Head Involution Defective (Hid)-triggered Apoptosis Requires Caspase-8 but Not FADD (Fas-associated Death Domain) and Is Regulated by Erk in Mammalian Cells*

The molecular machinery of apoptosis is evolutionarily conserved with some exceptions. One such example is the Drosophila proapoptotic gene Head involution defective (Hid), whose mammalian homologue is not known. Hid is apoptotic to mammalian cells, and we have examined the mechanism by which Hid induces death. We demonstrate for the first time a role for the extracellular signal-related kinase-1/2 (Erk-1/2) in the regulation of Hid function in mammalian cells. Bcl-2 and an inhibitor of caspase-9 blocked apoptosis, indicative of a role for the mitochondrion in this pathway, and we provide evidence for a role for caspase-8 in Hid-induced apoptosis. Thus, apoptosis was blocked by an inhibitor of caspase-8, deletion of caspase-8 rendered cells resistant to Hid-induced apoptosis, and Hid associated with caspase-8 in cell lysates. The Fas-associated death domain (FADD) was dispensable for the apoptotic function of Hid, indicating that Hid does not require extracellular death receptor signaling for the activation of caspase-8. In activated T cells, the cytokine interleukin-2 blocked caspase-8 processing and apoptosis, suggesting that survival cues from trophic factors may target a Hid-like intermediate present in mammalian cells. Thus, this study shows that Hid engages with conserved components of cellular death machinery and suggests that apoptotic paradigms characterized by FADD-independent activation of caspase-8 may involve a Hid-like molecule in mammalian cells.

In metazoans, apoptosis, together with cell proliferation and differentiation, regulates the balance between cell loss and cell gain required for tissue homeostasis and embryonic development (1). Basic mechanisms of apoptosis have been conserved throughout evolution from nematodes to humans (2, 3), and key intermediates belonging to the caspase family of cysteine proteases, proapoptotic members of the Bcl-2 family, and endogenous inhibitors of apoptosis have been identified in worms (4), flies (5), and mammals (6).

There are two major death pathways that are relatively well characterized in mammalian systems. These include extracellular receptor-mediated apoptotic signaling (7, 8) or death pathways principally integrated via the mitochondrion (9–11). These pathways usually culminate in the activation of the initiator caspase-8 and -9, respectively. Ligand-dependent death receptor oligomerization recruits cytoplasmic adapter proteins such as FADD,1 which in turn binds the pro-form of caspase-8 and triggers its activation. Caspase-9 is usually activated via the release of cytochrome c into the cytosol, an event regulated by members of the Bcl-2 family (12). Although death receptor-FADD interactions are the best understood mechanism of caspase-8 activation, recent studies have described alternative pathways that lead to the activation of caspase-8. Thus, apoptotic signaling via the B-cell receptor (13), antitumor drugs (14), and cytokines such as TGF-β (15) reportedly activate FADD-independent activation of caspase-8. The death domain in many members of the tumor necrosis factor receptor (TNFR) superfamily in mammals is present as the gene reaper in Drosophila, although a receptor-mediated death pathway is not known in flies. Caspase activity is inhibited by the inhibitor of apoptosis protein (IAP) class of proteins, which function by binding and inactivating processed effector and initiator caspases including caspase-3, -7, and -9. The IAP proteins have characteristic baculoviral IAP repeats that are required for their antiapoptotic function (16).

In Drosophila, head involution defective (Hid), a developmentally regulated gene (17), induces apoptosis by antagonizing Drosophila IAP (DIAP) (16, 18). Hid is apoptotic to mammalian cells, and apoptosis is inhibited by various antiapoptotic molecules that include baculoviral p35 (bp35), Bcl-xL, and Human X-linked IAP (XIAP) (19). The 43-KDa Hid protein has, in addition to the IAP-binding N terminus, five consensus p44/42 mitogen-activated protein kinase (MAPK) (Erk-1/2) phosphorylation domains (PX/S/T/P) (20) located between residues 121 and 257. Unlike other death genes identified in flies, expression of Hid mRNA is seen widely in various stages of development, regardless of cell death fate (20, 21). Smac/DIABLO (second mitochondrial activator of caspases/direct IAP-binding protein with low pI), believed to be the human equivalent of Hid in mammalian cells, has been recently identified (22, 23). However, unlike Hid, overexpression of Smac/DIABLO does not induce apoptosis in cells (23).

