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The proapoptotic function of *Drosophila* Hid is conserved in mammalian cells

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ABSTRACT **Three genes—*reaper*, *grim*, and *hid*—are crucial to the regulation of programmed cell death in *Drosophila melanogaster*. Mutations involving all three genes virtually abolish apoptosis during development, and homozygous *hid* mutants die as embryos with extensive defects in apoptosis. Although Hid is central to apoptosis in *Drosophila*, it has no mammalian homologue identified to date. We present evidence that expression of *Drosophila* Hid in mammalian cells induces apoptosis. This activity is subject to regulation by inhibitors of mammalian cell death. We show that the N terminus of Hid, which is a region of homology with Reaper and Grim, is essential for Hid's function in mammalian cells. We demonstrate that Hid is localized to the mitochondria via a hydrophobic region at its C terminus and functionally interacts with BclXL. This study shows that the function of Hid as a death inducer in *Drosophila* is conserved in mammalian cells and argues for the existence of a mammalian homologue of this critical regulator of apoptosis.**

The demise of cells by apoptosis is essential to the normal development and homeostasis of metazoan animals (1, 2). Critical molecular components controlling apoptosis show striking sequence similarity across wide evolutionary distances (1). However, there remain apoptosis-inducing genes in invertebrate model organisms—such as *Drosophila melanogaster*—that have yet to find relatives in vertebrate phyla.

Genetic studies have shown that three adjacent genes, *reaper* (3), *grim* (4), and *hid* (5), have central roles in the control of apoptosis induction in *Drosophila*. Flies homozygous mutant for a deletion that removes all three genes display virtually no apoptosis during development and die as embryos (3). Ectopic expression of any of these genes in *Drosophila* activates a caspase-dependent apoptotic pathway (4–7), and three caspases have been identified in *Drosophila* (8–10) to date. However, the exact mechanism of action of *reaper*, *grim*, and *hid* remains unclear.

Hid is a 410-aa protein expressed in a large number of *Drosophila* tissues during development. It has no significant homology to known proteins except at its N terminus, where it shows limited similarity to Reaper and Grim. Homozygous loss-of-function mutations in the *hid* gene are lethal, and mutant embryos show evidence of decreased apoptosis. Recently, it has been demonstrated that Hid-induced apoptosis is inhibited by Ras pathway activation (11). This is presumably effected by direct phosphorylation of Hid by the *Drosophila* homologue of mammalian mitogen-activated protein kinase p42/44, *rolled* (11). Ras pathway activation also suppresses Hid-induced apoptosis by down-regulating Hid expression (12). These findings suggest that Hid provides a mechanistic link between Ras pathway activation and the suppression of apoptosis.

Hid-induced apoptosis also is inhibited by a member of conserved class of inhibitors called Inhibitor of Apoptosis Proteins (IAPs), DIAP1. In *Drosophila*, homozygous *DIAP1* loss-of-function mutations are lethal, and ectopic expression of DIAP1 in the eye causes the persistence of supernumerary cells (13), implying reduced apoptosis during eye development. Heterozygous loss-of-function *DIAP1* mutations enhance the apoptotic effect of overexpression of Hid in the fly eye (13), whereas overexpression of DIAP1 inhibits Hid-induced apoptosis in the fly eye (11) and in cultured insect cells (14). The mechanism of inhibition of Hid-induced death by DIAP1 is not known. However, *in vitro* binding studies show that Hid binds to DIAP1 via a short N-terminal domain of Hid (14). The deletion of this domain, which is homologous to the same region of Reaper and Grim, also results in the loss of Hid's ability to induce apoptosis (14). *In vitro* assays show that some mammalian IAPs inhibit caspase 3 and 7 directly by binding to them (15–17).

In *Drosophila*, IAPs and mitogen-activated protein kinases inhibit Hid, and caspases effect its apoptotic activity. IAPs, mitogen-activated protein kinases, and caspases are highly conserved between *Drosophila* and vertebrates. We reasoned, therefore, that there may also be a functional homologue of Hid in higher organisms. If so, expression of *Drosophila* Hid may be able to initiate apoptosis in mammalian cells. Recently, the proapoptotic activities of Reaper and Grim have been shown in mammalian cells (18, 19). Here we show that expression of Hid potently induces apoptosis in human cells and can be inhibited by *Drosophila* and mammalian IAPs. Just as in insect cells, the induction of apoptosis in mammalian cells requires the DIAP1-interacting N-terminal domain of Hid. We demonstrate that Hid localizes to the mitochondria via a C-terminal hydrophobic domain, but this localization is not essential for its apoptotic function in the assays used here. BclXL both inhibits Hid's killing activity and disrupts its mitochondrial distribution. This demonstration of a specific mammalian apoptosis pathway activated by the expression of *Drosophila* Hid, argues for the existence a Hid homologue in vertebrates.

