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sickle, a Novel *Drosophila* Death Gene in the *reaper/hid/grim* Region, Encodes an IAP-Inhibitory Protein

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Summary

Inhibitors of apoptosis proteins (IAPs) interact with caspases and inhibit their protease activity, whereas the IAP-inhibitory proteins Smac/DIABLO in mammals and Reaper, Hid, and Grim in flies relieve IAP-mediated inhibition [1-5] to induce cell death. Here we describe the functional characterization of the novel *Drosophila* cell death protein Sickle (Skl), which binds to IAPs and neutralizes their apoptotic inhibitory activity. Skl exhibits no sequence homology to Reaper, Hid, Grim, or Smac/DIABLO, except within the 4 residue N-terminal IAP binding motif. Skl interacts with *Drosophila* and mammalian IAPs and can promote caspase activation in the presence of IAPs. Consistent with these findings, expression of Skl in *Drosophila* and mammalian cell lines or in *Drosophila* embryos induces apoptosis. Skl can also synergize with Grim to induce cell death in the *Drosophila* eye imaginal disc. Based on biochemical and structural data, the N terminus of Skl, like that of the mammalian Smac/ DIABLO, is absolutely required for its apoptotic and caspase-promoting activities and its ability to interact with IAPs. These findings point to conservation in the structure and function of the IAP-inhibitory proteins across species and suggest the existence of other family members.

Results and Discussion

Identification and Cloning of *Drosophila skl*

Proteins that relieve the IAP-mediated inhibition of caspases, known as IAP-inhibitory proteins, include Reaper, Hid, and Grim in *Drosophila* [6-8] and Smac/DIABLO in mammalian cells [9,10]. Recently, the human serine protease Omi/Htra2 was also identified as an IAP-inhibitory protein [11-14]. These proteins bind, via a conserved N-terminal IAP binding motif (IBM), to the same BIR domains of IAPs that inhibit caspases, suggesting that they relieve the caspase-inhibitory activity of IAPs by disrupting the caspase-IAP interaction [5,15,16].

To identify additional IAP-inhibitory proteins, we searched the entire public GenBank database for genes that encode proteins with a conserved IBM at their N termini. This resulted in identification of a novel *Drosophila* gene of unknown function mapping near the *reaper/grim/*

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Accession Numbers The *sickle* (*skl*) GenBank accession number is AY062250.

Supplementary Material
Supplementary Material containing Experimental Procedures is available at http://images.cellpress.com/supmat/supmatin.htm.

hid region of chromosome 3L (Figure 1B). This gene consists of one exon and encodes a 108 amino acid protein (Figure 1A). Based on the ability of this protein to induce cell death and to synergize with Grim, it was named Skl (see below). Sequence analysis of Skl revealed that its N-terminal 4 residues share significant homology with the N-terminal IBM of the *Drosophila* proteins Hid/Grim/Reaper and mature mammalian Smac/DIABLO and caspase-9-p12 (Figure 1C). Intriguingly, Skl has an overall acidic pI (pI ~4.5), which is different from those of Reaper, Hid, or Grim but very similar to that of Smac/DIABLO.

Expression of Skl during *Drosophila* Development

As a first step in determining the function of *skl* in *Drosophila*, we examined the expression of *skl* mRNA by RT-PCR analysis at different stages of *Drosophila* embryonic development. This analysis revealed that *skl* mRNA is developmentally regulated. It is initially present in small amounts in 4–14 hr embryos and in larger amounts in 14–21 hr embryos (Figure 1D). No *skl* mRNA was detected in adult male or female flies. Interestingly this temporal expression profile is similar to that of Reaper, suggesting that the two genes are under similar developmental regulation. It is noteworthy that most developmental programmed cell death in *Drosophila* occurs during this period of embryonic development [3], suggesting that Skl could play an important role in this process.

