced XLX degradation. Xenopus cytosol was supplemented with radiolabeled XLX produced in reticulocyte lysate. The full-length WT Reaper peptide was then added to this mixture in the presence of purified mitochondria or vesicles alone, a mixture of mitochondria and liposomes (B), or mitochondria and OMVs (C). Aliquots of each sample were withdrawn at the indicated time points and resolved by SDS-PAGE, and the fraction of XLX remaining was quantified using a PhosphorImager (expressed as a ratio relative to the 0-h time point).
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Mitochondrial Localization Is Not Sufficient to Confer DIAP1 Degradation—The data presented in Fig. 5 demonstrate that mitochondria are necessary to promote maximal in vitro IAP degradation; furthermore, previous work indicates that the GH3 domain, and by extension, mitochondrial Reaper, are necessary to promote DIAP1 degradation in intact fly cells. Moreover, we have reported previously that Reaper confers mitochondrial localization on DIAP1. However, it was not clear if the only contribution of Reaper to the induction of DIAP1 degradation came from this induced localization. To address this issue, we developed a DIAP1 degradation assay using cultured S2 cells. An HA-tagged DIAP1 expression construct was co-transfected into S2 cells with Reaper under the control of the inducible metallothionine promoter. After induction of Reaper expression, HA-DIAP1 degradation was monitored by immunoblotting for the HA epitope. If the only role of Reaper in the degradation process was to localize DIAP1 to mitochondria, then localizing DIAP1 to the MOM in the absence of Reaper might be sufficient to promote its turnover. This hypothesis was tested directly by appending the MOM tail anchoring sequence from Bcl-xl onto the carboxyl terminus of HA-DIAP1 (localization was verified by fluorescence microscopy (Fig. 6)). When this HA-DIAP1-xL chimera was expressed and localized to mitochondria in the absence of Reaper, this was not sufficient to promote its destabilization (Fig. 7A). However, co-expressing Reaper ΔGH3, which cannot localize to mitochondria and is a poor inducer of wild type DIAP1 degradation, allowed destabilization of mitochondrially targeted DIAP1 (Fig. 7, B and C). These data suggest that Reaper binding to DIAP1 through its IBM contributes to the IAP degradation in a manner distinct from its ability to bring DIAP1 to the mitochondria. Moreover, these observations confirm the conclusion that mitochondrial localization, rather than some other intrinsic feature of the GH3 domain, is important for Reaper-induced IAP degradation.

The ER Can Partially Substitute for Mitochondria in Reaper-induced DIAP1 Degradation—Given Reaper’s ability to induce cytochrome c release from mitochondria when expressed in heterologous systems, we speculated that the mitochondrial requirement in Reaper-induced DIAP1 degradation might reflect the involvement of a mitochondrially released factor in DIAP1 degradation. Alternatively, it might be that localization of DIAP1 at a membrane surface (perhaps to concentrate DIAP1 for trans-ubiquitination or to co-localize DIAP1 with a factor required for E3 ligase function; e.g. a specific E2 conjugase) was critical for its degradation. To distinguish between these possibilities, we sought to co-localize Reaper and DIAP1 at an intracellular membrane distinct from mitochondria to see if localization at other
membranes could, like forced mitochondrial localization, substitute for the Reaper GH3 domain in promoting IAP degradation. Therefore, we appended the ER targeting sequence of human cytochrome b₆ to the DIAPI carboxyl terminus. As expected, this sequence localized DIAPI away from mitochondria (Fig. 6). Moreover, expression of the Cb5-tagged DIAPI alone did not induce its turnover (Fig. 7A). Remarkably, however, this nonmitochondrial DIAPI remained at least partially susceptible to degradation induced by both WT and ΔGH3 Reaper (Fig. 7, B and C). These data strongly suggest that a high local concentration of DIAPI at a membrane is necessary but not sufficient for degradation. Because mitochondrial liposomes could not substitute for mitochondria in the in vitro IAP degradation assay, we speculate that a required co-factor for degradation might be present at both the mitochondrial and ER surface (and so either membrane will suffice). It is also possible that Reaper induces release of a factor present within both mitochondria and ER, so that either compartment can facilitate DIAPI degradation in vivo. In this regard, it is interesting to consider that OMVs alone did not enhance DIAP degradation (although they were able to compete with mitochondria and dampen their enhancement). It may be that peripherally associated proteins, subtle aspects of membrane bilayer structure, or particular protein conformations lost in OMV preparation are required for proper positioning of DIAPI for degradation. Alternatively, it may be that release of either mitochondrial or ER contents is important for Reaper-induced DIAPI degradation and that such released factors are absent from the OMV preparations. Nonetheless, our data demonstrate that juxtaposition of Reaper and DIAPI at intracellular membranes facilitates DIAPI degradation and that membranes can “substitute” for the GH3 domain in inducing DIAPI degradation. Moreover, the association of Reaper with mitochondria is driven in large part by GH3 interactions with lipids of the outer mitochondrial membrane.