MATERIALS AND METHODS

Plasmids. The ORF of Hid was amplified by standard PCR using Hid cDNA as a template and incorporating appropriate restriction sites and was subcloned into the mammalian expression vector pcDNA3 (Invitrogen). The sequence of the insert was ascertained by direct sequencing, and expression was confirmed by immunoblotting lysates of transfected HeLa cells (data not shown). Hid deletion mutants were generated

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Abbreviations: DIAP1, *Drosophila* inhibitor of apoptosis 1; IAP, inhibitor of apoptosis protein.

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by using PCR and cloned into pcDNA3. The β-galactosidase expression plasmid pCMV-lacZ and the plasmid pRK5-BclXLFlag were kindly provided by David Baltimore (California Institute of Technology, Pasadena, CA). The green fluorescent protein (GFP) reporter plasmid pEGFP-CMV was generated by subcloning the *Not*I/*Bgl*II fragment comprising the cytomegalovirus (CMV) promoter from pcDNA3 into the corresponding sites in the poly-linker upstream of pEGFP1 (CLONTECH). pcDNA3-p35 was generated by subcloning the ORF of p35 from pBSp35 into pcDNA3. The expression vector pcDNA3 XIAP-myc was a gift from John Reed (Burnham Institute, La Jolla, CA). pcDNA3-dIAP-Flag was generated by standard PCR techniques using custom primers incorporating the Flag epitope tag. pCMV-E1B19K was kindly provided by Eileen White (Rutgers University, New Brunswick, NJ) and pcDNA3 Caspase 9 (C287A) was a gift from E. Alnemri (Thomas Jefferson University, Philadelphia).

Cell Death Assays. Human HeLa and 293 cells were obtained originally from American Type Culture Collection, and were transiently transfected by using standard calcium phosphate techniques (20). Briefly, 2×10^5 cells were plated in each 35-mm well 12–16 hours before transfection. DNA/calcium phosphate precipitates were added to the medium, and 24 hours later, the cells were fixed with 1% paraformaldehyde for 10 minutes and then stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) for 4–16 hours. For experiments using the GFP reporter, cells were similarly fixed and incubated with Hoechst dye no. 33358 (5 μg/ml) in PBS for 15 minutes before viewing with a Zeiss Axiophot using a standard bandpass filter set. Stock solutions of benzyloxycarbonyl-Asp(Ome)-fluoromethyl ketone (BOC-D-fmk) and Cbz-Asp(Ome)-Glu-Val-Asp(Ome)-fluoromethyl ketone (Z-DEVD-fmk) (Enzyme Systems Products, Livermore, CA) were made in dimethyl sulfoxide.

Antibodies and Immunohistochemistry. We raised a mAb to Hid by immunizing BALB/cJ mice with full-length Hid protein fused to glutathione-*S*-transferase which was purified from bacteria by using standard techniques. Splenocytes were fused to SP2/O-AG14 cells (American Type Culture Collection). Hybridoma supernatants were screened by for Hid-specific activity by immunoblotting, and the mAb giving the strongest signal, CL1C3, was used for immunohistochemistry. Polyclonal antisera were raised by immunizing rabbits to a synthetic C-terminal peptide of Hid. The polyclonal antibody was used to label cells transfected with Hid dN14, as this deletion abrogated detection by CL1C3. Hid expressed in transfected cells was labeled with CL1C3 at a dilution of 1:1,000 and the polyclonal antibody at a dilution of 1:200. Antibody labeling was detected with FITC-conjugated goat-anti-mouse or goat-anti-rabbit antibodies. Nonspecific binding of the antibodies to vector-transfected cells was minimal. For mitochondrial labeling, MitoTracker red CMXRos (Molecular Probes) was added to the cells at a concentration of 50–100 nM for 30 minutes before fixation. Images were obtained by conventional indirect fluorescence microscopy using a Zeiss Axiophot, or with the DeltaVision microscope system (Applied Precision, Issaquah, WA).

RESULTS

Expression of Hid Induces Apoptosis in Mammalian Cells. HeLa and 293 cells were transiently transfected with a plasmid expressing Hid under the control of a constitutive viral promoter or with vector alone. These cell lines were chosen because their flat morphology allows the detection of apoptotic features. As early as 16 hours posttransfection, transfected HeLa cells started to display morphological features of apoptosis, including cell-body condensation, membrane blebbing (Fig. 1 *A* and *B*), and nuclear fragmentation (Fig. 1 *C–E*). By 24 hours, ≈33% of HeLa cells transfected with Hid demonstrated apoptotic features (Fig. 1*F*). Less than 5% of cells transfected with only the vector were apoptotic. Expression of Hid in 293 cells did not appear to induce more apoptosis than transfection with the vector alone (Fig. 1*F*).

