

Cloning and expression of apoptosis inhibitory protein homologs that function to inhibit apoptosis and/or bind tumor necrosis factor receptor-associated factors

(mammalian homolog of inhibitor of apoptosis protein/interleukin 1 β converting enzyme/FADD protein)

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ABSTRACT Baculovirus inhibitors of apoptosis (IAPs) act in insect cells to prevent cell death. Here we describe three mammalian homologs of IAP, MIHA, MIHB, and MIHC, and a *Drosophila* IAP homolog, DIHA. Each protein bears three baculovirus IAP repeats and an N-terminal ring finger motif. Apoptosis mediated by interleukin 1 β converting enzyme (ICE), which can be inhibited by *Orgyia pseudotsugata* nuclear polyhedrosis virus IAP (OpIAP) and cowpox virus crmA, was also inhibited by MIHA and MIHB. As MIHB and MIHC were able to bind to the tumor necrosis factor receptor-associated factors TRAF1 and TRAF2 in yeast two-hybrid assays, these results suggest that IAP proteins that inhibit apoptosis may do so by regulating signals required for activation of ICE-like proteases.

The mechanisms for apoptosis have been strongly conserved during evolution (1). For example, proteins resembling Bcl-2 can protect nematode, insect, and vertebrate cells from apoptosis (2–4), and cysteine proteases resembling interleukin 1 β converting enzyme (ICE) are required for apoptosis in both *Caenorhabditis elegans* and mammals (5–7). Although many apoptosis effector proteases and numerous stimuli that induce apoptosis have been found, little is known about the signaling and activation pathways that connect the cell-death stimuli to the apoptosis-effector mechanisms. We hoped that the study of viral anti-apoptosis proteins might reveal something about the intermediate steps of apoptosis signaling.

Apoptosis can be used as a defense against viruses, but many viruses carry genes for anti-apoptosis proteins, presumably to keep the host cell alive while the viruses replicate. Some viral anti-apoptosis proteins resemble known cellular proteins such as Bcl-2 (8). Others, such as the baculovirus p35 proteins, have no known cellular counterparts but can function in heterologous systems, such as nematodes and mammals, where they are thought to act as competitive inhibitors of ICE-like cysteine proteases (9–13). Miller and coworkers (14, 15) identified a family of proteins in baculoviruses they designated inhibitor of apoptosis proteins (IAPs) because these proteins could inhibit the apoptotic response of insect cells to viral infection. Viral IAP proteins typically have two N-terminal repeats designated baculovirus IAP repeats (BIRs) and a C-terminal RING finger domain.

The IAP protein from *Orgyia pseudotsugata* nuclear polyhedrosis virus (OpNPV) can inhibit ICE-mediated apoptosis in mammalian cells, so it must be able to interact with conserved components of the apoptotic mechanism (unpublished work). The discovery that NAIP, one of the candidate genes for spinal muscular atrophy, bore BIRs confirmed the existence of a mammalian IAP-like gene (16), but the function

of NAIP has not yet been determined. To see if there were other cellular IAP homologs and to determine if they function in cell death pathways, we undertook a search for genes encoding novel IAP proteins and tested their ability to inhibit apoptosis mediated by ICE and by FADD (a protein associated with the cytoplasmic domain of CD95). One IAP homolog gene was found in *Drosophila* (DIHA) and three IAP homolog genes were identified in mammalian cells (MIHA, MIHB, and MIHC). Sequence and functional analyses of the proteins encoded by these genes show that MIHA and MIHB can inhibit apoptosis and MIHB and MIHC can bind to the tumor necrosis factor (TNF) receptor-associated factors TRAF1 and TRAF2 (17).

