Cell Death and Differentiation (1998) 5, 569–576

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Anti-apoptotic potential of insect cellular and viral IAPs in mammalian cells

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Received 24.11.97; revised 2.2.98; accepted 26.2.98
Edited by G. Melino

Abstract

IAPs were identified as baculoviral proteins that could inhibit the apoptotic response of insect cells to infection. Of the viral IAPs, OpIAP and CpIAP can inhibit apoptosis, whereas AcIAP cannot. OpIAP and some mammalian homologues can inhibit mammalian cell death. Two mammalian IAPs bind to TNFRII associated factors (TRAFs), but the significance of this is unclear. Here we show that *Drosophila* cellular IAPs and two baculoviral IAPs (OpIAP and CpIAP) can inhibit mammalian cell death induced by overexpression of Caspases 1 and 2. IAPs must act on conserved components of the apoptotic mechanism, but as none of these IAPs could bind TRAF proteins, TRAFs are not likely to be important for IAP mediated apoptosis inhibition. As OpIAP protected against death induced by ligation of TNF receptor family members, but not by factor nor serum withdrawal from dependent cells, it can inhibit certain apoptotic pathways without affecting others.

Keywords: IAP; caspase; p35; *Drosophila*; cytokine; RING finger; programmed cell death

Abbreviations: IAP, inhibitor of apoptosis; BIR, baculovirus IAP-like repeat; TNF, tumor necrosis factor; TRAF, TNF receptor-associated factor; NGF, nerve growth factor; SEM, standard error of the mean; IL3, interleukin-3; GFP, green fluorescent protein; MIH, mammalian IAP homologue; MTT, dimethylthiazol dophenyltetrazolium bromide.

Introduction

Apoptosis is a physiological process by which unwanted cells are removed (Vaux and Strasser, 1996). It is used in normal circumstances during development and for homeostatic control of cell numbers. Apoptosis is also used as a defence mechanism to remove cells which have been altered in a way which renders them dangerous for the organism as a whole. Intracellular pathogens such as viruses require the host cell's synthetic machinery to replicate, so apoptosis can be used to halt their spread.

The mechanism of apoptosis is highly conserved throughout the metazoa. A family of a cysteine proteases termed caspases (Alnemri *et al*, 1996) appear to be the key apoptosis effector enzymes in nematodes, insects and mammals. For example, Ced-3, the nematode member of this family, is essential for programmed cell death in the worm *Caenorhabditis elegans* (Ellis and Horvitz, 1986; Yuan and Horvitz, 1990; Yuan *et al*, 1993). Several mammalian caspases have been described (reviewed by Alnemri, 1997), many of which trigger apoptosis when overexpressed, and some of which are essential for apoptosis in particular circumstances (Kuida *et al*, 1995, 1996). Caspases have been identified in insects (Ahmad *et al*, 1997; Fraser and Evan, 1997; Song *et al*, 1997), and inhibition of caspase activity in insect cells by baculoviral p35 prevents apoptosis (Bump *et al*, 1995; Clem *et al*, 1991).

Many viral anti-apoptosis proteins have been identified that prevent cell death by either inhibiting caspase activity, or blocking pathways that lead to caspase activation. Two types of apoptosis inhibitory molecules have been cloned from baculoviruses. p35 was initially described as a gene deleted from a mutant baculoviral strain for which infection was accompanied by massive cell death (Clem *et al*, 1991). p35 inhibits apoptosis by binding directly to and thereby inactiving the caspases (Bump *et al*, 1995; Xue and Horvitz, 1995).

The first members of the IAP family were identified in a search for novel baculoviral anti-apoptotic genes (Crook *et al*, 1993). These proteins have a carboxy terminal RING finger domain, and at their amino termini are two repeats of a motif designated baculovirus IAP-like repeat (BIR). IAPs from *Orgyia pseudotsugata* nuclear polyhedrosis virus and *Cydia pomonella* granulosis virus (OpIAP and CpIAP) could suppress the apoptosis caused by infection of insect cells with p35 deficient viruses (Birnbaum *et al*, 1994; Crook *et al*, 1993). Interestingly, a third baculoviral IAP from *Autographa californica* nuclear polyhedrosis virus (AcIAP) was incapable of inhibiting insect cell death (Birnbaum *et al*, 1994).

IAPs must interact with conserved components of the apoptotic mechanism, since OpIAP could inhibit death of mammalian cells due to overexpression of caspases, the CD95 signaling molecule FADD, or infection with Sindbis virus (Duckett *et al*, 1996; Hawkins *et al*, 1996).

While these experiments did not reveal which components of the apoptotic mechanism were affected by IAPs, they suggested that cellular IAP homologues may exist that regulate apoptosis in host organisms. Two endogenous insect IAPs have been cloned from *Drosophila* using a screen for repressors of REAPER-induced death in the fly eye (Hay *et al*, 1995). Lesions in DIAP-1 enhance the cell

death seen in the eyes of flies with enforced expression of the *Drosophila* cell death gene *rpr*. Overexpression of DIAP-1 and a second related gene, DIHA/DIAP-2/dILP prevented insect cell apoptosis triggered during development and by overexpression of the *Drosophila* cell death proteins REAPER and HID (Hay *et al*, 1995; Vucic *et al*, 1997). OpIAP truncation or mutated constructs were previously tested in insect and mammalian cells and the RING finger was found to be essential for optimal protective ability (Clem and Miller, 1994; Hawkins *et al*, 1996). By contrast, however, a DIAP-1 gene lacking the RING finger gave elevated levels of protection in the *Drosophila* eye compared to the full length gene (Hay *et al*, 1995).

