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The *Drosophila* Gene *hid* Is a Direct Molecular Target of Ras-Dependent Survival Signaling

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Summary

Extracellular growth factors are required for the survival of most animal cells. They often signal through the activation of the Ras pathway. However, the molecular mechanisms by which Ras signaling inhibits the intrinsic cell death machinery are not well understood. Here, we present evidence that in *Drosophila*, activation of the Ras pathway specifically inhibits the proapoptotic activity of the gene *head involution defective* (*hid*). By using transgenic animals and cultured cells, we show that MAPK phosphorylation sites in Hid are critical for this response. These findings define a novel mechanism by which growth factor signaling directly inactivates a critical component of the intrinsic cell death machinery. These studies provide further insights into the function of *ras* as an oncogene.

Introduction

The development and survival of multicellular organisms depend on the recruitment of a large number of different cell types into tissues and organs. A common principle for the development of tissues and organs appears to be the initial generation of an excess of cells prior to cell fate determination, after which surplus cells are removed by cell death. Physiological cell death occurs primarily through an evolutionarily conserved form of cell suicide called apoptosis. After development is complete, the survival of the organism depends largely on the maintenance and renewal of these cell types.

Apoptosis is regulated by a variety of extracellular and intracellular signals. In most tissues, cell survival is dependent on the constant supply of survival signals provided by neighboring cells and the extracellular matrix (Raff, 1992; Barres et al., 1993; Raff et al., 1993). Cells isolated in culture will undergo apoptosis in the absence of exogenous survival factors. In many cases, this form of cell death does not require protein synthesis, indicating that in these cells an intrinsic cell suicide program is present that operates by default unless active survival signaling suppresses it.

A number of peptide factors including the neurotrophins, insulin-like growth factor 1 (IGF-1), fibroblast growth factor (FGF), and epidermal growth factor (EGF) promote cell survival by suppressing the intrinsic cell death program (Raff et al., 1993; Gardner and Johnson, 1996; Parrizas et al., 1997; Yamada et al., 1997). The mechanisms by which survival factors inactivate the intrinsic cell death program are currently the subject of intensive investigation. The growth factors listed above bind to and activate receptor tyrosine kinases (RTKs) at the cell surface, which in turn stimulate the antiapoptotic activity of the proto-oncogene *ras* (reviewed in Downward, 1998). Ras controls the activity of a number of effector pathways, two of which result in activation of protein kinases known to mediate its antiapoptotic effect: the mitogen-activated protein kinase p42/44 (MAPK) of the ERK-type (extracellular signal-related kinase) via Raf (Xia et al., 1995; Gardner and Johnson, 1996; Parrizas et al., 1997) and the Akt kinase via Phosphoinositide 3-kinase (PI3-K; Yao and Cooper, 1995). Recently, it has been shown that activation of the antiapoptotic PI3-K/Akt-kinase pathway leads to phosphorylation of Bad, a proapoptotic member of the Bcl-2 family, resulting in its binding to 14–3–3 as an inactive complex (Datta et al., 1997; Del Peso et al., 1997). Activation of the Erk-type MAPK has been shown to be required to protect PC-12 cells from apoptosis induced by NGF withdrawal (Xia et al., 1995; Parrizas et al., 1997). However, a direct mechanistic link between the Raf/MAPK survival pathway and the cell death machinery has not been demonstrated thus far.

Molecular genetic analysis in the fruit fly *Drosophila melanogaster* might provide new insights into understanding the regulation of apoptosis by survival signaling pathways. Just like in vertebrates, large numbers of cells die during development of *Drosophila* (Abrams et al., 1993; Steller, 1995), and since many developmental mechanisms are conserved during evolution, it is likely that critical lessons learned by examination of survival signaling pathways in *Drosophila* will contribute significantly to our understanding of vertebrate survival signaling.

There is a *Drosophila* homolog of mammalian *N-ras*, *K-ras*, and *H-ras* termed Ras1 (Simon et al., 1991), and the *Drosophila* homolog of MAPK is encoded by the *rolled* (*rl*) locus (Biggs et al., 1994). The role Ras1/MAPK signaling plays in regulating cell proliferation and cell differentiation has been well established genetically (reviewed in Wassarman and Therrien, 1997). Recently, an important antiapoptotic function of Ras1 in *Drosophila* was revealed in cell ablation studies (Miller and Cagan, 1998) as well as by expressing genes that negatively regulate the Ras1 pathway in postmitotic cells (Sawamoto et al., 1998).

In *Drosophila*, molecular analysis has led to the isolation of three novel cell death genes, *reaper*, *head involution defective* (*hid*), and *grim*, which all appear to integrate different signals regulating apoptosis (White et al., 1994; Grether et al., 1995; Chen et al., 1996). Embryos homozygous mutant for these genes completely lack apoptosis (White et al., 1994; reviewed in McCall and Steller, 1997). When ectopically expressed, they induce apoptosis by activating a caspase pathway (Grether et al., 1995; Hay et al., 1995; Chen et al., 1996; White et al., 1996). The *reaper* and *grim* genes appear to be specifically expressed only in cells that are doomed to die

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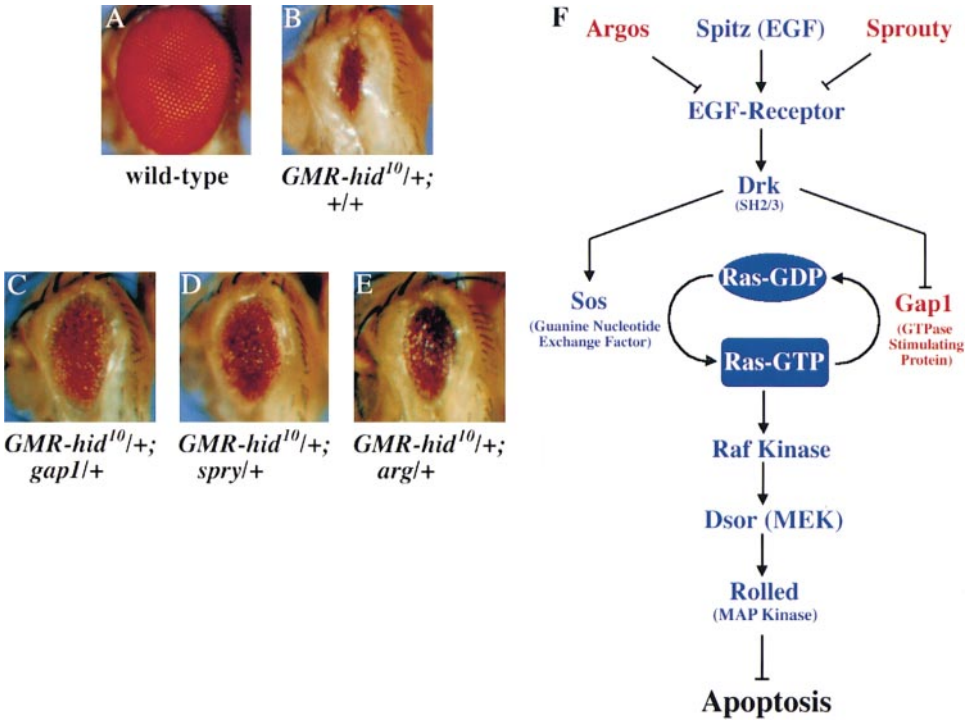

Figure 1. Mutations that Increase Ras1 Signaling Suppress Hid-Induced Apoptosis in the Compound Eye

gap1, *spry*, and *arg* encode genes that inhibit EGFR/Ras1 signaling. Mutations in these genes increase Ras1 signaling resulting in suppression of Hid-induced apoptosis. Flies in this and all other figures were incubated in parallel at 25°C throughout development. Compound eyes of females are shown. The genotypes of flies shown are indicated below each panel. All photographs were taken at the same magnification.
(A) Wild-type.
(B) Eye ablation phenotype caused by one copy of the *GMR-hid*^{10} transgene. Note the strong reduction in eye size in comparison to A.
(C–E) Dominant suppression of the *GMR-hid*^{10}-induced eye phenotype by lof mutations in *gap1*$^{21\text{-}1s}$, *spry*$^{28\text{-}4s}$, and *arg*$^{l\Delta 7}$.
(F) Schematic drawing of the EGFR/Ras1/MAPK signaling pathway and the relative position of the inhibitory genes *gap1*, *argos*, and *sprouty*. Arg and Spry are secreted polypeptides that inhibit EGFR activation. Gap1 promotes the GTPase activity of Ras1. Abbreviations used: EGF, epidermal growth factor; Drk, downstream of receptor kinase; Sos, Son of sevenless; Dsor, downstream suppressor of raf; MAPK, mitogen activated protein kinase; and MEK, MAPK-Erk kinase.