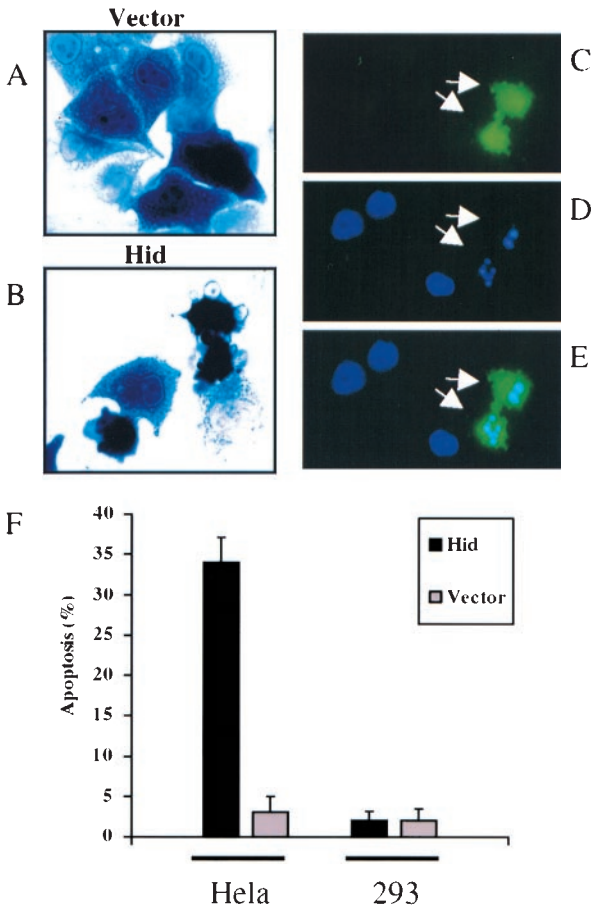

FIG. 1. Hid induces apoptotic morphology in HeLa cells. HeLa cells were transiently transfected with 0.5 μg of pcDNA3 empty vector (*A*) or 0.5 μg of pcDNA3 Hid (*B*) along with 0.5 μg of pCMV-lacZ. Twenty-four hours posttransfection, the cells were fixed, stained with X-Gal, and observed by using light microscopy. (*C–E*) HeLa cells were transfected with 0.5 μg of pcDNA3 Hid along with 0.5 μg of pEGFP-CMV. At 24 hours, they were fixed, and the cells were stained with Hoechst dye no. 33358 (5 μg/ml) for 30 minutes, washed, and observed by using fluorescence microscopy on a FITC filter set (*C*) and a blue filterset (*D*). (*E*) Artificially merged images. (*F*) Quantification of apoptosis: The indicated cell types were transfected with 0.5 μg of pcDNA3 Hid or pcDNA3 along with a 0.5 μg of pCMV-lacZ. Twenty-four hours posttransfection, the cells were fixed and stained with X-Gal. Two hundred blue cells were counted in each of two duplicate transfections in three independent experiments. The fraction of blue stained cells with apoptotic morphology as a percentage of all blue-stained cells was calculated. The percentage is expressed as the mean ± SEM.

Caspase Activation Is Required for Hid-Induced Apoptosis. To determine whether caspases were involved in the changes observed in Hid-transfected cells, we treated them with caspase inhibitors. The cell-permeant peptides BOC-D-fmk and Z-DEVD-fmk irreversibly inhibit cysteine proteases (21, 22). Z-DEVD-fmk is a potent inhibitor of caspase 3 and 7 (23). When added to the medium during Hid transfection, each substantially reduced the proportion of cells with apoptotic morphology (Fig. 2). BOC-D-fmk completely suppressed Hid-induced apoptosis whereas the inhibition by Z-DEVD-fmk was less pronounced, with a reduction in apoptosis from 32% to 8%. The concentrations used to achieve this inhibition were comparable to those reported to inhibit apoptosis induced by irradiation, etoposide, or dexamethasone in thymocytes (24).

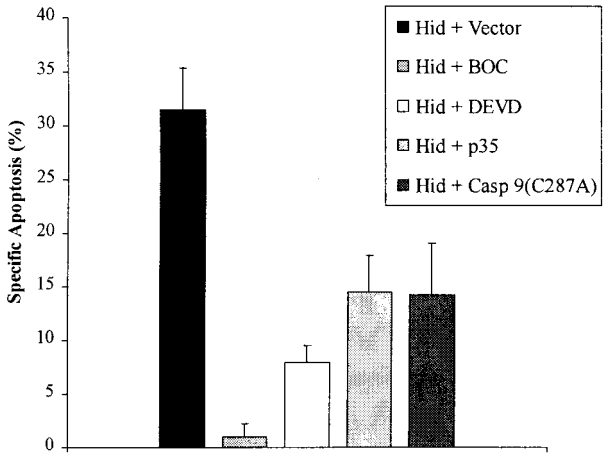

FIG. 2. Hid-mediated apoptosis is inhibited by caspase inhibitors. HeLa cells were transfected with 0.5 μg of pcDNA3 Hid together with 3 μg of vector of the indicated plasmid. In the experiments indicated, BOC-D-fmk was added to the medium to a concentration of 40 μM, or DEVD-fmk was added to the medium to a concentration of 100 μM 2 hours posttransfection. Apoptosis was determined as in Fig. 1*F*. "Specific apoptosis" refers to the percentage of apoptotic blue cells in each experimental condition minus the percentage of apoptotic blue cells in vector-transfected cells.