Five mammalian IAP genes have been identified. The first, NAIP, a candidate gene for Spinal Muscular Atrophy, has three BIRs but no RING finger (Roy *et al*, 1995). Three of the other mammalian IAPs, MIHA/hILP/xiap, MIHB/hiap-1/c-IAP1 and MIHC/hiap2/c-IAP-2 all have three BIRs and a RING finger (Rothe *et al*, 1995; Duckett *et al*, 1996; Liston *et al*, 1996; Uren *et al*, 1996). The fifth mammalian IAP, survivin, has a single BIR and no RING finger (Ambrosini *et al*, 1997). In this paper, the cellular IAPs will be referred to using the MIH/DIH nomenclature.

One group identified MIHB and MIHC by virtue of their association with TNFRII (Rothe *et al*, 1995). MIHB and MIHC interact indirectly with this receptor, via the signaling components TRAF1 and TRAF2 (Rothe *et al*, 1994). Since many members of the TNFR family of receptors are capable of signaling apoptosis, this interaction raised the possibility that IAPs may prevent apoptosis by modulating signals from them.

Much has been recently discovered about the components of the signal transduction pathways which emanate from these receptors. Signals from TNFRI can lead to activation of caspases and hence apoptosis, theoretically by two alternative sets of interacting molecules. One pathway from the receptor is via TRADD and FADD to Caspase-8 (Muzio *et al*, 1997; Srinivasula *et al*, 1996). The other, which involves TRADD, RIP and RAIDD, activates Caspase-2 (Duan and Dixit, 1997). Both TNFRI and TNFRII can signal NFκB activation via TRAF2, but this usually has a pro-survival effect (Liu *et al*, 1996; Rothe *et al*, 1994).

Cell viability can be regulated by signals from membrane receptors other than the TNF receptor family. For example, many cells require a continuous supply of cytokines for their survival. When growth factors are removed from such cells, they undergo apoptosis. The hemopoietin family of receptors signal via Jak pathways (reviewed by Ihle, 1996) but the way that factor deprivation results in cell death is not known.

Apoptosis due to loss of signals from hemopoietin receptors is often efficiently blocked by members of the Bcl-2 family of apoptosis inhibitors (Vaux *et al*, 1988), whereas in most cell types killing by TNF is relatively poorly blocked by Bcl-2 and its relatives (Huang *et al*, 1997; Strasser *et al*, 1995).

In an attempt to more fully characterize the cell death-inhibitory actions of the IAP family, we have compared the ability of baculoviral and endogenous insect members of the IAP family to suppress mammalian cell death induced by caspase overexpression, and explored the range of mammalian apoptotic stimuli against which one family member, OpIAP, can function. We have also assessed the ability of DIAP-1 and DIHA genes (both full length and lacking the RING domain) to prevent mammalian apoptosis, and tested endogenous and viral insect IAPs for their ability to bind TRAF proteins.

Results

Protection against mammalian cell death by baculoviral IAPs

To determine if the antiapoptotic activity of OpIAP, CpIAP and AcIAP in mammalian cells reflected their activity in insect cells, we tested their ability to protect against death induced by transient expression of Caspase-1 and Caspase-2 in HeLa cells using the caspase-lacZ fusion protein overexpression system described previously (Miura *et al*, 1993; Hawkins *et al*, 1996) (Figure 1). As in insect cells, OpIAP and CpIAP were able to promote survival, whereas AcIAP was not. Although these results do not indicate whether AcIAP will be able to inhibit apoptosis in some other system, they lend support to the notion that the targets of OpIAP and CpIAP have been highly conserved during evolution.

Analysis of anti-apoptotic potential of full length and truncated *Drosophila* IAPs in mammalian cells

When overexpressed in the eye, the two *Drosophila* IAPs protect insect cells from developmental death and that induced by overexpression of REAPER and HID (Hay *et al*, 1995). To determine whether, like the insect virus genes, they could function in mammalian cells, HeLa cells were cotransfected with the Casp-1/lacZ expression vector (Miura *et al*, 1993) and a plasmid expressing DIAP-1 or DIHA, or controls. Figure 2 shows that both of the *Drosophila* IAPs were capable of suppressing apoptosis induced by overexpression of Caspase-1.

Experiments in insect cells have shown that substitution of either the BIRs or the RING finger of OpIAP with those from AcIAP abolishes their protective ability (Clem and Miller, 1994). This suggests that both the BIRs and the RING finger domain of IAPs are required for activity. Consistent with this idea, removal of the RING finger of OpIAP also abolished its protective potential in mammalian cells. (Duckett *et al*, 1996; Hawkins *et al*, 1996). In the context of the *Drosophila* eye, however, a truncated DIAP1 lacking its RING finger domain functioned more effectively than wild type to inhibit developmental, HID and REAPER induced death (Hay *et al*, 1995).

