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The baculovirus anti-apoptotic protein Op-IAP does not inhibit *Drosophila* caspases or apoptosis in *Drosophila* S2 cells and instead sensitizes S2 cells to virus-induced apoptosis

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Abstract

The Op-IAP protein from the baculovirus *Orgyia pseudotsugata* M nucleopolyhedrovirus (OpMNPV) is highly effective at inhibiting apoptosis triggered by a variety of different stimuli in lepidopteran cells as well as in several different mammalian cell types, suggesting that it functions at a highly conserved step in the apoptotic pathway. However, the mechanism by which Op-IAP inhibits apoptosis is unclear. Since some IAP proteins can bind and inhibit caspases, we tested whether Op-IAP could inhibit the activity of caspases from *Drosophila melanogaster*. We found that recombinant Op-IAP protein was not able to bind or directly inhibit the activity of the *Drosophila* caspases DRONC, DrICE, or DCP-1 in vitro. In addition, expression of Op-IAP was unable to inhibit apoptosis triggered by either actinomycin D or UV light in *D. melanogaster* S2 cells. Surprisingly, Op-IAP expression in S2 cells enhanced apoptosis caused by baculovirus infection, but did not cause increased sensitivity to either actinomycin D or UV damage-induced apoptosis. The observation that Op-IAP cannot inhibit these insect caspases suggests that it functions by a mechanism that does not involve direct caspase inhibition.

Keywords: Apoptosis; Baculovirus; IAP protein; Op-IAP; Caspase; *Drosophila melanogaster*; DRONC; DrICE; DCP-1

Introduction

Apoptosis is a genetically controlled program of cell suicide. This form of programmed cell death is important for proper development during embryogenesis, tissue homeostasis, immune development, and function, and serves as a line of defense against viral infection (Horvitz, 1999; Jacobson et al., 1997; O'Brien, 1998; Rathmell and Thompson, 2002). A fine balance between cell death and life exists, and when components of the apoptotic program malfunction disease states can occur. An important family of proteins that regulate the cellular life–death balance is the inhibitor of apoptosis (IAP) family (Salvesen and Duckett, 2002). IAP proteins were first identified in baculoviruses and are now known to exist in a wide range of eukaryotes. They are characterized by the presence of an ~70 amino acid conserved zinc binding motif termed the baculovirus IAP repeat (BIR), which is involved in protein–protein interactions. Many IAPs also function as E3 ubiquitin ligases transferring ubiquitin to target proteins by means of a carboxy-terminal RING-finger (Jesenberger and Jentsch, 2002; Orlowski, 1999; Tyers and Willems, 1999; Yang et al., 2000).

Caspases, cysteine proteases that cleave their substrates following aspartic acid residues (Salvesen, 2002; Shi, 2002b), are present in metazoan cells in zymogen form consisting of a large and small subunit preceded by a prodomain. Caspases are activated in a hierarchical pattern with the upstream initiator or apical caspase processing itself after binding to adapter molecules. Once an apical caspase is activated, it then cleaves and activates downstream effector caspases, which are responsible for the

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destruction of the cell through cleaving various cellular components. The cellular IAPs are thought to serve as a type of rheostat, preventing caspase activation under normal conditions but allowing it to proceed when an appropriate signal is received (Miller, 1999). Some IAPs have been shown to directly bind to caspases through interactions within or immediately surrounding the BIR domains, and also to ubiquitinate caspases, targeting them for proteasomal degradation (Huang et al., 2000a; Suzuki et al., 2001; Wilson et al., 2002).

The apoptotic threshold is overcome in mammalian cells, in part, by the mitochondrial release of the pro-apoptotic IAP antagonist, Smac (Du et al., 2000; Liu et al., 2000; Srinivasula et al., 2000; Verhagen et al., 2000; Wu et al., 2000). Smac is released from the mitochondria along with other apoptotic factors, i.e., cytochrome *c* and Omi/HrtA2, which contribute to caspase activation. Cytochrome *c* binds to the adaptor molecule Apaf-1 and causes a conformational change that allows recruitment and activation of the apical caspase-9. Smac antagonizes IAPs by binding to them, thereby causing dissociation of the activated bound caspase from the IAP and apoptosis ensues.

On the other hand, apoptosis appears to be regulated somewhat differently in *Drosophila*, where cytochrome *c* release from mitochondria does not occur and is not necessary for caspase activation (Dorstyn et al., 2002, 2004; Varkey et al., 1999; Zimmermann et al., 2002). Instead, the apical caspase DRONC is constitutively active in *Drosophila* cells, and DIAP1 is required for cell viability because of its ability to promote DRONC ubiquitination and degradation (Muro et al., 2002; Rodriguez et al., 2002; Wilson et al., 2002). The *Drosophila* pro-apoptotic proteins Reaper, Hid, and Grim function similarly to Smac in that they are IAP antagonists and can therefore relieve the inhibitory activity of DIAP1 on DRONC (Goyal et al., 2000; Wang et al., 1999; Yoo et al., 2002). The available evidence also indicates that the lepidopteran apoptotic program is similar to that of *Drosophila*. Upon silencing of Sf-IAP in the lepidopteran cell line SF-21, the cells underwent spontaneous apoptosis suggesting that Sf-IAP is also required to inhibit a constitutively activated caspase, similar to DIAP1 in *Drosophila* (Muro et al., 2002). Therefore, superficially it may appear that IAPs are mostly similar in how they function, by directly binding and inhibiting active caspases. However, the IAPs have been implicated in other cellular processes, extending their function beyond mere caspase inhibition in the regulation of the apoptotic program (Birkey Reffey et al., 2001; Fraser et al., 1999; Li et al., 1998; Uren et al., 1999).

