

Bifunctional killing activity encoded by conserved reaper proteins

P Chen^{1,2}, S-I Ho^{1,2}, Z Shi¹ and JM Abrams^{*1}

¹ Department of Cell Biology, UT Southwestern Medical Center, Dallas, TX 75390, USA

² These authors contributed equally to this work

* Corresponding author: JM Abrams, Tel: +1-214-648-9226;

Fax: +1-214-648-8694; E-mail: john.Abrams@utsouthwestern.edu

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Abstract

***Drosophila* activators of apoptosis mapping to the Reaper region function, in part, by antagonizing IAP proteins through a shared RHG motif. We isolated Reaper from the Blowfly *L. cuprina*, which triggered extensive apoptosis in *Drosophila* cells. Conserved regions of Reaper were tested in the context of GFP fusions and a second killing activity, distinct from the RHG, was identified. A 20 amino-acid peptide, designated R3, conferred targeting to a focal compartment and promoted membrane blebbing. Killing by the R3 fragment did not correlate with translational suppression or with reduced DIAP1 levels. Likewise, R3-induced cell deaths were only modestly suppressed by silencing of *Dronc* and involved no detectable association with DIAP1. Instead, a second IAP-binding domain, distinct from the R3, was identified at the C terminus of Reaper that bound to DIAP1 but failed to trigger apoptosis. Collectively, these findings are inconsistent with single effector models for cell killing by Reaper and suggest, instead, that Reaper encodes conserved bifunctional death activities that propagate through distinct effector pathways.**

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Abbreviations: PCD, programmed cell death; Dark, *Drosophila* Apaf-1-related killer; Hid, head involution defective; Rpr, Reaper; IAP, inhibitor of apoptosis; RNAi, RNA interference; S2, Schneider cell line; Z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; RHG, Reaper Hid Grim motif; IBM, IAP-binding motif; GFP, green fluorescent protein

Introduction

The control of programmed cell death (PCD) in *Drosophila* is governed by genes mapping to a complex locus referred to as the Reaper region. Four activators of apoptosis map in this region: Reaper (Rpr), Grim, Hid and Sickie (Skl).^{1–6} Recently, a fifth inhibitor of apoptosis (IAP) antagonist, Jafrac2, has been reported, which also shares this N-terminal motif.⁷

Proteins encoded by these genes function, in part, by antagonizing the IAPs through a shared IAP-binding motif (IBM) at the amino terminus, also referred to as the RHG domain.^{6,8} Results from *in vitro* and *in vivo* studies established that IBMs mediate binding of Rpr, Skl, Grim, Hid proteins to the BIR2 domain of DIAP1,^{4–6,9} and it is thought that at least one consequence of this interaction serves to liberate active caspases from IAP-mediated inhibition.

Numerous observations are consistent with a caspase/DIAP1 liberation model for cell killing by Rpr-like proteins. First, the apoptotic action of Rpr-like proteins is effectively suppressed by caspase inhibitors and by DIAP1 in both cultured cells and in the animal (reviewed in Abrams¹⁰ and Hay¹¹). Second, removal of DIAP1 from cells^{12,13} or embryos^{14,15} promotes rapid cell death. Third, association between DIAP1 and Rpr-like proteins in a yeast system is sufficient to reverse the attenuation of caspase activity by DIAP1.¹⁴ Fourth, flies carrying DIAP1 mutations that selectively impair binding to Rpr and Hid exhibit resistance to apoptosis.^{16,17} And fifth, if fused to heterologous proteins, the IBM can elicit apoptotic cell death in cultured cells.^{18,19}

While the observations cited above support ‘caspase-liberation’ models, several findings are inconsistent with scenarios whereby IAPs define exclusive effectors of Rpr-like proteins. First, Reaper proteins that are deleted for the N-terminal IBM retain considerable killing activity.^{18,20–22} In the case of Grim, pro-death activity outside of the N-terminal IBM mapped to a stretch of residues termed the GH3 domain, which overlaps a common region shared among Rpr, Grim and Sickie, also referred to as the Trp-block.¹⁹ Second, a candidate effector protein, Scythe, binds to Rpr outside of the shared N-terminal IBM.^{23,24} Third, both Rpr and Grim can repress translation *in vitro* and, where tested, this activity mapped outside of the IBM.^{25,26} Finally, epistasis analyses in the animal¹⁵ and in cultured cells^{12,13} establish that apoptosis requires concurrent positive input from the Apaf-1/ced 4 protein Dark, together with the disruption of IAP–caspase complexes. Taken together, these observations implicate significant death-promoting effectors for Rpr-like proteins that may be distinct from members of the IAP gene family.

To further characterize the regulation and function(s) of Rpr-like proteins, we isolated the Rpr gene from the blowfly *Lucilia cuprina* (a species representing ~100 million years of phylogenetic distance from *Drosophila melanogaster*²⁷ and tested numerous Rpr variants for several apoptotic properties. These studies uncovered a conserved killing activity within Rpr, corresponding to a 20 amino-acid fragment termed R3. Results from RNAi studies indicate that killing by this fragment is distinct from the N-terminal IBM. Moreover, the R3 domain directed localization to a subcellular compartment that partitions to heavy membranes in cell fractionations, but is clearly distinct from mitochondria when observed *in situ*. When tested *in vivo*, killing by the R3 domain was not affected by RNAi-mediated silencing of DARK, and involved neither binding to, nor reductions of, the DIAP1 protein. Surprisingly,

a second motif at the extreme c terminus of the Rpr protein also conferred binding to DIAP1, but was insufficient to trigger apoptosis. Hence, *in vivo* complexes of Rpr-like proteins together with IAPs may also occur through surfaces mapping outside of the N-terminal IBM. Collectively, the findings are inconsistent with single effector models of cell killing by Rpr. Instead, our observations strongly implicate additional mechanisms of cell killing involving novel effectors.

Results

Reaper proteins are conserved among Dipteran insects

To identify conserved residues of Rpr, we screened a blowfly cDNA library using Rpr ORF sequences as a probe and identified a single orthologous clone out of 300,000 colonies. Blowfly Rpr is a 70 amino-acid protein. Among these, 38 residues are identical and five are similar to the fruit fly counterpart. Figure 1a aligns these proteins and includes the deduced Rpr protein found in the *D. pseudoobscura* genome (*Drosophila Genome Project*). The 15 residues at the N-terminus (referred to as the RHG domain) are highly conserved in all the three Rpr proteins. When expressed in *Drosophila* S2 cells, blowfly Rpr triggered extensive apoptosis with kinetics and death-associated morphological changes that were indistinguishable from those induced by *Drosophila* Rpr (Figure 1c).

