***Drosophila* grim induces apoptosis in mammalian cells**

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Genetic studies have shown that *grim* is a central genetic switch of programmed cell death in *Drosophila*; however, homologous genes have not been described in other species, nor has its mechanism of action been defined. We show here that *grim* expression induces apoptosis in mouse fibroblasts. Cell death induced by *grim* in mammalian cells involves membrane blebbing, cytoplasmic loss and nuclear DNA fragmentation. Grim-induced apoptosis is blocked by both natural and synthetic caspase inhibitors. We found that *grim* itself shows caspase-dependent proteolytic processing of its C-terminus *in vitro*. Grim-induced death is antagonized by bcl-2 in a dose-dependent manner, and neither Fas signalling nor p53 are required for *grim* pro-apoptotic activity. Grim protein localizes both in the cytosol and in the mitochondria of mouse fibroblasts, the latter location becoming predominant as apoptosis progresses. These results show that *Drosophila* *grim* induces death in mammalian cells by specifically acting on mitochondrial apoptotic pathways executed by endogenous caspases. These findings advance our knowledge of the mechanism by which *grim* induces apoptosis and show the conservation through evolution of this crucial programmed cell death pathway.

Keywords: apoptosis/bcl-2/*grim*/mitochondria/mouse

Introduction

Multicellular organisms eliminate unwanted or damaged cells by a regulated cell death process. Deviations from the physiological levels of cell death during adult life result in either proliferative or degenerative disorders (Thompson, 1995). Cell death is particularly relevant in several critical processes of development, such as organ and tissue shaping during morphogenesis and embryogenesis, tissue resorption during metamorphosis and neuronal selection (Jacobson *et al.*, 1997). Cell death during development occurs in a temporally and spatially reproducible pattern and is genetically controlled, and thus is regarded as programmed cell death (PCD). Most PCD processes, independently of the origin of the death

stimulus, are executed through a stereotyped pattern of cellular and biochemical events known as apoptosis (Wyllie *et al.*, 1980). Genetic analysis of PCD in the nematode *Caenorhabditis elegans* identified essential elements of the basic machinery for both the regulation and execution phases of apoptotic death (Ellis *et al.*, 1991), and showed for the first time their conservation throughout metazoan evolution (Vaux *et al.*, 1992). Presently, endogenous and viral molecules that promote, inhibit or are required for apoptosis have been found to be conserved from nematodes to humans (McCall and Steller, 1997). Among these, the activation of a particular group of conserved sequence-specific proteases, known as caspases (cysteine aspartases), is fundamental for the execution phase of the apoptotic programme (Nicholson and Thornberry, 1997). Apoptotic caspases can be activated in mammalian cells through at least two independent pathways: the pathway triggered by the activation of the transmembrane receptors Fas and tumour necrosis factor receptor (TNFR) (reviewed in Nagata, 1997), and a mitochondrial, bcl-2-sensitive pathway (reviewed in Kroemer, 1997).

Mitochondria integrate death signals from very diverse origins, such as genotoxic damage, cytotoxic stress, factor deprivation, glucocorticoid addition, heat shock and radiation. While many of such mechanisms constitute cellular responses to exogenous injury, others respond to events that operate *in vivo* to activate physiological PCD; however, the pathways that connect these events to the mitochondria remain unknown. Some of these pathways may involve the transcriptional activation of killer genes. In fact, even though several mammalian cell types can be induced experimentally to enter apoptosis without protein synthesis (Weil *et al.*, 1996), transcriptional activation of specific genes is absolutely required for natural PCD in both insect and vertebrate whole embryos (Tata, 1966; Oppenheim *et al.*, 1990; Schwartz *et al.*, 1990; Coucouvanis and Martin, 1995) and factor-deprived cultured neurons (Martin *et al.*, 1988). Much of the natural cell death occurring during insect and vertebrate development is likely to be mediated by the transcriptional activation of killer genes. Recent studies in the fly *Drosophila melanogaster* have uncovered three novel components of the genetic programme controlling PCD, *reaper*, *hid* and *grim*, whose transcriptional activation precedes, induces and is necessary for PCD by apoptosis (White *et al.*, 1994; Grether *et al.*, 1995; Chen *et al.*, 1996; Robinow *et al.*, 1997). The three genes map to a single genetic complex and function as death switches that are regulated at the level of transcription. Their ectopic activation triggers apoptosis in otherwise viable cells, and their inactivation prevents apoptosis of cells that would normally undergo PCD. Interestingly, their activation not only mediates programmed apoptotic events occurring