**DISCUSSION**

Although many proapoptotic signaling pathways and gene products are conserved between *Drosophila* and vertebrates, mitochondrial involvement in fly programmed cell death remains controversial. Cytosolic cytochrome c plays a well-documented role in vertebrate caspase activation (19, 41); however, studies in cultured *Drosophila* S2 cells and fly embryos fail to detect cytochrome c release during apoptosis (23, 42). Recent evidence suggests that neither cytochrome c nor other mitochondrial factors contribute to caspase activation observed in vitro within S100 lysate prepared from *Drosophila* S2 cells (43). However, published reports do not rule out a role for *Drosophila* mitochondria during in vivo cell death, and evidence suggests that mitochondria play an essential role during apoptosis. Reaper (24) and Grim (25) have been reported to localize to *Drosophila* mitochondria in cultured S2 cells in a GH3 domain-dependent manner. The pro-apoptotic protein Hid also appears to be mitochondrial (6, 24). An intact GH3 domain or forced localization of DIAPI to mitochondria or other intracellular membranes is required for Reaper-induced DIAPI degradation, potentially implicating mitochondrial factors during Reaper-induced DIAPI degradation. This study establishes that Reaper localization to mitochondria is mediated, at least in part, by phospholipids comprising the outer mitochondrial membrane; moreover, the Reaper-mitochondrion association is necessary for Reaper to exert its maximal proapoptotic activity. Our data do not rule out a role for Reaper-induced mitochondrial permeabilization in fly apoptosis but suggest that nonmitochondrial membranes are also able to at least partially support DIAPI degradation.

**Reaper Associates with the Outer Mitochondrial Membrane via GH3-Lipid Interactions**—Using biochemical fractionation, we localized a synthetic, full-length Reaper peptide to purified *Xenopus* and *Drosophila* mitochondria. Moreover, we have shown that endogenous Reaper co-fractionates with mitochondria prepared from fly embryos. Conflicting observations regarding Reaper localization to mitochondria have been reported. Claveria et al. (25) and Olson et al. (24) originally reported that GFP fusions of Grim and Reaper, respectively, localize to a subset of mitochondria within cultured S2 cells when observed using confocal microscopy. In contrast, Chen et al. (35) reported that Reaper fractionated with crude membranes from S2 cells; however, Reaper fused to GFP did not co-localize with mitochondria in that study when observed with confocal microscopy. We would argue that mitochondria that had “seen” Reaper in this study might have lost integrity (as caspase inhibitors do not appear to have been included in these assays). Our subfractionation data suggest that Reaper is embedded within the mitochondrial outer membrane. Although Reaper fused to GFP at its carboxyl terminus was previously found to fractionate with the crude membrane of S2 cells (35), we have further localized Reaper to highly purified mitochondria isolated from vertebrate and *Drosophila* model systems using caspase inhibitors throughout the preparation. In the vertebrate *Xenopus* egg extract system, wild type Reaper localizes to the heavy membrane fraction enriched in mitochondria and concentrates within the fraction composed of mitochondria purified over a Percoll gradient. When supplemented into crude S2 cell lysate, wild type Reaper and Reaper lacking its GH3 domain localized to the crude membrane fraction; however, most of the wild type Reaper, but not ΔGH3 Reaper, co-purified with mitochondria after Percoll gradient centrifugation. Full-length, synthetic ΔGH3 Reaper peptide fractionated to heavy membranous components distinct from mitochondria when added to crude *Drosophila* lysate. An attractive hypothesis, given our previous work documenting a role for Reaper at ribosomes, is that ΔGH3 Reaper preferentially binds ribosomes associated with rough ER contained within the *Drosophila* crude membrane fraction. Reaper inhibits its translation in vitro (44) and in *Drosophila* embryos (14). Furthermore, the Reaper ribosome-binding site maps amino-terminal to the GH3 domain. Thus, when Reaper is directed away from mitochondria by deletion of the GH3 domain, it may preferentially bind ribosomes present in *Drosophila* crude membranes.