Two distinctive domains in Rpr can induce cell death

Reaper is a potent apoptosis activator. The N-terminal 15 amino acids of Reaper shares homology with the N-terminus of other *Drosophila* death activators Hid, Grim and Sickie, and is referred to as the RHG domain²¹ or the IBM.⁶ Previous studies from our lab and others showed that expression of Rpr with or without the RHG domain could induce cell death.^{20,21} Based on homologies to Blowfly Rpr, we divided Rpr into four domains (Figure 1), designating them here as R1 (RHG domain), R2, R3 (spans the Trp block²² and GH3 domain²⁸) and R4. Sequences corresponding to these domains were linked in frame to GFP, and the fusion proteins were expressed in S2 cells to examine their ability to kill cells (Figure 1c). With one exception (see R3-GFP below), all fusion proteins were expressed at comparable levels and readily detected *in vivo* (as in Figures 3 and 5) or by Western blotting (e.g. Figures 4 and 6). The full-length Rpr-GFP fusion protein is a potent apoptosis activator with activity in this assay equivalent to both the *Drosophila* protein alone (unpublished data) or the blowfly protein (Figure 1c). R1-GFP promotes significant apoptosis, but with reduced severity and rate compared to Rpr-GFP (Figure 1c). These differences could possibly reflect the distinct aspects of signaling behavior or, alternatively, it is also possible that these differences reflect the distinct kinetics of protein accumulation, localization, etc. Despite the high degree of sequence preservation, the R2 domain did not trigger appreciable levels of cell death. In

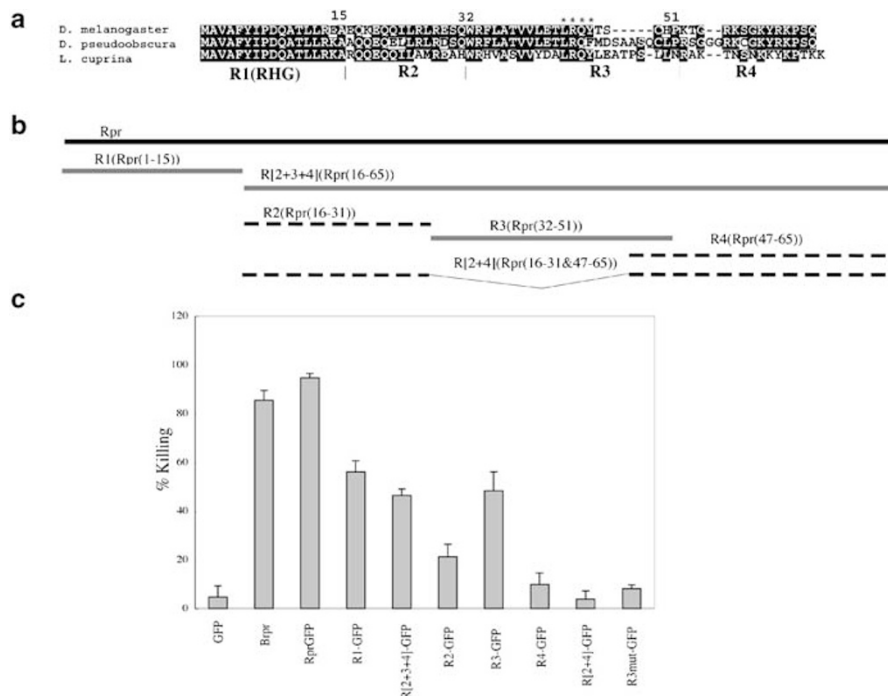


Figure 1 (a) Alignment of RPR proteins from the fruit fly *D. melanogaster*, the blowfly *L. cuprina* and *D. pseudoobscura*. Black boxes identify identities, and gray boxes show similarities. The four domains of Rpr (R1, R2, R3 and R4) and their boundaries are indicated. R1 is often referred to as RHG domain, and R3 is also called Trp block. The four conserved residues that are mutated in R3mut-GFP (see (c)) are labeled with *. (b) Schematics of various Rpr-GFP constructs used in this study. The shade of the bars represents the relative killing potency of the constructs, black indicates a strong apoptotic activity. Broken bars indicate regions that are negative for killing. (c) The killing efficiency of GFP, full-length Blowfly Rpr, and various full-length or truncated Rpr-GFP fusion proteins after 12 h of expression. The percent of killing is calculated by comparing LacZ-positive cells with and without copper induction

contrast, substantial cell killing was provoked by expression of the R3 domain. In these assays, R3-GFP induced about 50% cell death after 12 h, with kinetics similar to either the RHG domain alone (R1-GFP) or the full-length Reaper without the RHG domain R[2 + 3 + 4]. This observation was particularly striking in light of the fact that R3-GFP was consistently underexpressed relative to all other members of this series (see Figure 6). To test if the conserved residues within the R3 domain were required for killing activity, we produced a mutated version of R3-GFP that substitutes alanines for residues 43–46, which notably map outside of the GH3 domain recently described by Olson *et al.*²⁸ When tested in parallel with other active constructs, this mutated R3 domain (R3mut-GFP) variant exhibits no killing activity even though levels of protein produced from this plasmid were significantly more abundant than the wild-type form (data not shown). Assays with a Rpr variant that lacks both the RHG and R3 domains R[2 + 4]-GFP were completely devoid of cell-killing activity and, likewise, the R4 domain alone was also negative. Together, these data highlight pro-death activity encoded by Rpr that is distinct from the RHG motif and maps to a conserved set of residues defined by the R3 fragment.

To characterize the signaling pathways associated with the RHG and R3 domains of Rpr, we used RNA interference (RNAi) to test whether reduced levels of candidate effectors (e.g. Dark, Dronc, Debcl or Buffy) might impact cell killing provoked by either R1-GFP or R3-GFP. As shown in Figure 2a, reductions in the level of *dark* partially suppressed R1-induced cell death, while *dronc* dsRNA treatment completely prevented R1-induced death (Figure 2a). In contrast, RNAi directed against *dronc* had modest effects on R3-induced cell killing (Figure 2b). Also, no significant effects were observed with dsRNAs corresponding to *dark* or to the *Drosophila* Bcl-2 family members *debcl/dborg* and *buffy*. In related studies, we found that the caspase inhibitor Z-VAD effectively reversed cell killing induced by R1, but was ineffective in the context of cell killing by the R3 domain (Figure 2b). Consistent with these results, significant DEVDase activity was detectable in R1-GFP-expressing cells, but not in cells expressing the R3-GFP fusion (Figure 2c). However, compared to Rpr-GFP-transfected cells, DEVDase activity in R1-GFP transfectants was substantially lower and only detected after longer induction times. This attenuated caspase activity in R1-GFP cells might explain the reduced potency associated with R1-GFP relative to the full-length protein. By analogy to other IAP antagonists,²⁹ Rpr might also function as dimers or multimers and, consequently, a possible mechanism underlying reduced R1 activity might relate to failures in the oligomerization.