MATERIALS AND METHODS

cDNA Cloning. A human X chromosome genomic sequence-tagged site (GenBank no. L24579) was used to design PCR primers. A product was amplified and used to screen a human genomic DNA library (Stratagene). A fragment isolated from this library was used to probe a mouse liver cDNA library (Stratagene) at low stringency, yielding three murine cDNA clones that were designated mammalian IAP homolog A (MIHA). The human expressed sequence tag sequences (GenBank nos. R19628 and T96284) were used to design PCR primers that were used to generate probes for screening a human fetal liver cDNA library (Stratagene). The hybridizing cDNA clones were designated MIHB and MIHC, respectively. The *Drosophila* genomic sequence (GenBank code DROC-CAAT) was used to amplify a 900-bp PCR product from *Drosophila* cDNA, which was used to screen an oligo(dT)-primed *Drosophila* larval cDNA library constructed in Lambda ZAP (Stratagene). A 2-kb cDNA (DIHA) clone encoding all but the 16 N-terminal amino acids was isolated. A genomic fragment encoding these amino acids was amplified by PCR and used to complete the DIHA coding region.

RNA Analysis. Radiolabeled *mMIHA* (m, murine) and *GAPDH* (encoding glyceraldehyde-3-phosphate dehydrogenase) were hybridized at high stringency to a mouse tissue Northern blot bearing 5 μ g of total RNA per lane. A mouse multiple tissue Northern blot (Clontech) bearing 2 μ g of poly(A)⁺ RNA was probed with radiolabeled *hMIHC* (h, human) at low stringency, stripped, probed with *hMIHB* at low stringency, stripped again, and probed at high stringency with a β -actin probe according to the manufacturer's instructions.

Yeast Two-Hybrid System. The coding regions of MIHA, MIHB, and MIHC were amplified by PCR and subcloned into

Abbreviations: ICE, interleukin 1 β converting enzyme; IAP, inhibitor of apoptosis protein; BIR, baculovirus IAP repeat; TNF, tumor necrosis factor; TRAF, TNF receptor-associated factor.

Data deposition: The sequences reported in this paper have been deposited in the GenBank data base [accession nos. U36842 (MIHA), U37547 (MIHB), U37546 (MIHC), and U38809 (DIHA)].

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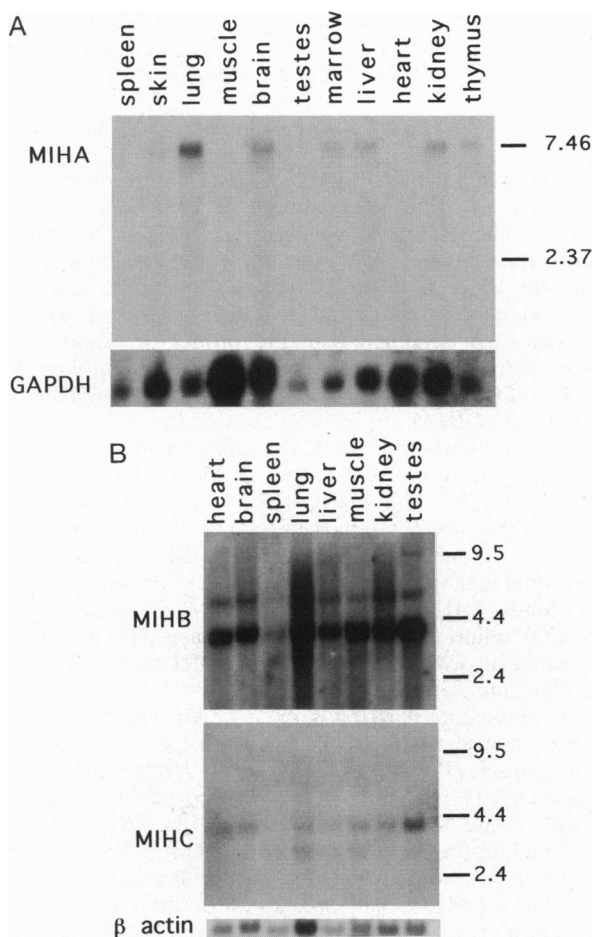


FIG. 2. Mammalian IAP homologs are expressed in a variety of tissues. (A) An adult mouse tissue total RNA Northern blot was probed with the *mMIHA* cDNA coding region at high stringency and a *GAPDH* probe to indicate loading. (B) An adult mouse tissue poly(A)⁺ RNA Northern blot (Clontech) was probed with the *hMIHB* cDNA coding region and the *hMIHC* cDNA coding region at low stringency. A β -actin probe was used to indicate loading.

transcripts may also represent other closely related mammalian IAP homologs.