during normal development, but also the apoptosis induced in response to exogenous agents, such as ionizing radiation, to which their promoter regions respond (Nordstrom *et al.*, 1996). Therefore, *reaper*, *grim* and *hid* play a pivotal role in integrating different death stimuli leading to insect cell death by apoptosis (reviewed in McCall and Steller, 1997). A similarly acting cell death regulator has not yet been identified in any vertebrate system, but *reaper* has been shown to induce apoptosis in an *in vitro* amphibian model (Evans *et al.*, 1997).

Despite their organization in a single genetic complex, the three gene products share no sequence similarity except for the partially conserved first 14 amino acids. Cooperativity has been reported among the three genes, but *grim* appears to be the most efficient cell death inducer (Chen *et al.*, 1996; Zhou *et al.*, 1997). While many other components of PCD pathways show a high structural and functional conservation throughout evolution, no *grim*-like gene has yet been isolated from any species but *Drosophila*, nor have conserved *grim* domains been described in other proteins. The mechanism of *grim* action also remains unknown.

Herein we demonstrate that *grim* induces apoptosis in 3T3 and primary mouse fibroblasts through a bcl-2-sensitive mechanism that triggers endogenous apoptotic caspases. Our findings show that the endogenous mitochondrial mammalian apoptotic machinery recognizes and responds correctly to *Drosophila* *grim*. These results strongly argue for the existence of endogenous *grim* homologues that may account for transcription-dependent PCD regulation in vertebrates.

Results

Grim induces death in mouse 3T3 fibroblasts

To test its ability to induce apoptosis in mammalian cells, we transiently expressed *Drosophila* *grim* in mouse 3T3 fibroblasts. The *grim*-coding sequence was inserted into a vector that provided doxycycline-inducible synchronous expression of the sequences inserted. To characterize *grim* expression, we developed an antiserum against the whole recombinant protein purified from *Escherichia coli*. Western blots detected a band of the expected size, specific for extracts from transfected cells, which coincided with the size of the endogenous *Drosophila* protein and of the recombinant *grim* protein purified from *E.coli* (Figure 1I). An additional smaller band specific for transfected cells was also observed. A similar downshift was observed for both bands in extracts from cells transfected with a truncated *grim* mutant lacking amino acids 2–14 (see below) (Figure 1I). This confirms that the detected bands correspond to the transfected *grim* and shows that the smaller form results from partial cleavage of the protein at its C-terminus. However, the smaller band was not observed when the concentration of the serine/cysteine protease general inhibitor phenylmethylsulfonyl fluoride (PMSF), included in the lysis buffer, was increased from the standard 1 mM to 2 mM (Figure 1I). We therefore conclude that *grim* proteolysis is taking place after cell lysis by a strong proteolytic activity present in the extracts. Immunofluorescence was used to identify *grim*-expressing cells in transfected cultures. We found that cells with high *grim* levels show morphological traits characteristic of

various phases of apoptosis including loss of substrate attachment, membrane blebbing and cytoplasmic loss (Figure 1A–H). Initially, when the predominant feature was membrane blebbing, nuclei were still negative for TUNEL staining (Figure 1B and F). Later, *grim*-expressing cells adhered to neighbouring cells, showed nuclear DNA condensation and became positive for TUNEL staining, indicating DNA fragmentation (Figure 1C, G and J). Finally, cytoplasm and nuclei disaggregated completely into apoptotic bodies, which remained strongly positive both for *grim* and TUNEL (Figure 1D, H and K).

To facilitate quantitation of the death induction effect, *grim* was co-transfected with the same expression vector driving LacZ expression. *Grim* expression resulted in the elimination of two-thirds of the transfected cells during the 24 h of culture after the addition of doxycycline (Figures 2A, and 3A and B). The reduction in blue cells specifically affected the high-LacZ-expressing cell class; >90% of cells in this class were killed in *grim*-transfected cultures (Figures 2B, and 3A and B). In agreement with the observed cell death effect, *grim*-transfected cultures showed a high percentage of blue cells with a characteristic apoptotic morphology (Figure 2C). All experiments were performed maintaining cells in 10% serum; lowering the serum concentration to 0.1% did not reduce the percentage of surviving cells (not shown). We conclude that *grim* kills 3T3 cells through apoptosis induction in a serum-insensitive manner.