Taken together, the data in Figures 1 and 2 confirm earlier studies regarding caspase-dependent effectors of Reaper function and, surprisingly, also suggest the existence of a noncanonical pathway of cell killing mediated through the R3 domain. It was recently reported that elements of the JNK pathway contributed essential functions during Rpr-induced apoptosis.³⁰ We therefore tested whether co-expression of the JNK phosphatase Puckered (Puc) might reverse cell killing by R1- or R3-induced cell death. Expression of this phosphatase in fly cells is thought to provide a universal block to JNK signaling and Puc effectively prevented cell death

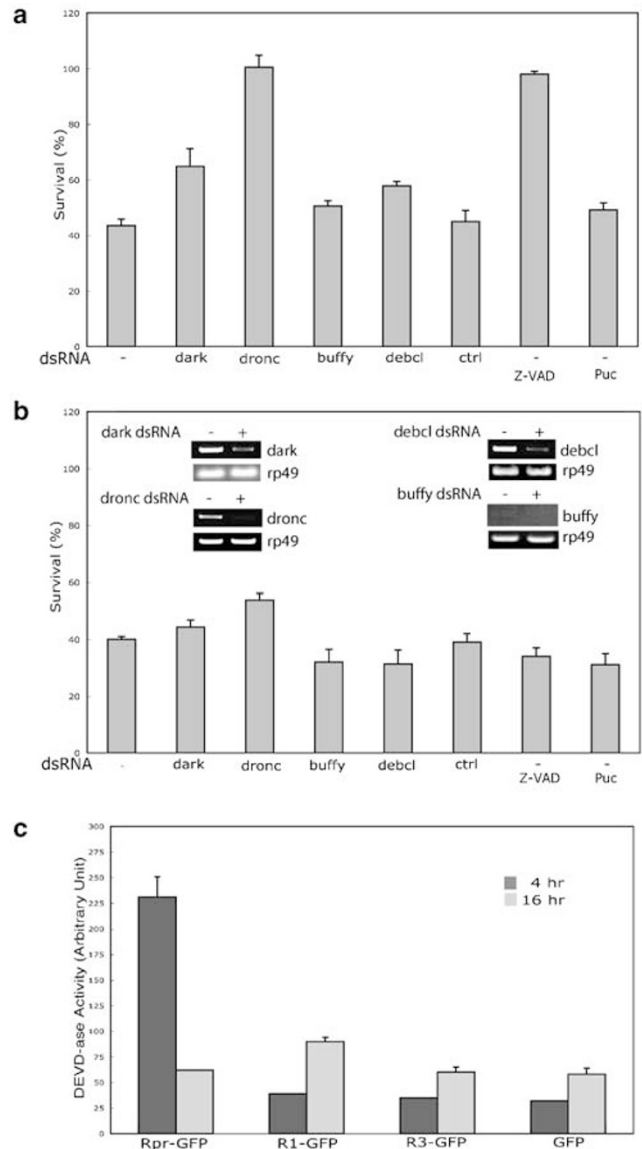


Figure 2 R1 and R3 domain of Rpr engage distinct death pathways. Cell killing by R1-GFP (a) or R3-GFP (b) was assayed in S2 cells treated with indicated dsRNA. DsRNA against a mouse protein CYP7A1 was used as control. The efficiency of RNAi was checked by RT-PCR, shown in (b). Note that R1-induced death requires the function of Dark and Dronc; in contrast, R3-induced death is not dependent on Dark or Dronc. Z-VAD peptide could block R1-induced cell death, but not R3-induced death. Co-expression of JNK phosphatase Puckered (Puc) had no effect on either R1- or R3-induced death but, in parallel assays, did reverse killing by the TNF homolog, Eiger.³¹ (c) DEVDase activity was measured in lysates from cells expressing Rpr-GFP, R1-GFP, R3-GFP and GFP for 4 or 16 h

induced by the TNF ligand ortholog (Eiger) in flies and in cultured S2 cells.^{31–33} Surprisingly, we found that Puc had no effect on apoptotic activity associated with either full-length Reaper,³¹ nor did we find any effect upon R1- or R3-induced killing (Figure 2) though, in parallel assays, Puc reversed virtually all cell-killing activity associated with Eiger-induced signaling.³¹ These data appear to exclude an essential role for Jnk signaling in cell killing by Rpr.

Subcellular distribution of Rpr is patterned by the R3 fragment

To determine the subcellular localization of Rpr and its derivatives, we directly visualized GFP patterns in cells expressing the fusion proteins. Prior to apoptotic cell death in Rpr-GFP-expressing cells, we observed a punctate distribution that is superimposed upon a uniform distribution of the protein throughout both cytoplasmic and nuclear compartments (Figure 3a). This distribution was unaffected by the caspase inhibitor Z-VAD (unpublished data) and was preserved during overt apoptosis, persisting even in cells that were clearly blebbing (Figure 3a, inset). R3-GFP showed a near exclusive punctate, or focal distribution (Figure 3c and d) and similar focal patterns were also observed for other R3-containing constructs, R[2+3+4]-GFP (Figure 3e) and R[3+4]-GFP (Figure 3g). In stark contrast, R1-GFP was uniformly distributed throughout the nuclear and cytoplasmic compartments (Figure 3b) and, likewise, all c-terminal Rpr fusions lacking the R3 fragment such as R[2+4]-GFP (Figure 3f) and R2-GFP (unpublished observation) and R4-GFP (Figure 3h) were also evenly localized. These data indicate that the R3 domain is necessary and sufficient for subcellular localization of Rpr.

The punctate patterns observed in Rpr-GFP, R3-GFP, R[3+4]-GFP and Rpr[2+3+4]-GFP cells could potentially indicate a mitochondrial association. We therefore fractionated cell lysates by differential centrifugation, validating each preparation by Western blotting for cytochrome *c*. Like cytochrome *c*, we found that full-length Rpr-GFP as well as R3-GFP fractionated almost exclusively to heavy membranes (Figure 4). In contrast, the R2-GFP fusion occurred preferentially in the cytosol, whereas the R1- and R4-GFP fusions were evenly distributed between heavy membrane and cytosolic fractions (Figure 4).