In this study, we have analyzed the mechanism of Hid-

1 The abbreviations used are: FADD, Fas-associated death domain; Hid, Head involution defective; Erk, extracellular signal-regulated kinase; pErk, phosphorylated Erk; MAPK, mitogen-activated protein kinase; MEK, MAPK/Erk kinase; CA-MEK, constitutively active MEK; MEK, MEK, MEK; DN-MEK, dominant negative MEK; TNFR, tumor necrosis factor receptor; IAP, inhibitor of apoptosis protein; IL, interleukin; FMK, fluoromethyl ketone; zVAD-FMK, z-Val-Ala-Asp (O-methyl)-fluoromethyl ketone; IETD-FMK, Ile-Glu-Thr-Asp-FMK; LEHD-FMK, Leu-Glu-His-Asp-FMK; PMA, phorbol 12-myristate 13-acetate; GPP, green fluorescent protein; EGFP, enhanced GFP; MBP, maltose-binding protein; FL, full length; Smac, second mitochondrial activator of caspases; DIABLO, direct IAP-binding protein with low pI; c, cytoplasmic.

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induced apoptosis by utilizing mammalian cell lines of lymphoid origin that differ in their sensitivity to Hid-induced apoptosis. We demonstrate Erk-mediated negative regulation of Hid function in mammalian cells and demonstrate that Erk did not regulate Smac/DIABLO-mediated potentiation of apoptosis in the same cells. Hid activates an apoptotic pathway dependent on caspase-8. We show that Hid associates with caspase-8, that the deletion of caspase-8 renders cells resistant to Hid, and that caspase-8 activation is independent of FADD-mediated signaling events. Caspase-8 processing in mitogen-stimulated T cells is inhibited by the trophic factor interleukin-2 (IL-2) via an Erk-dependent event, suggesting that a Hid-like molecule may be present in mammalian cells.

EXPERIMENTAL PROCEDURES

Cells and Reagents—Jurkat, a T lymphoblastoid line of human origin, and d11S, a murine T cell hybridoma, were used in all experiments. Activated T cells were generated as described before (24). The peptide inhibitors z-Val-Ala-Asp (O-methyl)-fluoromethyl ketone (zVAD-FMK), Ile-Glu-Thr-Asp-FMK (IETD-FMK), and Leu-Glu-His-Asp-FMK (LEHD-FMK) were obtained from Enzyme Systems Products (Dublin, CA). Hoechst 33342 and PMA were obtained from Sigma. Antibodies to green fluorescent protein (GFP), caspase-8, Erk-1, and p38MAPK were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to phosphorylated Erk and U0126 were from Cell Signaling Technology, Inc. (Beverly, MA), and antibodies to full-length Bid (BH3-interacting domain death agonist) and TNFR-1 and TNFR-2 were from R&D Systems (Minneapolis, MN). PD98059 and LY294002 were obtained from Calbiochem.