We examined both the mRNA and protein expression patterns in whole *Drosophila* embryos. As Figure 1E shows, expression in embryos is localized to specific regions of the head and to specific cells of the central nervous system (CNS). The CNS expression pattern is highly reminiscent of those of the cell death inducers Reaper [6] and Grim [8], while dorsal head staining may foreshadow that of Hid in the optic lobe region [7]. The similarity of the Skl expression pattern to those of the known cell death inducers (Reaper and Grim) suggests that it may act in concert with these proteins to orchestrate the complex pattern of apoptosis during development, particularly in the CNS.

Skl Binds and Inhibits IAP Function

The significant homology in the IBM among Skl, Reaper, Hid, and Grim, as well as the close proximity of their genes on chromosome 3L, suggests that Skl, like Reaper/Hid/Grim, might play an important role in developmental cell death in *Drosophila* by binding and inhibiting the activity of IAPs. To test this hypothesis, we analyzed the interactions of GST-tagged Skl, Grim, and Smac/DIABLO with *Drosophila* DIAP1 or DIAP2 or with their isolated BIR domains in vitro. As shown in Figure 2A, Skl, like Grim and Smac/DIABLO, was able to interact strongly and specifically with either of the full-length proteins or with the BIR2 of DIAP1 or BIR3 of DIAP2. However, unlike Grim or Smac/DIABLO, Skl was also able to interact with the BIR1 domain of DIAP1. Subsequent analysis of the interactions of N-terminal truncated Skl mutants with *Drosophila* DIAP1 or DIAP2 or with human XIAP revealed that deletion of the first 10 N-terminal residues of Skl (ΔN10), which contain the IBM, or mutation of the first Ala of the IBM to Met (A2M) completely prevented the interactions of Skl with these IAPs (Figure 2B). The above data indicate that Skl is a bona fide IAP binding protein and that its interactions with IAPs are mediated by its N-terminal IBM.

The ability of Skl to bind to IAPs suggests that it could neutralize the caspase-inhibitory activity of IAPs by binding to free IAPs or by disrupting already formed IAP-caspase complexes. Therefore, we tested the ability of purified Skl protein to inhibit the activity of IAPs in an in vitro caspase activation system. Addition of Skl to a reaction containing DIAP1 and the *Drosophila* caspase DCP-1 resulted in almost complete inhibition of DIAP1, as measured by the restoration of DCP-1 activity (Figure 2C, compare the reduction of caspase activity by DIAP1 alone to that of DIAP1 + Skl). The ability to counteract DIAP1 was completely dependent on the IBM of Skl, since deletion (ΔN10) or point mutation of the first Ala (A2M)

of this motif abolished the caspase-promoting activity (DIAP1 inhibition of DCP-1 is unaffected by these Skl mutants). Skl was also able to promote activation of human caspases in XIAP-containing 293 cellular S100 extracts almost to the same extent as Smac/DIABLO (Figure 2D). This activity was also dependent on the IBM of Skl, since deletion or mutation of this motif abolished its caspase-promoting activity. These observations suggest that the activity of Skl is evolutionarily conserved and that Skl, like Smac/DIABLO, is able to disrupt the interaction of caspases with IAPs to promote caspase activation.

Skl Induces Apoptosis in Cultured Cells and in *Drosophila*

The *Drosophila* Reaper, Hid, and Grim proteins are known to induce apoptosis in mammalian cells [17,18]. To determine the apoptotic activity of Skl compared to that of Reaper and Grim in mammalian cells, we transiently transfected human MCF-7 cells with C-terminal GFP-tagged Skl, Reaper, or Grim or the ΔN10 Skl mutant. As expected, WT Skl but not the ΔN10 mutant was able to induce apoptosis in MCF-7 cells to the same extent as Reaper and Grim (Figure 3A). This result indicates that Skl can induce apoptosis in mammalian cells, and that this activity is dependent on its ability to bind to IAPs. Like its activity in mammalian cells, Skl was also able to induce apoptosis in *Drosophila* S2 cells to a similar extent as Reaper (Figure 3B). This apoptosis was inhibited by the baculovirus caspase inhibitor p35, confirming that the apoptotic activity of Skl is caspase dependent.