The viral antiapoptosis protein p35 inhibits multiple caspases (25). Its expression can block apoptosis in *Caenorhabditis elegans* (26), *Drosophila* (5, 6), and mammals (27). To test the ability of p35 to inhibit Hid-induced apoptosis in mammalian cells, we cotransfected plasmids expressing both genes into HeLa cells. To ensure that most Hid transfectants would also be transfected with p35, a 6-fold excess of the latter plasmid was used. This resulted in a significant reduction in the amount of apoptosis, from 32% in cells transfected with Hid alone to 15% in those cotransfected with p35.

Caspase 9 activates caspases 3 and 7 to initiate apoptosis (28, 29). A dominant negative form of caspase 9, in which the active site is mutated, blocks apoptosis induced by overexpression of Apaf-1 or Bax (28, 29). To investigate whether this caspase was involved in Hid-mediated apoptosis, HeLa cells were cotransfected with plasmids expressing Hid and the dominant-negative caspase 9 (C287A). This resulted in a reduction of apoptosis (32% to 14%) (Fig. 2) comparable to that seen with cotransfection of p35. These data demonstrate that inhibition of caspase 9 by overexpression of a dominant-negative form was as effective at inhibiting apoptosis as inhibition of multiple caspases by p35.

Hid-Induced Apoptosis Can Be Inhibited by IAPs and Bcl-2 Family Members. Members of the *IAP* class of genes were also tested for their ability to block Hid-induced apoptosis. DIAP1 inhibits Hid-mediated apoptosis in *Drosophila* and has been shown to interact directly with Hid *in vitro* (13, 30). DIAP1 was therefore cotransfected with Hid to ascertain whether this inhibitory effect was conserved in mammalian cells. XIAP, a mammalian IAP that inhibits caspase 3 and 7 *in vitro* (16), was also tested for inhibitory activity against Hid-mediated apoptosis (Fig. 3*A*). A 6-fold excess of plasmid was used in these experiments. Significant inhibition of apoptosis was seen in the cells cotransfected with DIAP1 (29% to 9%). Noticeable, but weaker attenuation of apoptosis was seen in cells cotransfected with XIAP.

The Bcl2 family comprises a set of molecules of which some have potent antiapoptotic effects (31). The antiapoptotic members include BclXL (32) and the adenoviral protein E1B19k (33). Although homologues of this family exist in *C. elegans* and mammals, no *Drosophila* counterpart has yet been identified. It was therefore of interest to ascertain whether the

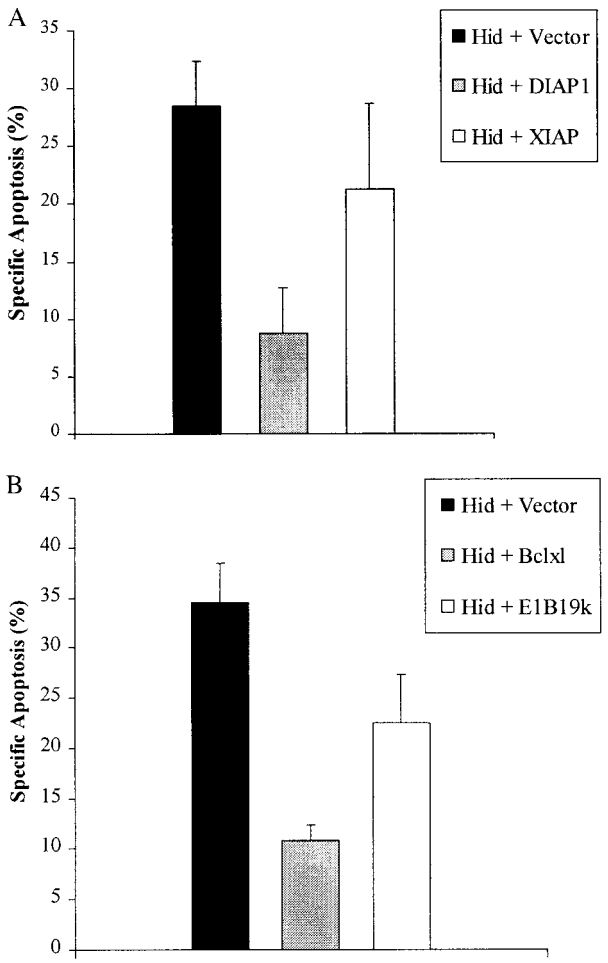

FIG. 3. Hid is inhibited by IAPs and Bcl2 family members. pcDNA3 Hid (0.5 μg) was cotransfected with 3 μg of the indicated plasmids expressing IAPs (*A*) or Bcl2 family members (*B*). Specific apoptosis was determined as in Fig. 2.

apoptosis pathway triggered by Hid in mammalian cells was susceptible to Bcl2-family inhibition. BclXL showed a pronounced effect on reducing Hid-induced apoptosis (35% to 11%), whereas E1B19k showed a more modest effect (to 23%)(Fig. 3*B*). These results demonstrate that Bcl2-type antiapoptotic genes can inhibit Hid-induced apoptosis in mammalian cells.