Plasmids—Hid cDNA was obtained from Kristin White (Harvard Medical School, Boston, MA) (21). The Hid construct was made by PCR amplification of the open reading frame, which was cloned into the Bgl-11 and Kpn-1 sites of the pEGFP-N3 vector (CLONTECH, Palo Alto, CA) with GFP in-frame in the C terminus. The bvp35 plasmid was a kind gift of Charlie Zacharchuk (NCI, National Institutes of Health). Constitutively active or dominant negative MEK-1 plasmids were originally from the laboratories of M. J. Weber (University of Virginia Health Sciences Center, Charlottesville, Virginia) and C. J. Marshall (Institute of Cancer Research, London, UK) and were obtained from Shahid Jameel (International Center for Genetic Engineering and Biotechnology, New Delhi, India). The Smac plasmid was from X. Wang (University of Texas, Southwestern Medical Center at Dallas, Dallas TX and obtained from Dr. Santhosh (Rajiv Gandhi Center for Biotechnology, Thrivunananthapuram, India).

Transient Transfection of Cells—A total of 3–5 × 10⁶ cells were transfected by electroporation at 250 mV and 960 microfarad as described previously (25). Cells were routinely transfected with 5 μg of relevant DNA unless specified otherwise. The total amount of DNA across all transfection groups in an experiment was kept constant and adjusted when required by additional amounts of the pEGFP-N3 plasmid.

Treatment with Modulators—In all experiments with peptide inhibitors of caspases or kinase inhibitors, agents were added to cells soon after transfection. In the experiments with PMA and U0126, U0126 was added to the cells immediately after transfection followed by the
addition of PMA after 45 min. This protocol was followed to allow U0126 to enter cells prior to the relatively rapid activation of Erk triggered by PMA.

**Assays for Apoptotic Nuclear Damage**—Apoptotic damage was assessed using Hoechst 33342 by fluorescence microscopy as described (26). GFP-positive cells were viewed under a blue filter, and nuclear morphology of GFP-positive cells was scored using the UV filter.

**Interactors of Hid**—Hid protein tagged with maltose-binding protein (MBP) was added to lysates made from 3 × 10^6 Jurkat cells. MBP-tagged Hid and associated proteins were pulled down using amylose beads, and after thorough washing, they were analyzed by Western blot analysis. Total cell lysates were prepared in SDS sample buffer (62.5 mM Tris-HCl, 2% w/v SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1% bromphenol blue), vortexed to reduce sample viscosity, denatured by boiling, and then cooled on ice. Samples were resolved on 10 or 12% SDS-PAGE gels, transferred onto Hybond-ECL nitrocellulose membrane (Amersham Biosciences). Proteins were detected by chemiluminescence according to the manufacturer's instructions (Pierce).

**T Cell Apoptosis Assays**—Freshly activated T cells were washed and cultured as such to induce spontaneous death, which could be blocked if the growth factor interleukin-2 was added at the initiation of culture. When used, the blocking antibodies to TNFR-1 or TNFR-2 were also added at the initiation of culture. For activation-induced cell death, freshly activated cells were cultured overnight in the presence of IL-2 before being used for the assay. 0.4 × 10^6 cells/ml were cultured for 18–20 h on dishes that had been precoated with 10 μg/ml anti-CD3 (clone 2C11). In all experiments with T cell blasts, apoptotic nuclear damage was assessed using Hoechst 33342.

**RESULTS**

**T Cell Lines Demonstrate a Differential Susceptibility to Hid-induced Apoptosis**—In a screen of mammalian cell lines, we observed that transient overexpression of full-length Hid (Fig. 1A, Hid-FL) induced dose-dependent apoptotic nuclear damage in Jurkat T cells but not in the d11S T cell line (Fig. 1B, solid bars). Although concentrations as low as 1 μg of Hid-FL triggered death in Jurkat cells, d11S cells were resistant to the apoptotic effects of Hid at concentrations as high as 20 μg (Fig. 1C, inset). Since Hid was tagged to GFP, the detection of GFP at the appropriate molecular weight by Western blot analysis, 8–10 h after transfection (Fig. 1C, inset), confirmed expression of the Hid protein in these cells. This time point was determined based on experiments that indicated near maximal levels of GFP expression by flow cytometry (data not shown). Since both d11S and Jurkat cells expressed comparable levels of the transfected gene (indicated by GFP), it appeared that