(White et al., 1994; Chen et al., 1996; Robinow et al., 1997). In contrast, *hid* is expressed in many cells that live as well as in cells that undergo apoptosis (Grether et al., 1995). This observation suggests that efficient posttranslational survival mechanisms operate in these cells to protect them from *hid*-induced apoptosis.

Here, the strong eye ablation phenotype caused by expressing *hid* under the control of an eye-specific promoter was used to perform a genetic screen aimed to identify components that regulate and mediate Hid activity. Mutations in genes that regulate the EGF receptor (EGFR)/Ras1 pathway were recovered as strong suppressors of Hid-induced apoptosis. The survival effect of the EGFR/Ras1 pathway is specific for Hid-induced apoptosis since neither Reaper- nor Grim-induced apoptosis is affected by the EGFR/Ras1 pathway. We show further in vivo and in cultured cells that the Ras1 pathway inhibits Hid activity apparently by direct phosphorylation of Hid by MAPK. We conclude that the *hid* gene in *Drosophila* provides a mechanistic link between the survival activity of Ras1 and the apoptotic machinery.

Results

Ectopic expression of *hid* under the control of the eye-specific glass multimer reporter construct (pGMR, Hay et al., 1994; construct designated *GMR-hid*) that drives expression in virtually all cells of the developing eye, beginning at the onset of differentiation in the morphogenetic furrow (Ellis et al., 1993), results in eyes that are severely reduced in size and devoid of most normal ommatidial morphology (Figure 1B; Grether et al., 1995). The severity of this eye ablation phenotype is dosage-sensitive; that is, flies carrying two copies of the *GMR-hid* transgene have significantly smaller eyes than flies carrying only one copy (data not shown). The correlation between the degree of *hid* activity and the strength of the induced phenotype suggests that a 50% reduction in the dose of a gene involved in *hid*-mediated apoptosis should result in the visible modification of the eye phenotype caused by *GMR-hid*. Therefore, using the sensitized *GMR-hid* background a genetic F1 screen was performed to isolate mutations in genes that dominantly suppress the *GMR-hid*-induced eye ablation phenotype. A similar approach has been highly successful for defining a genetic pathway for R7 cell fate determination in the *Drosophila* eye (Simon et al., 1991; Dickson et al., 1996; Karim et al., 1996). Dominant suppressors were scored by looking for enlarged eye size compared to the unmodified *GMR-hid* phenotype (see Figure 1 for examples) and are expected to carry mutations in genes that are positively required for Hid activity. In this way,

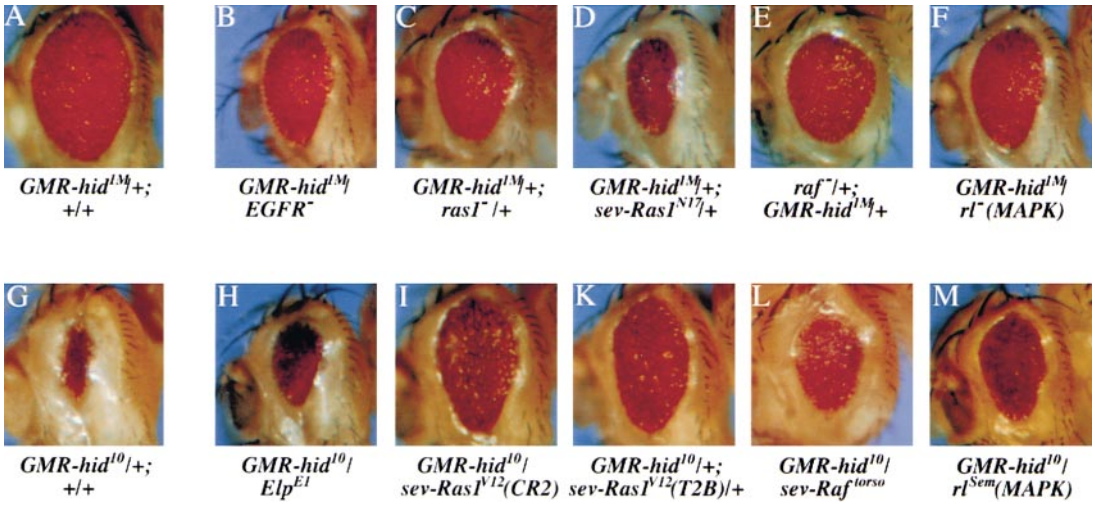

Figure 2. Genetic Interaction of EGFR/Ras1/MAPK Pathway Mutants with *GMR-hid*

The mild eye ablation phenotype of *GMR-hid*^{1M} (A) was used to score for enhancement caused by lof mutants of *EGFR* (B), *ras1* (C), *raf* (E), and *rl*/MAPK (F) or caused by the dominant negative *sev-Ras1*^{N17} transgene (D). Note the smaller eyes in (B)–(F) compared to the unmodified *GMR-hid*^{1M} eye. The *sev-Ras1*^{N17} transgene behaves as the strongest enhancer (D). The eye phenotypes of heterozygous *EGFR*$^{-}$, *ras1*$^{-}$, *raf*$^{-}$, and *rl*$^{-}$/MAPK flies alone are phenotypically wild-type (data not shown). The dominant negative *sev-Ras1*^{N17} allele alone produces a mild rough eye phenotype as the result from the loss of R7 cells (Karim et al., 1996); the eye size, however, is not affected by *sev-Ras1*^{N17}.

The strong eye ablation phenotype of *GMR-hid*^{10} (G) was used to score for suppression caused by gof mutants and transgenes of *EGFR* (*Elp*^{E1}, H), *sev-Ras1*^{V12} (I and K), *sev-Raf*^{torso} (L), and *rl*^{Sem}/MAPK. The strongest suppression is seen with the two *sev-Ras1*^{V12} transgenes used (*CR2* and *T2B*). The genotypes of flies shown are indicated below each panel.

about 300,000 mutagenized F1 progeny were screened and a total of 120 dominant suppressors was isolated (J. A., K. M., and H. S., unpublished data).

Mutations in Components of the EGFR/Ras/MAPK Pathway Were Recovered as Dominant Modifiers in the *GMR-hid* Screen

Among known genes, five loss-of-function (lof) alleles of each *gap1* and *sprouty* (*spry*) were recovered as strong suppressors of *GMR-hid* in the screen (Figures 1C and 1D). *gap1*, encoding a GTPase activating protein, was originally identified as a negative regulator of R7 photoreceptor development (Gaul et al., 1992) and appears to function by stimulating the GTPase activity of Ras1 causing Ras1 to hydrolyze GTP to GDP and thereby returning it to its inactive conformation (see Figure 1F). *Sprouty* was originally identified as an inhibitor of tracheal branching by antagonizing the *Drosophila* FGF RTK pathway and encodes a novel, presumably secreted polypeptide (Hacohen et al., 1998). Both genes are believed to negatively regulate RTK/Ras1 signaling (Figure 1F). Another gene, *argos* (*arg*), known to negatively regulate EGF receptor (EGFR) signaling (Freeman et al., 1992; Okano et al., 1992), was also found to suppress the *GMR-hid*-induced eye ablation phenotype (Figure 1E).