Mitochondrial Localization of Hid. Given the functional interaction between Hid and BclXL, a protein that can target the mitochondria (34, 35), we were interested in determining the cellular localization of Hid. We raised a mAb to full-length Hid protein and used it to label transfected cells immunohistochemically (Fig. 4). Hid immunostaining was predominantly punctate and perinuclear. To identify better the subcellular distribution of Hid, transfected cells were colabeled with a fluorescent dye that accumulates inside mitochondria. The pattern of mitochondrial staining was very similar to that of Hid. Merged images (Fig. 4*C*) of Hid- and mitochondrially stained cells showed a striking concordance in the distribution of these two stains. This result demonstrates that *Drosophila* Hid localizes to mitochondria when expressed in mammalian cells. Further magnified views of dually stained cells shows that, although the pattern of staining is very similar, it is not overlapping; rather, the Hid-staining appears on the outside of the mitochondrion whereas the mitochondrial dye labels the inner portion (Fig. 4*D*, arrow). Of interest, despite the lack of Hid-induced apoptosis in 293 cells, its distribution in these cells was also mitochondrial (data not shown).

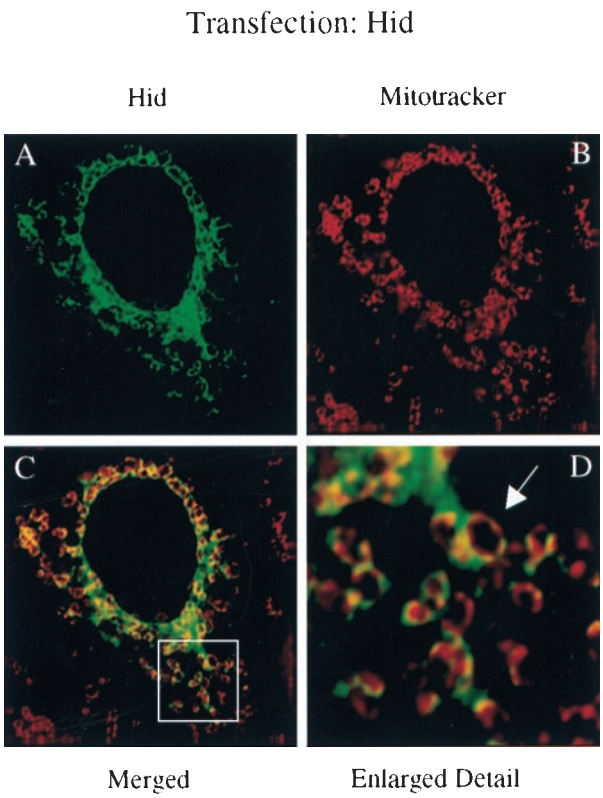

FIG. 4. Hid localizes to the mitochondria. HeLa cells were transfected with pcDNA3 Hid. Twenty-four hours posttransfection, the cells were stained with 100 nM MitoTracker for 30 minutes, fixed, and immunostained with a mAb to Hid (CL1C3) and a FITC-conjugated secondary antibody. *A–C* shows a single HeLa cell visualized with indirect fluorescence microscopy filtered to allow FITC (*A*), MitoTracker (*B*), or both (*C*) to be seen. (*D*) Enlarged detail of the area indicated by the white box in *C*.