As it was unclear whether these different requirements for the RING finger domain reflected different actions of particular IAP proteins, or was due to differences in the cellular contexts in which they had been tested, we compared them directly using the same assay in which the deleted OpIAP construct was inactive (Hawkins *et al*, 1996).

As shown in Figure 2, removal of the RING finger from either of the *Drosophila* IAPs decreased the efficacy of their protection, with DIAP1 being more strongly affected than DIHA. These results are consistent with a model in which the BIRs are the key functional parts of the IAP proteins, but the requirement for the RING finger domain is only partial, and depends on the cellular context.

Determination of the range of apoptotic stimuli inhibited by OpIAP

Ligation of TNF and CD95 receptors OpIAP was the most efficient inhibitor of apoptosis in the assays we had performed previously (Hawkins *et al*, 1996; Uren *et al*, 1996), so it was chosen to determine which apoptotic signaling pathways utilize components which are susceptible to IAP inhibition. MCF-7 mammary carcinoma cells were transfected with a vector bearing the lacZ gene and either empty vector, p35 or OpIAP plasmids. Following transfection, the cells were either incubated with normal medium or treated with TNF at two doses, for either 24 h (Figure 3A) or 48 h (Figure 3B). The viability of the β-galactosidase positive cells was then scored. As shown in Figure 3, both p35 and OpIAP conferred substantial protection against TNF-mediated death in this cell line.

A subline of HeLa cells, which die in response to ligation of CD95, were used to assess whether OpIAP could inhibit apoptosis triggered in this way. Cells

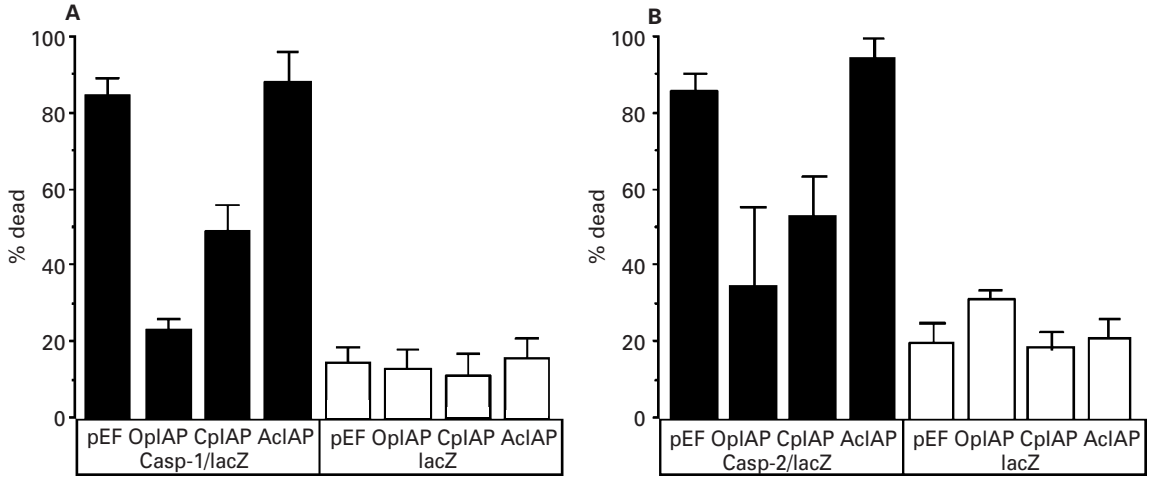

Figure 1 OpIAP and CpIAP but not AcIAP can prevent mammalian cell death induced by overexpression of Caspass 1 and 2. HeLa cells were transiently transfected with coded transfection mixtures of lipofectamine with either Casp-1/lacZ (**A**) or Casp-2/lacZ plasmids (**B**) and pEF with either no insert, or the coding regions of OpIAP, CpIAP or AcIAP. Parallel transfections were performed using the lacZ only plasmid insted of the caspase/lacZ fusion plasmids to assess background cell death casued by the transfection procedure. After 18 h the cells were fixed, stained and scored visually for viability. As in all Figures, error bars represent two standard errors of the mean (S.E.M.)