The mammalian IAP homologs were tested for their ability to prevent apoptosis due to two stimuli: overexpression of p32 ICE and overexpression of FADD. Transfection of cells, such as HeLa cells with constructs expressing the precursor of the cysteine protease ICE, have previously been shown to cause cell death exhibiting all of the classic features of apoptosis, including DNA degradation (6, 22).

As the ICE precursor protein is enzymatically inactive when translated, the mechanisms that process it must be constitutively active in the cells, presumably at a low level that is insufficient to cause apoptosis without the introduction of large amounts of ICE precursor. Baculoviral *IAP* can prevent apoptosis due to transfection of *p32ICE*, as can other anti-apoptosis genes such as *bcl-2*, *crmA*, and *p35* (refs. 6 and 12; Hawkins and Vaux, unpublished work). We tested MIHA, MIHB, and MIHC to determine whether they too could block apoptosis caused by ICE overexpression. HeLa cells were cotransfected with a plasmid bearing an ICE-lacZ fusion construct together with plasmids encoding the IAP homologs or controls. The cells were stained with X-Gal to identify those that had been transfected; these were assessed visually for viability. As shown in Fig. 3A, MIHA, MIHB, and OpIAP significantly reduced the amount of death caused by ICE, whereas MIHC did not provide detectable protection.

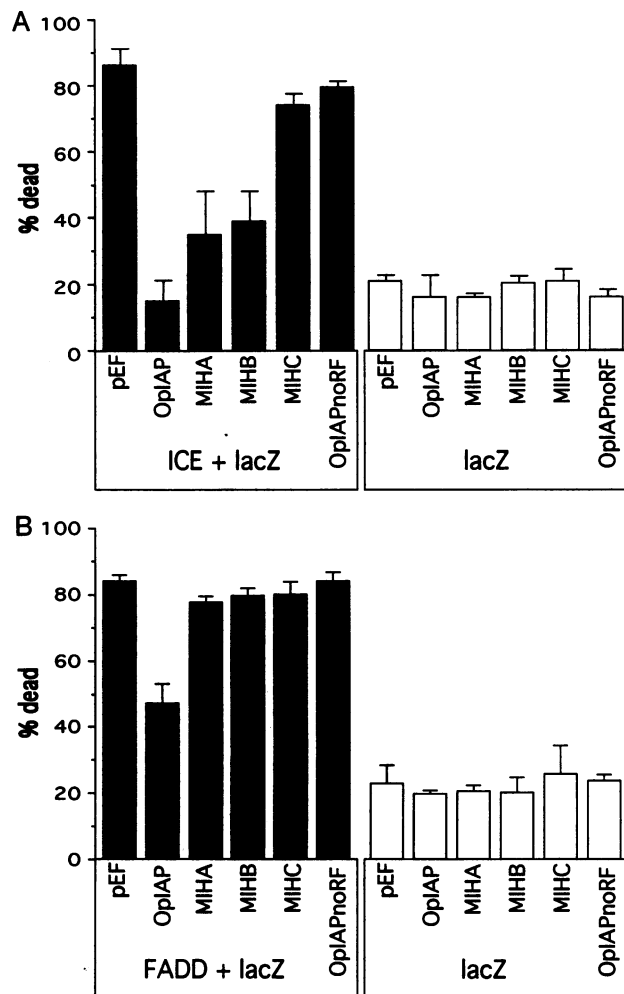


FIG. 3. MIHA and MIHB protect against death induced by overexpression of ICE but not FADD. (Upper) Induction of apoptosis by transfection with ICE. Columns 1–6 (solid bars) indicate the percentage of dead cells cotransfected with *p32ICE-lacZ* fusion plasmid and the plasmids bearing either the IAP homologs or controls. Death of cells cotransfected with *lacZ* only, together with the same test plasmids, is shown in columns 7–12 (open bars) and indicates the amount of cell death due to the transfection procedure itself. (Lower) Induction of apoptosis by transfection with FADD. Plasmids encoding the IAP homologs were cotransfected with a *lacZ* vector and a construct bearing the FADD coding region (columns 1–6). As with the ICE experiment, background death was monitored in a parallel set of cultures (columns 7–12). In both ICE and FADD experiments, each column represents the average of three separate transfections conducted in parallel; on average, >400 cells were counted in each transfection. Error bars indicate ± 2 SEM; randomly coded assays were read blind.