The conserved N-terminal region of grim is not necessary for apoptosis induction or execution

The only conserved sequence found in *grim* is a 14 amino acid N-terminal motif, also present in *hid* and *reaper* (Chen *et al.*, 1996). To test the relevance of this motif in our system, a truncated form of *grim* lacking amino acids 2–14 was expressed (*grim*^Δ, Figure 1I). The mutant form retained most of the *grim* killing ability, but cells appeared to tolerate slightly higher levels of the mutant protein without entering apoptosis (Figure 2A–C). We used time-lapse videomicroscopy to characterize and compare the death-inducing effects of wild-type and mutant *grim* forms. Video analysis showed that between 4 and 10 h after doxycycline addition, the rate of cell death in *grim*-transfected cultures was 14 times higher than in control-transfected cultures, and demonstrated no differences between the wild-type and mutant forms (Figure 2D). In addition, we determined the mean length of the blebbing period as a reference measure for the duration of the execution phase of apoptosis induced by both transfected forms. Again, no differences were observed between the mutant and wild-type forms (Figure 2E). We conclude that the conserved N-terminal domain is not required for either the initiation or the execution of apoptosis by *grim*. The same conclusion was reported when a similar *grim* truncation mutant was introduced in insect cells (Vucic *et al.*, 1998).

Caspase activity is required to execute grim-induced cell death

To test the involvement of caspases in *grim*-induced death, we used specific inhibitors together with LacZ co-transfection. Addition of the cell-permeable broad specificity caspase inhibitor zVAD.fmk to the culture

Fig. 1. Grim induces apoptosis in mouse 3T3 fibroblasts. The pictures show immunofluorescent detection of grim expression and DNA fragmentation by TUNEL staining. Grim expression is shown in red, TUNEL staining in green and Hoechst-stained nuclei in blue. (A and E) Grim-positive cells presenting apoptotic morphology (arrowheads) and a matching DIC + fluorescence-composed image, respectively. (B–D) Cells representative of the successive steps of the apoptotic process. (F, G and H) DIC + fluorescence-composed images matching respectively, (B), (C) and (D). (J and K) DIC + Hoechst-composed images matching, respectively, (G) and (H). (I) Western blot detection of grim in whole protein extracts from *Drosophila* embryos (d), grim protein purified from *E.coli* extracts (p), and extracts from 3T3 fibroblasts transfected with either full-length grim (g), grim Δ (g Δ) or the corresponding empty vectors (c) from cells lysed in either 1 or 2 mM PMSF as indicated.

medium rescued all the LacZ-expressing cells from grim-induced death (Figures 3E and F, and 4A). At the same time and in agreement with previous evidence from insects (Chen *et al.*, 1996), the wide-range natural caspase inhibitor p35 also rescued cells from grim-induced death (Figures 3G and 4A). In contrast, the group II-caspase inhibitor DEVD.cho only showed a residual rescuing capacity (Figures 3D and 4A). In agreement with this result, the natural group II caspase inhibitor OpIAP also showed a very limited rescuing capacity (Figures 3C and 4A). Increasing the molar ratio of OpIAP-expressing vector up to 3:1 versus the grim-expressing vector did not improve the viability of grim-transfected cells (data not shown). The group I caspase inhibitor YVAD.cho was unable to prevent grim-induced death, either alone or in combination with DEVD.cho (Figures 3H and L, and 4A). Increasing YVAD.cho and DEVD.cho concentrations up to 250 μ M did not elicit cell rescue by these inhibitors (data not shown). Western blot analysis showed that transfected grim was expressed at high levels in cultures that were either incubated or co-transfected with the different inhibitors (Figure 4B), confirming that inhibition of caspase activity counteracts apoptosis execution by blocking a step downstream of grim action.