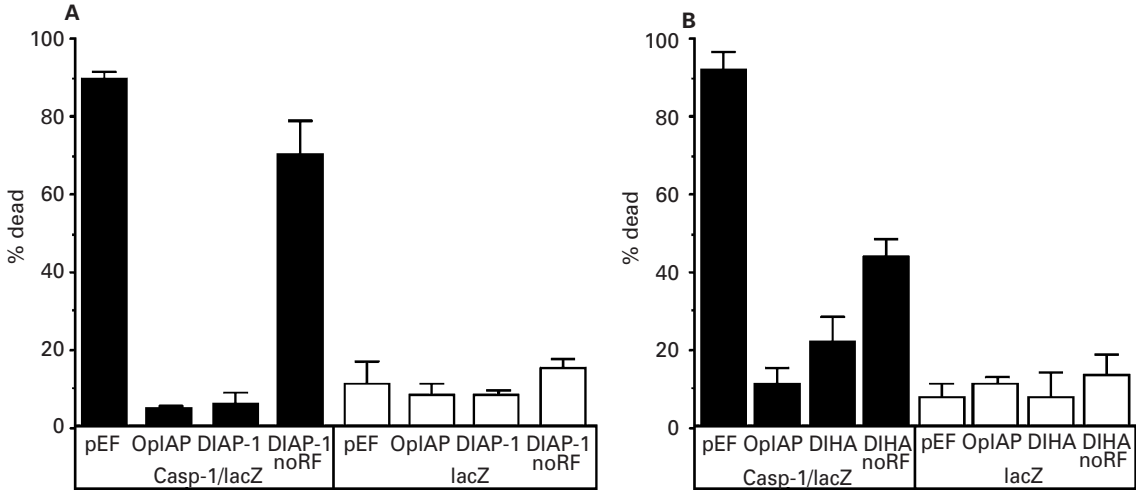

Figure 2 DIAP1 and DIHA can prevent mammalian cell death induced by Caspase-1, and truncated forms lacking the RING finger are less potent than full length genes. HeLa cells were transfected with Casp-1/lacZ and pEF vector containing either no insert, OpIAP or full length or truncated DIAP1 (**A**), or full length or truncated DIHA (**B**). Parallel transfections were performed using the lacZ plasmid instead of the caspase fusion plasmids to assess background cell death. After 18 h the cells were fixed, stained and scored. Error bars represent 2 × S.E.M.

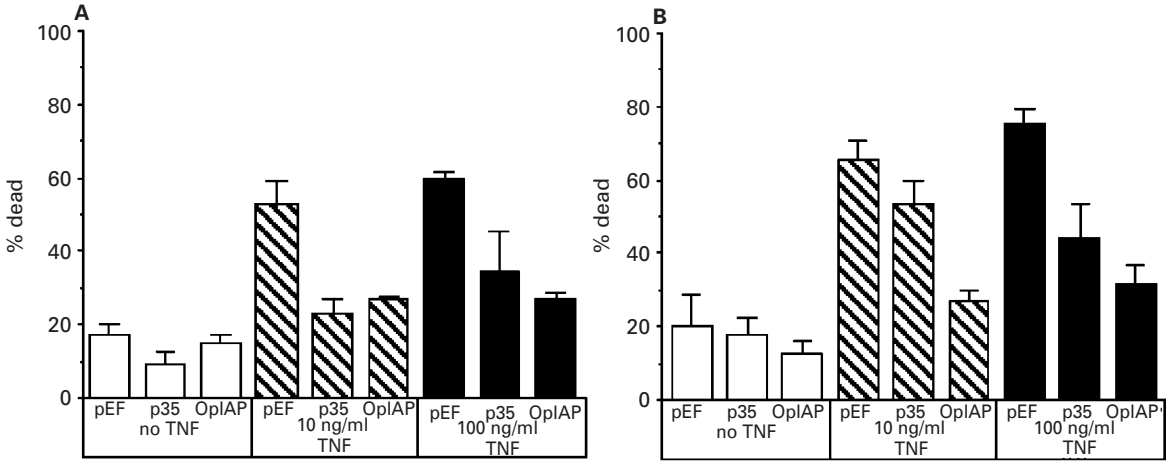

Figure 3 OpIAP can prevent TNF mediated mammalian cell death. MCF7 cells were transiently transfected with lacZ plasmid and pEF vector containing either no insert, p35 or OpIAP. After 18 h the medium was replaced with medium containing either no TNF, 10 ng/ml or 100 ng/ml TNF. After a further 24 h (**A**) or 48 h (**B**), the cells were fixed, stained and scored for viability. Error bars represent 2 × S.E.M.

transfected with plasmids encoding lacZ and apoptosis inhibitors were incubated for 48 h either with normal medium or medium containing anti-Apo-1 antibody. The β-galactosidase positive cells were then scored for viability. While most of the cells transfected with empty vector died, the cells transfected with plasmids encoding p35 survived (Figure 4). Cells receiving the OpIAP plasmid exhibited increased survival compared to the control cells, but this protection was not as efficient as that conferred by p35.

Factor or serum withdrawal The myeloid FDC-P1 cell line, which is dependent on IL-3 or GM-CSF for survival (Dexter *et al*, 1980) was used to test the ability of OpIAP to inhibit death induced by factor starvation. For these experiments, clonal lines stably expressing OpIAP were generated. Levels of expression were determined by Northern blot (Figure 5A).

As shown in Figure 5B, all of the OpIAP expressing lines died when cultured in the absence of IL-3. Furthermore, the rate of cell death was the same as for the parental cells and a Bax-expressing line. OpIAP must target a different apoptosis pathway to Bcl-w, as this Bcl-2 family protein was able to confer significant protection against growth factor withdrawal (Gibson *et al*, 1996).