Enforced expression of the CD95-associated protein FADD also causes cell death (21, 23). We cotransfected HeLa cells with three plasmids: a FADD expression construct, a plasmid carrying the *lacZ* gene, and vectors encoding the mammalian IAP homologs or OpIAP. OpIAP provided partial protection against FADD, but it was not as effective as it was against ICE (compare column 2 in Fig. 3A and B). We could not detect any reduction in the amount of FADD-induced cell death by MIHA, MIHB, or MIHC.

Two of the mammalian IAP homologs, MIHB and MIHC, have also been isolated independently by M. Rothe (personal communication) as part of a protein complex that binds to the cytoplasmic domain of TNF-R2 (p75) together with TRAF1 and TRAF2 (17). We used the yeast two-hybrid system (24) to test the ability of all three mammalian IAP homologs and viral

Table 1. Yeast two-hybrid assays for binding between TRAF1, TRAF2, TRAF3, and mammalian IAP homologs

Transformant		Growth on Trp ⁻ , Leu ⁻ , His ⁻ medium	Colony color
DNA-binding hybrid	Activation hybrid		
OpIAP	TRAF1	—	—
MIHA	TRAF1	—	—
MIHB	TRAF1	+	++
MIHC	TRAF1	+	++
<i>c-jun</i>	TRAF1	—	—
OpIAP	TRAF2	—	—
MIHA	TRAF2	—	—
MIHB	TRAF2	+	+
MIHC	TRAF2	+	+
<i>c-jun</i>	TRAF2	—	—
OpIAP	TRAF3	—	—
MIHA	TRAF3	—	—
MIHB	TRAF3	—	—
MIHC	TRAF3	—	—
<i>c-jun</i>	TRAF3	—	—
OpIAP	<i>fos</i>	—	—
MIHA	<i>fos</i>	—	—
c-IAP1	<i>fos</i>	—	—
MIHC	<i>fos</i>	—	—
<i>c-jun</i>	<i>fos</i>	+	+++

The yeast strain HF7c was cotransformed with constructs that express fusion proteins between the GAL4 DNA-binding domain and the IAP family members or controls, and vectors that encode fusions between the TRAF proteins or controls and the GAL4 activation domain. Expression from the *his* and *lacZ* reporter genes (which indicates interactions) was analyzed by growth of double transformants on medium lacking histidine and blue staining of colonies with X-Gal. *c-jun* and *fos* were used as control genes encoding interacting proteins in the DNA-binding vector and activation vector, respectively.

OpIAP to bind TRAF1, TRAF2, and TRAF3, a related protein also known as CD40BP/CRAF-1/CAP1 (18, 25, 26). As shown in Table 1, yeast cotransfected with MIHB or MIHC together with TRAF1 or TRAF2, but not TRAF3, were rendered His⁺ and LacZ⁺, confirming the observations of M. Rothe (personal communication). In contrast, no interactions were detectable in this system between any of the TRAFs tested and OpIAP or MIHA. These results show that MIHB and MIHC can bind to TRAF1 and TRAF2, but suggest that OpIAP and MIHA interact with other proteins or that OpIAP and MIHA do not function in the yeast assays the same way as they do in mammalian cells.

DISCUSSION

We have described three novel mammalian IAP proteins and an IAP homolog from *Drosophila*. The amino acid sequence of these proteins shows considerable conservation between flies and mammals, with all four coding regions containing three BIR and one RING finger motifs. Thus, in addition to the Bcl-2 family and the ICE family, a third family of proteins has been found whose structure and function are evolutionarily conserved in physiological cell death pathways. Much of what we do know about apoptosis has come from studying inhibitors that viruses use to prevent defensive cell death. This work was undertaken with the hope that study of viral IAPs and their cellular counterparts would reveal something about the less well-characterized stages of the cell death process.