**FIG. 2.** Erk regulation of Hid-induced apoptosis in d11S cells. A, 10^6 or 5 × 10^5 d11S (lanes 1 and 3) or Jurkat cells (lanes 2 and 4) were lysed and immunoblotted with an antibody to phospho-Erk (panel 1). The same blot was stripped and reprobed for total Erk (panel 2). B, Western blot analysis of endogenous pErk in d11S cells left untreated (lane 1) or treated with 20 μM U0126 (lane 2) or 25 μM PD98059 (lane 3) or 10 μM LY294002 (lane 4) for 2 h. C, percent apoptotic damage after 18 h in d11S cells transfected with pEGFP-N3 (open bars) or Hid (black bars) and left untreated (medium) or cultured in the presence of either of the two inhibitors shown in the panel. Results derived from three to five separate analysis have been shown.

**FIG. 3.** Effect of modulating Erk on Hid-induced apoptosis. A, i–iii. Western blot analysis of pErk, total Erk, and total p38 MAPK. B, Western blot analysis of PMA-treated Jurkat cells. Total lysates of Jurkat cells transfected with Hid-FL and untreated (lane 1), or with PMA (lane 2), were examined for phospho-Erk (panel 1, inset), phospho-p38 MAPK (panel 2, inset), and total Erk (panel 3, inset). C, apoptotic effect on Jurkat cells transfected with pEGFP-N3, Hid, Hid + CA-MEK-1, or Hid + DN-MEK-1 in the presence or absence of PMA. All experiments were performed three-five times.
resistance to apoptosis could not be attributed to the instability of the Hid protein in d11S cells. The differential effect of Hid on these two cell lines was sustained if a non-tagged construct of Hid was co-transfected with GFP (data not shown). Fig. 1D shows the induction of apoptotic nuclear morphology (Fig. 1D, iv) in cells expressing Hid (Fig. 1D, iii and iv) as opposed to the normal nuclei (Fig. 1D, ii) seen in the cells expressing GFP alone (Fig. 1D, i and ii). Both cell lines were equally susceptible to the proapoptotic molecule Bax, suggesting that d11S cells were specifically resistant to Hid-induced apoptosis (Fig. 1E).

These experiments showed that Hid was not generally cytotoxic and that its activity was regulated in mammalian cells. We then tested whether molecules that regulate Hid function in flies were also conserved in mammalian cells. Members of the Ras family of protein kinases are key manipulators of many apoptotic pathways (27), and the Erk pathway regulates Hid-induced apoptosis in flies either via transcriptional mechanisms or via modification of the Hid protein (20, 21). Hid has five consensus p44/42 MAPK (Erk-1/2) phosphorylation domains (PX(S/T/P)) (20) located between residues 121 and 257. In subsequent experiments, we tested whether negative regulation of Hid function by Erk signaling was also seen in mammalian cells.

Erk Blocks Hid-induced Apoptosis in Mammalian Cells—We compared levels of endogenous, phosphorylated Erk (pErk) in both Hid-resistant (d11S) and Hid-sensitive (Jurkat) cells. As shown in Fig. 2A, d11S cells (lanes 1 and 3) expressed higher levels of endogenous pErk as compared with Jurkat cells (upper panel, lanes 2 and 4), although total Erk protein was detected in both cell lines (Fig. 2A, lower panel). The antiapoptotic role of Erk was examined by utilizing two reagents that can inhibit MEK1/2, the kinase that phosphorylates Erk (28). Both U0126 (Fig. 2B, lane 1) following a 2-h treatment in d11S cells. An inhibitor of phosphatidylinositol 3-kinase, LY294002 (Fig. 2B, lane 4), was without effect on pErk in the