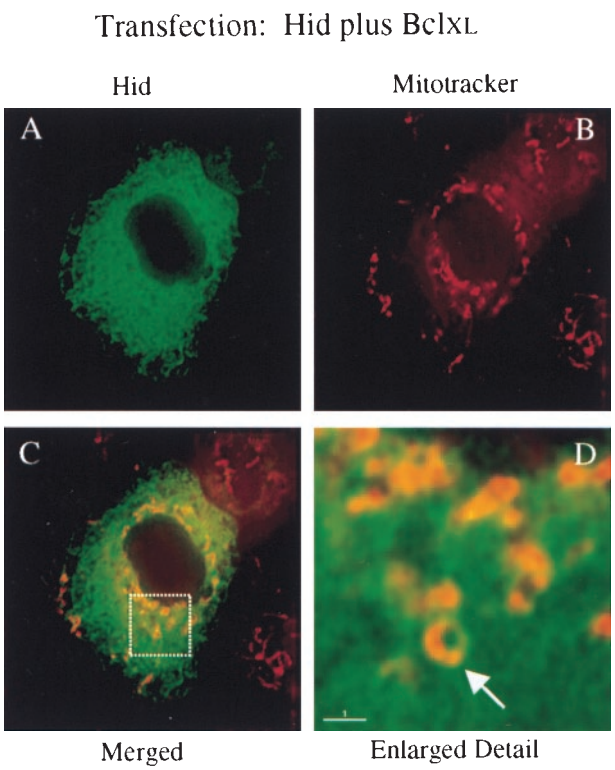

FIG. 5. BclXL disrupts the mitochondrial localization of Hid. HeLa cells were cotransfected with pcDNA3 Hid and pRK5 BclXL. Twenty-four hours posttransfection, the cells were fixed and stained as in Fig. 4. (*A–C*) A single HeLa cell visualized as in Fig. 4. (*D*) Enlarged detail of the area indicated by the white box in *C*.

Because the mitochondrial localization of Hid had not been previously demonstrated in insect cells, we expressed Hid by transient transfection in the insect cell line, SF9. This cell line is susceptible to apoptosis from Hid overexpression (data not shown). Cells colabeled with mitochondrial dye and Hid antibody again showed a predominantly mitochondrial pattern of Hid staining (data not shown).

To assess the effect of apoptosis inhibition on the pattern of Hid staining, immunohistochemistry was performed on HeLa cells cotransfected with Hid and BclXL. The mitochondrial localization of Hid was disrupted in these cells (Fig. 5, arrow), and Hid fluorescence was found in a diffuse pattern, suggestive of cytoplasmic distribution (Fig. 5). This effect was not seen in cells cotransfected with p35, DIAP1, or XIAP or in those treated with BOC-D-fmk (data not shown).

The C Terminus of Hid Is Required for Mitochondrial Localization but Not for Cell Killing. To investigate which portions of the Hid molecule were required for its proapoptotic activity and subcellular localization, we studied two Hid mutant proteins encoded by alleles A206 and A329. These mutations in the *hid* gene locus were induced in flies by chemical mutagenesis, and they cause a mild reduction in Hid function in *Drosophila* (36). Each mutation is the result of a single nucleotide change that causes a premature stop codon at amino acid position 261 and position 304 in alleles A206 and A329, respectively (Fig. 6*A*) (5). Both of these prematurely truncated proteins induced apoptosis in HeLa cells at levels comparable to that caused by wild-type Hid (see Fig. 6*B*). This may be because of the high levels of Hid expression achieved in HeLa cells. A reduction of Hid function that may be sufficient to reduce its proapoptotic activity in *Drosophila* cells may not be noticeable in HeLa cells because of the large amounts of Hid protein expressed.

Immunohistochemistry of cells transfected with each of these mutants, however, showed a marked alteration of cellular localization. Whereas levels of expression were comparable, the mitochondrial targeting of wild-type Hid was completely lost, and the mutant Hid-transfected cells showed a diffuse cytoplasmic pattern of staining (Fig. 7). Although Hid appears to have neither a signal sequence nor a mitochondrial localization signal, close scrutiny of the C terminus reveals a stretch of hydrophobic residues (amino acid position 393–409). Deletion of these residues was sufficient to abolish mitochondrial localization (Fig. 7). However, this mutation did not impair apoptosis induction (Fig. 6*B*). These results suggest that when expressed at high levels in HeLa cells, Hid does not require mitochondrial localization to effect cell death. However, the fact that mutations that delete the C terminus of Hid were identified as loss-of-function in *Drosophila* suggests that this domain, and possibly mitochondrial localization, is important to Hid's proapoptotic function.

The N Terminus of Hid Is Required for Its Ability to Induce Apoptosis. Sequence analysis of Hid, Reaper, and Grim reveals similarity restricted to their N-terminal 14 amino acids (5). We found that deleting residues 2–14 of Hid abolished its ability to initiate apoptosis in mammalian cells (Fig. 6*B*). Immunostaining of mutant-transfected cells showed levels of expression comparable to cells transfected with wild-type Hid (Fig. 7). The deletion did not impair the mutant's ability to localize to the mitochondria (Fig. 7). Because the deleted region is that required for DIAP1 binding (14), one interpretation of this result is that binding to IAPs—presumably endogenous mammalian IAPs in these experiments—is essential for Hid's ability to induce cell death in HeLa cells.