We tested the ability of Skl to induce apoptosis in *Drosophila* embryos. This was achieved by ectopically expressing the *skl* coding region using a heat-inducible transgene. As shown in Figures 3C and 3D, many embryonic cells were induced to undergo apoptosis following *skl* expression, as visualized by acridine orange staining [19]. Furthermore, we found that coexpression of Skl and Grim in the eye discs of transgenic flies results in a dramatic reduction of eye size (Figure 3E), indicating that the induction of ectopic cell death during eye development can be enhanced by coexpression of two IAP inhibitors. Taken together, the above data clearly demonstrate that Skl is an apoptotic protein, which exerts its activity by binding to IAPs and/or disrupting caspase-IAP complexes and that it can act together with other IAP inhibitors to produce a maximal apoptotic effect.

Structure of DIAP1-BIR2 Bound to a Skl N-Terminal Peptide

To further examine the interactions between DIAP1 and Skl, we crystallized the second BIR domain (BIR2) in complex with an N-terminal 10 residue peptide of Skl. The structure was determined at 2.1 Å resolution (Figure 4). The final atomic model of DIAP1-BIR2 contains residues 215–316, comprising six α helices, a three-stranded β sheet, and a zinc atom chelated by 3 Cys and 1 His residues (Cys263, Cys266, His283, and Cys290) (Figure 4A). This structure is nearly identical to that complexed with Hid or Grim peptide, with root-mean-square deviation (RMSD) of less than 0.2 Å for all aligned Cα atoms. Binding by the Skl peptide, however, differs from that by a Hid or Grim peptide (Figure 4A). In contrast to the conserved DIAP1 binding by 7 residues in the Grim or Hid peptide, only 4 N-terminal residues of the Skl peptide bind a shallow surface groove on DIAP1 (Figures 4A and 4B), consistent with limited sequence homology between Skl and the other three *Drosophila* death proteins.

Binding to the DIAP1-BIR2 domain results in the burial of 600 $Å^2$ of surface area for the Skl peptide (Figure 4B). The recognition involves both hydrogen bond networks and van der Waals contacts between 4 hydrophobic residues on the peptide and conserved DIAP1 residues (Figure 4C). Similar to the Smac/DIABLO-XIAP interactions, the N terminus of Skl is positioned in an acidic environment (Figure 4C), in which 3 charged residues (Asp277, Gln282, and Glu314) in DIAP1 play an essential role in binding the peptide. The amino group of Ala1 donates two hydrogen bonds to the surrounding residues Asp277 and Gln282, while the carbonyl group accepts two hydrogen bonds from the side chains of Trp286 and Glu314 (Figure 4C). These

central interactions are buttressed by additional hydrogen bonds and hydrophobic contacts (Figure 4C).

In conclusion, we have identified and characterized a novel *Drosophila* cell death protein named Skl. Skl belongs to the growing family of IAP binding proteins and exerts its activity through a highly conserved mechanism of interaction of its N terminus with a surface groove on the BIR domain of IAPs. This interaction results in disruption of the caspase-IAP interaction, culminating in induction of apoptosis. The restricted expression of Skl at early stages of *Drosophila* development and its ability to induce apoptosis in transgenic *Drosophila* embryos suggest that Skl might be an important regulator of programmed cell death during *Drosophila* development. The observed synergistic effect of coexpression of Skl and Grim in the eye imaginal disc of transgenic *Drosophila* suggests that Skl may function together with the other *Drosophila* IAP-inhibitory proteins to regulate apoptosis during development. In the absence of Reaper, Hid, and Grim, physiological levels of Skl alone may not be sufficient to bind all IAPs and induce cell death during *Drosophila* development in most tissues. This could explain the reported lack of developmental cell death in the *Drosophila* H99 deletion mutant, which removes the *reaper*, *hid*, and *grim* genes but not the *skl* gene ([6] and data not shown). Further, some cell death is unaffected by the H99 deficiency [20], and *skl* function may be sufficient for apoptosis in such cases.