Here we have demonstrated that MIHA and MIHB, like the baculoviral OpIAP, significantly reduce apoptosis caused by transfection of HeLa cells with the ICE precursor (Fig. 3A), thus establishing a role for mammalian IAP homologs in regulating apoptosis. In this assay, the cowpox protein CrmA can also inhibit apoptosis induced the same way by acting late

in the pathway as a competitive inhibitor of the ICE protease (6, 27). The baculovirus anti-apoptosis protein p35 acts similarly to block the activated effector protease (12, 13). Bcl-2 and some of its homologs can counter apoptosis mediated by ICE and its relatives (6), but their mechanism of action is unknown. How then do the IAPs inhibit apoptosis? While it is possible that they act like CrmA and p35 to block the active protease, we think it is more likely that they operate at an earlier stage to prevent activation of ICE, which must be cleaved from its precursor and assembled into a tetramer before it can function (28, 29).

It is curious that MIHA and MIHB could inhibit apoptosis, but MIHC, which resembles MIHB much more closely than MIHA does, appeared to be inactive. It is unlikely that this is due to an inadvertent mutation of the *MIHC* coding region, as independently cloned *MIHB* and *MIHC* cDNAs in a different expression construct gave the same results [data not shown; c-IAP1 (*MIHB*) and c-IAP2 (*MIHC*) constructs provided by M. Rothe]. The differing behavior of MIHB and MIHC can be attributed to either a quantitative difference in protein stability, affinity for targets, or threshold for activity, or MIHB and MIHC are qualitatively different. It is also possible that some IAP molecules have no role in regulating apoptosis. For example, *Autographa californica* NPV encodes an IAP (AcIAP) that does not block apoptosis, but it may have another function (30).

ICE is implicated in apoptosis caused by CD95 ligation, TNF, TRADD, and FADD (7, 21, 31, 32). OpIAP protects better against apoptosis caused by transfection with ICE than against FADD. MIHA and MIHB do not block FADD-induced apoptosis but can reduce apoptosis caused by ICE (Fig. 3). One possible explanation for this variability is that ICE may be activated differently in the two assays. When cells are transfected with ICE precursor, it must become activated by constitutive activation signals that are insufficient to cause apoptosis before transfection. It may be easier for IAPs to overcome these signals than to overcome the higher level of activation signals caused by transfection with FADD.

MIHB and MIHC can bind to TRAF1 and TRAF2 in yeast two-hybrid assays, and MIHB and MIHC have been found in protein complexes with the TNF-R2 cytoplasmic domain in mammalian cells (M. Rothe, personal communication). Therefore, it is possible that some IAPs act to mediate or modulate receptor signaling. Several members of the TNF family of receptors can transmit life-or-death signals to a cell when bound by their cognate ligands. CD95 and TNF-R1 (p55) can send death signals via their associated proteins, FADD, RIP, and TRADD (21, 23, 32, 33). Although binding of TNF-R2 is not usually associated with induction of apoptosis, in some circumstances it too can send a death signal (34–36), which may require TRAF proteins. Curiously, however, TRAF proteins have not yet been shown to regulate cell death signals, but they are required for activation of NF- κ B by TNF-R2 (37). A role for IAPs in receptor signaling is consistent with an upstream model for IAPs, where IAPs regulate signals required for the processing and activation of cysteine proteases rather than binding to and inhibiting them as do crmA and p35.

MIHA and OpIAP did not interact with TRAF1, TRAF2, or TRAF3 in the yeast two-hybrid assays. This suggests that TRAF binding ability of IAPs may not correlate with their anti-apoptotic activity, although conditions in yeast may not accurately reflect conditions in mammalian cells. A more interesting possibility is that there are other cellular targets of MIHA and OpIAP (and perhaps MIHB and MIHC) which mediate their anti-apoptotic function. These may be novel TRAF proteins or unrelated molecules.

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