The Op-*iap3* gene is found in the baculovirus *Orgyia pseudotsugata* M nucleopolyhedrovirus (Op*M*NPV) and was one of the first *iap* genes identified (Birnbaum et al., 1994). Using RNA-mediated interference, it was demonstrated that the Op-IAP protein is required to prevent apoptosis during OpMNPV infection of LD652Y cells, derived from the lepidopteran insect *Lymantria dispar* (Means et al., 2003). Exogenous expression of Op-IAP has also been reported to inhibit apoptosis in both lepidopteran and mammalian cells. More specifically, in addition to LD652Y cells, Op-IAP has been shown to potently protect cells derived from *Spodoptera frugiperda* (SF-21 cells), HeLa cells, 293 (human embryonic kidney) cells, Chinese hamster ovary cells, and transgenic murine thymocytes from undergoing apoptosis induced by multiple stimuli (Clem and Miller, 1994; Hawkins et al., 1996, 1998; Manji et al., 1997; Robles et al., 2002; Vucic et al., 1997, 1998a). Although Op-IAP has been studied for more than 10 years and can protect a number of cell types from numerous death signals, its mechanism of anti-apoptotic function is not very well understood.

Op-IAP binds to the *Drosophila* pro-apoptotic proteins Hid, Reaper, and Grim, and inhibits apoptosis caused by overexpression of these proteins in SF-21 cells (Vucic et al., 1997, 1998a; Wright and Clem, 2002). These pro-death proteins all harbor what has been termed an IAP binding motif consisting of four amino acids at their amino terminus immediately following the initiating methionine (Shi, 2002a). The interaction between Op-IAP and the IAP binding motif of Hid occurs only after the methionine is removed by a metalloprotease, presumably a methionine aminopeptidase (Wright and Clem, 2002). Furthermore, the interaction between Op-IAP and the amino terminus of Hid is conserved in mammals, i.e., the interaction between the mammalian X-linked IAP (XIAP) and the mammalian IAP antagonist Smac. However, there are Op-IAP mutants that bind Hid normally but are unable to block Hid-induced apoptosis (Vucic et al., 1998b; Wright and Clem, 2002), leading to the conclusion that Op-IAP must possess other mechanisms of apoptosis inhibition.

The conventional wisdom of IAP function, as stated above, is that IAPs protect cells by inhibiting caspase activity through direct binding in the caspase active site. However, Op-IAP cannot inhibit the activity of the mammalian apical caspases-2, -8, or -9, nor the effector caspases-3, -6, or -7 in vitro (Huang et al., 2000b; Wilkinson et al., 2004), even though it can inhibit apoptosis in mammalian cells (Hawkins et al., 1996, 1998; Huang et al., 2000b). Expression of Op-IAP can inhibit, either directly or indirectly, a caspase-like activity in SF-21 cells which has been attributed to an unidentified apical caspase termed Sf-caspase-X, but available evidence indicates that Op-IAP functions upstream of Sf-caspase-X (LaCount et al., 2000; Zoog et al., 2002). Op-IAP has also been shown to possess E3 ubiquitin protein ligase activity and can promote ubiquitination of itself and of Hid (Green et al., 2004).

To further address the question of whether Op-IAP can directly inhibit caspases, we chose to examine *Drosophila* caspases since lepidopteran apical initiator caspases have not been identified to date, and Op-IAP is unable to inhibit the activity of the lepidopteran effector caspase Sf-caspase-1 (Manji et al., 1997; Seshagiri and Miller, 1997). Op-IAP

failed to bind to or inhibit the protease activity of the *Drosophila* caspases DRONC, DrICE, or DCP-1 in vitro. However, in the course of characterizing the ability of Op-IAP to directly inhibit *Drosophila* caspase activity, we found that Op-IAP could not inhibit apoptosis in *Drosophila* S2 cells. Furthermore, Op-IAP expression sensitized S2 cells to apoptosis induced by baculovirus infection, but not to apoptosis stimulated by actinomycin D or UV-induced damage.

Results

Op-IAP cannot inhibit Drosophila caspase activity in vitro

To determine whether Op-IAP can directly inhibit *Drosophila* caspases, we expressed a GST-Op-IAP fusion protein, which has an N-terminal GST tag, in bacteria and purified it using glutathione-conjugated beads. As controls, we also expressed and purified GST and GST-DIAP1 in a similar fashion. We also expressed the *Drosophila* caspases DRONC-His_6, DrICE-His_6, and DCP-1-His_6 in bacteria and purified them using cobalt resin. DRONC is an apical caspase responsible for activating downstream effector caspases, while DrICE and DCP-1 are effector caspases whose protease activity is thought to lead directly to the death of the cell. To measure caspase activity, we used the fluorogenic caspase substrates Ac-IETD-AFC (an apical caspase substrate recognized by DRONC) or Ac-DEVD-AFC (an effector caspase substrate recognized by DrICE and DCP-1).

When increasing concentrations of GST or GST-Op-IAP were preincubated with DRONC-His_6, DrICE-His_6, or DCP-1-His_6, caspase activity was not inhibited (Fig. 1), indicating that Op-IAP was unable to directly inhibit the activity of any of these caspases in vitro. GST-DIAP1 inhibited DCP-1-His_6 activity in vitro, but failed to inhibit