Results related to those presented here have been obtained by two other groups and are reported in accompanying papers (see Christich et al. [21] and Wing et al. [22] in this issue of *Current Biology*).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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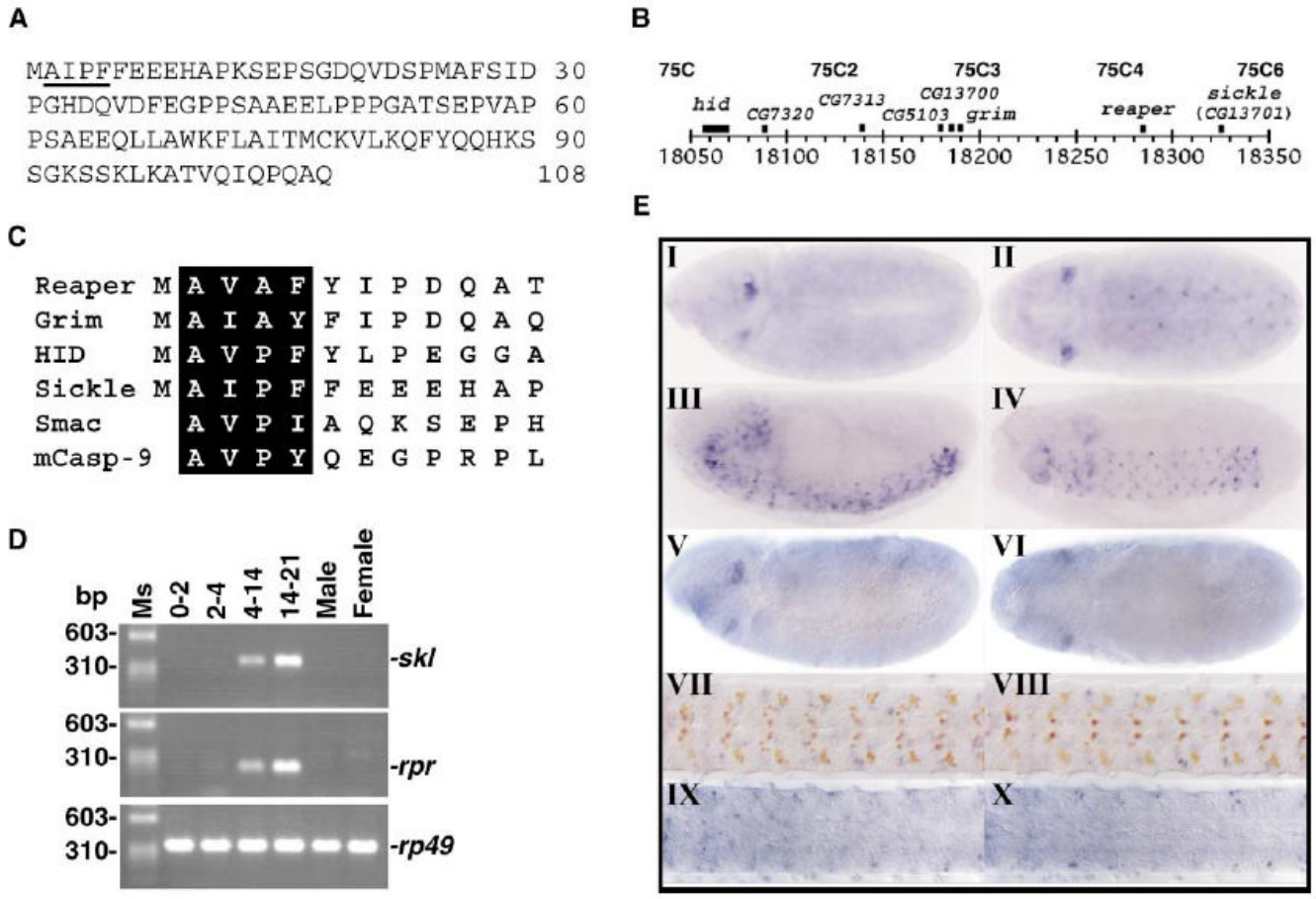

Figure 1. Sequence and Expression of Skl in *Drosophila*

(A) Predicted amino acid sequence of *Drosophila* Skl. The 4 residue N-terminal IAP binding motif (AIPF) is underlined.