Using the biochemically tractable *Xenopus* egg extract model system, we characterized the Reaper-mitochondrion association. Subfractionation of purified mitochondria revealed that Reaper resides in the MOM. Furthermore, the Reaper-mitochondrion association was nonperipheral. Once bound to mitochondria, Reaper could not be extracted by treatment with highly basic buffer, and mitochondrial Reaper became resistant...
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to protease digestion indicating that it is not solvent-accessible. Intact proteins embedded within the MOM are not required for binding because Reaper retains the ability to associate with mitochondria that have been predigested with protease and could associate with protein-free liposomes in a GH3 domain-dependent manner. These data are consistent with models suggesting that Reaper binds mitochondria by using the hydrophobic face of the GH3 domain to insert into the lipid bilayer of the MOM. Proteins embedded or peripherally associated with the MOM are not required for Reaper to target or bind mitochondria. Although we cannot rule out a role for mitochondrial proteins in stabilizing Reaper-mitochondrial interactions, they do not seem to be fundamentally required for binding. Our data are consistent with previous reports showing that the GH3 domain alone can target GFP to mitochondria (24, 25). Consistent with a role for the GH3 domain in mediating lipid, rather than protein interactions, we have never been able to identify GH3-binding proteins, despite extensive efforts to do so (data not shown). Evidence presented here suggests that Reaper binds mitochondria in a manner distinct from tBid. The pro-apoptotic, BH3 only, Bcl-2 family member tBid targets the MOM by directly associating with the mitochondrial specific phospholipid cardiolipin enriched within contact sites (38). We originally hypothesized that Reaper targets mitochondria by associating with a lipid microdomain in a similar manner; however, experimental evidence fails to support this hypothesis. Liposomes derived from mitochondrial phospholipids contain all lipid species in their native molar ratios; however, lipid microdomains such as contact sites are scrambled during liposome preparation. We hypothesize that tBid could not bind to liposomes used in this study because mitochondrial contact sites were not preserved. In contrast, Reaper bound these liposomes indicating that specific mitochondrial lipid microdomains are not necessary for Reaper to associate with mitochondria. Further characterization of the Reaper-mitochondrion interaction is needed to more precisely determine the mechanism by which Reaper recognizes the mitochondria. But our data suggest that tight association of the GH3 domain with the MOM lipid bilayer helps locate Reaper to the mitochondria.

Mitochondria and Reaper-induced IAP Degradation—The available data suggest that Reaper must bind mitochondria to exert its maximal proapoptotic activity in both vertebrate and fly systems. Although we cannot rule out the release of critical apoptotic factors from mitochondria by Reaper in intact fly cells (as in vertebrate cells), our data suggest a role for mitochondrial participation in Reaper function, even without invoking mitochondrial permeabilization.

