Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 4974–4978, May 1996
Cell Biology

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 1β converting enzyme/FADD protein)

ANTHONY G. UREN, MIHA PAKUSCH, CHRISTINE J. HAWKINS, KIRSTEN L. PULS, AND DAVID L. VAUX

The Walter and Eliza Hall Institute of Medical Research, Post Office Royal Melbourne Hospital, Victoria 3050, Australia

Communicated by G. J. V. Nossal, The Walter and Eliza Hall Institute of Medical Research, Victoria, Australia, January 17, 1996 (received for review December 14, 1995)

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 1β 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 1β 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 DROCCAAT) 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 gylceraldhyde-3-phosphate dehydrogenase) were hybridized at high stringency to a mouse tissue Northern blot bearing 5 μg of total RNA per lane. A mouse multiple tissue Northern blot (Clontech) bearing 2 μg 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

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Abbreviations: ICE, interleukin 1β 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*)].

the pGBT9 vector (Clontech) such that the proteins would be expressed as in-frame fusions with the GAL4 DNA-binding domain. The *OpIAP* gene from the *Hin*dIII site 17 codons upstream of the initiating ATG was also subcloned into the pGBT9 vector so that an in-frame fusion would result. TRAF1, TRAF2, and TRAF3 expression vectors have been described (17, 18) and were kindly provided by M. Rothe (Tularik, South San Francisco, CA). Vectors with the coding regions of c-*jun* in pGBT9 and *fos* in pGAD424 were used as controls for the detection of interacting proteins. The yeast strain HF7c was transformed with these plasmids by using the lithium acetate protocol (19).

Transient Transfection Assays. A 1.8-kb coding fragment from a *MIHA* cDNA clone was subcloned into pEF, a derivative of the pEFBOS vector (20) modified by D. Huang (The Walter and Eliza Hall Institute). *Pfu* DNA polymerase (Stratagene) was used to amplify the coding regions of *MIHB* and *MIHC*, which were subcloned into pEF. The p32ICE–*lacZ* fusion plasmid pβactM11Z was kindly provided by J. Yuan (6). The FADD expression construct FADD-AU1 was obtained from V. Dixit (21). The coding regions of *bcl-2, crmA,* and *p35* from AcNPV and *IAP* from OpNPV were inserted into the pEF vector. A fragment encoding β-galactosidase was expressed from the mouse cytomegalovirus promoter. The truncated *OpIAP* plasmid was constructed by digestion of the pEF vector containing full-length *IAP* with *Nru*I and *Sma*I, and religating. This deleted sequences 3′ of the *Nru*I site in the *OpIAP* gene that encode the RING finger domain.

Subconfluent cultures of HeLa cells grown in RPMI medium 1640 with 10% fetal calf serum were transfected with 0.1 μg of *ICE–lacZ* with 1 μg of test plasmid in 3 μl of Lipofectamine (GIBCO) per well in 12-well tissue culture plates. To assess protection against FADD, HeLa cells were transfected with 0.1 μg of *lacZ* plasmid, 0.45 μg of FADD expression plasmid, and 0.45 μg of test plasmid. In control experiments, 0.1 μg of *lacZ* plasmid and 1 μg of test plasmid were used. After 16-hr incubation, the cells were fixed in 2% formaldehyde plus 0.2% glutaraldehyde for 5 min and stained for β-galactosidase expression with 0.1% 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM $MgCl_2$ in PBS. The blue cells were scored visually as alive or dead. All scoring was carried out blind on randomly coded wells.

RESULTS

To identify cellular IAP homologs, we undertook data base searches (Jan.–June 1995) for genes encoding novel IAP proteins. The searches revealed a *Drosophila* genomic sequence and a number of mammalian sequences that resembled either the BIRs or the RING finger domain of viral IAPs. These sequences were used to generate probes by PCR. Libraries were screened with the probes to isolate cDNA clones, which we designated mammalian IAP homologs A, B, and C (*mMIHA*, *hMIHB*, and *hMIHC*) and *Drosophila* IAP homolog A (*DIHA*).

Fig. 1 compares the predicted amino acid sequences for DIHA (predicted molecular mass, 55 kDa), MIHA (56 kDa), MIHB (70 kDa), and MIHC (68 kDa). Start codons were chosen as the most 5′ methionine with upstream, in-frame stop codons. All four proteins bear three BIR repeats in the N-terminal half and a single RING finger domain close to the C terminus. MIHB and MIHC are the most closely related, with 73% amino acid identity. MIHA shares 43% identity and 62% similarity with MIHB and MIHC. DIHA has 35% identity and 56% similarity with MIHC.