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**Fig. 4. Effect of Erk signaling on potentiation of UV-induced apoptosis by Smac/DIABLO.** A, Jurkat cells transfected with control vector pEGFP-N3 or Smac/DIABLO (10 μg of each) co-transfected with or without CA-MEK. Cells were cultured overnight to allow for expression of transfected genes. Transfected groups were untreated or exposed to UV light for 6 h in the absence (experiment 1, Exp1) or presence (experiment 2, Exp2) of serum and apoptotic nuclear morphology assessed after 4 h. The graph shows results of two representative experiments. In B, Jurkat cells were transfected with 10 μg of pEGFP-N3 or Smac/DIABLO and cultured overnight. Transfected groups were exposed to UV and then cultured in the presence or absence of PMA (10 ng/ml). Apoptotic nuclear damage in all experimental groups was assessed 4 h after UV irradiation.

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**Fig. 5. Effect of caspase inhibitors on Hid-induced apoptosis and interaction of Hid with caspase-8.** Apoptotic damage after 15 h in the following conditions. A, Jurkat cells transfected with control vector or Bcl-2 or bvp35 (open bars), or Hid + Bcl-2, or Hid + bvp35 (black bars). In B, Jurkat cells transfected with 5 μg of pEGFP-N3 (open bars) or Hid (black bars) were left untreated (control) or treated with 10 μM IETD-FMK or LEHD-FMK or ZVAD-FMK. C, wild type Jurkat cells, Jurkat cells deficient in caspase-8 (Caspase 8−/−), or FADD (FADD−/−) were transfected with 5 μg of pEGFP-N3 or 5 μg of Hid. In D, total lysates of Jurkat cells transfected with 5 μg of pEGFP-N3 (lane 1) or Hid (lane 2) and cultured for 10 h were probed for Bid (panel 1) or total p38 MAPK (panel 2) by Western blot analysis. In E, MBP-tagged Hid protein or MBP alone were incubated with amylose beads for 1 h, and the Hid-MBP or MBP bound to amylose beads was incubated in Jurkat cell lysate. The amylose bead-Hid-MBP complex and associated proteins were analyzed using Western blot. The figure shows an interaction of Hid-MBP with caspase-8, cIAP-1, and cIAP-2 (lane 2), and MBP alone (lane 1) shows no association with these proteins. Expression of antiapoptotic proteins in Jurkat whole cell lysates (WCL) is also shown. Experiments were performed a minimum of three times.
same experiment and served as a specificity control. The inhibitors were used to probe the functional role of the kinase in Hid-induced apoptosis. At concentrations that reduced phosphorylation of Erk, both inhibitors revealed a sensitivity to Hid-induced apoptosis in d11S cells (Fig. 2C). These inhibitors were without effect on Hid-induced killing in Jurkat cells (data not shown). To rule out the possibility that the protective effect of Erk was an artifact of the d11S cell line, we tested the effect of activating Erk on Hid-induced apoptosis in Jurkat cells.

Effect of Modulating Erk on Hid-induced Death in Jurkat Cells—Phorbol esters such as PMA activate Erk (31), and in Jurkat cells, 10 ng/ml PMA increased pErk (Fig. 3A, i and iv), which was blocked by UO126 (Fig. 3A, iv, lane 3). In functional assays, the same concentration of PMA reduced Hid-induced death close to background levels (Fig. 3B), and pretreatment with UO126 prior to addition of PMA reversed the protective effect of this agent on Hid-induced apoptosis (Fig. 3B) in Jurkat cells. These experiments suggest that PMA could act at the level of MEK1/2 since its effect was blocked by UO126 in cells. PMA has pleiotropic effects on cells, and in subsequent experiments, we used other approaches to confirm the antiapoptotic role of Erk in this system.