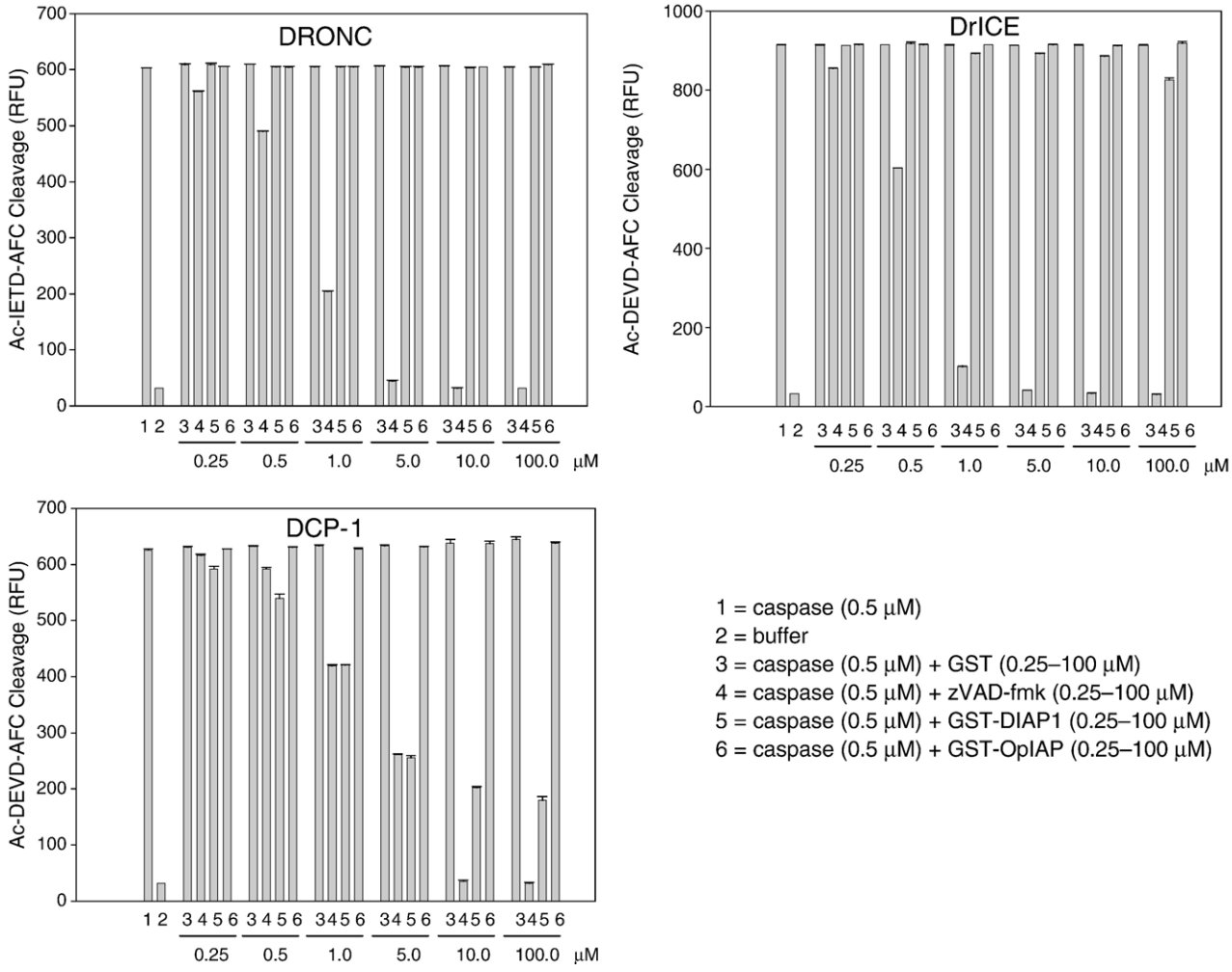

Fig. 1. Op-IAP does not inhibit *Drosophila* caspase activity in vitro. Recombinant DRONC-His_6, DrICE-His_6, or DCP-1-His_6 protein was incubated for 30 min either alone or with increasing concentrations of the recombinant proteins GST, GST-DIAP1, or GST-Op-IAP, or the caspase inhibitor Z-VAD-FMK. Fluorogenic caspase substrates were then added (Ac-IETD-AFC to the DRONC reactions and Ac-DEVD-AFC to the DrICE and DCP-1 reactions) and caspase activity was assessed by measuring relative fluorescence units (RFU) after 1 h of incubation. In reactions labeled "buffer", substrate was incubated in buffer alone. The results shown are the means ± standard error of three independent assays.

DRONC-His_6 or DrICE-His_6 activity, in agreement with previous reports (Chai et al., 2003; Hawkins et al., 1999, 2000). As expected, all three caspases were inhibited by the pan-caspase inhibitor Z-VAD-FMK.

Op-IAP cannot bind caspases in vitro

In order to understand why Op-IAP could not inhibit any of the *Drosophila* caspases tested in vitro, we hypothesized that Op-IAP could not directly bind to the caspases themselves. To address our hypothesis, we performed co-immunoprecipitation experiments where equivalent concentrations of GST-DIAP1 or GST-Op-IAP were mixed with similar concentrations of DRONC-His_6, a catalytically inactive DRONC mutant (DRONC-His_6(C318S)), DCP-1-His_6, or DrICE-His_6, and incubated for 1 h to allow association. Following incubation, samples were immunoprecipitated with anti-His antibody and precipitated proteins were analyzed by Western blot analysis using anti-DIAP1 or anti-Op-IAP antibodies. As shown in Fig. 2, DRONC-His_6, DRONC-His_6(C318S), DCP-1-His_6, and DrICE-His_6 bound to GST-DIAP1 but none of the caspases tested could bind to GST-Op-IAP. To insure that the GST-Op-IAP fusion protein was properly folded, we performed circular dichroism analysis of purified GST-Op-IAP, which revealed the presence of alpha helices and beta sheets (data not shown). In addition, GST-Op-IAP was able to bind a peptide containing the amino terminus of Hid (Fig. 2B). These results, in addition to the fact that GST-Op-IAP purified under similar conditions has in vitro ubiquitin ligase activity (Green et al., 2004), strongly support the presence of a correctly folded protein in our assays.

Op-IAP cannot protect Drosophila S2 cells against UV- or actinomycin D-induced apoptosis

In the course of examining the ability of Op-IAP to inhibit *Drosophila* caspases, we asked whether or not expression of Op-IAP could inhibit apoptosis in *Drosophila* S2 cells. To test this, we transiently co-transfected *Drosophila* S2 cells with plasmids encoding HA-tagged Op-IAP (HA-Op-IAP) and enhanced green fluorescent protein (eGFP) and then UV-irradiated the cells 20 h later. Transfected (green) cells were scored at 2 h and 24 h after UV treatment, and cell viability was calculated by dividing by the number of transfected cells left at 24 h by the number present at 2 h. S2 cells were efficiently protected by overexpression of HA-DIAP1, but HA-Op-IAP was unable to protect S2 cells from UV-induced apoptosis even though it was expressed at levels equivalent to DIAP1 (Fig. 3A and data not shown). Although unable to protect S2 cells, HA-Op-IAP protected SF-21 cells against UV-induced apoptosis (Fig. 3A) as has been demonstrated previously (Manji et al., 1997).