The message for *mMIHA* is about 7.5 kb, and it is expressed in most mouse tissues with the exception of skeletal and cardiac muscle (Fig. 2*A*). A cDNA of *hMIHB* hybridized at low stringency to two messages of ≈4.0 kb and ≈5.5 kb on a mouse multiple tissue Northern blot analysis (Fig. 2*B*). The upper, less abundant transcript was expressed least in the spleen and skeletal muscle and at higher levels in all other tissues analyzed. The more abundant ≈4.0-kb transcript was expressed at lowest levels in the spleen and at highest levels in the testes. An additional transcript of ≈9.5 kb was detected in the testes but not seen in other tissues. A full-length *hMIHC* cDNA probe hybridized at low stringency to two messages of ≈3.0 and ≈4.0 kb (Fig. 2*C*). It is possible that the upper (4.0 kb) transcript is the same as that detected by the *MIHB* probe. Some of the

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     1                                                  50                                                 100
MIHB MHKTASQRLF PGPSYQNIKS IMEDSTILSD WTNS.NKQKM KYDFSCELYR MSTYSTFPAG VPVSERSLAR AGFYYTGVND KVKCFCCGLM LDNWKLGDSP
MIHC .......... ........MN IVENSIFLSN LMKSANTFEL KYDLSCELYR MSTYSTFPAG VPVSERSLAR AGFYYTGVND KVKCFCCGLM LDNWKRGDSP
MIHA                      MTFNSFEGT RTFVLADTNK DEEFVEEFNR LKTFANFPSS SPVSASTLAR AGFLYTGEGD TVQCFSCHAA IDRWQYGDSA
DIHA                                                MT ELGMELESVR LATFGEWPLN APVSAEDLVA NGFFATGNWL EAECHFCHVR IDRWEYGDQV

     101                                                150                                                200
MIHB IQKHKQLYPS CSFIQNLVS. ASLGSTSKNT ..SPM..... ....RNSFAH SLSPTLEHSS LFSGSYSSLS PNPLNSRAVE DISSSRTNPY SYAMSTEEAR
MIHC TEKHKKLYPS CRFVQSLNSV NNLEATSQPT FPSSV..... ....TNS.TH SLLPGTENSG YFRGSYSNSP SNPVNSRANQ DFSALMRSSY HCAMNNENAR
MIHA VGRHRRISPN CRFINGFYFE NGAAQSTNPG IQNGQYKSEN CVGNRNPFAP DRPPETHADY LLRTGQVVDI SDTIYPRNP. .......... ..AMCSEEAR
DIHA AERHRRSSPI CSMV..... .......... .......... ..LAPNS.MC GNVPRSQESD NEGNSVV... .DSPESCSPD .......... ...LLLEANR

     201                                                250                                                300
MIHB FLTYHMWP.L TFLSPSELAR AGFYYIGPGD RVACFACGGK LSNWEPKDDA MSEHRRHFPN CPFL..... ...ENSL.ET LRFSIS.... .NLSMQTHAA
MIHC LLTFQTWP.L TFLSPTDLAK AGFYYIGPGD RVACFACGGK LSNWEPKDNA MSEHLRHFPK CPFI..... ...ENQLQDT SRYTVS.... .NLSMQTHAA
MIHA LKSFQNWPDY AHLTPRELAS AGLYYTGADD QVQCFCCGGK LENWEPCDRA WSEHRRHFPN CFFVLGRNVN VRSESGVSSD RNFPNSTNSP RNPAMAEYEA
DIHA LVTFKDWPN. PNITPQALAK AGFYYLNRLD HVKCVWCNGV IAKWEKNDNA FEEHKRFFPQ CPRVQMGPLI EFATGKNLDE LGIQ.PTTLP LRPKYACVDA

     301                                                350                                                400
MIHB RMRTFMYWPS SVPVQPEQLA SAGFYYVGRN DDVKCFCCDG GLRCWESGDD PWVEHAKWFP RCEFLIRMKG QEFVDEIQGR YPHLLEQLLS TSDTTGEENA
MIHC RFKTFFNWPS SVLVNPEQLA SAGFYYVGNS DDVKCFCCDG GLRCWESGDD PWVQHAKWFP RCEYLIRIKG QEFIRQVQAS YPHLLEQLLS TSDSPGDENA
MIHA RIVTFGTWTS SV..NKEQLA RAGFYALGEG DKVKCFHCGG GLTDWKPSED PWEQHAKWYP GCKYLLDEKG QEYINNIH.L THSLEESLGR T..... .A
DIHA RLRTFTDWPI S.NIQPASLA AAGLYYQKIG DQVRCFHCNI GLRSWQKEDE PWFEHAKWSP KCQFVLLDKG PAYVSEV... .......LAT TAA.....NA