(B) Map of *hid-grim-reaper-skl* genomic region. Locations of genes in polytene chromosome band 75C. Nucleotide and gene numbering are according to the Berkeley *Drosophila* Genome Project.

(C) Colinear alignment of the N-terminal sequences of *Drosophila* Skl, Reaper, Grim, and Hid and mouse caspase-9-p12 and Smac/DIABLO. The IAP binding motif is highlighted.

(D) RT-PCR analysis of *skl* mRNA expression in *Drosophila*. poly(A)$^+$ RNA samples from different stages of *Drosophila* embryonic development and from adult male and female flies were amplified by RT-PCR with primers specific for *skl* and *reaper* and then analyzed on an agarose gel and stained with ethidium bromide. The expression of RP49 was used as an internal control. Ms, molecular weight markers.

(E) Expression of *skl* mRNA and protein in *Drosophila* embryos. In (EI)–(EIV), (EVII), and (EVIII), in situ hybridization to *skl* mRNA (blue), and, in (EVII)–(EVIII), even-skipped protein staining (orange) as a spatial reference. (EV, EVI, EIX, and EX) Immunostaining with Skl antiserum. Anterior is to the left in all panels. (EI) *skl* mRNA is expressed strongly in the head region at stage 10 (lateral view). (EII) *skl* is also expressed near the midline at stage 10 (dorsal view). (EIII) *skl* mRNA is expressed in brain and CNS at stage 15 (lateral view). (EIV) Ventral view of stage 15 embryo. (EV and EVI) Expression pattern of Skl protein at stage 10, lateral and dorsal view, respectively. (EVII and EVIII) Dissected CNS from stage 15 embryos, in different focal planes. (EIX and EX) Skl protein expression in CNS at stage 15, in different focal planes.