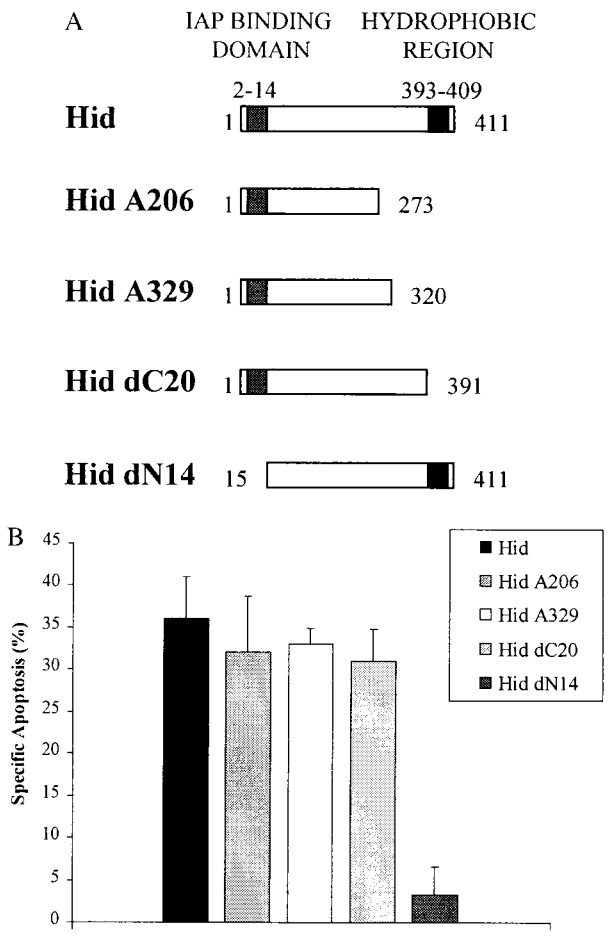

FIG. 6. Deletion analysis of Hid. (*A*) The horizontal bars represent Hid sequences present in deletion mutants. (*B*) HeLa cells were transfected with 0.5 μg of the indicated plasmids. Apoptosis was determined as for Fig. 2.

DISCUSSION

The *Drosophila* genes Reaper, Grim, and Hid are central to the control of apoptosis in flies (37, 38) and are potent inducers of cell death in insect cells (4, 5, 7). In this study, we show that Hid has an evolutionarily conserved ability to initiate apoptosis when expressed in mammalian cells.

Our experiments show several striking similarities between Hid-induced apoptosis in insect and mammalian cells. Just as in insect cells, Hid-induced apoptosis in mammalian cells depends on caspases. The viral protein p35, which inhibits multiple caspases (25, 26), and peptide inhibitors of caspases block Hid-mediated cell death. Moreover, cotransfection with the dominant-negative form of a single caspase, caspase 9, significantly reduces Hid-induced apoptosis. This result suggests that the activation of caspase 9 is an important element of Hid-induced apoptosis in mammalian cells. This observation is consistent with the putative role of caspase 9 as an "initiator" caspase (28, 29).

We also show that DIAP1 blocks Hid-induced apoptosis in mammalian cells. In insect cells, IAPs are important modulators of cell death (13, 14). Homozygous loss-of-function mutations in *DIAP1* are lethal, and mutant embryos die with evidence of increased apoptosis (L. Goyal, K. McCall, J. Agapite, E. A. Hartweig, and H.S., unpublished data). Overexpression of DIAP1 inhibits Hid-induced apoptosis in *Drosophila*(11, 13) and in cultured insect cells (14). DIAP1-mediated inhibition of Hid in mammalian cells indicates that Hid's proapoptotic activity is susceptible to similar regulation as in insect cells. Moreover, because a mammalian IAP (XIAP) also inhibits Hid function, IAP-mediated inhibition of Hid's activity may also be evolutionarily conserved.