     401                                                450                                                500
MIHB DPPIIHFGPG ESSSEDAVMM NTPVVKSALE MGFNRDLVKQ TVQSKILTTG ENYKTVNDIV SALLNAEDEK REEEKEKQAE EMASDDLSLI RKNRMALFQQ
MIHC ESSIIHFEPG EDHSEDAIMM NTPVINAAVE MGFSRSLVKQ TVQRKILATG ENYRLVNDLV LDLLNAEDEI REEERERATE EKESNDLLLI RKNRMALFQH
MIHA EKT.....PS LTKKIDDTIF QNPMVQEAIR MGFSFKDIKK TMEEKIQTSG SSYLSLEVLI ADLVSAQKDN TEDE..... .......... ..........
DIHA SSPTATA.PA PTLQADVLMD EAPA.KEALA LGIDGGVVRN AIQRKLLSSG CAFSTLDELL HDIFDDAGAG AALEVREPPE PSAP..... ..........

     501                                                550                                                600
MIHB LTCVLPILDN LLKANVINKQ EHDIIKQKTQ IPLQARELID TILVKGNAAA NIFKNCLKEI DSTLYKNLFV DKNMKYIPTE DVSGLSLEEQ LRRLQEERTC
MIHC LTCVIPILDS LLTAGIINEQ EHDVIKQKTQ TSLQARELID TILVKGNIAA TVFRNSLQEA EAVLYEHLFV QQDIKYIPTE DVSDLPVEEQ LRRLQEERTC
MIHA .......... .......... .......SSQ TSLQ..... .......... .......... .......... ..KDISTEEQ LRRLQEEKLC
DIHA ..FIEPCQAT TSKAASVPIP VADSIPAKPQ A......... .......... .........A EAVANISKIT DEIQKMSVAT PNGNLSLEEE NRQLKDARTC

     601                                                648
MIHB KVCMDKEVSV VFIPCGHLVV CQECAPSLRK CPICRGIIKG TVRTFLS*
MIHC KVCMDKEVSI VFIPCGHLVV CKDCAPSLRK CPICRSTIKG TVRTFLS*
MIHA KICMDRNIAI VFVPCGHLVT CKQCAEAVDK CPMCYTVITF KQKIFMS*
DIHA KVCLDEEVGV VFLPCGHLAT CNQCAPSVAN CPMCRADIKG FVRTFLS*
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FIG. 1. Comparison of deduced peptide sequences of IAP proteins. Comparison of MIHB, MIHC, MIHA, and DIHA. Amino acids shared by three or more of the proteins are in boldface type. Arrows indicate the three BIRs. The RING finger domain is indicated by a dashed arrow.

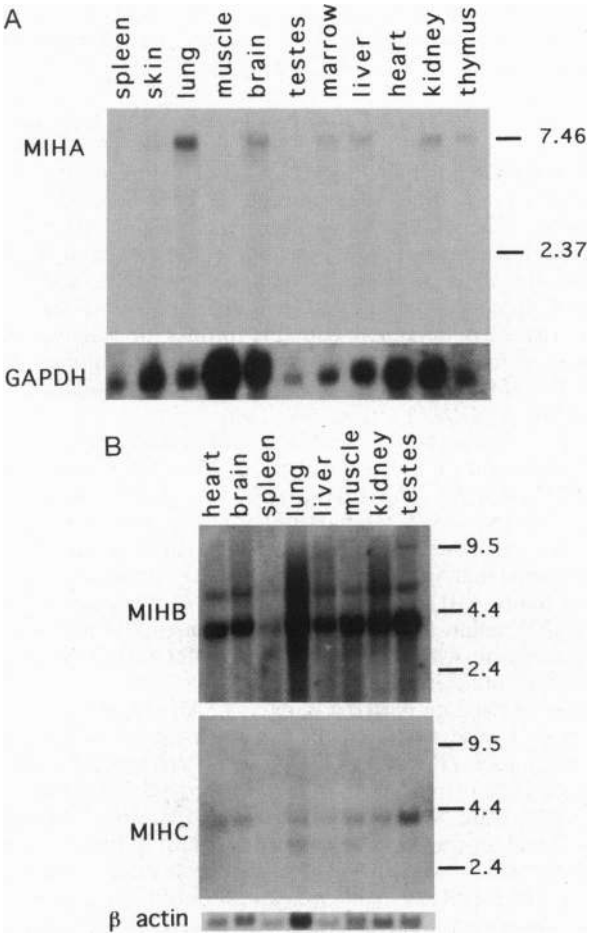

FIG. 2. Mammalian IAP homologs are expressed in a variety of tissues. (*A*) An adult mouse tissue total RNA Northern blot was probed with the *mMIHA* cDNA coding region at high stringency and a *GAPDH* probe to indicate loading. (*B*) An adult mouse tissue poly(A)$^+$ RNA Northern blot (Clontech) was 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. 3*A*, MIHA, MIHB, and OpIAP significantly reduced the amount of death caused by ICE, whereas MIHC did not provide detectable protection.