The broad specificity caspase inhibitors zVAD.fmk and p35 prevented grim cleavage under conditions that allowed

its proteolysis in cell extracts (Figure 4B). This result suggests that an endogenous caspase is responsible for grim cleavage. The similar shift observed in Western blot bands for both the cleaved and intact forms of the protein in the N-terminal deletion mutant showed that the cleaved portion of the protein was the C-terminus (Figure 1I). Three clustered aspartate residues near the grim C-terminus (positions 126, 128 and 129) could then be used by a caspase as a target sequence. The downshift predicted by digestion at these sites is between 1 and 1.3 kDa; however, the observed size for the short form is ~4 kDa less than that observed for the intact protein. Nevertheless, we have observed that C-terminal deletions of the protein provoke downshifts greater than expected, which are in the range of the observed downshift for the proteolysed form of the protein (data not shown). These results show that besides activating caspases, grim itself can be processed as a consequence of their proteolytic activity. Even though OpIAP and DEVD.cho did not block grim-induced apoptosis, they were both functionally active in repressing endogenous caspases in either treated or transfected cells, since they prevented grim cleavage (Figure 4B). Therefore, it is likely that caspases insensitive to OpIAP and/or DEVD.cho were sufficient to achieve grim-induced apoptosis.

Interestingly, zVAD-treated cells did initiate apoptosis

Fig. 2. Quantitation of cell death by LacZ co-transfection and time-lapse videomicroscopy. The frequency of total blue cells (A) or intense blue cells (B) was measured in co-transfections of LacZ vector plus empty (control), grim or grim Δ expression vectors. The moment of doxycycline addition (18 h after transfection) was taken as t_0 in the graphs. (C) The frequency of cells with apoptotic morphology within the blue cell class. Time-lapse videorecording was used for the observation of the transfected cultures. Initiation of membrane blebbing invariably led to cell death; cell death events were therefore scored at the initiation of blebbing (D). Average total cell numbers during filming time were 245, 258 and 239 for control, grim and grim Δ , respectively. (E) The average blebbing time for wild-type and mutant grim forms.

but were blocked at the blebbing stage, did not complete the apoptotic programme during the observation time, remained intact in the culture dish, did not become TUNEL-positive and did not suffer nuclear disaggregation (Figure 3I and J). The same cellular phenotype has been described previously for cells in which the apoptosis induced by c-myc, E1A and Bak had been blocked by zVAD (McCarthy *et al.*, 1997). In contrast, grim-expressing p35-rescued cells continued to divide at a similar rate to that of control p35-transfected cells and did not show membrane blebbing (Figure 3K), suggesting that p35 may inhibit apoptotic processes independent of the activity of caspases sensitive to zVAD inhibition. These results demonstrate that grim induces apoptosis by activating endogenous mammalian caspases.

Grim does not induce unspecific toxicity in mammalian cells

Overexpressed exogenous molecules can generate unspecific toxicity leading to a stress response, including apoptosis induction. In order to determine whether the observed induction of apoptosis by grim was originated through an unspecific toxic effect, we analysed two basic metabolic parameters of grim-transfected p35-rescued cells. We measured DNA and protein synthesis by the incorporation of [3 H]thymidine and [35 S]methionine. In spite of them expressing very high levels of grim (Figure 4B), we found that they synthesized DNA and protein at

the same rates as mock-transfected cells (Figure 5). Interestingly, simply p35-transfected cells showed protein synthesis rates similar to control and p35+grim-transfected cells, but showed very low levels of thymidine incorporation, suggesting that p35 inhibits DNA synthesis and that grim reverses this effect. Since caspase inhibition by p35 involves its cleavage, caspase activation by grim may lower the effective amount of p35, thereby allowing normal DNA synthesis rates. These results show that, as long as apoptosis is blocked by p35, even high levels of grim protein do not affect basic parameters of cell metabolism such as DNA or protein synthesis. We conclude that grim kills mouse fibroblasts by specifically inducing apoptosis pathways.

Death induction by grim is inhibited by bcl-2 and is independent of Fas signalling or p53 activity

Although the Fas pathway involves activation of an extracellular signalling mechanism, it has been shown to mediate the apoptosis-inducing effect of c-myc in fibroblasts by an autocrine mechanism (Hueber *et al.*, 1997). To test the implication of the Fas–FasL pathway in grim death induction, we transfected it into primary mouse embryo fibroblasts (MEFs) derived from *lpr* (Fas-deficient) mice. We found that the ability of grim to kill 3T3 fibroblasts was conserved in control wild-type MEFs and that cell survival was not increased when grim was transfected into *lpr/lpr* primary fibroblasts (Figure 6A).