Taken together, Figures 3 and 4 suggest that Rpr and the R3 derivative might target to subcellular sites (e.g. mitochondria), which preferentially sort to heavy-membrane fractions when cells are homogenized. To determine whether the R3-

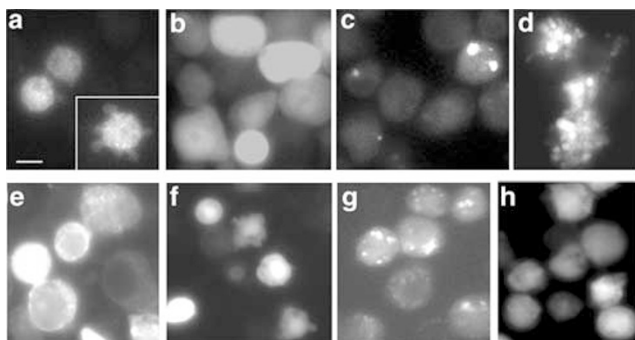


Figure 3 Compartmentalized localization of Rpr is dependent upon R3 domain. Conventional fluorescent light microscopy images of cells expressing Rpr-GFP (a), R1-GFP (b), R3-GFP (c, d), Rpr[2+3+4]-GFP (e), R[2+4]-GFP (f), R[3+4]-GFP (g) and R4-GFP (h). Bar, 10 μ m. Note that Rpr-GFP, R3-GFP, Rpr[2+3+4]-GFP and R[3+4]-GFP occur in spotted patterns superimposed upon an even distribution throughout the cytoplasm (a, c, e and g). In contrast, R1-GFP, R[2+4]-GFP and R4-GFP are evenly distributed throughout the cytoplasm. Panel (d) and inset in panel (a) show examples of apoptotic (blebbing) cells. A longer exposure time was used to visualize R3-GFP

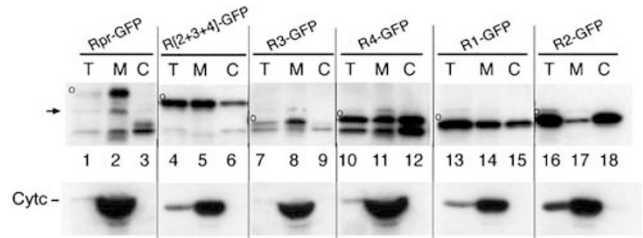


Figure 4 Rpr-GFP and R3-GFP proteins occur predominantly in heavy-membrane fractions. Total extracts (lanes labeled T) prepared from cells expressing the indicated GFP fusion protein were fractionated in sequential centrifugation steps into heavy membranes (lanes labeled M) and cytosol (lanes labeled C). All cells were treated with copper for 6 h except that Rpr-GFP cells were induced for 90 min; therefore, all lysates were made from cells harvested prior to the occurrence of extensive cell death. Proteins were separated by SDS-PAGE and blotted with anti-GFP (upper panel) to detect the Rpr fusion proteins, or anti-cytochrome *c* (bottom panel) to validate subcellular fractions. Open circles, placed at the upper left corner of indicated bands, mark the intact Rpr fusion product. The arrow indicates possible Rpr-GFP cleavage product (lanes 1 and 2)

GFP signal might co-localize with mitochondria *in vivo*, we used antisera directed against a mitochondrial transcription factor, dTFAM, which is a robust and reliable marker for mitochondria in fixed preparations of cultured *Drosophila* cells.^{34,35} The images shown in Figure 5 summarize findings from co-labeling experiments, where anti-GFP antibodies were used together with anti-dTFAM (Figure 5). Consistent with our direct observations of GFP-derived signal (see Figure 3), we found that R1-GFP was uniformly distributed throughout the cytoplasm, whereas Rpr and R3-GFP displayed extensive localization often manifested as punctate subcellular compartments within the cytoplasm. Importantly, the subcellular patterns observed for Rpr and R3 did not coincide with mitochondria and were clearly distributed in a pattern distinct from these organelles (Figure 5).

Rpr associates with DIAP1 through the RHG and R4 domains

The caspase antagonist DIAP1 is an important effector of Rpr function^{14,16,17} and previous *in vitro* studies found that the N terminus of Rpr can bind to the BIR2 domain of DIAP1.⁴ To extend these findings *in vivo* and further map portions of Rpr that promote IAP association, we tested our Rpr-GFP fusion series for association to the BIR1 and BIR2 domains of DIAP1 (Figure 6c) and for binding to native DIAP1 (Figure 6a). In these assays, the full-length Rpr fusion as well as the R1 fusion bound to the BIR2 but not the BIR1 domain of this protein (Figure 6c) and, likewise, both proteins co-immunoprecipitated with endogenous DIAP1 (Figure 6a, lanes 1–4). Surprisingly, these experiments uncovered a second DIAP1-binding domain within Rpr, since a variant lacking the R1 (RHG) domain, R[2+3+4], also bound to DIAP1 with avidity comparable to the R1 domain alone (Figure 6a, lanes 5, 6). This second DIAP1 association site mapped to the R4 domain of Rpr, since neither the R2 nor R3 domains associated with DIAP1 (Figure 6a, lanes 9–12) but R4-GFP and R[2+4]-GFP clearly did (Figure 6a, lanes 13–16). Moreover, although the R4 fragment did not induce cell death, it bound equally well to

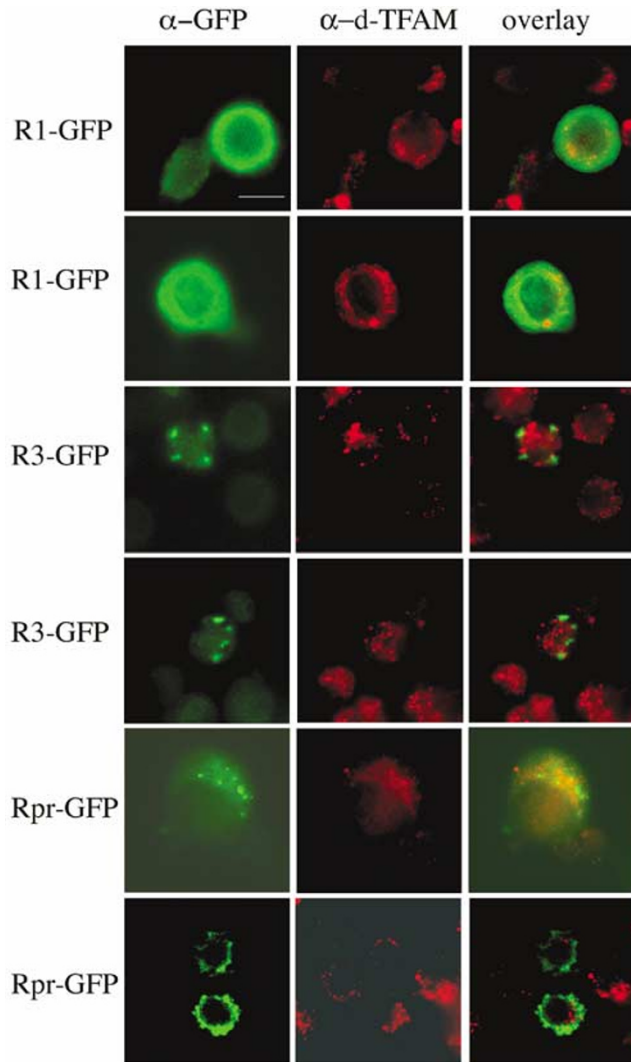


Figure 5 The R3 domain promotes localization of Rpr to a compartment distinct from mitochondria. Immunostaining for GFP (green) and the mitochondrial transcription factor d-TFAM (red) in cells expressing Rpr-GFP, R1-GFP or R3-GFP. The localized signals corresponding to Rpr-GFP and R3-GFP occur in a cytoplasmic compartment distinct from mitochondria. Bar, 10 μ m