The neuronal cell line PC12 was used to investigate the ability of OpIAP to inhibit apoptosis induced by serum starvation. When undifferentiated, these cells die in response to serum withdrawal, as shown in Figure 6. Cells were cotransfected with either the empty vector (pEF), or plasmids encoding apoptosis inhibitors, together with a Green Fluorescent Protein (GFP) vector as a marker of transfection. The green cells were sorted by flow cytometry and cultured for 2 days in medium either containing or lacking serum. The number of viable cells was then determined by an MTT assay, which stains cells with active mitochondria (Figure 6). Bcl-2 and (to a lesser extent) p35 protected PC12 cells from this apoptotic

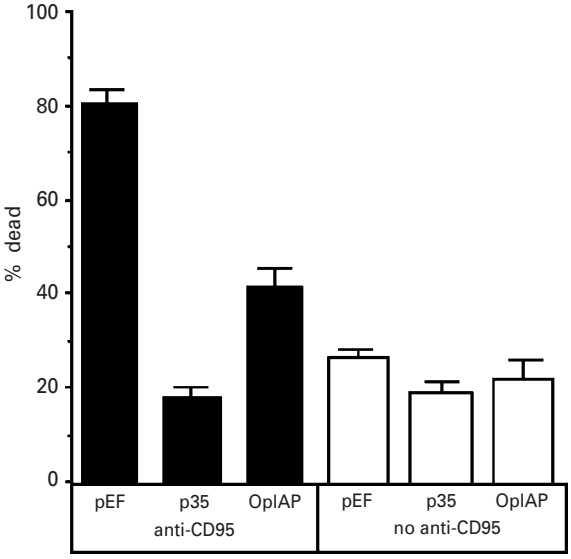

Figure 4 OpIAP inhibits CD95-mediated apoptosis. HeLa cells were transfected with lacZ plasmid together with pEF vector containing either no insert, p35 or OpIAP. After 18 h the medium was replaced with either normal medium, or medium containing anti-CD95 antibody at 1 μg/ml. After a further 48 h the cells were fixed, stained and scored for viability. Error bars represent 2 × S.E.M.

stimulus, as has been previously reported (Mah *et al*, 1993). OpIAP was not able to protect the cells from death due to serum withdrawal. This suggests that OpIAP interferes with different components of death signaling pathways than p35 and Bcl-2.

TRAF binding

The finding that two of the mammalian IAPs, MIHB and MIHC, could interact with components of the TNFRII signaling

machinery hinted that this may be the way by which IAPs inhibit cell death (Rothe *et al*, 1995). While the finding that OpIAP can inhibit TNF and CD95 mediated death is consistent with this idea, earlier work had shown that neither OpIAP nor MIHA could interact with TRAF1, 2 nor 3 (Uren *et al*, 1996). To establish whether the other baculoviral IAPs or the *Drosophila* IAPs could bind to the TRAFs, we analyzed them using the yeast two hybrid system. As shown in Figure 7, unlike MIHB and MIHC, none of the endogenous insect or viral IAPs tested could bind to TRAF1, TRAF2, TRAF3 in yeast.

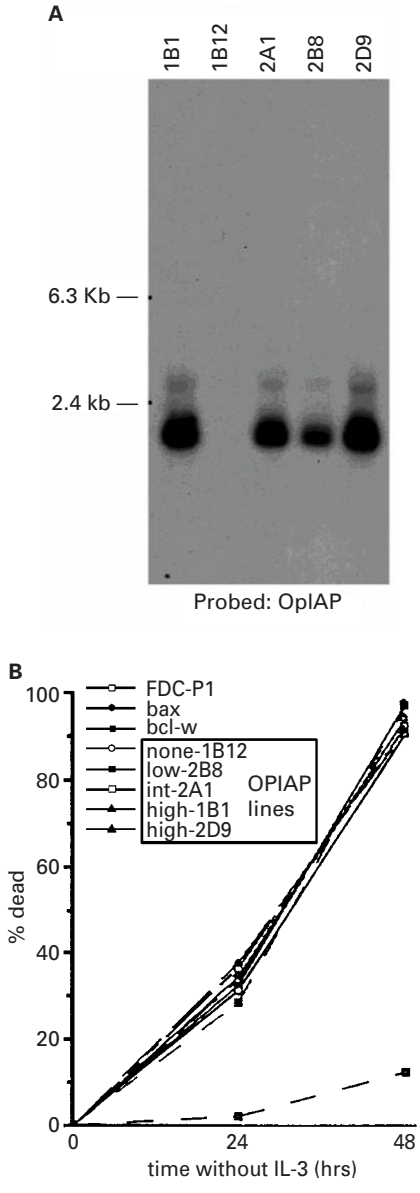

Figure 5 Stable FDC-P1 lines express a range of levels of OpIAP mRNA but were not protected from death induced by factor withdrawal. (**A**) Northern analysis of total RNA from stable FDC-P1 lines probed with OpIAP. (**B**) Wild-type FDC-P1 cells or lines stably expressing various levels of OpIAP, Bcl-w or Bax were incubated in medium in the presence or absence of IL-3. The cells assayed for viability at 24 and 48 h using PI exclusion followed by flow cytometric analysis

Discussion

The ability of the insect and baculoviral IAPs to inhibit apoptosis in mammalian cells corresponded to their ability to inhibit the death of insect cells (Birnbaum *et al*, 1994; Clem and Miller, 1994; Hay *et al*, 1995; Vucic *et al*, 1997). This presumably indicates that in these two systems IAPs interfere with homologous components of the apoptosis mechanism.