Since both PD98059 and UO126 can inhibit kinases other than MEK1/2, a constitutively active form of MEK1 (S218/222D, CA-MEK) (32) was tested for its effect on Hid-triggered apoptosis. As shown in Fig. 3C, overexpression of this construct blocked Hid-induced death to the same levels as those achieved by PMA. In addition, overexpression of a dominant negative form (S217/A, DN-MEK) of this kinase (33) reversed the protective effect of PMA on Hid-induced death (Fig. 3C), an observation consistent with the data using UO126 (Fig. 3B). Thus, Hid-induced apoptosis was suppressed by the hyperactivation of Erk as the protective effect of PMA was reversed by the genetic approaches and pharmacological reagents that block phosphorylation of Erk in our system.

Potentiation of UV-induced Apoptosis by Smac/DIABLO Is Not Regulated by Erk Signaling—Smac/DIABLO (a mammalian protein with some similarity to Hid) is a mitochondrial localized protein that can bind IAP and potentiate apoptosis induced by diverse stimuli (22, 23). Both Smac/DIABLO and Hid function by relieving the inhibitory effect of IAP on caspases. Therefore we tested whether Erk signaling similarly inhibited Smac/DIABLO potentiation of UV-induced apoptosis in Jurkat cells. As shown in Fig. 4, overexpression of Smac/DIABLO increased apoptosis triggered by UV irradiation in these cells. Overexpression of CA-MEK (Fig. 4A) or PMA (Fig. 4B) at concentrations that blocked the apoptotic function of Hid did not block the proapoptotic effect of Smac/DIABLO. Since serum can protect cells during the process of irradiation, we show data using protocols in which cells are irradiated in the absence (experiment 1) or presence (experiment 2) of serum containing medium for the duration of UV exposure. Following the exposure, cells are washed and continued in culture in complete medium.

Hid Activates a Caspase-dependent Pathway of Apoptosis in Mammalian Cells—In subsequent experiments, we investigated the involvement of caspases in Hid-induced apoptosis. In accordance with the literature, Hid-induced apoptosis in Jurkat cells was blocked by the antiapoptotic protein Bel-2 and the baculoviral pan-caspase inhibitor bvp35 (Fig. 5A) (19). Death was blocked by the broad spectrum caspase inhibitory peptide ZVAD-FMK (34, 35), the caspase-9 inhibitory peptide LEHD-FMK, and somewhat unexpectedly by the peptide IETD-FMK, a more specific inhibitor of caspase-8 (Fig. 5B). Previous studies have shown that Hid activates a caspase-9-dependent apoptotic death pathway (19), and our data with LEHD-FMK and inhibition by Bcl-2 are consistent with this observation. However, the indication of caspase-8 functioning as a rate-limiting step in this pathway was unexpected, and we used additional approaches to confirm this observation.

Caspase-8 Is Required for Hid-induced Apoptosis—To confirm the role of caspase-8 in this pathway, Jurkat cells that have a deletion of caspase-8 were tested for their susceptibility to Hid. As shown in Fig. 5C (Caspase 8−), a Jurkat line deficient for caspase-8 (J1 9.2) was resistant to Hid, implicating caspase-8 in Hid-induced apoptosis. Minimal activation of caspase-8 cleaves the proapoptotic molecule Bid, which translocates to the mitochondrion and results in the activation of caspase-9 (36). Hid-induced apoptotic death was accompanied by a loss of full-length Bid protein, which indicated a possible truncation of the full-length Bid protein (Fig. 5D, lane 2-Hid) in Jurkat cells. It may be noted that at the time we detect the loss of Bid, total protein expression of p38MAPK is unchanged in Hid-treated cells. We also tested for an interaction of Hid with caspase-8. Lysates of Jurkat cells were incubated with purified Hid protein, and the associated complex was isolated by immunoprecipitation of Hid. These experiments revealed an interaction of Hid with caspase-8, IAP-1, and IAP-2 (Fig. 5E). We did not detect interactions with Bid/Bcl-2 in these experiments (data not shown). All these proteins are expressed in Jurkat cells (Fig. 5E, WCL).