both the BIR1 and BIR2 domains of DIAP1 (Figure 6c). In contrast, neither the R2 nor the R3 fragments associated with DIAP1 protein (Figure 6a, lanes 9–12) or its BIR domain derivatives (Figure 6c), despite the fact that R3 can induce cell death and that both R3 and R2 fragments share well-conserved residues with the blowfly Rpr. It is worth noting here that, regardless of the fusion protein expressed or death signaling status, levels of native DIAP1 protein were similar in all samples examined (Figure 6b).

Rpr does not influence bulk translation *in vivo*

When added to cell-free translation systems, recombinant Rpr exerts a suppressive effect upon bulk translation rates *in vitro*.^{25,26} This observation suggests a scenario whereby Rpr might downregulate DIAP1 levels (and possibly other short-lived anti-death proteins) through general effects upon the

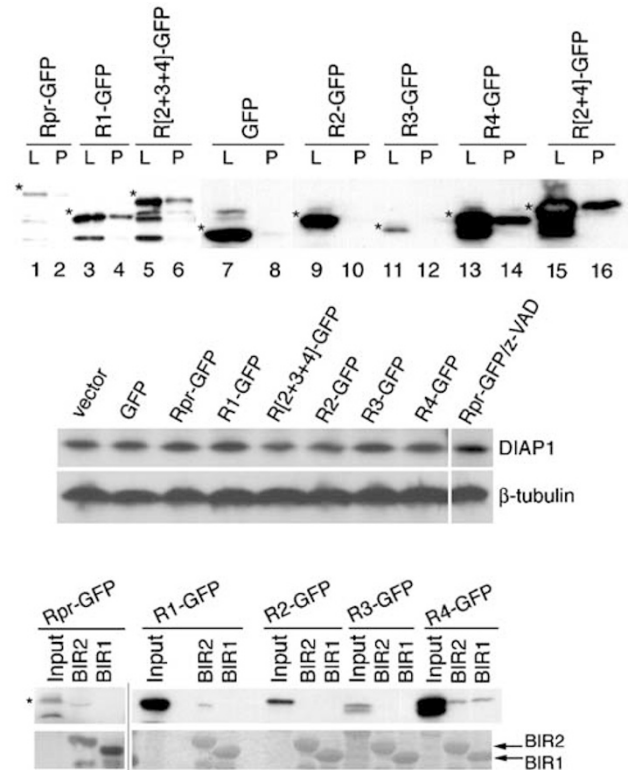


Figure 6 Association of Rpr with DIAP1 is mediated through RHG and R4 domains. (a) Lysates prepared from S2 cells expressing the indicated GFP fusion proteins were immunoprecipitated with anti-DIAP1 antibody. Immunoprecipitated protein complexes (lanes labeled with P) and the original lysates (lanes labeled with L) were immunoblotted using anti-GFP antibody. Note that, in contrast to either the R2 or R3 domains, both the RHG and R4 domains co-immunoprecipitate with endogenous DIAP1. (b) Levels of DIAP1 in cells expressing GFP, Rpr-GFP and various derivatives were determined by Western analyses (top panel). Induction conditions were the same as those were used in (a), that is, all cells were treated with copper for 6 h except that Rpr-GFP cells were induced for 90 min. The level of β -tubulin was shown for loading control. (c) Lysates of S2 cells expressing indicated GFP fusion proteins were incubated with glutathione beads bearing GST-BIR1 or GST-BIR2. Proteins bound to the beads were resolved using SDS-PAGE and were blotted with anti-GFP (upper panel). The GST fusion proteins were visualized by ponceau S staining (lower panel). Note that the RHG specifically binds to the BIR2 domain of DIAP1, whereas the R4 binds to both the BIR1 and BIR2 domains. In contrast, the R3 and R2 domains show no interaction with either BIR domain, even after the blots were extensively overexposed (unpublished data). The asterisk at the upper left corner of marked bands in (a) indicates the Rpr-GFP band

translation machinery. To test whether this activity might account for death effector functions encoded by Rpr *in vivo*, we assayed global translation rates in cells expressing Rpr-GFP or the R1 and R3 derivatives (Figure 7). In these experiments, equal numbers of cells were plated and the incorporation of ³⁵S-labeled methionine was measured over a 6 h post-induction period (overt apoptosis triggered by R1 and R3 occurs after more than 8 h of induction). To authenticate these assays and insure that our studies could effectively detect reductions in bulk translation, samples treated with cyclohexamide were also included. We also included the caspase inhibitor Z-VAD where noted, to insure against the trivial loss of material from the induction of cell death. Compared to cells expressing GFP alone, we found no significant effects upon translational efficacy with the R1 or R3

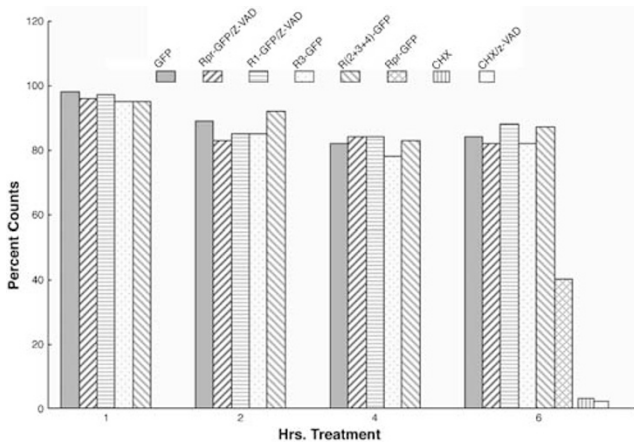


Figure 7 Rpr expression does not suppress bulk translation *in vivo*. After Rpr-GFP and various fusion proteins were expressed for the indicated time, cells were labeled with ^{35}S -methionine for 20 min. TCA precipitable counts in the cells were measured and normalized against counts from cells without copper treatment. Cells treated with cyclohexamide (CHX) for 6 h were used to validate the assay. Z-VAD was added prior to copper or CHX treatment where indicated. Transfection efficiencies in these experiments ranged from 50 to 80%

derivatives. We also tested cells that were induced for Rpr-GFP but rescued from death by incubation with Z-VAD. In these samples, translation rates remained unchanged even after 6 h of Rpr expression (without Z-VAD, overt apoptosis occurs in these samples at ~ 4 h). Full-length Rpr-GFP cells that were not treated with Z-VAD showed a substantial reduction in labeled counts, which is likely accounted for by cells lost to apoptosis in the cultures. While these experiments indicated no clear connection between Rpr signaling and bulk translation *in vivo*, they do not exclude the possibility that caspases promote suppressed translation, nor they do not exclude possible effects relating to the translation of specific proteins.