The wild-type function of the genes *gap1*, *spry*, and *arg* is required to inhibit EGFR/Ras1 signaling. Mutations in any one of these genes increase the signaling strength of the EGFR/Ras1 pathway. Thus, recovery of mutants in these genes as potent suppressors of the *GMR-hid*-induced eye phenotype indicates that the EGFR/Ras1-signaling pathway has an antiapoptotic effect by inhibiting *hid* activity. To test this notion, we studied the consequence of both lof and gain-of-function (gof) mutants of components of the Ras1 pathway on *GMR-hid*. Two different *GMR-hid* transgenic lines were used in this analysis. *GMR-hid* line 1M (*GMR-hid*^{1M}) causes a mild eye ablation phenotype (Figure 2A) and was used to score for enhancement by Ras1 pathway mutants. *GMR-hid* line 10 (*GMR-hid*^{10}) causes a strong eye ablation phenotype (Figure 2G) and was used to score for suppression by Ras1 pathway mutants.

The results obtained are consistent with our previous findings. Reduction of Ras1 pathway activity leads to enhancement of *GMR-hid*-induced killing activity. Lof alleles of the *EGF receptor*, *ras1*, *raf*, and *rolled* (*rl*), the *Drosophila* MAPK homolog (referred to as *rl*/MAPK), enhance *GMR-hid*^{1M}-induced apoptosis (Figure 2). The strongest enhancement of *GMR-hid*^{1M}, however, was caused by a dominant negative allele of *ras1*, *ras1*^{N17}, placed under eye-specific *sevenless* promoter control (*sev-Ras1*^{N17}; Karim et al., 1996; Figure 2D).

The opposite effect was observed when gof alleles of *EGFR*, *ras1*, *raf*, and *rl*/MAPK were tested against *GMR-hid*^{10}. The gof alleles of both Ras1 and Raf are transgenes that were placed under control of the eye-specific promoter of the *sevenless* gene (*sev-Ras1*^{V12}, *sevRaf*^{torso}). Their gof character was determined by the ability to induce supernumerary R7 cells in the absence of RTK signaling (Dickson et al., 1992; Fortini et al., 1992). The *sev-Ras1*^{V12} transgenes contain a valine for glycine substitution at residue 12 that renders Ras1 constitutively active and bypasses the requirement for RTK activation (Fortini et al., 1992). Two independent *sev-Ras1*^{V12} transformants, designated CR2 and T2B (Karim et al., 1996), showed very strong suppression of the *GMR-hid*^{10}-induced eye phenotype (Figures 2I and 2K).

Activating Raf in *sev-Raf*^{torso} is achieved by targeting Raf to the membrane by fusing the kinase domain of Raf to the transmembrane and extracellular domain of the RTK torso (Dickson et al., 1992). The *sev-Raf*^{torso} transgene tested in this study shows a strong suppression of *GMR-hid*^{10} (Figure 2L).

Whereas the gof alleles of Ras1 and Raf are transgenes, the gof alleles of the EGFR (*Elp*^{E1}) and of *rl*/MAPK (*rl*^{Sem}, Sem-Sevenmaker) are mutations in the endogenous genes (Baker and Rubin, 1989; Brunner et al., 1994a). For instance, the RTK independent activation by the *Sevenmaker* allele of *rl*/MAPK is caused by a single amino acid substitution (Asp to Asn at position 334; Brunner et al., 1994a). Thus, it appears that the strong suppression of *GMR-hid*^{10} by *rl*^{Sem}/MAPK (Figure 2M) is not caused by a simple overexpression effect but rather by specific activation of the *rl*^{Sem}/MAPK gene product. We also saw strong suppression of *GMR-hid*^{10}-induced apoptosis by expression of *secreted spitz*, the activated form of the EGF ligand encoded by the *spitz* gene (data not shown; Schweitzer et al., 1995).

In the *GMR-hid* suppression assay, we detected a slightly stronger suppression of *hid*-induced apoptosis by the *sev-Ras1*^{V12} transgenes compared to the suppression obtained by *rl*^{Sem}/MAPK (compare Figures 2I and 2K with Figure 2M). This suggests that in addition to MAPK signaling, other Ras1-dependent survival mechanisms may operate to inactivate *hid* activity. Active, GTP-bound Ras transduces signals through multiple intracellular targets including (among others) Raf (at the apex of the MAPK pathway), the p110 catalytic subunit of PI3-Kinase (activating the Akt-1 kinase), and Ral.GDS, the exchange factor for Ral.GTPases (Figure 3A; see Introduction). We investigated the relative contributions made by each of these effectors on suppression of *hid*-induced apoptosis. We used partial lof mutants located in the Ras effector loop that each activate only one of the downstream pathways mentioned above. Each mutant resides in a constitutively activated Ras1^{V12} background, such that the mutant Ras1^{V12S35} interacts with Raf but fails to interact with PI3-K or Ral.GDS; Ras1^{V12G37} interacts with Ral.GDS, but not with Raf or PI3-K; and the Ras1^{V12C40} mutant interacts with PI3-K, but not with Raf or Ral.GDS (Figure 3A; White et al., 1995; Rodriguez-Viciana et al., 1997; Karim and Rubin, 1998). We used the GAL4-UAS system (Brand and Perrimon, 1993) to express wild-type Ras1 and the Ras1 mutants under *sev* promoter control (*sev-GAL4*) specifically in eye imaginal discs. This analysis showed that the Raf/MAPK branch provides the major suppression of *hid*-induced apoptosis (Figure 3D). The Ral.GDS effector pathway failed to contribute to the survival activity of Ras1 (Figure 3E). A moderate suppression of *hid*-induced apoptosis was provided by the PI3-K/Akt-kinase branch (Figure 3F), consistent with its previously reported antiapoptotic function (Yao and Cooper, 1995; Kennedy et al., 1997; Staveley et al., 1998). Thus, it appears that Ras1 mediates its survival activity largely through the MAPK pathway, supplemented by a minor component of the PI3-K/Akt-kinase branch.

To further characterize the survival activity of the EGFR/Ras1/MAPK pathway on Hid-induced cell death, we sought an alternative assay in a different developmental context. Induction of *hid* under control of a heat

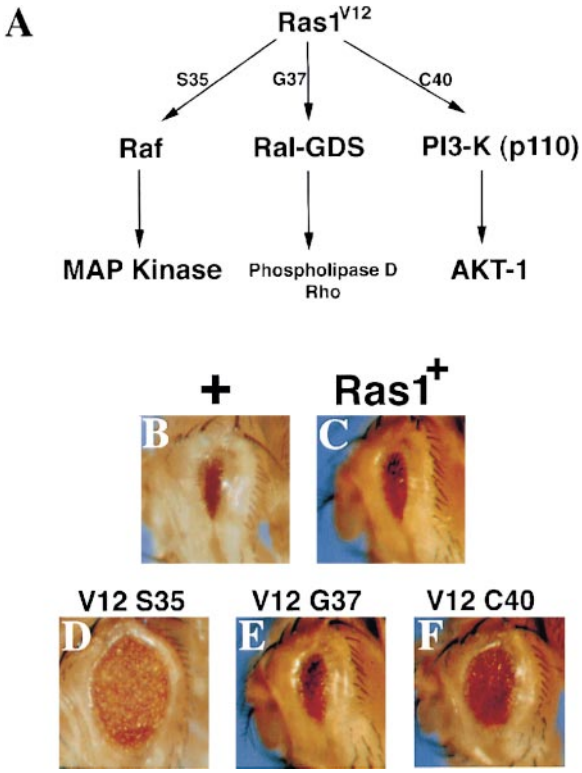

Figure 3. The Raf/MAPK Effector Branch of Ras1 Is the Major Pathway for Suppression of *GMR-hid*

(A) Schematic outline of the downstream effector pathways of Ras1 and of the effector loop mutations in Ras1 leading specifically to activation of only one effector branch. For instance, Ras1^{V12S35} interacts with Raf, but not with Ral-GDS and PI3-K; Ras1^{V12G37} only interacts with Ral-GDS; Ras1^{V12C40} only interacts with the p110 subunit of PI3-K.