Because the presence of an epitope tag can interfere with the ability of Op-IAP to protect mammalian cells (Colin

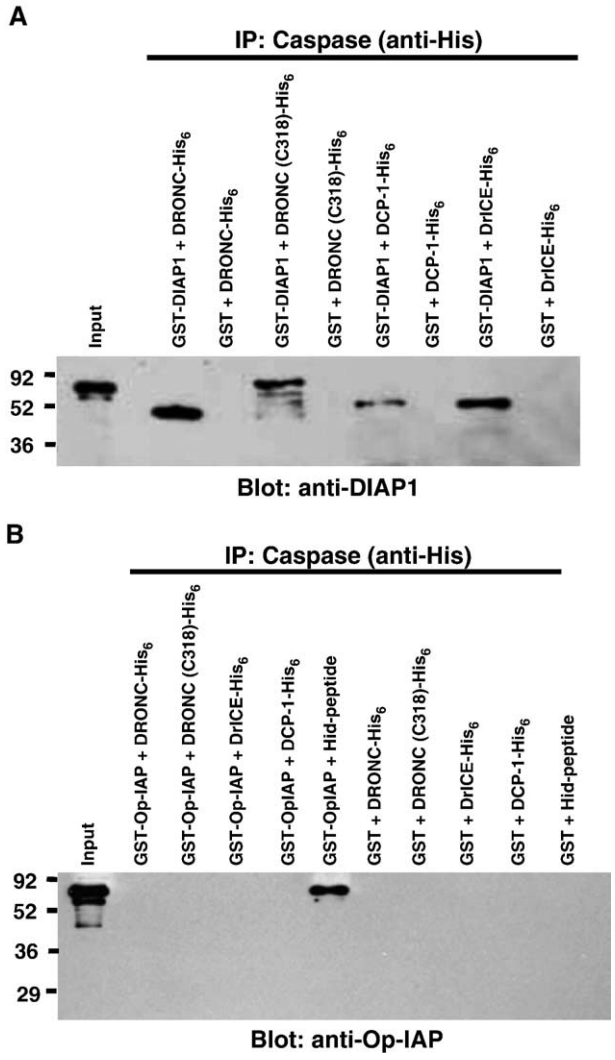

Fig. 2. Op-IAP does not bind *Drosophila* caspases in vitro. Recombinant GST-DIAP1 (A) or GST-Op-IAP (B) fusion protein was incubated with DrICE-His_6, DCP-1-His_6, DRONC-His_6, or catalytically inactive DRONC-His_6(C318S), and the reactions were then subjected to pull-down assay with anti-His antibody or with Hid peptide. Interacting proteins were analyzed by Western blot using anti-DIAP1 (A) or anti-Op-IAP (B) antibodies. The lanes labeled "Input" contain 10% of the protein used in each pull-down reaction.

Duckett, personal communication), we tested whether Op-IAP could protect S2 cells when the HA-tag was removed. As shown in Fig. 3B, the presence or absence of the HA-tag on Op-IAP did not affect the ability of Op-IAP to protect S2 cells, although removal of the HA-tag did cause a slight increase in the activity of Op-IAP in SF-21 cells, as well as that of DIAP1 in S2 cells. Results similar to those shown in Figs. 3A and B were obtained when actinomycin D was used to stimulate apoptosis (data not shown).

Op-IAP sensitizes Drosophila S2 cells to baculovirus-induced apoptosis

As a different method of expressing Op-IAP in S2 cells, we constructed a recombinant of the baculovirus *Auto-*

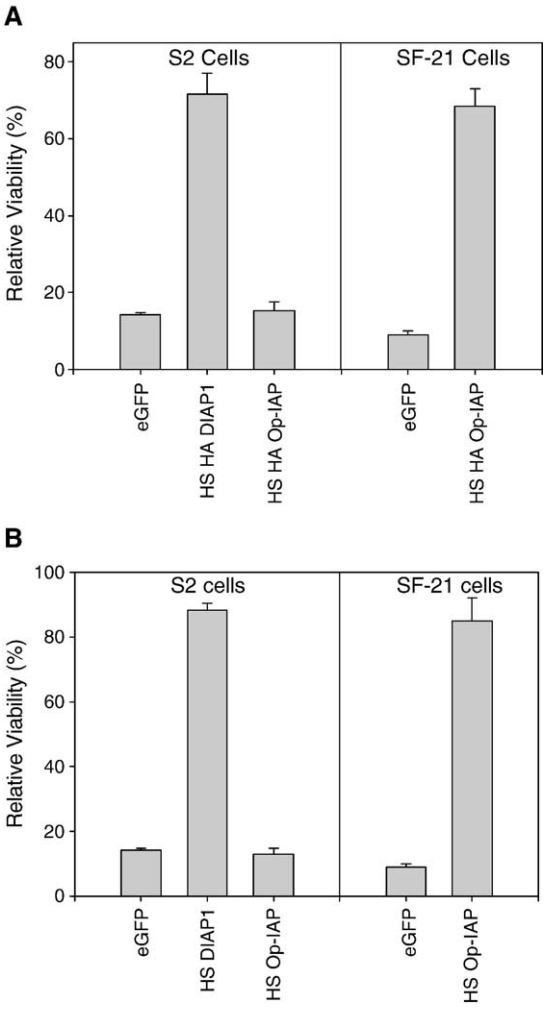

Fig. 3. Op-IAP does not inhibit apoptosis in *Drosophila* S2 cells. (A) A plasmid expressing eGFP was transfected into S2 or SF-21 cells either alone or together with the indicated plasmids expressing HA-DIAP1 or HA-Op-IAP from the heat shock (HS) promoter. The cells were UV irradiated and the relative viability of the cells 24 h later was determined as described in Materials and methods. HA-Op-IAP and HA-DIAP1 were expressed in S2 cells at roughly equal levels as judged by Western blotting with anti-HA (data not shown). (B) Removing the HA epitope tag had no effect on the ability of Op-IAP to block UV-induced apoptosis in S2 cells but had a slight positive effect in SF-21 cells (compare A and B).