Fig. 3. Grim-induced apoptosis requires caspase activity. The pictures show X-Gal staining of 3T3 fibroblast cultures transfected with LacZ-expressing vector and either empty or grim-expressing vector as indicated. Vectors expressing p35 or OplAP under the control of the elongation factor-1 (EF-1) promoter were co-transfected, when indicated, in a 1:1 ratio to the grim-expressing vector. After transfection, 50 μ M zVAD.fmk, DEVD.cho or YVAD.cho was added to the culture medium as indicated. Cells were processed for LacZ staining 24 h after doxycycline addition.

Fas signalling is, therefore, not required for grim pro-apoptotic activity, suggesting that grim activates a mitochondrial death pathway.

One of the endogenous pathways leading to mitochondrial-mediated cell death is that triggered by p53 activation in response to DNA damage (Clarke *et al.*, 1993; Lowe *et al.*, 1993; Polyak *et al.*, 1997). We transfected grim into p53 null mutant MEFs to determine whether p53 mediated grim-induced apoptosis. Cell viability was not increased in p53-deficient primary fibroblasts as compared with control wild-type MEFs (Figure 6A), showing that grim does not require p53 activity to induce apoptosis.

Mitochondrial pathways leading to apoptosis induction are generally inhibited by the anti-apoptotic protein bcl-2. To test whether grim apoptosis induction was sensitive to bcl-2 levels, we co-transfected it with grim into 3T3 fibroblasts. Overexpression of bcl-2 significantly reduced grim killing efficiency, increasing the relative viability of grim-transfected cells, without affecting the efficiency of grim expression (Figure 6B). The bcl-2 rescue effect was dose sensitive, and was suppressed by increasing the amount of grim-expressing vector (Figure 6B). These results show that the balance between grim and bcl-2 determines whether or not the cell enters into apoptosis, and suggest that grim activates death by interacting with components of the mitochondrial pathways.

Grim progressively localizes in mitochondria during apoptosis progression

The sensitivity of the grim-induced death to bcl-2 levels suggested that grim acts by activating a mitochondrial pathway. To determine the site of grim action, we explored its subcellular localization in transfected cells that do not yet show morphological symptoms of apoptosis. Pre-apoptotic fibroblasts simultaneously show grim in two different cytoplasmic localizations: diffuse cytosolic and a punctate pattern. However, while cells simply grim-transfected showed almost exclusively a cytosolic localization, cells treated with zVAD.fmk showed predominantly the punctate pattern (Figure 7). The same pattern is observed in grim-expressing p35-rescued cells (data not shown). Co-localization of grim protein with a mitochondrial-specific antibody showed that the punctate pattern corresponded to mitochondria (Figure 7). In contrast to the pre-apoptotic cells, blebbing cells simply grim-transfected, while still presenting the diffuse localization, also consistently showed the mitochondrial localization (Figure 7). Again, zVAD-blocked blebbing cells showed almost exclusively a mitochondrial expression pattern (Figure 7). We conclude that grim localizes initially in the cytoplasm but accumulates progressively in the mitochondria, correlating with apoptosis progression. It is possible that grim translocation to mitochondria is the

Fig. 4. Quantification of cell rescue capacity of natural and synthetic caspase inhibitors. The bar graph (A) shows the survival ratio of cells transfected under the experimental conditions shown in Figure 3. The survival ratio was calculated as the ratio between the frequency of blue cells found in grim- and empty vector-transfected cultures in each experimental condition. Western blot detection of p35- and OplAP-transfected proteins is shown above. (B) Western blot detection of grim protein in 3T3 fibroblasts transfected under control conditions and cultured in the presence of the different caspase inhibitors or co-transfected with either p35 or OplAP, as indicated.

event that triggers the apoptotic pathway. In that case, the increased mitochondrial localization of grim in zVAD-treated cells would be the result of the apoptosis blockade by zVAD at a point downstream of grim incorporation in the mitochondria.