In both in vitro egg extracts and in intact fly cells we found that Reaper localizes DIAP1 to the MOM for subsequent degradation; however, the precise functional role of mitochondria during DIAP1 degradation remains to be elucidated. It is still formally possible that Reaper localizes DIAP1 to mitochondria and concomitantly causes MOM permeabilization to release a factor necessary for DIAP1 turnover. However, data in Fig. 7B argue against this possibility because cytosolic ΔGH3 Reaper, which has no apparent capacity to permeabilize mitochondria, is sufficient to induce degradation of DIAP1 forced to localize at the MOM by the hydrophobic tail of Bcl-xL. Moreover, forced localization of DIAP1 to the MOM by the targeting sequence of human cytochrome b₆ also renders it at least partially susceptible to Reaper-induced degradation, even in the absence of the GH3 domain. Taken together, we believe that these data make it more likely that intracellular membranes, not necessarily the MOM, act as an essential scaffold to promote DIAP1 degradation, perhaps by facilitating ubiquitination in trans. However, it is still not entirely clear why OMVs were unable to facilitate Reaper-induced DIAP1 degradation, although the native structure of the mitochondrial membrane may have been perturbed in the preparation of the OMVs. A high local concentration of DIAP1 alone is not sufficient to induce degradation as DIAP1 localized to the MOM and the ER persists in the absence of Reaper. Upon binding the Reaper IBM, DIAP1 may change conformation to facilitate its degradation (for example, by allowing docking of an E2 conjugase); however, our data cannot rule out the possibility that Reaper may serve as an adaptor to bring DIAP1 into close proximity with an E2 conjugase resident on the MOM. Two Drosophila proteins, Morgue (45) and Ubcd1 (46), that could potentially serve as ubiquitin conjugases for DIAP1, have been reported in the literature; it is possible that these proteins act at the mitochondrial surface. It is not known if they might also act at the ER, where DIAP1 targeting also facilitated its Reaper-induced degradation.

These findings raise the perplexing question of whether the ability of Reaper to permeabilize mitochondria (and induce cytochrome c release) in vertebrate cells reflects any important function of Reaper in fly cells. Although mitochondrial permeabilization does not seem to be required for Reaper-mediated IAP inhibition, and may facilitate but is not required for IAP degradation, it remains possible that Reaper-induced mitochondrial permeabilization might, in some other way, enhance apoptosis in vivo. In support of this are recent findings of the ability of Reaper to permeabilize mitochondria and alter mitochondrial morphology in Drosophila S2 cells (26, 27). Knockdown of the dynamin-related mitochondrial fission protein Drp1 inhibited Reaper permeabilization and cytochrome c release. Moreover, Drp1 RNA interference correlated with a decrease in Reaper-induced cell death, suggesting that Reaper-mediated mitochondrial disruption may be a contributing factor in Drosophila apoptosis.

It is interesting to note that the 16-65 Reaper protein lacking an IBM, and therefore unable to modulate IAPs, has weak but detectable apoptotic activity in flies (47). Similarly, 16-65 Reaper retains the ability to permeabilize mitochondria and induce death upon heterologous expression. Indeed, although Chen et al. (35) failed to observe Reaper co-localization with mitochondria, they did observe that ectopic expression of a Reaper fragment that spans the GH3 domain causes cell death in S2 cells. Because this Reaper fragment could not directly engage DIAP1, it is tempting to speculate that this fragment kills by compromising mitochondrial function. Perhaps Reaper can employ dual mechanisms to ensure cell death; Reaper may act rapidly by directly displacing active caspases from DIAP1 and coordinately promoting DIAP1 degradation. As these processes take place at the mitochondria, prolonged Reaper-mitochondrial association permeabilizes Drosophila mitochondria to ensure irreversible cell death. Future studies will be needed
to elucidate the precise role of mitochondria during DIAP1 degradation and to understand more fully the potential role of mitochondria in *Drosophila* apoposis.