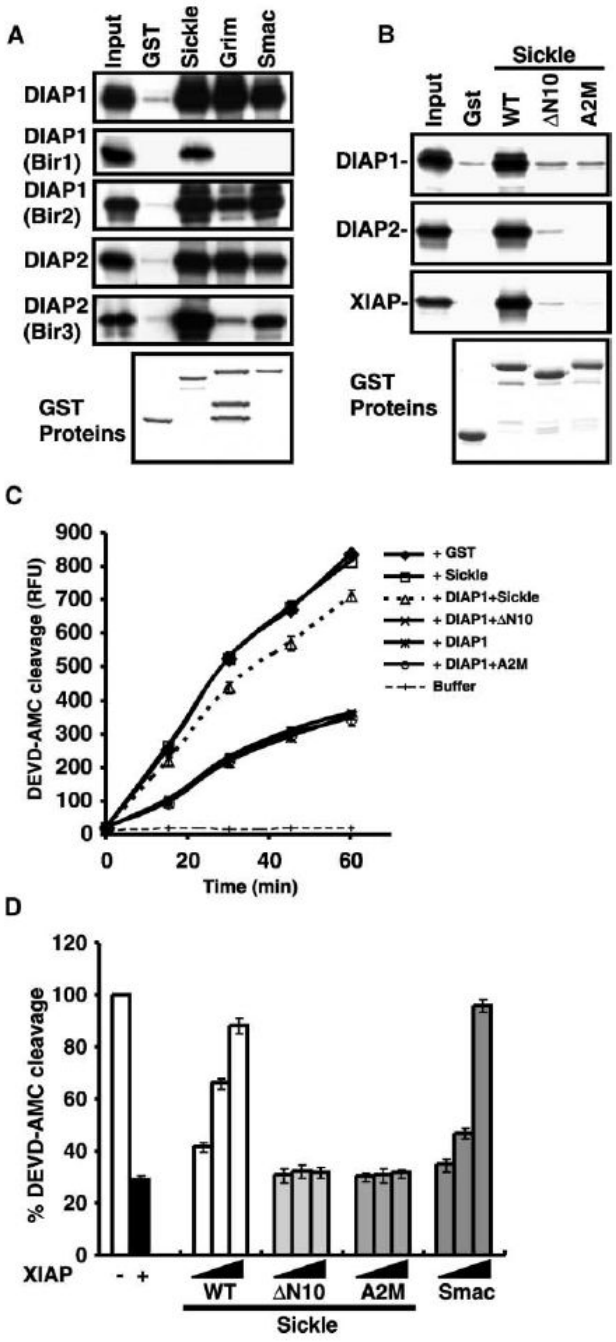

Figure 2. Skl Binds IAPs and Inhibits IAP Activity In Vitro

(A) Interaction of Skl with DIAP1 and DIAP2 and their isolated BIR domains. C-terminal GST-tagged Skl, Grim, or Smac/DIABLO or GST protein was immobilized onto glutathione-Sepharose resin. The bound resins were incubated with in vitro-translated ^{35}S-labeled DIAP1, DIAP2, or their isolated BIR domains, washed extensively, and then analyzed by SDS polyacrylamide gel electrophoresis and autoradiography. GST was used as a negative control (second lane). (Lower panel) Coomassie-stained gel of the immobilized GST-fusion proteins. (B) Interaction of Skl and N-terminal truncated Skl mutants with *Drosophila* DIAP-1 or DIAP2 or human XIAP. C-terminal GST-tagged WT Skl or ΔN10 or A2M mutants were incubated with ^{35}S-labeled DIAP1, DIAP2, or human XIAP and then analyzed as in (A).

(C) Skl neutralizes the inhibitory activity of DIAP1. Purified *Drosophila* DCP-1 (0.2 nM) was mixed with DIAP1 (0.25 μM), and the mixtures were then incubated with purified Skl, Skl-ΔN10, or Skl-A2M (0.5 μM). The reactions were carried out in the presence of the peptide substrate DEVD-AMC (50 μM) for the indicated times, and substrate cleavage was measured by luminescence spectrometry. Caspase activity in all samples is represented as relative fluorescence units (RFU). Note that the activities of Skl and GST alone (controls) are indistinguishable, as are those of DIAP1 alone and DIAP1 with the Skl mutants.
(D) Skl neutralizes the inhibitory activity of human XIAP. 293T S100 extracts were mixed with purified XIAP (20 nM) and then stimulated with cytochrome c plus dATP in the presence of increasing amounts of Smac/DIABLO, Skl, Skl ΔN10, or Skl-A2M (50, 100, and 500 nM). The reactions are carried out in the presence of DEVD-AMC as a substrate, and the caspase activities were represented as percent DEVD-AMC cleavage.