Discussion

Our results show that the novel cell death gene *grim*, recently isolated from *Drosophila*, induces apoptosis in mouse fibroblasts. As reported in insects (Chen *et al.*, 1996; Vucic *et al.*, 1998), the apoptotic process induced by grim is executed through caspase activity. One possible explanation for this result would be that grim kills mammalian cells by an unspecific toxicity effect resulting in caspase activation. Such a mechanism would imply that p35-rescued cells are doomed cells that cannot die because their apoptosis execution machinery is blocked. However, p35-rescued cells continue dividing normally and do not show reduction in DNA or protein synthesis. Therefore, grim acts by specifically inducing apoptotic pathways, rather than by an unspecific toxicity effect on mammalian cells. We found that the protein is present in a cytoplasmic diffuse localization and progressively translocates to mitochondria during apoptosis progression. Grim may therefore operate by activating death pathways transduced by the mitochondria, but we cannot exclude that the cytosolic form of grim might be required for, or enhance events of the apoptosis induction or execution phase. It is then possible that mitochondrial components of the mammalian cellular apoptotic machinery recognize the *Drosophila* grim protein and respond by activating the apoptotic programme, as they would after the proper endogenous

Fig. 5. Grim-expressing p35-rescued cells do not show symptoms of cellular stress. Graphs (A) and (B) show, respectively, the [³H]thymidine and [³⁵S]methionine incorporation rates by cells transfected with the LacZ-expressing vector and p35 and grim empty vectors (control), p35-expressing vector and grim empty vector (p35), or p35- and grim-expressing vectors (grim + p35). The horizontal axis shows the number of cells lipofected in each assayed culture well. Cells received 12 h pulses of either isotope starting 30 h after lipofection. Serum-starved and cycloheximide-treated cells were used as negative controls for the incorporation of [³H]thymidine and [³⁵S]methionine, respectively. Replica wells were stained for LacZ to determine the actual transfection level under each experimental condition. The percentage of transfected cells did not vary among the different cell concentrations and were as follows: control, 52 ± 4%; p35, 46 ± 6%; grim + p35, 49 ± 5%. CHX, cycloheximide.

Fig. 6. Death induction by grim is counteracted by bcl-2 expression and does not require either Fas signalling or p53 activity. **(A)** The survival ratio of grim-transfected p53- (*p53*^{-/-}) or Fas- (*lpr/lpr*) deficient MEFs and the respective control MEFs of the same genetic background. **(B)** The survival ratio of 3T3 fibroblasts co-transfected with grim and LacZ expression vectors, and either empty or hbcl-2-pEF-BOS expression vectors. The grim-expressing vector was included at different molar ratios to the bcl-2-expressing vector as indicated. The survival ratio was calculated as described in the legend to Figure 4. Western blot detection of bcl-2 and grim proteins in each experimental condition is shown above the corresponding bars. Both proteins were detected on the same filter by consecutive incubation with bcl-2 and grim antibodies, respectively.

stimulus. In good agreement with this view, grim action is counteracted by the overexpression of the anti-apoptotic factor bcl-2, which generally inhibits all mitochondrial death pathways. bcl-2 inhibits grim-induced death in a dose-dependent manner, suggesting that they mutually antagonize to either promote or inhibit caspase activation. A bcl-2-like molecule has not yet been isolated in *Drosophila*, but mammalian and nematode members of this family have been shown to rescue ced-3- and reaper-induced apoptosis in cultured fly cells (Hisahara *et al.*, 1998). One possibility is that grim promotes cytochrome *c*

release from the mitochondria, as has been shown for reaper in an *in vitro* amphibian system (Evans *et al.*, 1997). In our hands, however, reaper does not induce apoptosis in mouse fibroblasts (C.Clavería and M.Torres, unpublished), arguing for a mechanism of action or regulation different from that of grim.

We also found that grim is susceptible to *in vitro* proteolysis by a caspase activity triggered during the apoptotic process. As grim is responsible for caspase activation, its processing by caspases may constitute a regulatory loop. This loop may function as either a positive or a negative feedback loop that controls grim activity during physiological PCD. Although we have no evidence for the operation of this mechanism *in vivo* we cannot exclude the possibility of its relevance under as yet unexplored physiological conditions.

Our results show that the activity of caspases insensitive to OpIAP, DEVD.cho and YVAD.cho is sufficient to execute the apoptosis induced by grim. Caspase 9, which has been shown to transduce apoptotic mitochondrial signals (Li *et al.*, 1997; Hu *et al.*, 1998; Pan *et al.*, 1998), may be one of those activated by mitochondrial grim. Nevertheless, group II caspases are activated as well, since grim cleavage is inhibited by caspase inhibitors specific for this group and we cannot exclude that activation of group II caspases may also serve to execute grim-induced apoptosis. Inhibitor of apoptosis proteins (IAPs), the natural inhibitors of group II caspases, have been shown to inhibit apoptosis by directly binding to grim, reaper and hid in a lepidopteran cell line (Vucic *et al.*, 1997, 1998). In contrast, we observe that neither OpIAP nor DEVD block grim-induced death. Our results, nevertheless, show that group II caspases are activated, suggesting that some of the grim-induced caspase activation cascades are similar between mouse fibroblasts and cultured lepidopteran cells, whereas others may differ. It is possible that the particular caspase activation cascade triggered by grim depends more on the cell type in which it is expressed rather than on an intrinsic specificity of its action.