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REFERENCES

1. White, K., Grether, M., Abrams, J., Young, L., Farrell, K., and Steller, H. (1994) *Science* 264, 677–683
2. White, K., Tahaoglu, E., and Steller, H. (1996) *Science* 271, 805–807
3. Grether, M. E., Abrams, J. M., Agapite, J., White, K., and Steller, H. (1995) *Genes Dev.* 9, 1694–1708
4. Chen, P., Nordstrom, W., Gish, B., and Abrams, J. M. (1996) *Genes Dev.* 10, 1773–1782
5. McCarthy, J. V., and Dixit, V. M. (1998) *J. Biol. Chem.* 273, 24009–24015
6. Haining, W. N., Carboy-Newcomb, C., Wei, C. L., and Steller, H. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 4936–4941
7. Claveria, C., Albar, J. P., Serrano, A., Buesa, J. M., Barbero, J. L., Martinez-A, C., and Torres, M. (1999) *EMBO J.* 18, 7199–7208
8. Evans, E. K., Kuwana, T., Strum, S. L., Smith, J. J., Barbero, J. L., Martinez-A, C., and Torres, M. (1999) *EMBO J.* 18, 7199–7208
9. Danial, N. N., and Korsmeyer, S. J. (2004) *Cell* 116, 205–219
10. Rodriguez, A., Oliver, H., Zou, H., Chen, P., Wang, X., and Abrams, J. M. (1999) *Nat. Cell Biol.* 1, 272–279
11. Goyal, L., McCall, K., Agapite, J., Hartweg, E., and Steller, H. (2000) *EMBO J.* 19, 589–597
12. Yan, N., Huh, J. R., Schirf, V., Demeler, B., Hay, B. A., and Shi, Y. (2006) *J. Biol. Chem.* 281, 369–376
13. Olson, M. R., Holley, C. L., Yoo, S. J., Huh, J. R., Hay, B. A., and Kornbluth, S. (2003) *J. Biol. Chem.* 278, 4028–4034
14. Yoo, S. J., Huh, J. R., Muro, I., Yu, H., Wang, L., Wang, S. L., Feldman, R. M., Clem, R. J., Miller, H. A., and Hay, B. A. (2002) *Nat. Cell Biol.* 4, 416–424
15. Holley, C. L., Olson, M. R., Colon-Ramos, D. A., and Kornbluth, S. (2002) *Nat. Cell Biol.* 4, 439–444
16. Lisi, S., Mazzon, I., and White, K. (2000) *Genetics* 154, 669–678
17. Yokokura, T., Dresnek, D., Huseinovic, N., Lisi, S., Abdelwahid, E., Bangs, P., and White, K. (2004) *J. Biol. Chem.* 279, 52603–52612
18. Jiang, X., and Wang, X. (2004) *Annu. Rev. Biochem.* 73, 87–106
19. Wei, M. C., Zong, W. X., Cheng, E. H., Lindsten, T., Panoutsokopoulou, V., Ross, A. I., Roth, K. A., MacGregor, G. R., Thompson, C. B., and Korsmeyer, S. J. (2001) *Science* 292, 727–730
20. Varkey, J., Chen, P., Jemmerson, R., and Abrams, J. M. (1999) *J. Cell Biol.* 144, 701–710
21. Arama, E., Agapite, J., and Steller, H. (2003) *Dev. Cell* 4, 687–697
22. Dorstyn, L., Read, S., Cakouros, D., Huh, J. R., Hay, B. A., and Kumar, S. (2002) *J. Cell Biol.* 156, 1089–1098
23. Zimmermann, K. C., Ricci, J. E., Droin, N. M., and Green, D. R. (2002) *J. Cell Biol.* 156, 1077–1087
24. Olson, M. R., Holley, C. L., Gan, E. C., Colon-Ramos, D. A., Kaplan, B., and Kornbluth, S. (2003) *J. Biol. Chem.* 278, 44758–44768