grapha californica MNPV (Ac*M*NPV) that lacked the caspase inhibitor *p35* but expressed HA-tagged Op-IAP from a *Drosophila* hsp70 promoter (vHAOp-IAP). Ac*M*NPV is able to enter *Drosophila* cells and although some viral genes are expressed, no viral replication occurs (Carbonell et al., 1985; Lee et al., 2000; Morris and Miller, 1992, 1993). However, genes under the control of the *Drosophila* hsp70 promoter are highly expressed. Mutants of AcMNPV that lack the *p35* gene induce apoptosis in several different lepidopteran cell lines, as well as in *Drosophila* DL-1 cells (Clem, 2001; Zoog et al., 2002). We thus used this recombinant virus as a vector for expression of HA-Op-IAP in S2 cells.

Infection of S2 cells with a virus containing the *p35* gene (vHSGFP) did not induce apoptosis, while infection of cells with a virus lacking the *p35* gene (vHSGFP/P35del) caused a reduction in viability of around 50% at 30 h post-infection (Fig. 4A). However, it was immediately clear from observing the cells that apoptosis was more prevalent in vHAOp-IAP-infected cells. Measurement of the viability of S2 cells infected with vHAOp-IAP indicated that the number of viable cells remaining at 30 h post-infection was only around 10% (Fig. 4A). Cell death did not start to occur until around 24 h post-infection, but Op-IAP was expressed at high levels in cells infected with vHAOp-IAP prior to this time (Fig. 4B). Dying cells infected with either vHSGFP/P35del or vHAOp-IAP exhibited typical morphological characteristics of apoptosis, including cell blebbing and nuclear fragmentation (data not shown). Both apical and effector caspase activities (as measured by cleavage of IETD-AFC and DEVD-AFC, respectively) were also increased in cells infected with vHAOp-IAP when compared to vHSGFP/P35del and vHSGFP, with activities of both types of caspases being highest in vHAOp-IAP-infected cells (Fig. 4C). To rule out the possibility that the differences in the amount of apoptosis observed were due to differences in the abilities of the viruses to infect the cells, the infection rates for all three viruses were determined. Immunofluorescence using an antibody against the viral IE1 protein at 12 h post-infection indicated that between 98% and 99% of cells were infected by each virus (Fig. 4D, panels a–f). Staining was not observed in mock-infected cells (Fig. 4D, panels g and h) or when infected cells were stained with secondary antibody alone (data not shown).

Finally, we explored the possibility that Op-IAP may sensitize these cells to other apoptotic stimuli in addition to viral infection. S2 cells were transiently transfected with a control plasmid or with Op-IAP- or DIAP1-expressing plasmids and then treated with UV radiation or actinomycin D. Since the levels of UV and actinomycin D that we normally use result in almost complete cell death (Fig. 3), we used lower doses of each stimulus that only induced apoptosis in 40–60% of untransfected cells. As shown in Fig. 5, Op-IAP expression did not cause increased apoptosis in response to either stimulus, indicating that the sensitizing effect of Op-IAP on apoptosis was stimulus-dependent.

Discussion

In this study, we found that Op-IAP was unable to inhibit the activity of the *Drosophila* caspases DRONC, DrICE, or DCP-1 in vitro, nor could it bind to any of these caspases. Op-IAP also failed to inhibit apoptosis in *Drosophila* S2 cells induced by UV irradiation, actinomycin D, or infection with *p35* mutant AcMNPV. Surprisingly, not only was Op-IAP unable to inhibit apoptosis in S2 cells induced by these stimuli, it actually sensitized S2 cells to virus-induced apoptosis, but not to apoptosis