Caspase-8 is usually activated via ligand-dependent oligomerization of cell surface receptors belonging to the superfamily of TNFR. There are no models of death receptor-induced apoptosis in flies that have been described thus far, whereas receptor-independent activation of caspase-8 has been described in some apoptotic paradigms in mammalian cells (13–15). We used a Jurkat cell line with a deletion of the death adaptor protein FADD (J12.1) to test the involvement of death receptor signaling in Hid-induced apoptosis. As shown in Fig.
5C, FADD negative cells (FADD−) remained susceptible to Hid-induced apoptosis.

These experiments indicate that Hid-triggered apoptosis is dependent on caspase-8 and that apoptosis is suppressed by MEK1/2 signaling in mammalian cells. In the d11S cell line, inhibition of Erk rendered cells susceptible to Hid-induced apoptosis. We tested whether in these experimental conditions caspases functioned as effectors of apoptosis in the d11S cell line as well. Both PD98059 (Fig. 6A) and UO126 (Fig. 6B) revealed a sensitivity to Hid-induced apoptosis in the d11S cell line. Apoptosis triggered by Hid in these conditions was blocked by the broad spectrum caspase inhibitor bvp35 as well as the peptide inhibitors of caspase-8 or -9 (Fig. 6B), thereby recreating the pathway described in Jurkat cells (Fig. 6, A and B).

Erk Regulation of Apoptotic Death in Activated T Cells—Regulation by Erk and the activation of caspase-8 appear to be consistent features of the Hid-induced apoptotic pathway in lymphocytes. Trophic factors regulate survival of many cell types, and activated T cells undergo apoptosis in the absence of exogenous survival factors such as the cytokine IL-2 (37). As shown in Fig. 7, A–C, apoptosis induced by the withdrawal of IL-2 in T cell blasts is independent of Fas and TNFR-mediated signaling. Thus, activated T cell blasts generated from splenocytes from normal C57Bl/6 (triangles) or mutant Fas (C57Bl/6lpr/lpr squares) mice undergo apoptosis in the absence of IL-2 (No IL-2, solid lines A and B), which is not blocked by soluble blocking antibodies to TNFR1 (Fig. 7A) or TNFR2 (Fig. 7B). The levels of apoptosis were comparable in both normal and lpr/lpr mice. The experiment in panel C confirms that T cell blasts from lpr/lpr mice are resistant to Fas ligand-Fas mediated apoptosis induced by cross-linking the T cell receptor complex. When cultured in the absence of IL-2 (Fig. 7D), activated T cells undergo apoptosis characterized by the processing of caspase-8, assessed by the loss of the full-length 55-kDa pro-form over time. The progressive disappearance of the pro-form of caspase-8 correlated with increased levels of apoptotic damage (Fig. 7D, values below the blot) and preceded the loss of caspase-3 in these cells. The loss of caspase-8 was blocked by...
IL-2 or PMA (Fig. 7E). Both reagents also induced the phosphorylation of Erk in these cells (Fig. 7E). Furthermore, the inhibition of caspase-8 processing and the antiapoptotic effect of IL-2 (Fig. 7, F and G) or PMA (Fig. 7G) was reversed by UO126, indicating a role for Erk in the regulation of this caspase in T cells.

**Discussion**

In this study, we demonstrate that Hid-induced apoptosis is dependent on both caspase-8 and -9 and, as demonstrated in flies, the apoptotic pathway is regulated by Erk in mammalian cells as well. The antiapoptotic function of Erk in this death pathway was inferred from the following experiments: a cell line that was resistant to Hid-induced apoptosis had high constitutive expression of pErk, and inhibition of MEK-1/2 revealed sensitivity to Hid-induced apoptosis in these cells (Fig. 2). In the Jurkat cell line, a constitutively active form of MEK1 blocked Hid-induced apoptosis (Fig. 3C), the phorbol ester PMA blocked Hid-induced apoptosis, and protection was reversed by UO126 (an inhibitor of MEK) or a dominant negative MEK construct, identifying MEK as a key component of the PMA-induced antiapoptotic pathway.