(B) Eye ablation phenotype caused by *GMR-hid*^{10} *sev-GAL4*.

(C) Wild-type Ras1 does not modify the *GMR-hid*^{10} eye phenotype.

(D) Activation of the Raf/MAPK pathway by Ras1^{V12S35} results in strong suppression of *GMR-hid*^{10}. The quantatively stronger suppression seen in this experiment compared to Figure 2M is due to stronger expression of Ras1^{V12S35} caused by the GAL4/UAS system.

(E) The Ral.GDS effector pathway fails to suppress *GMR-hid*^{10}.

(F) Activation of the PI3-K/Akt pathway by Ras1^{V12C40} results in moderate suppression of *GMR-hid*^{10}.

The genotypes are: (B) *GMR-hid*^{10} *sev-GAL4*/+, (C) *GMR-hid*^{10} *sev-GAL4*/*UAS-Ras1*$^{+}$, (D) *GMR-hid*^{10} *sev-GAL4*/*UAS-Ras1*^{V12S35}, (E) *GMR-hid*^{10} *sev-GAL4*/*UAS-Ras1*^{V12G37}, and (F) *GMR-hid*^{10} *sev-GAL4*/*UAS-Ras1*^{V12C40}.

shock promoter (*hs-hid*) very efficiently causes organismal lethality (Grether et al., 1995). Only about 2% of the animals containing a *hs-hid* transgene survive to adulthood compared to control (non-*hs-hid*) flies after they received a 30 min heat shock at 37°C during the first instar larval stage (Figure 4, see Experimental Procedures for details). A 40 min heat shock under the same conditions was sufficient to kill all *hs-hid*-containing animals.

However, in a heterozygous mutant background for the genes *gap1*, *spry*, and *arg*, the *hs-hid*-induced lethality is strongly reduced such that approximately 20%–40% of *hs-hid* transgenic animals survive even after a 60 min heat shock at 37°C during first instar larval stage (Figure 4). An even more striking rescue is observed if

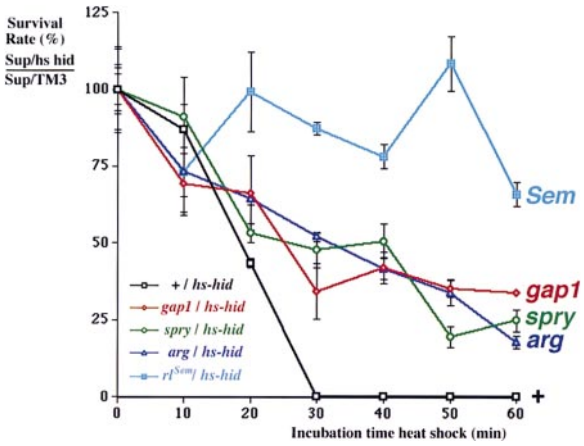

Figure 4. Dominant Suppression of *hs-hid*-Induced Lethality

Heat shock induction of *hid* via a *hs-hid* transgene during first instar larvae causes strong organismal lethality (Grether et al., 1995). After 30 min of heat shock, only 2% of the *hs-hid* animals survive compared to control (non-*hs-hid*) animals (see Experimental Procedures for details). Lof mutations in *gap1*, *spry*, and *arg* protect against *hs-hid*-induced lethality, such that about 20%–40% of Sup/*hs-hid* animals survive even a 60 min heat shock at 37°C. The gof mutation rl^{Sem}/MAPK has the strongest survival activity against *hs-hid*-induced lethality. Approximately 70% of rl^{Sem}; *hs-hid* animals survive a 60 min heat shock at 37°C.

The genotype of flies analyzed in this assay are +/*hshid*, $gap1^{21\text{-}1s}$/*hs-hid*, $spry^{28\text{-}4s}$/*hs-hid*, $arg^{\Delta 7}$/*hs-hid*, and rl^{Sem}/+; *hs-hid*/+. The results shown represent the average of three independently performed experiments.

the *hs-hid* suppression assay is performed with the gof rl^{Sem}/MAPK allele (see above; Brunner et al., 1994a). After 60 min of heat shock 70%–80% of *hs-hid* transgenic flies survive (Figure 4).

In summary, the observed genetic interaction strongly suggests that activation of the EGFR/Ras1/MAPK pathway inactivates the death-inducing ability of the pro-apoptotic gene *hid*.

The EGFR/Ras1/MAPK Pathway Acts Specifically on Hid and Does Not Influence Reaper- and Grim-Induced Killing

We also studied the influence of the EGFR/Ras1/MAPK pathway on the other two known death effector genes in *Drosophila*, *reaper* and *grim*. In Figure 5, we compare the enhancing and suppressing effects of the dominant negative $sev\text{-}Ras1^{N17}$ allele and the gof $sev\text{-}Ras1^{V12}$ allele, respectively, on *GMR-reaper*- and *GMR-grim*-induced eye phenotypes. These two Ras1 alleles gave the strongest effects on *GMR-hid*-induced apoptosis (Figures 2D, 2I, and 2K). However, the eye ablation phenotypes of both *GMR-reaper* and *GMR-grim* are not significantly affected by the Ras1 mutants (Figure 5). This finding is further confirmed by analysis of other pathway mutants (data not shown). Thus, in summary, the antiapoptotic survival activity of the Ras1 pathway is predominantly mediated by specific inactivation of the death effector gene *hid*. In the following, we address the molecular mechanisms of this effect.

Alteration of MAPK Sites of Hid Blocks the Survival Abilities of $Ras1^{V12}$ and Rl^{Sem}/MAPK in SL2 Cells

Since in both the *GMR-hid* and the *hs-hid* suppression assays, *hid* is placed under heterologous promoter control, we assumed that the EGFR/Ras1/MAPK pathway suppresses *hid* activity at a posttranslational level. The presence of five MAPK phosphorylation consensus sites in the *hid* protein (Figure 6) suggested the possibility that Hid is a direct target of MAPK phosphorylation. Three known phosphorylation targets of MAPK in *Drosophila* are Yan, which contains eight phosphorylation consensus sites (Rebay and Rubin, 1995), D-Jun (three consensus sites, Peverali et al., 1996), and Pointed P2 (one such sequence, Brunner et al., 1994b). Whereas D-Jun and Pointed P2 are activated via phosphorylation by MAPK, the Yan protein is inactivated in response to MAPK signaling (Brunner et al., 1994b; Rebay and Rubin, 1995; Peverali et al., 1996). By analogy to Yan, we considered that phosphorylation of Hid protein by MAPK leads to its inactivation and is the cause for the observed genetic effects.

To investigate this hypothesis, we used in vitro mutagenesis to replace the phospho-acceptor residues of the consensus sites with a nonphosphorylatable amino acid, alanine. If downregulation of *hid* activity occurs via phosphorylation by MAPK, then removal of the phosphorylation sites should result in a mutant form of Hid that fails to respond to Ras1/MAPK signaling. We constructed two different MAPK deficient mutants of *hid*.