25. Claveria, C., Caminero, E., Martinez-A, C., Campuzano, S., and Torres, M. (2002) *EMBO J.* 21, 3327–3336
26. Abdelwahid, E., Yokokura, T., Krieser, R. J., Balasundaram, S., Fowlie, W. H., and White, K. (2007) *Dev. Cell* 12, 793–806
27. Goyal, G., Fell, B., Sarin, A., Youle, R., and Siriram, V. (2007) *Dev. Cell* 12, 807–816
28. Jonas, E. A., Hickman, J. A., Chachar, M., Polster, B. M., Brand, T. A., Fannjiang, Y., Ivanovska, I., Basanez, G., Kinnally, K. W., Zimmerberg, J., Hardwick, J. M., and Kaczmarek, L. K. (2004) *Proc. Natl. Acad. Sci. U. S. A.* 101, 13590–13595
29. von Ahsen, O., Renken, C., Perkins, G., Kluck, R. M., Bossy-Wetzel, E., and Newmeyer, D. D. (2000) *J. Cell Biol.* 150, 1027–1036
30. Schwarze, S. R., Weindruch, R., and Aiken, J. M. (1998) *Free Radic. Biol. Med.* 25, 740–747
31. Pedersen, P. L., Greenawalt, J. W., Reynafarje, B., Hullihen, J., Decker, G. L., Soper, J. W., and Bustamente, E. (1978) *Methods Cell Biol.* 20, 411–481
32. Kuwana, T., Mackey, M. R., Perkins, G., Ellisman, M. H., Latterich, M., Schneider, R., Green, D. R., and Newmeyer, D. D. (2002) *Cell* 111, 331–342
33. Colon-Ramos, D. A., Shenvi, C. L., Weitzel, D. H., Gan, E. C., Matts, R., Cate, J., and Kornbluth, S. (2006) *Nat. Struct. Mol. Biol.* 13, 103–111
34. Kaufmann, T., Schlipf, S., Sanz, J., Neubert, K., Stein, R., and Borner, C. (2003) *J. Cell Biol.* 160, 53–64
35. Chen, P., Ho, S. I., Shi, Z., and Abrams, J. M. (2004) *Cell Death Differ.* 11, 704–713
36. Robey, R. B., and Hay, N. (2005) *Cell Cycle* 4, 658–658
37. Kim, T.-H., Zhao, Y., Ding, W.-X., Shin, J. N., He, X., Seo, Y.-W., Chen, J., Rabinowich, H., Amoscato, A. A., and Yin, X.-M. (2004) *Mol. Biol. Cell* 15, 3061–3067
38. Lutter, M., Fang, M., Luo, X., Nishiijama, M., Xie, X.-S., and Wang, X. (2000) *Nat. Cell Biol.* 2, 754–761
39. van Meer, G. (2005) *EMBO J.* 24, 3159–3165
40. van Meer, G. (1989) *Annu. Rev. Cell Biol.* 5, 247–275
41. Liu, X., Kim, C. N., Yang, J., Jemmerson, R., and Wang, X. (1996) *Cell* 86, 147–157
42. Dorstyn, L., Mills, K., LaZebinik, Y., and Kumar, S. (2004) *J. Cell Biol.* 167, 405–410
43. Means, J. C., Muro, I., and Clem, R. J. (2005) *Cell Death Differ.* 13, 1222–1234
44. Colon-Ramos, D. A., Irusta, P. M., Gan, N. C., Schwartz, L. M., White, K., and Aiken, J. M. (2002) *Nat. Cell Biol.* 4, 687–697
45. Wing, J. P., Schreader, B. A., Yokokura, T., Wang, Y., Andrews, P., Huseinovic, N., Dong, C. K., Ogdaul, J. L., Schwartz, L. M., White, K., and Nambu, J. R. (2002) *Nat. Cell Biol.* 4, 451–456
46. Ryooh, H. D., Bergmann, A., Gonon, H., Ciechanover, A., and Steller, H. (2002) *Nat. Cell Biol.* 4, 432–438
47. Wing, J., Zhou, L., Schwartz, L., and Nambu, J. (1998) *Cell Death Differ.* 5, 930–939