There has been some controversy regarding the requirement for the RING finger domain of IAPs for their ability to inhibit cell death. Data from experiments using OpIAP, both in insect (Clem and Miller, 1994) and mammalian cells (Hawkins *et al*, 1996) indicate that the RING finger is required for efficient protection. While we have found that the RING domain of MIHA is necessary for maximal activity (data not shown), Devereaux *et al* found that the RING finger of MIHA was dispensable for protection against apoptosis induced by expression of Bax (Deveraux *et al*, 1997). In the context of the *Drosophila* eye, however, the protective ability of DIAP1 is enhanced by removal of its RING finger domain (Hay *et al*, 1995).

In this paper we compared protection of full length *Drosophila* IAPs and the truncated forms lacking the RING fingers. In a system in which truncated OpIAP provided negligible protection, truncated DIAP1 and DIHA proteins (that conferred increased function in the *Drosophila* eye) gave drastically reduced protection. These results imply that the regulatory effect of the RING finger of the *Drosophila* IAPs is specific either to the cell type or the stimuli (developmental, irradiation, REAPER and HID overexpression induced death *versus* caspase overexpression), but is not a property of the proteins themselves. This may signify a difference in the components of the death signal transduction pathway(s) with which the IAPs interact in these situations, or different effects on protein stability of removing the RING domain in different cell types. While the impact of removal of the RING finger differs from system to system, in all cell types examined the BIRs are required for anti-apoptotic activity, indicating that they are the key functional domain of IAP proteins.

OpIAP was able to inhibit some pathways leading to cell death, but not others. In myeloid cells OpIAP was incapable of preventing death induced by IL-3 withdrawal, and in PC12 cells it could not protect against death due to serum starvation. OpIAP did, however, protect MCF7 cells from death caused by exposure to TNF, and was capable of rescuing most transfected HeLa cells from CD95-mediated apoptosis.

Levels of expression of untagged OpIAP in the stable cell lines were determined by Northern blot, as antibodies against OpIAP are not available, and tagged OpIAP proteins have significantly reduced protective ability in caspase overexpression assays (data not shown). Despite our inability to quantify OpIAP protein expression in the FDC-P1 stable cell lines, the vector used to generate these lines does function in other assays, implying that most likely OpIAP protein is present in the FDC-P1 cells, but not protective against factor withdrawal induced death.