Discussion

Comparisons among the Rpr genes found in the *D. melanogaster*, *L. cuprina* and *D. pseudoobscura* genomes highlight conserved motifs spanning evolutionary distances of ~ 100 million years. Among these, the RHG motif is clearly the most well-conserved domain, since absolute identity spanning the first 13 residues is shared among all the three species. Divergent variants of this motif are found at the N terminus of other *Drosophila* activators of apoptosis (e.g. *grim*, *hid*, *skl* and *jafrac2*) and, where tested, they exhibit similar DIAP1-binding properties.^{4,18} Hence, the invariant nature of the N-terminal 13 residues in Rpr – so precisely preserved across such broad evolutionary distances – indicates that stringent selective pressures beyond IAP association may operate to conserve this exact residue profile at the N terminus of Rpr proteins.

In several biological models, the N-terminal RHG motif of Rpr is sufficient but not necessary for promoting cell death,^{20,21,36,37} suggesting that at least one additional domain, within the C-terminal 50 residues of this protein, is competent to provoke death signaling. Here we show that RHG-independent cell killing encoded by Rpr can be delimited

to 20 residues comprising the R3 fragment. These residues evidently account for killing associated with the entire C-terminal 50 residues of Rpr, since R[2 + 4]-GFP evoked no cell death and levels of cell killing provoked by R3 were equivalent to those induced by the entire C-terminal portion of the protein (R[2 + 3 + 4]-GFP). Moreover, four well-conserved residues within this domain (LRQY) are essential for cell killing, since the R3-mut (Figure 1c) was completely inactive. Since these essential residues (43–46) map outside of the Rpr GH3 domain (residues 32–42) recently studied by Olson *et al.*,²⁸ at least some activities associated with the R3 fragment are likely to be distinct from the GH3 domain, which is required for efficient IAP degradation and killing by the full-length protein (Olson *et al.* did not test whether the Rpr GH3 was sufficient to trigger cell death). While the R3 fragment triggered extensive membrane blebbing (Figure 3d), expression of R3 did not provoke other hallmarks characteristic of apoptosis. For example, in contrast to either the R1 fragment or the full-length protein, R3 did not provoke significant DEVDase activity, and killing by this domain was not reversed by Z-VAD. Hence, death signaling triggered by the R3 domain might involve a subset of caspases with specificities distinct from DEVDase or, alternatively, R3-induced killing could perhaps reflect a form of caspase-independent cell death. Our gene-silencing studies (Figure 2) were consistent with both scenarios, since neither *dark* nor the Bcl2 orthologs (*buffy* and *debc1*) were rate-limiting components required for R3-induced death. In contrast, since dsRNA targeting *dronc* was modestly protective, it is likely that R3-induced death signaling is at least partially dependent upon this apical caspase. These results are consistent with a report from Wing *et al.*,²² where forced expression of Rpr without the RHG domain (RprC) was only partially repressed by dominant-negative Dronc. Likewise, we found that the RprC-induced eye phenotype was unaffected in flies homozygous for a hypomorphic allele of *dark* (P Chen and J Abrams, unpublished observations).

Given that DIAP1 is an important proximal effector of Rpr, we tested the possibility that the R3 fragment might define an additional interface for binding to DIAP1. We found no evidence that the R3 fragment of Rpr could associate with either the BIR1 or the BIR2 domain of DIAP1, nor did we uncover any association between R3 and the full-length DIAP1 protein. Hence, killing by the R3 domain does not involve binding to, or repression of, DIAP1. At the same time, the studies also implicate additional, yet to be characterized, effectors of Rpr that might be conserved beyond the insect lineage. For instance, human 293 cells were also effectively killed by a Rpr variant lacking the RHG motif and, unlike full-length Rpr, this variant no longer bound to the human IAP protein c-IAP1.³⁸ Collectively, these findings are incompatible with single effector models for cell killing by Rpr and suggest, instead, that Rpr might encode bifunctional death activities that propagate through distinct effector pathways, one which requires IAP associations and one which does not.

One incidental and unexpected observation from the studies shown in Figure 6 is that the levels of DIAP1 protein remained unchanged (Figure 6). This result is surprising, since notable, yet modest effects upon DIAP1 immunoreactivity are often observed in animal studies.^{39–43} Reduced DIAP1 levels have also been documented in cultured

Drosophila cells, but importantly, these changes occurred 8–24 h after induction of Rpr and, presumably, coincident with (if not subsequent to) the onset of apoptosis.^{28,44} In contrast, the studies in Figure 6 sampled DIAP1 levels 1.5 h after induction of Rpr, at a time when we could detect associations between these proteins and yet 30 min prior to the overt morphological onset of apoptosis. These studies suggest that at least some apoptotic pathways do not require significant reductions in DIAP1 levels.

The studies described above strongly indicate that the R3 fragment kills without physically engaging DIAP1. However, at the same time, we also uncovered a previously unknown association between the C-terminal R4 domain of Rpr and DIAP1. For example, R4-GFP, R[2+4]-GFP and R[2+3+4]-GFP fusions readily co-precipitated with native DIAP1, while other GFP fusions did not (Figure 6). These findings are reminiscent of observations relating to Grim, where C-terminal fragments of this protein lacking the RHG motif also retained IAP-binding activity.¹⁸ When extended to *in vitro* systems, we found that the R4 domain bound equally well to either BIR domain of DIAP1, an unusual property not typically found among IAP antagonists. Thus, Rpr might potentially contact DIAP1 through both the N-terminal RHG motif and an additional binding interface formed by residues mapping within the R4 domain. It is also worth noting here that the R4 domain was completely inert in tests for cell killing, even though it binds the BIR domains of DIAP1. From this result, it seems probable that the act of simply binding to BIR domains itself (especially to the BIR2 of DIAP1) may not be synonymous with caspase activation or apoptosis. This conclusion is supported by studies on the RHG domain of Sickie, which similarly bound the BIR2 domain of DIAP1 but was not sufficient to promote apoptosis.⁴