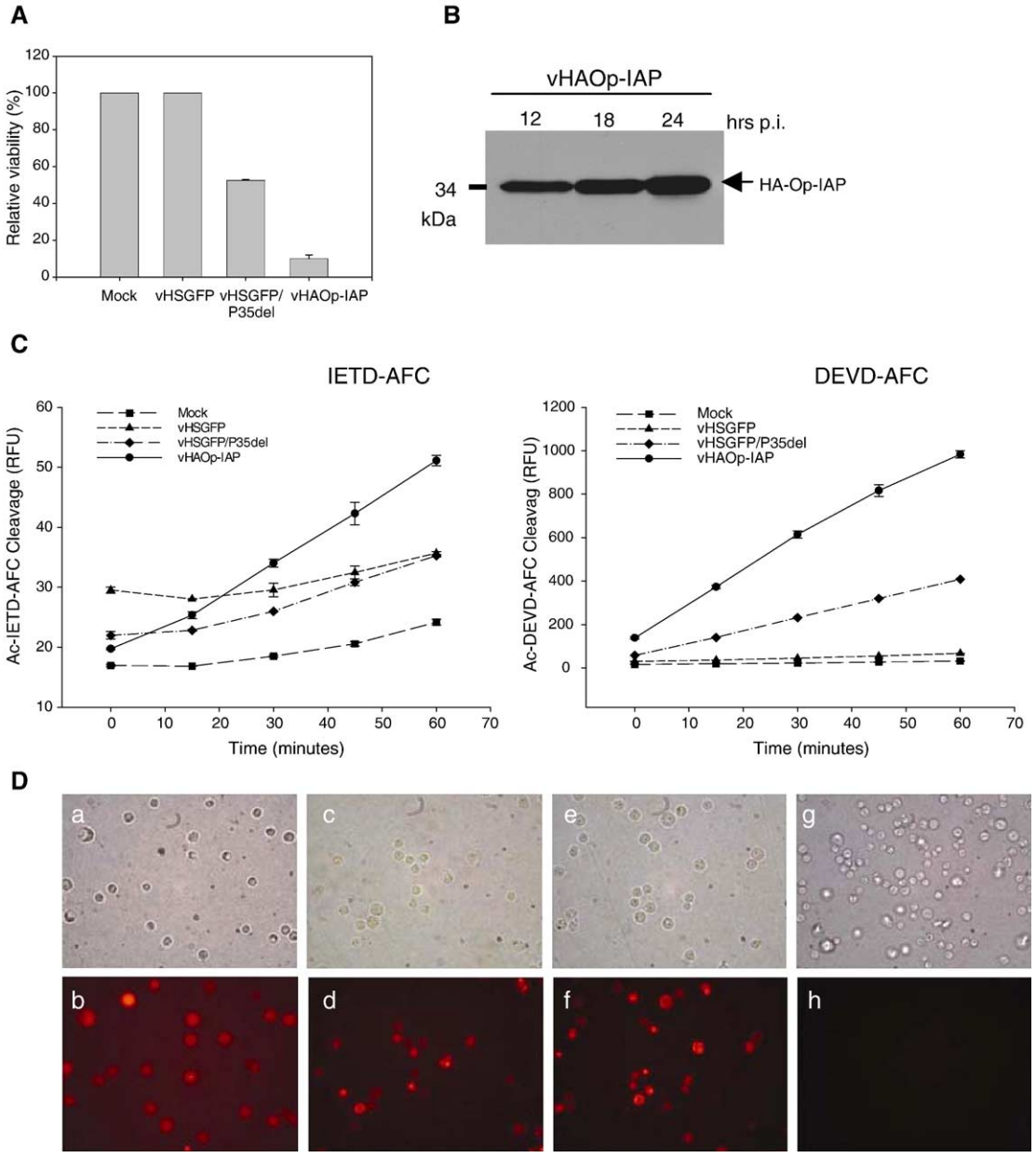

Fig. 4. Op-IAP sensitizes *Drosophila* S2 cells to virus-induced apoptosis. (A) S2 cells were infected with the indicated viruses, and at 30 h post-infection the percent viability relative to the mock-infected control was determined. (B) S2 cells were infected with vHAOp-IAP and at 12, 18, and 24 h post-infection cells were harvested and analyzed by Western blot using an anti-HA antibody. (C) S2 cells were infected with the indicated viruses and at 30 h post-infection the cells were harvested and caspase activity was quantified using the caspase substrates Ac-IETD-AFC (left panel) or Ac-DEVD-AFC (right panel). (D) S2 cells were infected with vHSGFP/P35del (a–b), vHAOp-IAP (c–d), vHSGFP (e–f), or mock infected (g–h), and at 12 h post-infection the cells were stained with antibody against the viral IE1 protein and fluorescent secondary antibody. In panels a, c, e, and g, the cells were viewed under normal light, while in panels b, d, f, and h, the cells were viewed under fluorescence.

induced by the other stimuli. Although these results may seem unusual, it is not unheard of for proteins that regulate apoptosis to have opposite activities under different circumstances. For example, the mammalian anti-apoptotic proteins Bcl-2 and Bad are normally anti-apoptotic and pro-apoptotic, respectively. However, under certain circumstances, both proteins can have the opposite function (Cheng et al., 1997; Fannjiang et al., 2003; Seo et al., 2004). In addition, both nuclear factor-kappaB (NF-κB) and c-Jun N-terminal kinase (JNK) signaling can be anti-apoptotic or pro-apoptotic, depending on the context of the cells and the organism (Liu and Lin, 2005; Shishodia and Aggarwal, 2004). Although Op-IAP is not normally found in S2 cells, these observations may prove useful because they provide clues pertaining to the biochemical function of Op-IAP.

DIAP1 is known to inhibit apical and effector caspases by different mechanisms. Using conditions similar to what

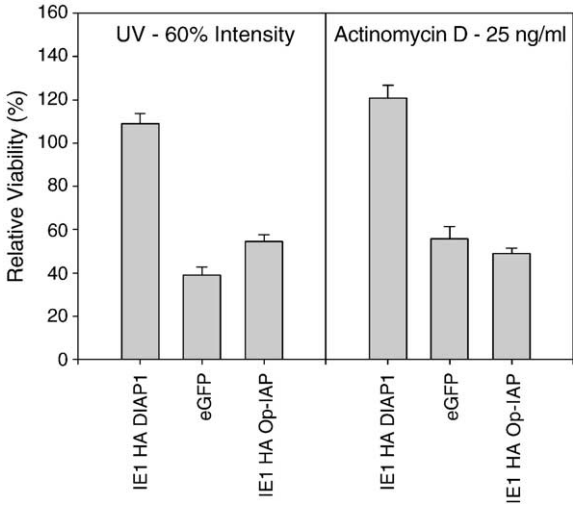

Fig. 5. Op-IAP does not sensitize S2 cells to other apoptotic stimuli. S2 cells were transfected with vector expressing eGFP alone or together with vectors expressing DIAP1 or Op-IAP from the viral IE1 promoter and then treated with low doses of UV or actinomycin D that only induced 40–60% apoptosis in control cells. The relative viability of the transfected cells was determined 24 h later as described in Materials and methods. HA-DIAP1 and HA-Op-IAP were expressed in roughly equivalent amounts (data not shown).

we have used, Hawkins et al. (2000) found that DIAP1 directly inhibited the enzymatic activity of DCP-1 in vitro, but did not inhibit the activity of DrICE. It was later shown that inhibition of DrICE by DIAP1 is improved if the first 20 amino acids of DIAP1, which form an auto-inhibitory domain, are removed (Yan et al., 2004). On the other hand, DIAP1 does not directly inhibit DRONC activity and instead assists in the ubiquitination and degradation of DRONC (Chai et al., 2003; Wilson et al., 2002). Op-IAP also has E3 ubiquitin ligase activity and can ubiquitinate the Hid protein (Green et al., 2004), and the RING motif of Op-IAP is important for its anti-apoptotic function (Clem and Miller, 1994; Vucic et al., 1998b; Wright and Clem, 2002). However, because Op-IAP cannot interact with DRONC or any of the other caspases tested, it is unclear at this time whether ubiquitination of caspases has any bearing on the anti-apoptotic activity of Op-IAP.