Erk is a member of the family of MAPKs downstream of Ras and is recruited into multiple signaling cascades (27). Prosurvival functions have been described for other members of this family of kinases (26, 38), and their function is conserved across various species of metazoans (39). Hid function is negatively regulated by Ras through the Drosophila Erk homologue, rolled, which down-regulates Hid expression (21) and inactivates Hid by phosphorylation (20). Our data are consistent with the latter study and show that despite enforced expression of Hid, apoptotic activity was regulated by endogenous Erk in mammalian cells. The involvement of this key regulatory event laid the foundation for the analysis of the mechanism by which Hid triggered apoptosis in mammalian cells. Our experiments show that Smac/DIABLO-induced potentiation of death is not regulated by Erk signaling (Fig. 4), consistent with the absence of conventional Erk phosphorylation sites on this molecule.

That Hid-induced death may be integrated by the mitochondrion, culminating in the activation of caspase-9, has been reported earlier (19). Activation or recruitment of caspase-9 requires the formation of an apoptogenic complex that includes co-factors dATP, Apaf-1, and cytochrome c (40). Apoptogenic complex formation is regulated by antiapoptotic members of the Bcl-2 family, which regulate the release of cytochrome c. Inhibition of Hid-induced apoptosis by Bcl-2, bdp35, and LEHD-FMK is consistent with a role for the mitochondrion in this pathway. However, the following experiments, unexpectedly, also indicated a role for caspase-8 in this pathway: Hid-induced apoptosis was blocked by a peptide inhibitor specific for this caspase; enforced expression of Hid triggered the truncation of Bid, a preferred substrate for caspase-8, and more conclusively, a Jurkat mutant with a deletion for caspase-8 was resistant to Hid-induced apoptosis. Furthermore, purified Hid protein, when used as a probe, immunoprecipitated caspase-8, cIAp-1, and cIAp-2 from Jurkat cell lysates. We did not detect an interaction between Bid/Bcl-xL, although these proteins are expressed in Jurkat cells. The latter experiments are consistent with the model that Hid promotes caspase activity by binding and antagonizing IAP function. Thus, we propose that Hid activates caspase-8, thereby triggering a cascade that involves the mitochondrion, culminating in the activation of caspase-9 and the death of cells.

Caspase-8 activation is linked to the oligomerization of death receptors and recruitment of adaptors containing death domains. However, no death domain has thus far been identified in Hid, prompting us to determine whether caspase-8 was activated by recruitment of the adaptor protein FADD. Since Jurkat cells with a deletion in the FADD gene were susceptible to Hid-induced apoptosis, our data suggest a FADD-independent mechanism of caspase-8 activation described in other systems (13–15).

Caspases similar to caspase-8 (DREDD, Ref. 41) and caspase-9 (DRONC, Ref. 42) have been described in the apoptotic machinery in Drosophila. However, death receptor signaling has not so far been demonstrated in flies. It should be noted that a deficiency that removed the DREDD gene suppressed Hid-induced apoptosis in the fly eye (43). The mechanism of Hid-induced apoptosis and its regulation appears to be highly conserved in mammalian cells (19). Our analysis in mammalian cells proposes a model of Hid interaction with the caspase-network and members of the Bcl-2 family that may likely be conserved in flies as well.

Cell survival in most tissues is dependent on survival cues from neighboring cells or the extracellular matrix. Thus, it is of interest to understand the mechanisms by which molecules such as trophic factors block intrinsic death programs. EGF signaling via Erk has been reported to block Hid-induced apoptosis in Drosophila (44). Our experiments show that caspase-8 is an intermediate in a death pathway in T cells where both caspase processing and apoptosis are blocked by MEK1/2. Thus, inhibition of a Hid-like molecule may be a conserved component of trophic factor-mediated survival in Drosophila and mammalian cells.

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