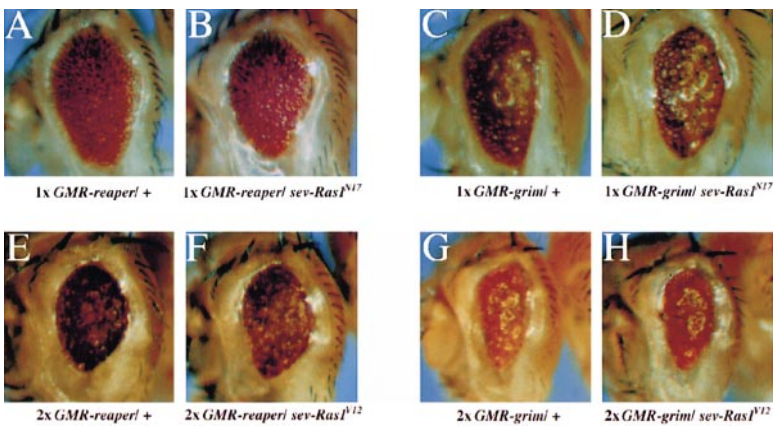

Figure 5. *GMR-reaper*- and *GMR-grim*-Induced Eye Phenotypes Are Not Affected by EGFR/Ras1/MAPK Signaling

The mild eye ablation phenotypes caused by one copy of *GMR-reaper* (A) and *GMR-grim* (C) were used to score for an enhancement by the dominant negative Ras1 allele $sev\text{-}Ras1^{N17}$ (B and D). Stronger eye ablation phenotypes were produced by two copies of either *GMR-reaper* (E) or *GMR-grim* (G) and were used to test for suppression by the gof Ras1 allele $sev\text{-}Ras1^{V12}$ (F and H). Both Ras1 transgenes, $sev\text{-}Ras1^{N17}$ and $sev\text{-}Ras1^{V12}$, do not or only weakly modify either the *GMR-rpr*- or *GMR-grim*-induced eye phenotypes. These Ras1 transgenes show striking effects on *GMR-hid*-induced apoptosis (see Figures 2D and 2I). Other EGFR/Ras1/MAPK pathway mutants also fail to show a genetic interaction with *GMR-reaper* and *GMR-grim* (data not shown). We conclude that the antiapoptotic activity of the EGFR/Ras1/MAPK specifically counteracts *hid*-induced apoptosis. The genotypes of flies shown are indicated below each panel.

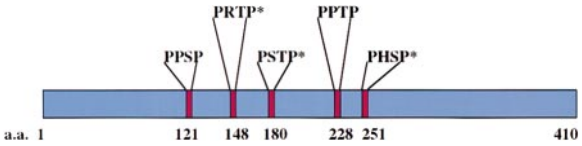

Figure 6. MAPK Phosphorylation Consensus Sites in Hid

The *hid* gene encodes a novel protein of 410 amino acid residues (Grether et al., 1995). The five MAPK phosphorylation consensus sites are indicated. The consensus site is defined as Pro-X-Ser/Thr-Pro, where X can be any residue exept Pro (Clark-Lewis et al., 1991). In hid^{Ala5}, all five phospho-acceptor residues are changed to a nonphosphorylatable residue, Ala. In hid^{Ala3}, only Thr-148, Thr-180, and Ser-251 are changed to Ala (indicated by asterisks), since the consensus sites of the remaining two phospho-acceptor residues, Ser-121 and Thr-228, contain a Pro in the X position.

In hid^{Ala5} all five Ser/Thr were changed to Ala. In hid^{Ala3} only Thr-148, Thr-180, and Ser-251 were changed to Ala (indicated with asterisks in Figure 6).

First, we tested the biological activity of these mutant constructs in a rapid cell culture assay. Schneider line 2 cells (SL2), a *Drosophila* cell line (Schneider, 1972), were transiently transfected with hid^{wt}, hid^{Ala3}, and hid^{Ala5}. The genes were expressed under control of the constitutively active *ie1* promoter from baculovirus (Jarvis et al., 1996). Transfection of the mutant constructs resulted in much stronger killing activity than transfection of the wild-type construct (Figure 7A). Therefore, the two *hid* mutants behave as gof alleles of *hid*. To explain the gof characteristics of the *hid* mutants in SL2 cells we assume that they are not responsive to Ras1/MAPK signaling due to the change of the MAPK phosphorylation sites of Hid. The components of the Ras1 pathway are present in SL2 cells as shown by transcriptional assays of other target genes of the pathway like *pointed* and *yan* (O'Neill et al., 1994; Rebay and Rubin, 1995). Survival factors provided by the medium may trigger activation of the Ras1 pathway leading to inactivation of Hid^{wt}. The Hid mutants, however, are not responsive to MAPK signaling anymore and thus behave as more efficient inducers of cell death in these cells.

To test the survival requirement of Ras1/MAPK on Hid-induced cell death, we transiently cotransfected the *hid* constructs with cDNAs encoding $Ras1^{V12}$ and RI^{Sem}/MAPK under *ie1* promoter control into Schneider SL2 cells. Consistent with the genetic findings, both $Ras1^{V12}$ and RI^{Sem}/MAPK suppress cell death induced by Hid^{wt} in SL2 cells (Figures 7B and 7C). The survival rescue provided by $Ras1^{V12}$ is quantitatively much stronger than that provided by RI^{Sem}/MAPK. Increasing concentrations of $Ras1^{V12}$ in this assay resulted in an up to 5-fold increase in the number of surviving SL2 cells, whereas RI^{Sem}/MAPK reduces the killing activity of Hid^{wt} only about 2-fold (Figures 7B and 7C). This difference in the survival abilities of Ras1 and RI/MAPK has previously been observed in the *GMR-hid* suppression assay (Figure 2) and might reflect activation of the PI3-K/Akt effector branch of Ras1 as seen in Figure 3.

In the cotransfection experiment performed with the mutants Hid^{Ala3} and Hid^{Ala5}, the survival ability of $Ras1^{V12}$ on the mutants is significantly weaker as compared to Hid^{wt}. The number of surviving SL2 cells is increased only 2.5-fold in the $Hid^{Ala3/5}$ experiment compared to a

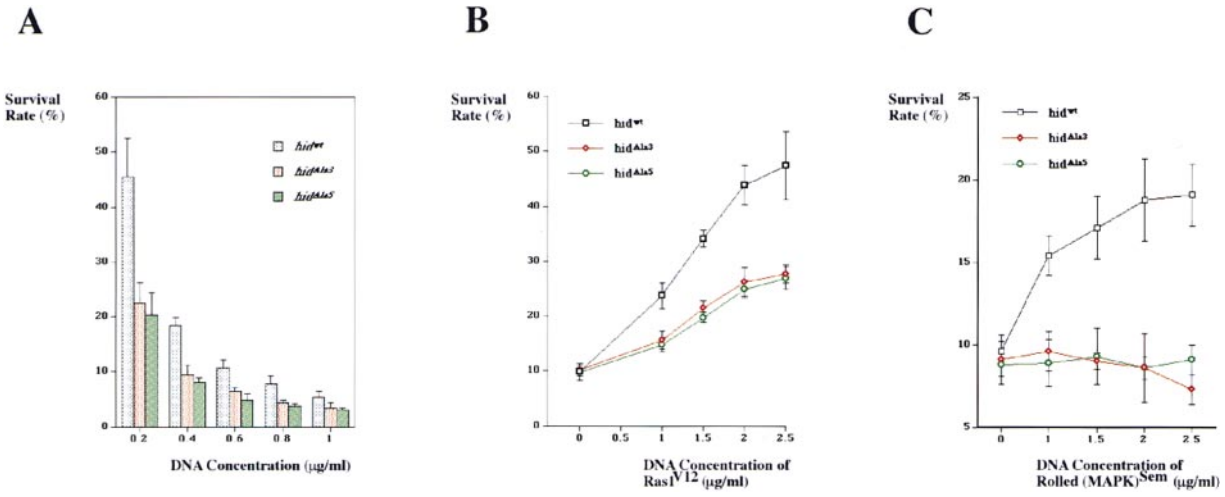

Figure 7. Effects of MAPK Phosphorylation in SL2 Cells

SL2 cells were transiently cotransfected with the indicated constructs. For details see Experimental Procedures.

(A) The indicated amount of DNA of the Hid constructs was transfected into SL2 cells and tested for their killing activity. The two MAPK site deficient Hid mutants induce apoptotic death more efficiently than does wild-type Hid.