Grim expression in mammalian cells may mimic the activation of endogenous killer genes in response to different death stimuli that are transduced through mitochondrial pathways such as factor deprivation. It is important to consider that grim induces death in a completely autonomous manner and it is not affected by high serum levels. This contrasts with the observation that death induction by other regulatory molecules such as c-myc, E1A, Bak, c-fos, E2F or BRCA-1 is enhanced by serum deprivation (Evan *et al.*, 1992; Rao *et al.*, 1992; Smeysne *et al.*, 1993; Chittenden *et al.*, 1995; Shao *et al.*, 1996; Bossy-Wetzel *et al.*, 1997) and is blocked by specific serum components (Harrington *et al.*, 1994). Insensitivity to serum deprivation suggests that grim may act either independently or downstream of the checkpoints at which the cell integrates internal and external signals to determine its entry into apoptosis. This view is in agreement with a grim action downstream in the pathway of factors that promote cell survival during *in vitro* culture or normal development.

One of the most striking observations concerning the role of *Drosophila reaper* is its activation upon irradiation of fly embryos (Nordstrom *et al.*, 1996). In addition, flies bearing a deletion of *reaper*, *hid* and *grim* are able to

Fig. 7. Grim subcellular localization. Confocal optical sections detecting the subcellular localization of grim (red), mitochondria (green) and co-localization of both signals (orange–yellow), as indicated. Stainings were performed on grim-transfected cells in the absence or presence of zVAD.fmk, as indicated.

withstand increased doses of irradiation without entering apoptosis. These observations situate these genes in the pathways responding to radiation-induced cell damage. In mammalian cells, this pathway can be transduced by p53, which, in response to DNA damage, activates the transcription of factors that promote the production of reactive oxygen species (Polyak *et al.*, 1997). Our results show that grim action does not require p53 activity; however, we cannot exclude that endogenous grim-like molecules may be transcriptionally activated by p53 in response to DNA damage. While *reaper* promoter regions that mediate transcriptional activation in response to UV radiation have been identified in the fly, it is not known how this activation is achieved. It is likely that regulators, responding to cellular damage as p53 does in mammalian cells, activate *reaper* promoter transcription in *Drosophila*; however, a p53 homologue has not yet been identified in the fly.

Altogether, our results strongly argue for the existence of mammalian homologues of *grim* that may act similarly to their insect counterpart, thus accounting for transcription-dependent PCD in mammals. We currently are attempting to clone *grim* mammalian homologues to determine their involvement in mammalian PCD.

Materials and methods

Expression vectors

Grim-coding sequences were obtained by RT–PCR from Canton-S 12–24 h *Drosophila* embryos using primers pairs G1–G3 for grim and

G2–G3 for grim^Δ: G1, TTCCTTCCGCGGCCGCCATGGCCATCG-CCTATTTTCAT; G2, TTCCTTCCGCGGCCGCCATGGCCAGAAGCT-ATCAGCA; G3, CGGGATCCTTAGTTCTCCTTGGAGGTG. Primers introduced *NcoI*–*Bam*HI sites that were used to clone the fragments in pET-11d (Novagen, Abingdon, UK). Sequencing confirmed the published coded protein sequence (Chen *et al.*, 1996). A novel expression vector was constructed from the retroviral vector pBPSTR1 (Paulus *et al.*, 1996) by substitution of tTA for rTA. LacZ-, grim- and grim^Δ-coding sequences were cloned into the responding cassette of the new vector and sequenced again before their use for lipofection. Human bcl-2- and baculovirus OpIAP- and p35-coding fragments were cloned in the pEF-BOS expression vector (Mizushima and Nagata, 1990).