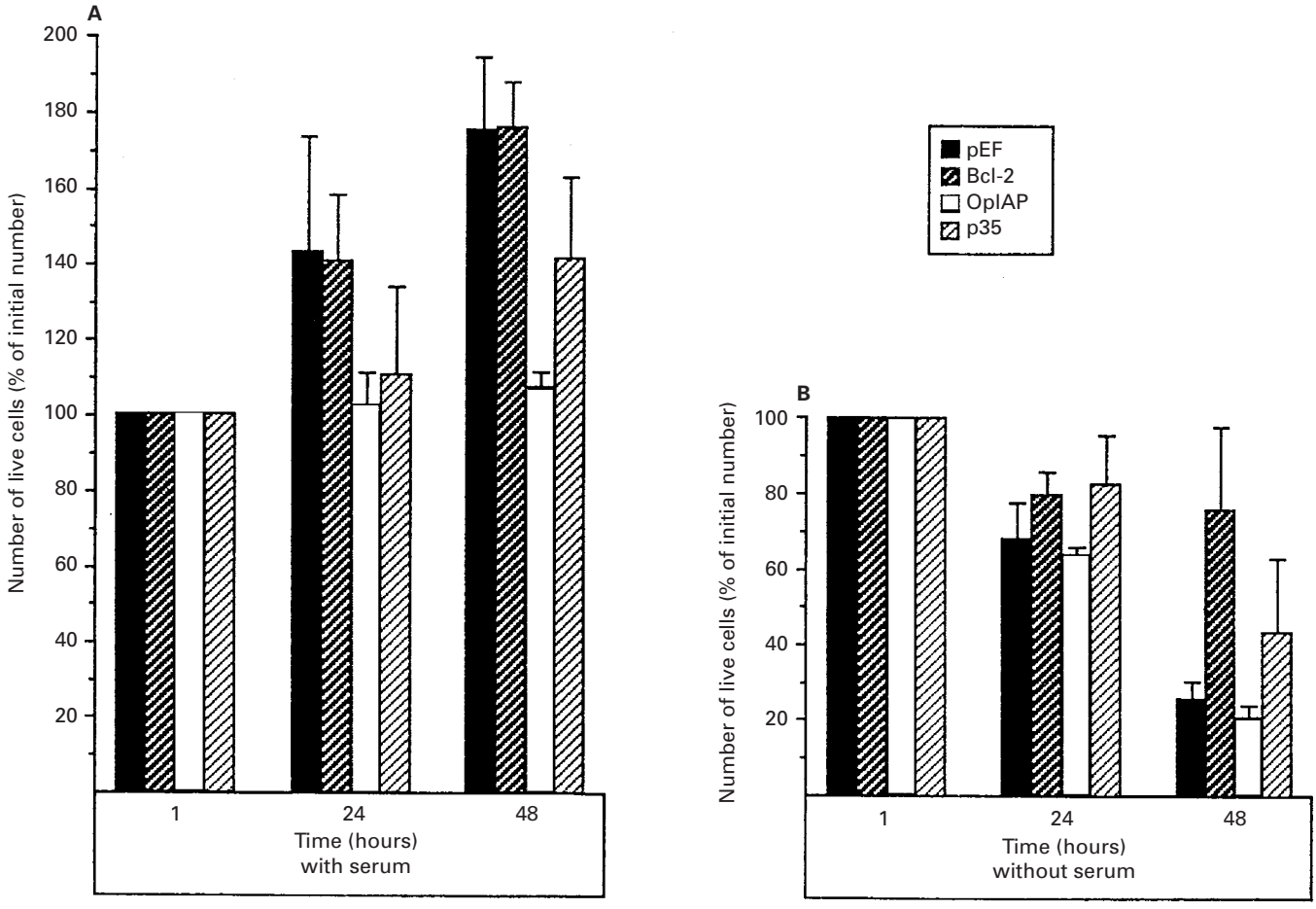

Figure 6 OpIAP does not protect PC12 cells from apoptosis triggered by serum starvation. PC12 cells were transfected with a plasmid expressing GFP along with the pEF vector with either no insert, p35, Bcl-2 or OpIAP. The green cells were sorted by flow cytometry. The following day they were cultured in medium either containing or lacking serum for 2 days. Viability was assessed by propidium iodide exclusion. Error bars represent 2 × S.E.M.

It is noteworthy that OpIAP behaved differently to Bcl-2 proteins and to p35, perhaps indicating differences either in the mechanisms of action or in specificity for substrates of these molecules. Bcl-2 and its relatives confer substantial protection against factor and serum withdrawal induced death in a variety of cell types (Vaux *et al*, 1988), but in most cell types offer little protection against apoptosis triggered by ligation of members of the TNF receptor family (Huang *et al*, 1997; Strasser *et al*, 1995). Interestingly, mammalian IAPs can prevent death induced by overexpression of the Bcl-2 antagonists Bak, Bik and Bax (Deveraux *et al*, 1997; Orth and Dixit, 1997). One interpretation of these two sets of data would be that factor withdrawal from dependent cells, although inhibitable by Bcl-2, does not require the action of Bak, Bik nor Bax, and hence cannot be blocked by overexpression of OpIAP. Recently published data from transgenic studies would support this hypothesis (Knudson and Korsemeyer, 1997).

The finding that of the eight IAPs tested to date, only MIHB and MIHC can bind to the TRAFs makes the hypothesis that the IAPs inhibit apoptosis by altering TRAF signaling seem unlikely.

The nature of the mechanism of action of the IAPs remains unresolved. Papers published recently present conflicting evidence on whether IAPs inhibit steps upstream of caspase activation or act downstream to directly bind to and inhibit activated caspases. Manji *et al* showed that OpIAP was unable to inhibit the active baculoviral host caspase, SfCasp-1 (Manji *et al*, 1997), whereas subsequent papers concluded that MIHA, B and C (XIAP, c-IAP-1 and c-IAP-2) directly bind and inhibit mammalian caspases 3 and 7 (Deveraux *et al*, 1997; Roy *et al*, 1997), but not to caspases 1, 6 or 8. It seems unlikely that different members of a family of genes so conserved in sequence and function would have different modes of action. It is possible that, as postulated by Deveraux *et al*, different members may be active against varying subsets of caspases (Deveraux *et al*, 1997). If this is true, it would argue that OpIAP's target in its host is an enzyme other than SfCasp-1. Both OpIAP and MIHA (X-IAP) can inhibit death induced by expression of pro-caspase-1 (Hawkins *et al*, 1996; Duckett *et al*, 1996; Uren *et al*, 1996). As MIHA does not bind to active caspase-1 (Deveraux *et al*, 1997), it, and by inference OpIAP, are likely to act by preventing signals that activate

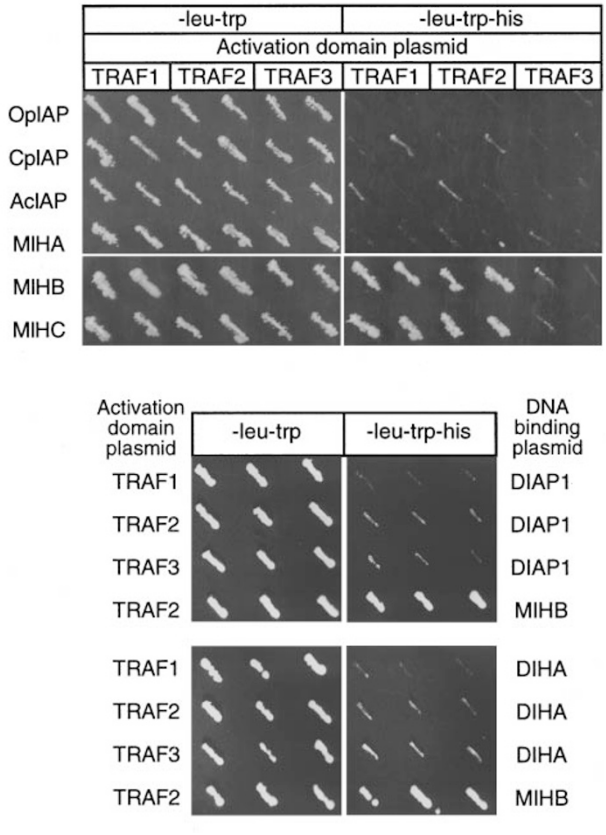

Figure 7 Neither the insect viral nor endogenous IAPs interact with TRAFs 1, 2 and 3. Yeast were cotransformed with TRAF and IAP plasmids, and the ability of the double transformants to grow on medium lacking histidine was determined. Only yeast containing MIHB or C and TRAF1 or 2 could grow on these plates, indicating that only these pairs of genes encode proteins which interact

pro-caspase-1 or by blocking caspases activated by caspase-1.