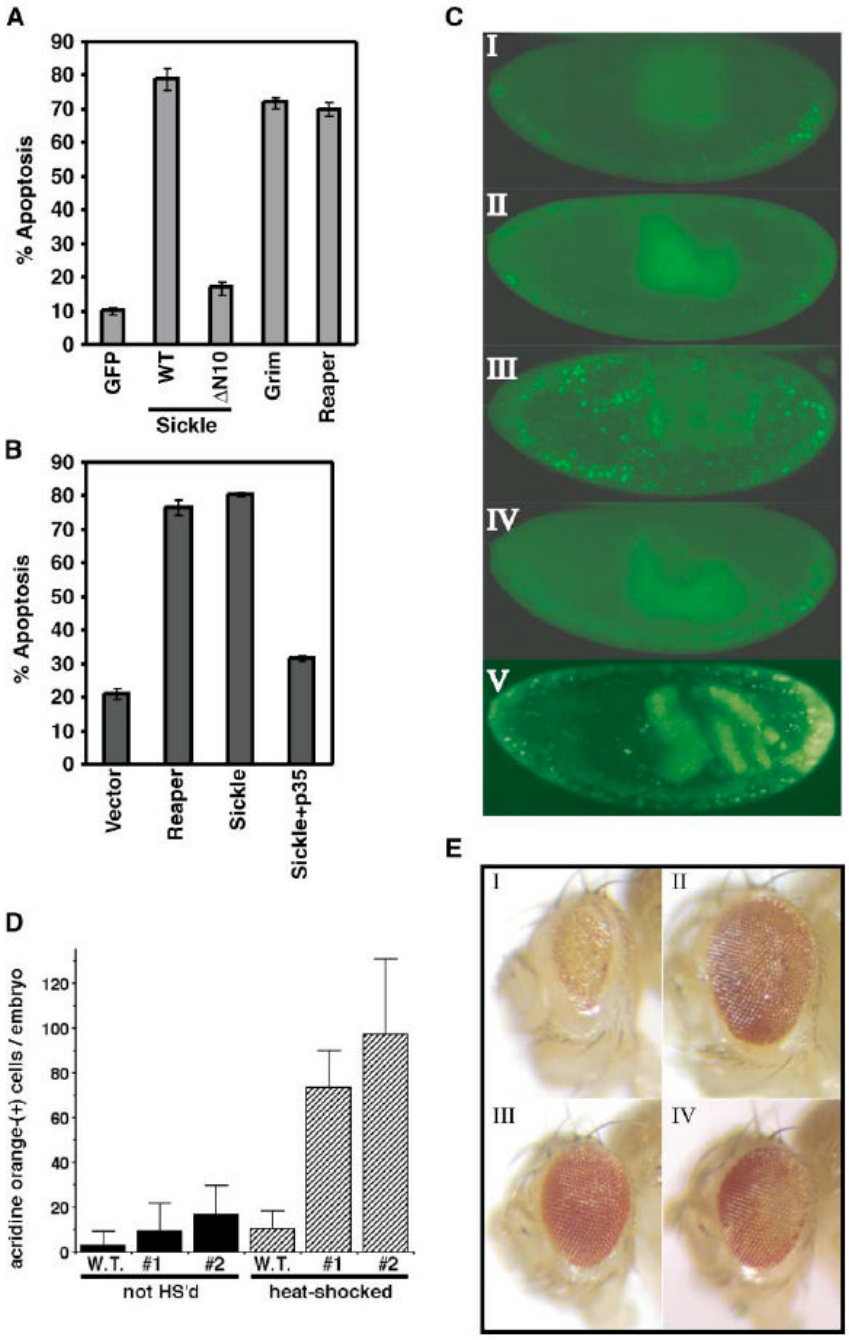

Figure 3. Skl Is an Apoptotic Protein

(A and B) Ectopic expression of Skl induces apoptosis in human MCF-7 and *Drosophila* S2 cells. MCF-7 or S2 cells were transfected with the indicated constructs and then assayed for apoptosis as described in Supplementary Material.

(C) Ectopically expressed Skl can cause cell death. Transgenic flies were generated carrying the *skl* coding region under the control of a heat shock (hs) promoter. Embryos were collected and stained with acridine orange, as described in Supplementary Material. (CI) Control heat-shocked wild-type (transgenic recipient strain); (CII and CIV) two independent transgenic lines, not heat shocked; (CIII and CV) the same two lines, heat shocked to induce Skl expression, as described in Supplementary Materials.

(D) Embryos (20) from each of the above lines, as well as from the recipient strain without heat shock, were selected at random, and the number of strongly staining cells was counted. The graph shows the average and standard deviation for each population.
(E) Skl and Grim synergize to induce cell death in the eye imaginal disc. (EI) Grim and Skl together: *GMR-grim/GMR-Gal4; UAS-skl/UAS-skl*. (EII) Grim alone: *GMR-grim/GMR-Gal4*; +/+. (EIII) Skl alone: *CyO/GMR-Gal4; UAS-skl/UAS-skl*. (EIV) Grim alone (*UAS-skl* inactive): *GMR-grim/CyO; UAS-skl/UAS-skl*. The genotypes refer to P element transgenes, except the *CyO* balancer chromosome. GMR is an eye disc-specific promoter, while UAS responds to the Gal4 activator. Similar results were obtained with each of the five independent *P*[*UAS-skl*] insertion lines tested, except that one line showed an effect with Skl alone (genotype III).