Residues within the R3 fragment were clearly necessary for compartmentalized localization and, conversely, the R3 is also sufficient for punctate localization, since this fragment on its own conferred a focal subcellular pattern to the heterologous GFP protein (Figures 3 and 5). Corroborating these findings were results from cell fractionation studies where, again, the R3 domain was clearly sufficient – in this case – to drive the GFP protein into heavy-membrane fractions. Like the full-length fusion, and in stark contrast to fusions lacking R3, all R3-containing proteins were notably, if not exclusively, enriched in heavy-membrane fraction. These observations, combined with earlier reports on the localization patterns of Grim¹⁹ and Hid,¹⁸ prompted us to determine whether the R3 fragment might direct localization to the mitochondrial compartment. To address this issue, we used an antibody directed against the *Drosophila* TFAM, a resident protein of the mitochondria.^{34,35} Compared with other methods that rely upon pH across mitochondrial membranes (e.g. Mitotracker), we found that staining with anti-dTFAM was less variable and more robust as a marker for mitochondria in SL2 cells, possibly because the signal labels a protein that exclusively resides in the mitochondrial matrix. Contrary to expectations, our observations were inconsistent with a mitochondrial localization. Instead, we found that full-length Rpr, and every derivative retaining the R3 fragment, was partially (e.g. R[3+4] and R[2+3+4]) or exclusively (e.g. R3-GFP) directed to cytoplasmic foci that were clearly distinct from

mitochondria. While the precise identity of this compartment is not known, we note that similar foci were observed by Lois Miller and colleagues as they studied Rpr distribution in lepidopteran cells⁴⁵ and, whatever their nature, residents of these foci are likely to fractionate to heavy membranes (Figure 4).

Recently, Claveria *et al.*¹⁹ reported on a domain in Grim, designated GH3, with cell-killing properties similar to those we describe here for the R3 fragment of Rpr. The GH3 and R3 span regions of shared residues with Skl (residues 70–90) and were originally noted by Wing *et al.*²² as the ‘trp block’ sequence, shared among the Rpr and Grim proteins. In functional studies, the R3 fragment in Rpr and the GH3 domain in Grim share notable properties. First, both are important for complete cell death signaling by their respective proteins since removal of each compromises killing efficacy. Second, both R3 and GH3 domains are sufficient for killing activity that is evidently unrelated to IAP proteins.¹⁹ Third, both R3 and GH3 domains conferred subcellular targeting activity and in neither case were these activities affected by caspase inhibitors. Notwithstanding these similarities, there are also important differences between the R3-induced death and death activities reported for the GH3 domain. For instance, p35 effectively rescued GH3-induced death, at least in cultured cells,¹⁹ but caspase inhibitors did not prevent R3-induced cell death and, similarly, p35 only marginally suppressed killing induced by a C-terminal fragment of Rpr when expressed as a transgene.²² Another difference between the behaviors of these domains relates to subcellular localization. In contrast to the R3 from Rpr, the GH3 domain conferred localization to mitochondria. One possible way to reconcile this distinction postulates that the R3 and GH3 domains encode information sufficient to target distinct subcellular compartments. Alternatively, the apparent discrepancy could, in part, relate to matters of interpretation and the markers used. For example, in their studies, Claveria *et al.* did not observe a complete coincidence of signals but, instead, they describe an occasional colocalization with mitochondria visualized with Mitotracker.¹⁹ Hence, the GH3 might promote associations with only a subset of these organelles. In addition, there were many GH3 foci that did not co-label with mitochondria¹⁹ and, like the R3 foci documented here, the nature of this compartment remains to be characterized. Olson *et al.*²⁸ also reported that the GH3-like region from Rpr conferred a mitochondrial localization, but like the studies from Claveria *et al.* only a subset of the mitotracker signal appeared coincident with the fusion protein. Hence, we suspect that the reliance on Mitotracker in dying L2 cells (see above) could be an important distinction between studies reported by others and the findings presented here.

The experimental model used here draws on observations derived from forced expression of studies and, consequently, the extent to which these observations shed light upon authentic signaling triggered by Reaper in a native, physiologic context becomes an important question. While it is possible that R3-induced killing reflects a nonphysiological outcome of forced expression, we favor the alternative view for the following reasons. First, pro-death functions associated with induced expression of GFP fusions are not commonly encountered and, in fact, many derivatives of Rpr

(as well as other proteins) were completely inert in these assays. Second, any and all fusion proteins that retained the R3 domain also retained killing activity. Third, cell killing by the R3 domain was associated with some features of apoptosis, such as membrane blebbing, and a partial requirement upon the apical caspase *Dronc*. Fourth, excessive levels of R3 expression were not necessary to provoke cell death and, in fact, both R1-GFP and R3-GFP were equally potent for killing (Figure 1), despite the fact that R3-GFP is consistently the least abundant of all fusion constructs (Figures 4 and 6).

Our findings argue against a vital role for components of the Jnk pathway as effectors of R3- or Rpr-induced cell killing (see also Kauppila *et al.*³¹). *Scythe* also qualifies as a potential effector of R3,²³ since the *Xenopus* ortholog of this protein binds Rpr outside of the RHG motif. However, neither forced expression of *Drosophila* *Scythe* nor dsRNAs targeting this protein significantly modified cell killing by the R3 domain (unpublished data). Given that Rpr can suppress mRNA translation *in vitro*,^{25,26} we also tested whether the R3 domain might exert suppressive effects upon protein translation (Figure 7). In these experiments, we uncovered no evidence for translational repression *in vivo*, either in association with the full-length protein or with the R3 domain. Hence, effectors that inhibit bulk translation are unlikely to account for R3-induced cell death.

In closing, we consider properties associated with fragments of Rpr that are distinct from full-length protein. For example, the R4 domain binds to both the BIR1 and BIR2 domains while the full-length protein is selective for BIR2. In addition, *dark* RNAi and the caspase inhibitor peptides (e.g. Z-VAD) were unable to prevent R3-induced cell deaths and yet these same agents effectively block apoptosis triggered by the full-length Rpr protein. At face value, these observations seem contradictory only if one assumes that Rpr is unaltered throughout the apoptotic process. However, since Rpr is modified during cell death (see Olson *et al.*⁴⁶), one potential way to reconcile these observations supposes that the action of caspases could expose caspase-independent activities embedded within the Rpr protein. A useful precedent here is tBid, a caspase-derived product that promotes caspase-independent activities leading to cell death.^{47,48} Hypothetically, if the Rpr were cleaved during apoptosis, proteolysis might liberate residues within the R3 domain, exposing them to death effectors distinct from caspase enzymes. Consistent with this scenario, we typically observe derivatives of the full-length protein which could reflect proteolytic cleavage N-terminal of the R3 domain (for example, see lane 1, Figure 4). Direct assessment of this scenario will require antibodies capable of detecting native Rpr protein.

Materials and Methods

Isolation of Blowfly rpr cDNA

A 200 bp DNA fragment corresponding to Rpr ORF was used to screen a *Lucilia cuprina* (blow fly) 0–7 h embryonic cDNA library in Lambda ZAP II (kindly provided by Dr Phil Batterham, Department of Genetics, University of Melbourne, Australia). In all, 10 clones were isolated from ~300 000 clones. One clone was identified to contain an ORF homologous to Reaper after sequencing.