The differing ability of OpIAP and p35 to inhibit apoptosis of PC12 cells induced by serum withdrawal suggests that if OpIAP is a caspase inhibitor, it binds to a different set of caspases than p35. If the IAPs act by directly binding to active caspases, their ability to inhibit apoptosis may also depend on their stability and the abundance of the caspase. Differing observations in different cell types may therefore also reflect the stoichiometric ratios of proteins within each cell type.

Materials and Methods

Constructs

The cDNAs for OpIAP, CpIAP and AcIAP were a gift from Lois Miller (University of Georgia). The TRAF plasmids were provided by Mike Rothe (Tularik Inc). The pEF vector was provided by David Huang (WEHI). The yeast two hybrid system and the vector pGBT9 were from the Matchmaker kit (Clontech). The Casp-1/lacZ (pβactM11Z) and Casp-2/lacZ (pβactH37Z) plasmids were provided by Junying Yuan (Harvard) (Miura *et al*, 1993; Wang *et al*, 1994).

Details of the pEF, pEF-OpIAP and LacZ plasmids have previously been reported (Hawkins *et al*, 1996), as have the OpIAP, MIHA, MIHB, MIHC GBT plasmids (Uren *et al*, 1996).

CpIAP, AcIAP, DIAP1, and DIHA cDNAs were amplified by PCR to generate products bearing mammalian consensus Kozak sequences and *Bam*HI and *Xba*I restriction sites to allow subcloning into the vector pEF. For insertion into the yeast vector pGBT, cDNAs were amplified with primers to allow inframe insertion into *Eco*RI and *Bam*HI sites. Primers designed to insert a termination codon after amino acid 381 of DIAP-1 were used to generate DIAP-1noRF and after amino acid 442 of DIHA to generate pEF-DIHAnoRF. Constructs were verified by sequencing. Details of primers used in making these constructs will be provided upon request.

Expression studies

Yeast two hybrid analysis and lipofectamine mediated transient transfections were performed as described previously (Hawkins *et al*, 1996). Cells were cultured in RPMI with 10% FCS unless otherwise specified. All cells were transfected with coded mixtures of lipofectamine (Gibco) and DNA such that scoring of the assays was performed blind. Error bars on the figures represent two standard errors of the mean (S.E.M.). For the caspase death assays, the caspase/lacZ fusion plasmid and the test plasmid or control were transfected in a ratio of 1 : 10 to ensure lacZ positive cells contained the second plasmid.

MCF7 cells were transfected with a 1 : 10 ratio of lacZ to test plasmid, then 1 day later the medium was replaced with RPMI/10% FCS with either none, 10 or 100 ng/ml TNF. Either 24 or 48 h later the cells were fixed and stained for β-galactosidase activity using Xgal as previously described (Hawkins *et al*, 1996; Miura *et al*, 1993), and scored visually for viability.

For the CD95 ligation assay, HeLa cells were transfected with a 1 : 10 ratio of lacZ to test plasmid, then 1 day later the medium was replaced with either RPMI/10% FCS or medium containing 1 μg/ml anti-Apo1 (a kind gift from Peter Krammer). After 48 h the cells were fixed and scored for viability.

FDC-P1 cells were transfected with the pEF-OpIAP plasmid, and stable clones were selected using puromycin (2 μg/ml). These lines were analyzed by Northern blot to determine the levels of OpIAP. Following culturing in medium either containing or lacking IL-3, the viability of the cells was determined by flow cytometry for exclusion of propidium iodide (2 μg/ml).

PC12 cells were electroporated with a total amount of DNA of 15 μg, comprising pEGFP-C1 (Clontech) and either empty pEF vector, pEF-OpIAP, pEF-Bcl-2 or pEF-p35, in a ratio of 1 : 10, then plated in DME with 10% FCS and 5% horse serum for 48 h. The cells were then harvested using trypsin, resuspended in medium containing 2 μg/ml propidium iodide and sorted by flow cytometry for green viable cells. Following overnight culture, the cells were removed using trypsin, washed twice in medium lacking serum, and replated in either DME with serum (10% FCS+5% horse serum) or DME without serum, in Terisaki plates. After 1, 24 or 48 h, cell viability was assessed by incubating with MTT for at least 15 min at 37°C.

Acknowledgements

We wish to thank Junying Yuan, Peter Krammer, David Huang, Lois Miller, and Mike Rothe for reagents. This work was supported by a grant to DLV by the Anti-Cancer Council of Victoria.

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