Although Op-IAP could not inhibit any of the *Drosophila* caspases tested, we still cannot formally rule out the possibility that a lepidopteran caspase exists that Op-IAP can physically bind to and inhibit or ubiquitinate. For example, Op-IAP may directly inhibit the as yet unidentified apical Sf-caspase-X. However, we consider this unlikely for several reasons. First, compelling data exist suggesting that Op-IAP inhibits the apoptotic program in SF-21 cells at a point in the pathway prior to the activation of Sf-caspase-X (Zoog et al., 2002). Second, in *Drosophila*, DRONC is the only apical caspase activated following a variety of apoptotic stimuli, and Op-IAP does not bind to or inhibit DRONC. Third, Op-IAP inhibits apoptosis in mammalian cells, but its ability to do so correlates with its ability to bind to Smac, and it does not directly inhibit any of several mammalian caspases that have been tested (Wilkinson et al., 2004). Thus the possibility that Op-IAP functions by inhibiting caspases in lepidopteran cells seems highly unlikely, given that it does not directly inhibit caspases from another type of insect and that it functions in mammalian cells by a mechanism that does not involve direct caspase inhibition.

We also found that Op-IAP failed to inhibit apoptosis in S2 cells induced by baculovirus infection. This result is similar to a report by Zoog et al. (2002), in which Op-IAP failed to inhibit apoptosis induced by infection of a different *Drosophila* cell line, DL-1 cells, with a baculovirus lacking *p35*. In addition to this, however, we found that Op-IAP actually sensitized S2 cells to baculovirus-induced apoptosis. The fact that Op-IAP did not sensitize S2 cells to UV or actinomycin D-induced apoptosis suggests that virus infection may stimulate a different apoptotic pathway than these other stimuli. Although the mechanism by which Op-IAP sensitizes S2 cells to baculovirus-induced apoptosis is unknown, it is possible that the Op-IAP protein is influencing signal transduction pathways, such as the NF-κB signaling pathway, which can be either pro-apoptotic or anti-apoptotic, depending on the cell type and the organism. Op-IAP expression has been shown to induce NF-κB signaling in mammalian cells (Robles et al., 2002, 2003), and NF-κB signaling can be pro-apoptotic in *Drosophila* (Georgel et al., 2001). Thus further experiments examining how Op-IAP expression influences these types of signaling pathways may prove illuminating.

Op-IAP appears to inhibit apoptosis in mammalian cells through titration of Smac and cannot directly inhibit any of several mammalian caspases that have been tested (Wilkinson et al., 2004). This result, along with the results here showing that Op-IAP is unable to inhibit apoptosis in *Drosophila* and is unable to directly bind to and inhibit *Drosophila* caspases, suggests that Op-IAP blocks apoptosis in lepidopteran cells by a mechanism that does not require direct caspase inhibition. However, conclusive evidence awaits the identification of apical caspases in lepidopteran insects.

Materials and methods

Cell lines and viruses

S. frugiperda IPLB-SF-21 (SF-21) and *Trichoplusia ni* (TN-368) cells were maintained in TC-100 medium (Invitrogen) supplemented with tryptose broth and 10% fetal bovine serum (Invitrogen) (complete TC-100). *Drosophila melanogaster* S2 cells were maintained in Schneider's *Drosophila* media (Invitrogen) supplemented with 10% fetal bovine serum. Cells were incubated at 27 °C and

propagated as previously described (O'Reilly et al., 1992). Construction of the vHSGFP strain of Ac*M*NPV has been described previously (Clarke and Clem, 2002), as has the construction of the Ac*M*NPV *p35* deletion virus vHSGFP/P35del (Clarke and Clem, 2003). The recombinant virus vHAOP-IAP was constructed by homologous recombination of Ac*M*NPV sequences in the pHSEpiOp-IAP shuttle vector (Vucic et al., 1997) with sequences flanking the polyhedrin region of vHSGFP/P35del, which replaced the eGFP coding sequence with that of Op-IAP under hsp70 promoter control.

Plasmid construction

Plasmids expressing HA-OpIAP, HA-DIAP1, and eGFP under the control of the *Drosophila* hsp70 heat shock promoter have previously been described (Clem and Miller, 1994; Vucic et al., 1997, 1998a; Wright and Clem, 2002). For expression driven by the baculovirus IE1 promoter, HA-Op-IAP and HA-DIAP1 were amplified by PCR and cloned downstream of the IE1 promoter in the pIE1^{hr}/PA vector (a gift from Paul Freisen, University of Wisconsin-Madison). For glutathione-S-transferase (GST)-tagged Op-IAP, the Op-IAP coding sequence was PCR-amplified and introduced into the GST expression vector pGEX3X (Amersham Pharmacia). A similar plasmid expressing GST-DIAP1 was provided by Bruce Hay (California Institute of Technology) (Hawkins et al., 1999). As in Hawkins et al. (1999), the coding sequences for DCP-1 (initiating at codon 31) and DrICE (initiating at codon 81) were PCR-amplified and cloned into the pET23a(+) (Novagen) vector. These plasmids thus expressed versions of DCP-1 and DrICE lacking the prodomain and containing a C-terminal His_6 tag. pET23a-DRONC and pET23a-DRONC (C318S) were provided by Bruce Hay (Hawkins et al., 2000).

Expression and purification of recombinant IAPs and caspases

GST-DIAP1, GST-Op-IAP, DRONC-His_6, DRONC (C318S)-His_6, DrICE-His_6, and DCP-1-His_6 were purified from BL21(DE3)pLysS (Novagen) *E. coli* by inoculating 1 l of LB medium with 50 ml of an overnight culture that had been grown with shaking at 37 °C. The inoculated 1-l culture was then grown at room temperature until the OD_{600} reached 0.4, followed by induction of protein expression by addition of IPTG to a final concentration of 0.1 mM and incubation for an additional 1 h. The cells were lysed by sonication and the protein was purified by incubating the bacterial lysate with either glutathione-conjugated beads for GST-DIAP1 and GST-Op-IAP, or with Talon resin (BD Clontech) for the His_6-tagged caspases for 1 h with rocking at 4 °C. The buffers and storage protocols used were as described (Hawkins et al., 1999).