Plasmids

PCR fragments corresponding to full-length Rpr or truncated Rpr with in-frame fusion to GFP protein were cloned into pRmHa.3 to generate pMt-rpr-GFP, pMt-rpr (2-15)-GFP, etc. All the plasmids were confirmed by sequencing. Expression of *puckered* was driven by MTAL-puc,³¹ a plasmid containing a *puckered* cDNA subcloned into the pRmHa.3 vector.

dsRNA synthesis

Fragments of 500–700 bp DNAs corresponding to *Dark*, *dronc*, *buffy*, *debel* sequences were amplified by PCR from cDNA templates. Each primer used in the PCR contained a T7 polymerase binding site followed by gene-specific sequences. The PCR products were purified and used to synthesize dsRNA using the MEGACSCRIPT T7 transcription kit (Ambion). In all, 2 μ g of dsRNA was analyzed by 1% agarose gel to ensure that the majority of the dsRNA existed as a single band.

Transfection, RNAi and cell-killing assay

Schneider L2 (L2) cells were cultured and transfected using Cellfectin (Invitrogen) as in Chen *et al.*⁴⁹ Cell-killing assays were done as described.²⁰ RNAi were performed essentially as described in Kauppila *et al.*³¹ Briefly, 3 μ g of various Rpr-GFP or pMt-GFP constructs were co-transfected with 0.2 μ g of pAct-lacZ into 1 million cells. For RNAi experiments, 1 day before transfection, 1 ml of 8×10^5 /ml S2 cells were plated in CCM3 medium (Hyclone). dsRNA (15 μ g) was added immediately to the cell suspension, and gently mixed before cells attached to the plate. The cells were incubated at 25°C for 30 min followed by addition of 1 ml of CCM3 containing gentamicin. The cells were transfected as above the next day, and 15 μ g dsRNA was added to the cells after transfection medium was removed. At 36 h after transfection, each well of the cells was split into two identical halves, and copper (0.7 mM) was added to one of the split. When caspase inhibitors were used, 50 μ M of the indicated inhibitor was added to the cells before copper. Cells were fixed and stained for LacZ-positive cells 12 or 16 h later. Blue cells in induced and control halves of the transfected cells were counted. Cell survival was calculated as the percent of blue cells in treated cells relative to the control cells. The data showed were the average of three independent experiments. Stably transfected cell lines were cultured by co-transfecting 0.2 μ g pCohygro and 3 μ g experimental plasmids into 10^6 Schneider L2 cells.

Co-immunoprecipitation, GST-BIR binding assays and Western analysis

In all, 10 million stably transfected cells were treated with copper for 4 h, and lysed in 600 μ l lysis buffer (0.5% Triton X-100 in buffer A⁵⁰ without DTT) on ice for 30 min, then centrifuged at $12\,000 \times g$ for 15 min. The resulting supernatants (cell lysates) were incubated with 30 μ l protein A/G plus-Agarose beads (Santa Cruz Biotechnology) for 1 h at 4°C. The supernatants were subsequently incubated with 4 μ g Anti-DIAP1 (a gift from Dr. Kristin White) 1 h at 4°C before 30 μ l Agarose beads was added, and rotated overnight. Beads are washed six times with lysis buffer + 120 mM NaCl, then mixed with SDS sample buffer and boiled for 5 min. The proteins were resolved on a 16% SDS-polyacrylamide gel, transferred to PVDF membrane, and blotted with anti-GFP (Covance). For GST-BIR binding assays, 600 μ l cell lysates were rotated with glutathione beads loaded with GST-BIR1 or GST-BIR2 for 2 h. The beads were then washed with lysis buffer six times, and bound proteins were analyzed by Western blot using anti-GFP.

Subcellular fractionation

In all, 10^7 cells were harvested by centrifugation, and cell pellets were resuspended in 200 μ l buffer A in 250 mM sucrose, and incubated on ice for 15 min. Cells were then homogenized by 10 strokes using a Teflon homogenizer, and homogenates were centrifuged twice at $500 \times g$ for 10 min. The supernatants (lysates) were centrifuged at $10\,000 \times g$ for 15 min at 4°C, the resulting pellets were heavy-membrane fractions containing mitochondria. The supernatants were further spin at $100\,000 \times g$ for 1 h. The resulting supernatants (S-100) were cytosol.

Immunofluorescence

For direct visualization of GFP, cells grown on coverslips were fixed with 4% formaldehyde for 30 min, washed with PBS several times and mounted onto slides. For double labeling of GFP and mitochondria, various S2 cells expressing GFP fusion proteins were grown on coverslips, fixed with methanol/water/acetic acid (95:4:1, by vol) for 15 min, and permeabilized with cold methanol for 10 min. They were subsequently incubated with both mouse anti-GFP (1:500, Covance) and rabbit anti-dTFAM (1:500, kindly provided by Yasuo Kitagawa) for 1 h. After four washes with buffer A, the coverslips were incubated with FITC-conjugated anti-mouse IgG (Jackson; 1:500 in buffer A) and Rhodamine-conjugated anti-rabbit IgG for 1 h, then washed four times with PBS, and mounted in fluoromount-G medium.

Caspase assays

Cell extracts were prepared by lysing cells expressing various GFP fusion proteins in Buffer A (20 mM HEPES-KOH pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride) supplemented with 0.5% Triton X-100. Cellular debris and lipids were separated from the soluble fraction by two 15 min spin at $15\,000 \times g$ at 4°C, and the protein concentrations were measured and normalized by addition of buffer A. A 25 μ g aliquot of protein extract was incubated with 10 μ M Ac-DEVD-AFC (Calbiochem) substrate in a final volume of 100 μ l in a 96 microtiter plate. Fluorescence was monitored over time with excitation at 360 nm and emission at 465 nm in a SpectraFluor Plus plate reader (Tecan).

Metabolic labeling of proteins

To monitor the bulk translation in S2 cells, transiently transfected S2 cells were divided into six wells in 12-well plates at a density of 10^6 /ml, treated with 50 μ M Z-VAD and/or 10 μ M cyclohexamide where indicated, and copper was added for various times (1, 2, 4, 6 h). Cells were incubated with 0.1 mCi/ml ^{35}S -met (ICN) in methionine free Sf900 II medium (Invitrogen) for 20 min before they were washed with cold Sf900 II medium, and lysed in 150 μ l of buffer A with 0.5% TX-100 supplemented with protease inhibitor cocktail (Roche). TCA precipitable counts in 50 μ l of lysates were quantified by scintillation counting. Data shown were the average of counts from two aliquots of each cell lysate.

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