Cell culture and lipofection

NIH-3T3 fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum. Cells were lipofected as recommended by the manufacturer (Gibco, Gaithersburg, MD). For co-transfections, the different expression or empty plasmids were added at equal molar ratios, except when indicated. At 18 h after lipofection, doxycycline was added to a final concentration of 2 µg/ml. At the indicated times, cells were fixed in 0.2% glutaraldehyde, washed in phosphate-buffered saline (PBS) and X-gal-stained following standard protocols.

Measurement of DNA and protein synthesis

Cells were seeded in 96-well culture plates and lipofected with the different expression vectors. For thymidine incorporation, 1 µCi of [methyl-³H]thymidine (sp. act. 2.0 Ci/mmol, Amersham) was added per well 30 h after lipofection. Incorporation of radiolabelled thymidine was analysed 12 h later. Starved replicas were totally deprived of serum 12 h before labelling started. For methionine incorporation, 1 µCi of [³⁵S]methionine (sp. act. >1000 Ci/mmol, Amersham) was added per well 30 h after lipofection. A period of 12 h of incorporation was allowed before measurement of protein synthesis. Replicas were assayed in the presence of 10 µg/ml cycloheximide. The results from three

independent experiments for each condition and cell concentration were averaged.

Antibodies

The rabbit anti-grim antibody was obtained against the full-length grim protein purified from *E. coli* extracts. The rabbit anti-OpIAP antibody was obtained against a synthetic peptide corresponding to OpIAP amino acids 65–81 coupled to keyhole limpet hemocyanin (KLH). In both cases, rabbits were immunized according to standard protocols by multiple intradermic injections. The total IgG fraction from grim antiserum was purified on a protein G-coupled affinity column by FPLC. Anti-hbcl-2 mouse monoclonal antibody was purchased from DAKO (Glostrup, Denmark). Chicken anti-p35 antiserum was purchased from Promega (Madison, WI). Anti-mitochondrial antibodies were obtained from the serum of a patient with primary biliary cirrhosis, shown to recognize the E2 polypeptide of the mammalian mitochondrial pyruvate dehydrogenase complex (G.Roy, personal communication).

Immunofluorescence and TUNEL assay

For immunofluorescence and TUNEL, cells were cultured in microscope slide chambers (Cultek). Cells were washed in PBS, fixed in 4% paraformaldehyde for 15 min at room temperature, pre-incubated in 10% goat serum, 0.1% Tween-20 in PBS for 1 h, incubated in 10 µg/ml anti-grim IgG for 1 h, washed three times in PBS-Tween and incubated for 1 h in Cy3-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA). TUNEL was performed using the MEBSTAIN Apoptosis kit as recommended by the manufacturer (Immunotech) and developed with streptavidin–fluorescein. For double mitochondrial and grim staining, anti-mitochondrial serum was used as a primary antibody at a 1:1000 dilution and revealed with an anti-human Cy2-conjugated secondary antibody (Amersham) diluted 1:400, and anti-grim IgGs were revealed with biotinylated anti-rabbit secondary antibodies and streptavidin–Cy3. Optical sections were obtained using an Ar–Kr laser and a TCS-NT Leica confocal imaging systems.

Western blot

Transfected cells were lysed 24 h after doxycycline addition by incubating for 30 min at 4°C in SDS sample buffer in the presence of either 1 or 2 mM PMSF. Proteins were separated by SDS–15% PAGE and transferred to membranes which were then probed with the following antibodies: anti-grim, 3 µg/ml total IgG; anti-hbcl-2 monoclonal antibody diluted 1:1000; anti-p35 whole serum diluted 1:1000; and anti-OpIAP whole serum diluted 1:300. In all cases, sample loading was corrected according to the percentage of blue cells observed, such that the signal obtained is normalized for the effective number of transfected cells in each experimental condition.

Time-lapse videomicroscopy

Cells were maintained in a 37°C chamber in HEPES-buffered culture medium and recorded with a digital video camera controlled by a computerized support. Time-lapse video was controlled by Scion-image (NIH-image for PC) software, set to record one image every 30 s for 6 h. Digital videos were mounted and viewed for a total duration of 1 min (360×).

Mouse strains

MEFs were prepared by standard protocols from 13.5 day post-coitum from *p53*^{−/−} (Donehower *et al.*, 1992) and *lpr/lpr* mouse embryos and from embryos of their respective similar genetic backgrounds. MEFs were lipofected two passages after the first plating.

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