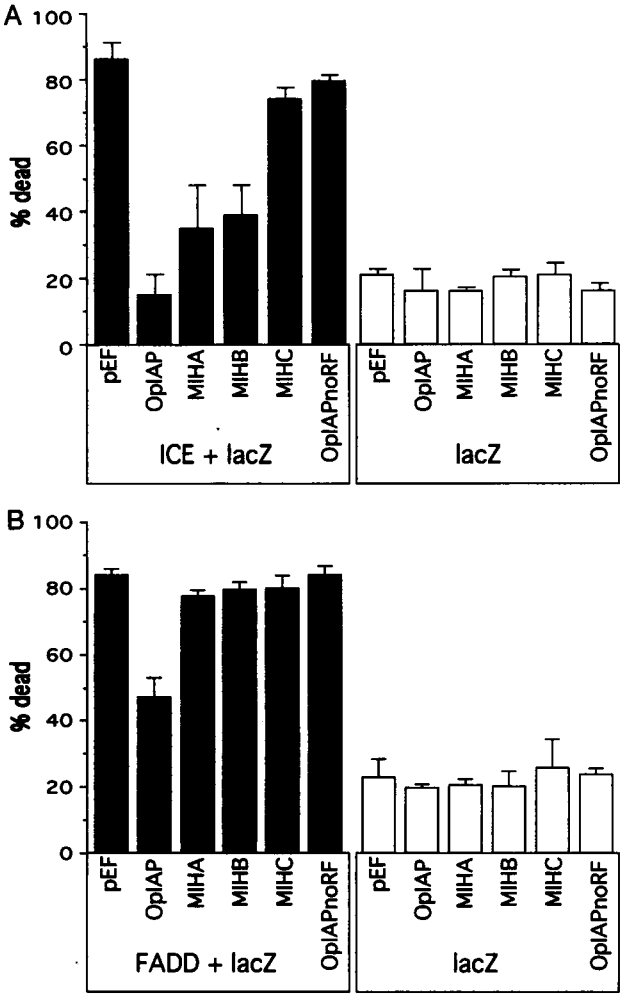

FIG. 3. MIHA and MIHB protect against death induced by overexpression of ICE but not FADD. (*Upper*) Induction of apoptosis by transfection with ICE. Columns 1–6 (solid bars) indicate the percentage of dead cells cotransfected with *p32ICE–lacZ* fusion plasmid and the plasmids bearing either the IAP 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 MIH proteins 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. 3 *A* 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			
DNA-binding hybrid	Activation hybrid	Growth on Trp$^-$, Leu$^-$, His$^-$ medium	Colony color
OpIAP	TRAF1	—	—
MIHA	TRAF1	—	—
MIHB	TRAF1	+	++
MIHC	TRAF1	+	++
c-*jun*	TRAF1	—	—
OpIAP	TRAF2	—	—
MIHA	TRAF2	—	—
MIHB	TRAF2	+	+
MIHC	TRAF2	+	+
c-*jun*	TRAF2	—	—
OpIAP	TRAF3	—	—
MIHA	TRAF3	—	—
MIHB	TRAF3	—	—
MIHC	TRAF3	—	—
c-*jun*	TRAF3	—	—
OpIAP	*fos*	—	—
MIHA	*fos*	—	—
c-IAP1	*fos*	—	—
MIHC	*fos*	—	—
c-*jun*	*fos*	+	+++

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. 3*A*), 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-κB 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 interestingly 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.

We thank Mike Rothe (Tularik) for discussions of unpublished results and provision of TRAF1 and TRAF2 yeast two-hybrid constructs and c-IAP1 and c-IAP2 expression constructs. We are indebted

to G. Hacker for advice and discussions, R. Clem and L. Miller for *OpIAP* cDNA, W. Alexander for mRNA, D. Huang and J. M. Adams for eukaryotic expression vectors, J. Yuan for the *ICE-lacZ* expression plasmid, and V. M. Dixit and H. M. Hu for the FADD and TRAF3 expression constructs. D.L.V. was supported by an Investigator Award from the Cancer Research Institute of New York and the Dunlop Fellowship from the Anti-Cancer Council of Victoria.

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