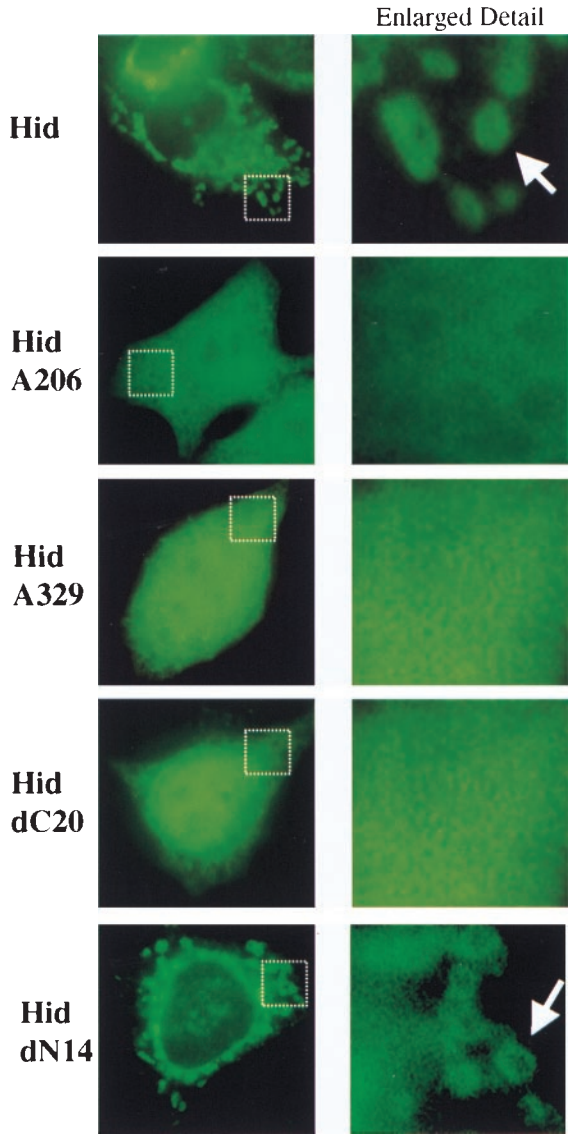

FIG. 7. Deletion of the C terminus abrogates mitochondrial localization. HeLa cells were transfected with the indicated plasmids. Twenty-four hours posttransfection, the cells were fixed and immunostained with CL1C3 (Hid, Hid A206, Hid A329, and Hid dC20) or a polyclonal Hid antibody (Hid dN14) and a FITC-conjugated secondary antibody. The enlarged details are of the areas indicated by the respective white boxes. Arrows indicate individual mitochondria.

Despite the similarities between the activity of Hid in insect and mammalian cells, a general concern with heterologous expression experiments is that apoptosis may be the result of nonspecific stress or toxicity. However, several arguments weigh against the idea that the apoptosis caused by Hid expression in mammalian cells is only because of the presence of a foreign protein.

First, genetic data firmly establish Hid as an important inducer of cell death in *Drosophila* (5, 11, 13). Therefore, it is reasonable to attribute cell death in a heterologous cell type to the specific activity of Hid. Second, our data show that, unlike HeLa cells, 293 cells are not susceptible to apoptosis, despite robust levels of Hid expression and proper localization of the protein; this argues strongly against a general toxic effect of Hid expression on mammalian cells. Finally, deletion of 14 amino acids at the N terminus of Hid abrogates its cell-killing

activity in both mammalian and insect cells. Significantly, the mutant protein is still expressed and properly localized, indicating that the mutation does not disrupt its structure or reduce its stability. Because this truncation removes a very small part of the protein, it is unlikely that it would significantly alter any toxic property of the protein. Rather, cell death must fail to occur because the N-terminal truncation specifically disrupts the normal cell-killing activity of Hid both in insect and mammalian cells.

The N terminus of Hid mediates binding to DIAP1 in *in vitro* studies (14). Given that the N-terminal domain of Hid is necessary both for Hid's proapoptotic activity and for binding to DIAP1 in insect cells, Hid's interaction with DIAP1 may be necessary for activation of apoptosis. Genetic analyses of Hid/DIAP1 interactions in *Drososphila* indicate that this binding blocks the anti-apoptotic activity of the inhibitor, thus activating apoptosis (L. Goyal, K. McCall, J. Agapite, E. A. Hartwieg, and H.S., unpublished data). A conserved interaction between *Drosophila* Hid and mammalian IAPs might therefore account for Hid's ability to initiate apoptosis in mammalian cells. According to this model, the resistance of 293 cells to Hid-induced apoptosis may be caused by the expression of an IAP that cannot be inhibited by Hid.

We show that Hid localizes to the mitochondria in insect and mammalian cells. In vertebrates, several important regulators of apoptosis also localize to the mitochondria (39). Antiapoptotic members of the Bcl2 family, such as BclXL, localize to the mitochondria via a C-terminal hydrophobic domain (39), and transition of the proapoptotic Bax to the mitochondria is associated with apoptosis (35, 40–42). We have shown that Hid also has a hydrophobic domain at its C terminus that is necessary for mitochondrial localization. The functional significance of this localization is suggested by the finding that coexpression of BclXL results both in inhibition of Hid function and in the loss of Hid's mitochondrial localization. However, Hid-induced apoptosis in HeLa cells is not affected by removal of the C terminus, with consequent loss of mitochondrial targeting. This may be a function of the high levels of Hid expression in HeLa cells. In *Drosophila*, analogous mutations in *hid* are hypomorphic but significantly reduce function (36). Therefore, mitochondrial localization of Hid appears to be important under normal physiological conditions, perhaps by increasing the amount of Hid in the same cellular compartment as its downstream effectors. The high levels of Hid expressed in HeLa cells may make such a targeting mechanism less important.

In summary, we have shown that the expression of *Drosophila* Hid initiates apoptosis in mammalian cells. Just as in insect cells, Hid-induced apoptosis is inhibited by IAPs, is effected through caspases, and requires Hid's N terminus. The presence of a Hid-activated apoptosis pathway in mammalian cells, regulated and executed by conserved molecular components, argues strongly for the existence of a vertebrate Hid homologue.

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