(B) Comparison of the rescuing activity of $Ras1^{V12}$ on Hid^{wt}-, Hid^{Ala3}-, and Hid^{Ala5}-induced apoptosis in SL2 cells. Constant amounts of DNA of the Hid constructs were cotransfected with increasing amounts of $Ras1^{V12}$ as indicated. Hid^{wt}-induced apoptosis is efficiently blocked by $Ras1^{V12}$, such that the survival rate goes up to about 50%. In contrast, the rescuing activity of $Ras1^{V12}$ on Hid^{Ala3}- and Hid^{Ala5}-induced apoptosis is partially blocked. Only about 28%–30% of the cells survive. The amount of the Hid constructs (0.6 μg/ml Hid^{wt}; 0.4 μg/ml Hid^{Ala3} and Hid^{Ala5}) was determined by Figure 6A as the amount necessary to allow only 10% of SL2 cells to survive.

(C) Similar experiment to Figure 7B except that increasing amounts of RI^{Sem}/MAPK instead of $Ras1^{V12}$ were transfected with constant amounts of the Hid constructs (0.5 μg/ml each). The gof allele RI^{Sem}/MAPK behaves also in cultured cells as a suppressor of Hid^{wt}-induced apoptosis. Alteration of the MAPK sites in Hid completely abolishes the rescuing activity of RI^{Sem}/MAPK on Hid^{Ala3}- and Hid^{Ala5}-induced apoptosis.

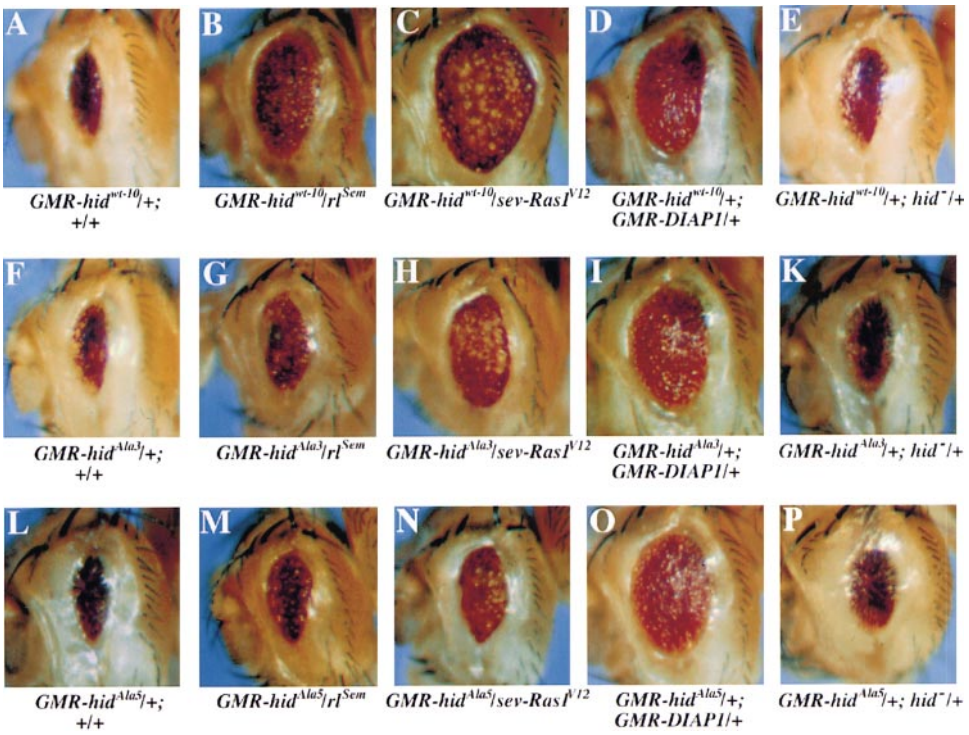

Figure 8. Transgenic Analysis of *GMR-hid*^{Ala3} and *GMR-hid*^{Ala5}

The unmodified eye phenotypes of *GMR-hid*$^{wt-10}$ (A), *GMR-hid*^{Ala3} (F), and *GMR-hid*^{Ala5} (L) are of similar strength allowing direct comparison of the rescuing abilities of *rl*^{Sem}/MAPK (B, G, and M), *sev-Ras1*^{V12} (line CR2; C, H, and N), *GMR-DIAP1* (D, I, and O), and *hid*$^{-}$ (E, K, and P). Note the block in the ability to suppress the eye ablation phenotype caused by the MAPK deficient *GMR-hid* transgenes by *rl*^{Sem}/MAPK (G and M) and *sev-Ras1*^{V12} (H and N), whereas expression of the cell death inhibitor DIAP1 suppresses *hid*-induced apoptosis independently of the MAPK phosphorylation sites (D, I, and O). The weak suppression observed for *GMR-hid*^{Ala3} and *GMR-hid*^{Ala5} by *rl*^{Sem}/MAPK (G and M) might be the result of inhibiting endogenous wild-type *hid* protein, since removal of one genomic copy of *hid* results in a weak suppression, too (K and P; see text for explanations). The genotypes of flies shown are indicated below each panel. The *hid* allele used in (E), (K), and (P) is *hid*$^{WR+X1}$.

5-fold increase in the Hid^{wt} experiment (Figure 7B). The weak rescue seen in this experiment might be the result of activation of the PI3-K/Akt-kinase effector branch (see Figure 3). However, alteration of the MAPK sites of Hid completely abrogates the survival ability of RI^{Sem}/MAPK on Hid-induced cell death in this assay (Figure 7C). The mutants Hid^{Ala3} and Hid^{Ala5} appear insensitive to MAPK signaling. These results indicate that changing the Ser/Thr residues in the MAPK sites of Hid is sufficient to render the *hid* protein insensitive to RI/MAPK signaling under these assay conditions and is consistent with our assumption that the MAPK sites are critical for the observed survival activity of Ras1/MAPK signaling on *hid*-induced apoptosis.

Transgenic Analysis of *GMR-hid*^{Ala3} and *GMR-hid*^{Ala5}

In order to study the *hid*^{Ala3} and *hid*^{Ala5} mutants in vivo we generated GMR based constructs, designated *GMR-hid*^{Ala3} and *GMR-hid*^{Ala5}, and established transgenic lines using P element mediated transformation. The genetic analysis performed with *GMR-hid*^{Ala3} and *GMR-hid*^{Ala5} is consistent with the findings in SL2 cells and strongly supports a model according to which the activity of the cell death regulator *hid* is modulated by MAPK signaling in vivo.

In total, six *GMR-hid*^{Ala3} and four *GMR-hid*^{Ala5} transgenic lines were obtained. While the strength of the eye ablation phenotype caused by *GMR-hid*^{wt} ranges from mild to severe defects (compare *GMR-hid*$^{wt-1M}$ with *GMR-hid*$^{wt-10}$ in Figure 2), all of the *GMR-hid*^{Ala3} and *GMR-hid*^{Ala5} lines produce a severe eye ablation phenotype (Figure 8), with some lines completely lacking eye structures (data not shown). These strong phenotypes are presumably caused by a failure of Ras1/MAPK signaling to suppress the MAPK deficient *GMR-hid* mutants, since Ras1 signaling plays an essential role during eye development (reviewed in Freeman, 1997).