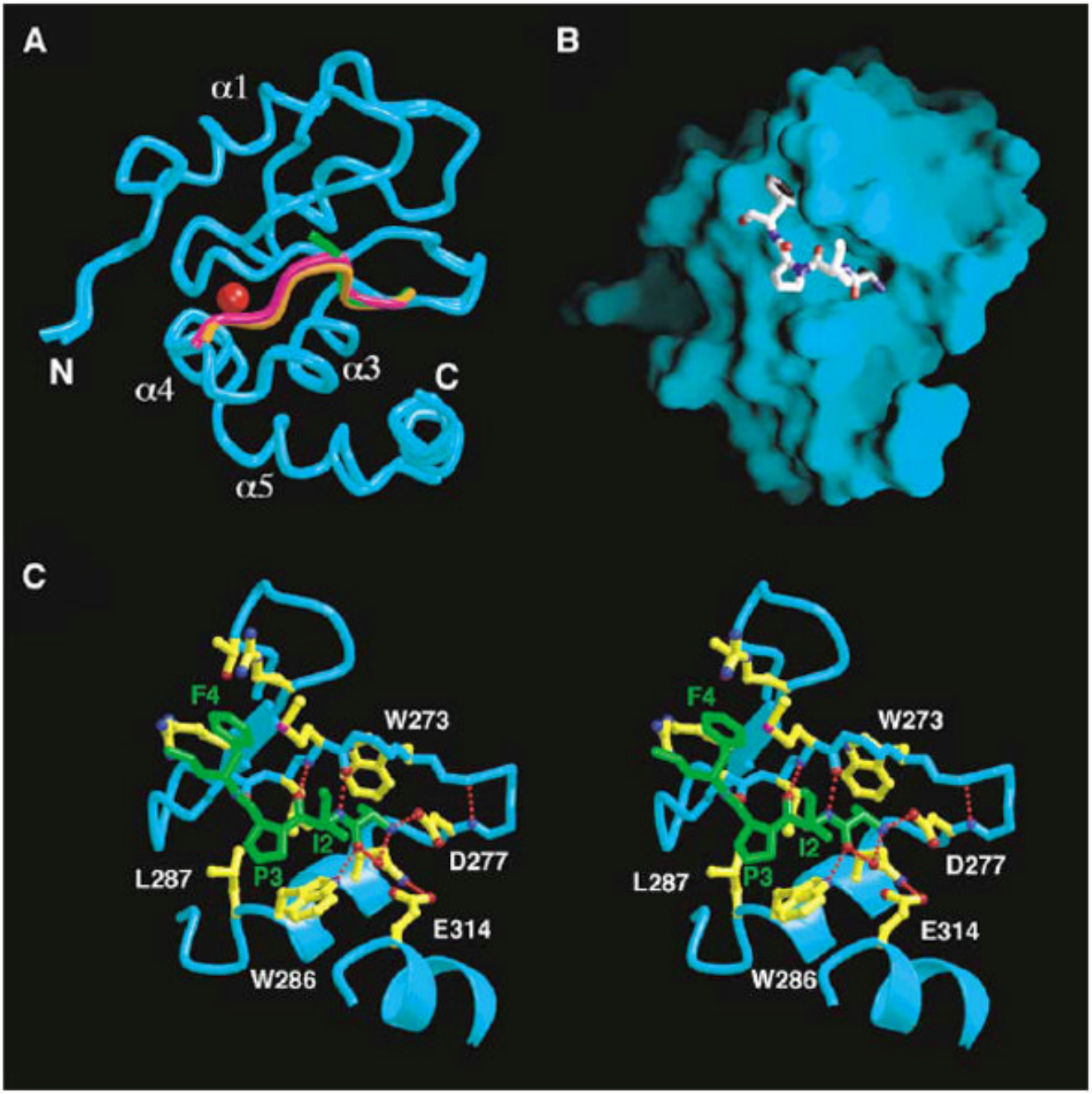

Figure 4. The N-Terminal Tetrapeptide of Skl Binds a Conserved Surface Groove on DIAP1-BIR2

(A) The structure of a DIAP1-BIR2 (cyan) bound to a Skl peptide (green) is superimposed with that complexed with a Hid (pink) or Grim (orange) peptide.

(B) Surface representation of DIAP1-BIR2 to highlight the binding of a Skl N-terminal tetrapeptide to a surface groove.

(C) Detailed atomic interactions at the BIR2-Skl interface. Conserved residues are labeled.