Caspase activity assay

Increasing concentrations of GST, GST-DIAP1, GST-Op-IAP, or Z-VAD-FMK were incubated with 0.5 μM caspase (DRONC-His_6, DrICE-His_6, or DCP-1-His_6) in caspase activity buffer (50 mM Hepes, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, 5 mM DTT) for 30 min at 30 °C. All caspase inhibitors and substrates were purchased from Enzyme Systems Products. Following incubation, the caspase-8 substrate, Ac-IETD-AFC (100 μM), was added to samples containing DRONC and the caspase-3 substrate, Ac-DEVD-AFC (100 μM), was added to samples containing DrICE or DCP-1. After 1-h incubation at 30 °C, caspase activity was determined as an increase in fluorescence detection caused by the enzymatic cleavage of the substrate and the release of AFC (7-amino-(trifluoromethyl)coumarin). When necessary, reactions were diluted to maintain enzyme activity in the linear range.

Caspase-IAP co-immunoprecipitations

Equal concentrations of protein were used (as determined by comparison with BSA standards on a coomassie stained gel). Recombinant GST-DIAP1 or GST-Op-IAP was added to DrICE-His_6, DCP-1-His_6, DRONC-His_6, or catalytically inactive DRONC-His_6(C318S) in 45 μl Buffer A (25 mM Tris–HCL, pH 8, 50 mM NaCl, 10 mM DTT) and incubated for 1 h at 30 °C. The reactions were then added to protein G beads that had been preincubated with anti-His antibody (His-Probe H-3, Santa Cruz Biotechnology) and rocked overnight at 4 °C. As a positive control, GST-Op-IAP was incubated with a biotinylated peptide consisting of amino acids 2–11 of the Hid protein that had been previously incubated with streptavidin beads as described (Wright and Clem, 2002). The beads were washed 3 times with Buffer A and bound protein was removed from the protein G beads or streptavidin beads by boiling the samples in Laemmli buffer for 5 min. The eluted protein was separated on a 15% SDS–PAGE gel, transferred to PVDF, and DIAP1 or Op-IAP protein was detected by Western blot using anti-DIAP1 monoclonal antibody (Muro et al., 2002) or anti-Op-IAP polyclonal antiserum (Means et al., 2003) at a 1:100 dilution followed by anti-mouse-HRP IgG antibody at 1:3000 for DIAP1 and anti-rabbit-HRP IgG antibody at 1:10,000 for Op-IAP.

Transfections and protection assays

3×10^6 S2 cells or 0.5×10^6 SF-21 cells were transfected with 3 μg of the indicated plasmid DNA along with 3 μg of a plasmid expressing eGFP. Cellfectin reagent (Invitrogen) was used for S2 cells and a liposome reagent (LaCount et al., 2000) was used for SF-21 cells. At 20 h post-transfection, the cells were treated with UV by placing the plates on a transilluminator for 10 min at an intensity of

8000 μW/cm^2 (312 nm wavelength) or actinomycin D was added at 500 ng/ml to induce apoptosis. 60% UV intensity and 25 ng/ml actinomycin D were used as suboptimal apoptotic treatments in the sensitization experiments. At 24 h post-treatment, viable eGFP-positive cells were scored and a percent relative viability was generated by dividing by the number of viable eGFP positive cells at 2 h after apoptosis induction. All protection assays were repeated twice and represent the mean ± standard error of three fields each from two wells.

Op-IAP sensitization of S2 cells to viral-induced apoptosis

Virus infections were conducted at a multiplicity of infection of 50 plaque-forming units per cell based on titers obtained by performing plaque assays with TN-368 cells. Virus in complete TC-100 was allowed to attach to cells for 2 h before being removed and replaced with complete TC-100. Viable, non-apoptotic cells (based on morphology) present at 30 h post-infection were enumerated by manually counting the cells present in 3 random fields of view at 400× magnification in duplicate wells. The percentage of viable cells remaining in each virus-infected sample was then generated by dividing the number of cells present by the number present in the mock-infected sample at the same time point (set at 100%). For the caspase assays, cells were harvested at 30 h post-infection and collected by centrifugation at 2880 × *g* for 5 min. The media were aspirated and the cells were resuspended in 100 μl of caspase activity buffer followed by 3 cycles of freeze–thaw to rupture the cells. The cellular debris was collected by centrifugation at 2000 × *g* for 2 min and the supernatant was transferred to a new tube. The supernatant was incubated at 37 °C for 20 min. 30 μl was removed and used to quantify caspase activity as described above.

Immunofluorescence assay

S2 cells were infected as described above and at 12 h post-infection the cells were washed with phosphate-buffered saline (PBS). All of the following washes were also done with PBS. The cells were then incubated 15 min with 1% bovine serum albumin in PBS (PBS–BSA). After 2 washes, the cells were fixed for 20 min in 2% paraformaldehyde in PBS. Following 3 washes, cells were permeabilized for 10 min with 0.2% Triton X-100 in PBS. Cells were washed 3 times, incubated with PBS–BSA, and washed 2 more times. Cells were then incubated for 1 h at 4 °C with anti-IE1 monoclonal antibody (kindly provided by Linda Guarino, Texas A and M University) diluted 1:100. Following 3 washes, cells were incubated again for 15 min with 1% PBS–BSA, washed 2 times, and incubated 30 min at 4 °C with goat anti-mouse IgG labeled with R-phycoerythrin (SouthernBiotech, Birmingham, AL) diluted 1:250. Stained cells were washed 3 times and observed with a Nikon TE200 microscope equipped with epifluorescence.

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