To further confirm this notion, we analyzed the effect of the gof *rl*^{Sem}/MAPK allele on *GMR-hid*^{Ala3} and *GMR-hid*^{Ala5}. Based on the results obtained in SL2 cells (see Figure 7) we did not expect to detect a suppression of the *GMR-hid*^{Ala3}- and *GMR-hid*^{Ala5}-induced eye phenotype by activated forms of MAPK. Several lines were tested and gave identical results. Flies expressing *GMR-hid*^{Ala3} and *GMR-hid*^{Ala5} in a *rl*^{Sem}/MAPK mutant background show a mildly suppressed eye phenotype (Figures 8G and 8M). The extent of this suppression is much weaker compared to the one obtained for *GMR-hid*$^{wt-10}$ (Figure 8B). Also, the *sev-Ras1*^{V12} transgenes largely fail to suppress the eye ablation phenotype caused by the MAPK site deficient *GMR-hid* transformants (Figures 8H and 8N). This result indicates that MAPK signaling inactivates the cell death inducing activity of *hid*. However, the *GMR-hid*^{Ala3}- and *GMR-hid*^{Ala5}-induced eye phenotypes are still partially suppressed by activated forms of MAPK. This partial suppression might be caused by

inactivation of the endogenous *hid* wild-type protein that is provided by the two genomic copies, which are widely expressed in the developing eye (Grether, 1994). Reduction of the endogenous *hid* gene dose by 50% (i.e., a heterozygous *hid* mutant background) resulted in weak suppression of the *GMR-hid^{wt}*, *GMR-hid^{Ala3}*, and *GMR-hid^{Ala5}* eye phenotypes (Figures 8E, 8K, and 8P). Thus, the endogenous *hid* gene adds to the full *GMR-hid*-induced eye phenotype. Since its gene product is expected to be fully responsive to Ras1/MAPK signaling, it is likely that the weak suppression of *GMR-hid^{Ala3}* and *GMR-hid^{Ala5}* by activated forms of MAPK is caused by inactivation of the endogenous *hid* gene.

In control crosses, we tested transgenes of and mutations in specific cell death genes that are not involved in Ras1/MAPK signaling. The well-characterized gene *diap1* (*Drosophila* inhibitor of apoptosis protein) under GMR promoter control (*GMR-DIAP1*, Hay et al., 1995) suppressed the eye ablation phenotypes caused by *GMR-hid^{wt}*, *GMR-hid^{Ala3}*, and *GMR-hid^{Ala5}* to a similar extent (Figures 8D, 8I, and 8O). Other control crosses included *GMR-p35*, a general inhibitor of apoptosis (Clem et al., 1991; Hay et al., 1994), dominant suppressors recovered in the *GMR-hid* suppressor screen, and mutations in *glass*, which encodes a transcription factor that activates transcription from the GMR promoter and is expected to influence GMR transgenes similarly. As with *GMR-DIAP1*, in these crosses, the eye ablation phenotypes caused by *GMR-hid^{wt}*, *GMR-hid^{Ala3}*, and *GMR-hid^{Ala5}* are suppressed to a similar extent (data not shown). The findings in these control crosses further support the notion that the failure to suppress *GMR-hid^{Ala3}* and *GMR-hid^{Ala5}* by active MAPK is due to the lack of MAPK phospho-acceptor sites in Hid.

In summary, our mutational analysis provides strong evidence that the MAPK phosphorylation consensus sites of Hid are critical for the observed survival activity of the Ras1/MAPK pathway on Hid-induced apoptosis. Thus, it appears that active MAPK suppresses Hid by direct phosphorylation.

Discussion

Signaling via RTKs and the Ras/Raf/MAPK pathway has been implicated in the suppression of programmed cell death (reviewed in Downward, 1998). Recently, genetic evidence for the antiapoptotic function of the EGFR/Ras1/MAPK pathway in *Drosophila* has been provided (Miller and Cagan, 1998; Sawamoto et al., 1998). In this paper we reveal a molecular mechanism by which the EGFR/Ras1/MAPK pathway delivers an antiapoptotic signal via direct inhibition of a component of the intrinsic cell death machinery in *Drosophila*, *hid*.

hid is an important inducer of programmed cell death in *Drosophila* (Grether et al., 1995). *hid* mutant embryos have decreased levels of apoptosis and extra cells in the head. In ectopic expression studies, *hid* behaves as a very potent inducer of cell death (Grether et al., 1995; this study). However, the expression of the *hid* gene is not restricted to cells that are doomed to die; *hid* is also expressed in many cells that live (Grether et al., 1995). This observation, together with the efficient induction of apoptosis by *hid* in our assays, indicates that there must be very efficient posttranslational mechanisms present in these cells to protect them from *hid*-induced apoptosis.

Activation of the EGFR/Ras1/MAPK Pathway Delivers an Antiapoptotic Signal by Inhibition of *hid* Activity

In this paper we present genetic evidence that signaling via the EGFR/Ras1/MAPK pathway promotes survival by directly blocking *hid* from inducing apoptosis (Figure 9). It is striking that the survival activity of the EGFR/Ras1/MAPK pathway is specific for Hid, since we detected little or no effects on the other two known cell death regulators in *Drosophila*, *reaper* and *grim* (Figure 9). In our assays, *hid*-induced apoptosis, using either *GMR-hid* or *hs-hid*, is very efficiently blocked by mutations in genes that lead to overactivation of the EGFR/Ras1/MAPK pathway. This includes lof mutations in genes, which negatively regulate the pathway, such as *gap1*, *argos*, and *sprouty* (Figure 1), as well as gof mutations in components of the EGFR/Ras1/MAPK pathway such as *Elp*, *Ras1^{V12}*, *Raf^{torso}*, and *rl^{Sem}*/MAPK (Figure 2). In order to explain the observed genetic effects on *hid*-induced apoptosis by EGFR/Ras1/MAPK signaling, we propose a posttranslational inactivation mechanism of *hid* protein for three reasons. First, in our assays, *hid* is under control of heterologous promoters (GMR, hs, and ie1) such that a potential regulation at the level of the endogenous *hid* promoter cannot fully account for the observed genetic effects. However, such a mechanism might operate under different circumstances (see below). Second, since similar rescuing effects are not observed with *GMR-reaper* and *GMR-grim*, a transcriptional regulation at the level of the GMR promoter (and presumably the hs promoter) can be excluded as well. Third, there are five MAPK phosphorylation consensus sites present in the *hid* protein (Grether et al., 1995). In contrast, neither *reaper* nor *grim* proteins possess MAPK phosphorylation consensus sites (White et al., 1994; Chen et al., 1996). Our mutational analysis strongly suggests that the MAPK phosphorylation sites of Hid are critical for the response to EGFR/Ras1/MAPK signaling in both transgenic animals and cultured cells.

The EGFR/Ras1/MAPK signaling pathway has been implicated in controlling cell proliferation, cell differentiation, and cell death in both vertebrates and invertebrates (Wassarman and Therrien, 1997; Downward, 1998). Recently, Karim and Rubin (1998) showed that ectopic expression of activated Ras1 (Ras1^{V12}) during early imaginal disc development in *Drosophila* (during a stage when the imaginal discs are actively proliferating) induces ectopic cell proliferation and hyperplastic growth. Therefore, we were concerned that the observed survival activity of EGFR/Ras1/MAPK signaling on *hid*-induced apoptosis might actually be the result of a compensatory mitogenic effect of Ras1^{V12} rather than the result of a direct inhibition of the apoptosis inducing activity of *hid*. However, we excluded this possiblity for a number of reasons. First, a mitogenic response to the *sev-Ras1^{V12}* transgenes used in this study has not been observed, presumably because the cells in which

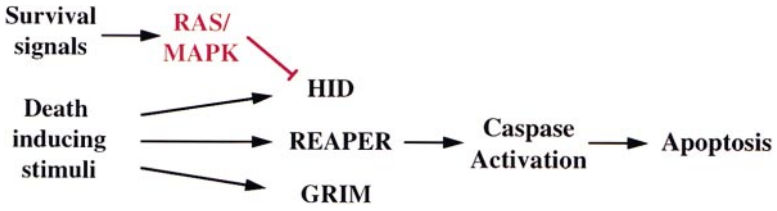

Figure 9. Specific Inhibition of Hid-Induced Apoptosis by Ras1/MAPK-Dependent Survival Pathways

The apoptotic inducers *hid*, *reaper*, and *grim* link the apoptotic program with many different death-inducing stimuli in *Drosophila*; these include cell lineage, cell–cell interactions, ecdysone, block of cell differentiation, deprivation of trophic factors, and radiation. Induction of apoptosis proceeds via a conserved caspase pathway. However, only the activity of *hid* is specifically inhibited by survival factors, which activate the Ras1/MAPK pathway.

$Ras1^{V12}$ is expressed are postmitotic (Karim and Rubin, 1998). Second, apoptosis induced by MAPK deficient mutants of *hid* is less well suppressed by Ras1 signaling. Third, if a mitogenic effect of the activated EGFR/Ras1/MAPK pathway accounts for the strong suppression of *GMR-hid*, then a similar effect should be detectable on *GMR-reaper*- and *GMR-grim*-induced apoptosis. Finally, in control transfection experiments in SL2 cells, a potential mitogenic effect of $Ras1^{V12}$ did not compensate for *hid*-induced cell death (data not shown). Thus, we conclude that the suppression of *hid*-induced apoptosis is the result of direct inhibition of the cell death–inducing activity of *hid* by the activated EGFR/Ras1/MAPK pathway.

We propose a model according to which the cell death–inducing activity of the Hid protein is specifically inactivated via phosphorylation of the *hid* protein by activated MAPK. The mutational analysis performed in transgenic animals and SL2 cells strongly supports such a mechanism. Unfortunately, in vitro kinase assays using recombinant Hid as a substrate have been hampered by its insolubility (unpublished data).

Our data show that phosphorylation of Hid by activated MAPK inhibits the cell killing activity of Hid, and we believe that this powerful control mechanism operates in most cells at all times (we tested different *Drosophila* tissues, developmental stages, and cultured cells). However, *hid* activity also appears to be regulated by the Ras1 pathway at the transcriptional level. Overexpression of $Ras1^{V12}$ during embryogenesis results in specific downregulation of *hid* mRNA expression (Kurada and White, 1998 [this issue of *Cell*]). Expression of *reaper*, *grim*, and *diap1* are not affected by this treatment. Thus, these findings suggest a regulation of *hid* activity by the Ras1 pathway at two different levels. We propose that in an acute response, *hid* protein is directly inactivated by phosphorylation via activated MAPK. This mechanism explains why the large number of cells that express *hid* are able to survive. In a second response, *hid* mRNA expression is downregulated by the Ras1 pathway (Kurada and White, 1998). In this way, the Ras1 pathway ensures that a cell that is selected to live will both inhibit existing *hid* protein pools and subsequently downregulate the transcription of *hid* and, thus, gain a safe distance from the "threshold of death." Taken together, both mechanisms can explain how cells that receive sufficient survival signals are stably selected to live.

Ras1-Dependent Inactivation of *hid*: Implications for Oncogenesis

Recent evidence suggests that the failure of cells to undergo apoptotic cell death might be involved in the pathogenesis of a variety of human diseases, including cancer, autoimmune diseases, and viral infections (reviewed in Thompson, 1995). Mutational activation of *ras* oncogenes is associated with about 30% of all human tumors (Bos, 1989). An open question in cancer research is why transformation usually requires two or more cooperative oncogenic events to induce a neoplastic lesion. Harmful cells that have acquired genetic alterations that predispose them to uncontrolled cell proliferation are usually detected by the organism and subsequently removed by apoptosis. Thus, apoptosis appears to be a critical process that protects the organism from harmful cells. However, tumor cells as well as metastatic cells have a decreased ability to undergo apoptosis (Hoffman and Liebermann, 1994). The findings in this paper define a potential mechanism by which activated *ras* oncogenes decrease the susceptibility of cells to die by inactivation of a critical component of the intrinsic cell death machinery.

Although there are no mammalian homologs of *Drosophila hid* known to date, genetic and biochemical studies on *hid* might provide an important paradigm for the antiapoptotic function of *ras* oncogenes. In preliminary experiments, we found that *hid* kills mammalian cells very efficiently (N. Haining, A. B., and H. S., unpublished data), indicating that the mechanisms leading to *hid*-induced apoptosis might be conserved between vertebrates and invertebrates. Even if there is no *hid* homolog at the structural level, we propose that in mammals other cell death–inducing gene(s) exist that act as functional homologs to *hid* and that are similarly responsive to *ras*-induced inactivation. Thus, studies performed in *Drosophila* might provide new insights into the function of *ras* as an oncogene.

Experimental Procedures

Fly Stocks

The following mutant and transgenic fly strains were used for phenotypical analysis and genetic interactions: *GMR-hid*, *hs-hid* 3, hid^{WR+X1} (Grether et al., 1995), $GMR\text{-}hid^{10}$ *sev-GAL4* (this study), *GMR-rpr* (White et al., 1996), *GMR-grim* (Chen et al., 1996), *GMR-DIAP1* 3–1 (Hay et al., 1995), $gap1^{21\text{-}1s}$ and $spry^{28\text{-}4s}$ (this study) $arg^{l\Delta7}$ (Freeman et al., 1992), $EGFR^{-} = flb^{f2}$ (Nüsslein-Volhard et al., 1984), ras^{DC40b} and $raf^{l1\text{–}29}$ (Hou et al., 1995), rl^{10a} (Peverali et al., 1996), Elp^{E1} (Baker and Rubin, 1989), $sev\text{-}Ras1^{V12}$ (Fortini et al., 1992), $sev\text{-}Ras1^{N17}$ (Karim et al., 1996), $sev\text{-}Raf^{torso}$ (Dickson et al., 1992), rl^{Sem}/MAPK (Brunner et al., 1994a), $UAS\text{-}Ras1^{+}$, $UAS\text{-}Ras1^{V12S35}$, $UAS\text{-}Ras1^{V12G37}$, $UAS\text{-}Ras1^{V12C40}$ (Karim and Rubin, 1998). Flies carrying $GMR\text{-}hid^{Ala3}$ and $GMR\text{-}hid^{Ala5}$ were generated by P element–mediated transformation. The $GMR\text{-}hid^{10}$ *sev-GAL4* line was obtained by meiotic recombination. All crosses were performed at 25°C.

hs-hid Suppression Assay

Offspring of crosses between *hs-hid*/TM3 and Sup/TM3 (Sup = $gap1^{21\text{-}1s}$, $spry^{28\text{-}4s}$, $arg^{l\Delta7}$) were heat shocked at 37°C during first instar

larval stage for 10, 20, 30, 40, 50, or 60 min. After recovery, the crosses were incubated at 25°C. After the flies eclosed, the ratio between *hs-hid*/Sup and Sup/TM3 animals was determined. For rl^{Sem}/MAPK, the procedure was similar except that the ratio between rl^{Sem}/+;*hshid*/+ and rl^{Sem}/+;+/TM3 was scored. In the control experiment, offspring of a cross between *hs-hid*/TM3 and +/+ animals were treated as described above, and the ratio between *hs-hid*/+ and +/TM3 animals was determined. The results presented in Figure 4 represent the average of three independently performed experiments.

Molecular Biology

In vitro mutagenesis of the MAPK phosphorylation consensus sites in *hid* was performed using PCR with specifically designed primers. Incorporation of the mutation was confirmed by sequencing. The constructs were subcloned into pGMR1 (Hay et al., 1994) for P element–mediated transformation and pIE1-3 (Novagen, Jarvis et al., 1996) for SL2 cell transfection experiments. The cDNAs encoding $Ras1^{V12}$ and rl^{Sem}/MAPK were cloned into pIE1-3 using convenient restriction sites for cell transfections.

SL2 Cell Transient Transfection Experiments

SL2 cells (Schneider, 1972) were grown in Schneider's *Drosophila* Medium (GIBCO–BRL) supplemented with 10% NCS. In each experiment, 100 ng/ml of the reporter plasmid pIE1-3-LacZ (kindly provided by Zhiwei Song) was transfected. Differences in the amount of tester plasmids were compensated for by the addition of empty vector pIE1-3. In three independent experiments, transfections were performed using the Cellfectin reagent according to the manufacturer's instructions (GIBCO–BRL) for 5 hr in serum-free medium in 24 well dishes in quadruplicates. Twenty four hours after transfection, cells were fixed and stained, and the number of surviving cells was determined.

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