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A conserved family of cellular genes related to the baculovirus *iap* gene and encoding apoptosis inhibitors

Colin S.Duckett[1–5], Victor E.Nava[6], Richard W.Gedrich[2,3], Rollie J.Clem[6], Jennifer L.Van Dongen[2,3], Molly C.Gilfillan[5], Helena Shiels[5], J.Marie Hardwick[6] and Craig B.Thompson[1–5,7]

[1]Howard Hughes Medical Institute, [2]Gwen Knapp Center for Lupus and Immunology Research, [3]Departments of Medicine, [4]Molecular Genetics and Cell Biology and [5]The Committee on Immunology, The University of Chicago, 924 East 57th Street, Chicago, IL 60637 and [6]Neurovirology Laboratories, Johns Hopkins University School of Medicine, Meyer 6-181, Baltimore, MD 21287, USA

[7]Corresponding author

The baculovirus inhibitor of apoptosis gene, *iap*, can impede cell death in insect cells. Here we show that *iap* can also prevent cell death in mammalian cells. The ability of *iap* to regulate programmed cell death in widely divergent species raised the possibility that cellular homologs of *iap* might exist. Consistent with this hypothesis, we have isolated *Drosophila* and human genes which encode IAP-like proteins (dILP and hILP). Like IAP, both dILP and hILP contain amino-terminal baculovirus IAP repeats (BIRs) and carboxy-terminal RING finger domains. Human *ilp* encodes a widely expressed cytoplasmic protein that can suppress apoptosis in transfected cells. An analysis of the expressed sequence tag database suggests that h*ilp* is one of several human genes related to *iap*. Together these data suggest that *iap* and related cellular genes play an evolutionarily conserved role in the regulation of apoptosis.

Keywords: apoptosis/*iap*/molecular cloning/programmed cell death/viruses

Introduction

The control of host cell translation and often the control of replication are integral parts of the life cycle of a virus. However, recent evidence suggests that most eukaryotic cells respond to viral disruption of normal cellular physiology by undergoing programmed cell death (apoptosis) (White, 1993). To counteract this, many viruses have evolved mechanisms to block host cell death (for reviews, see Clem and Miller, 1994a; White and Gooding, 1994). In several cases, viral genomes have been found to contain genes whose products interact with proteins that play a central role in regulating cell survival. For example, the product of the *crm*A gene isolated from the cowpox virus can inhibit apoptosis through its ability to inhibit members of the interleukin-1β-converting enzyme (ICE) protease family (Miura *et al.*, 1993; Yuan *et al.*, 1993; Gagliardini *et al.*, 1994; Komiyama *et al.*, 1994; Kumar *et al.*, 1994; Wang *et al.*, 1994; Fernandes-Alnemri *et al.*, 1995; Los *et al.*, 1995; Tewari and Dixit, 1995). The E1B 19 kDa protein of adenovirus can also protect cells from death induced by a wide variety of stimuli (Debbas and White, 1993; Sabbatini *et al.*, 1995). The ability of E1B 19 kDa to protect cells from apoptosis correlates with its ability to bind to members of the *bcl-2* family that promote cell death (Chiou *et al.*, 1994; Farrow *et al.*, 1995).

Insect baculoviruses contain at least two independent genes that promote the survival of the infected host cell, *p35* (Friesen and Miller, 1987; Clem *et al.*, 1991; Hershberger *et al.*, 1992, 1994; Clem and Miller, 1993; Kamita *et al.*, 1993) and *iap* (Crook *et al.*, 1993; Birnbaum *et al.*, 1994). Recombinant baculoviruses lacking both genes induce accelerated host cell death leading to severely impaired virus production (Clem and Miller, 1994b). Although *p35* and *iap* have no sequence similarity, they are functionally equivalent in the context of the virus. When provided in *trans* either gene can protect host cells from death induced by a baculovirus lacking both genes (Clem and Miller, 1994b). This suggests that *p35* and *iap* exert their effects at key points in the cellular apoptotic pathway. Consistent with this view is the discovery that *p35* can confer protection from cell death in mammalian cells (Rabizadeh *et al.*, 1993; Beidler *et al.*, 1995), an observation which reflects the high degree of evolutionary conservation of the apoptotic cell death pathway (Vaux *et al.*, 1994; White *et al.*, 1994). Recently, p35 has been shown to act by blocking the activity of members of the ICE family of cysteine proteases (Bump *et al.*, 1995; Xue and Horvitz, 1995). Whether IAP also blocks an evolutionarily conserved step in apoptosis has not been determined.

The *iap* genes isolated from different baculoviruses all display two distinct structural features. The first of these is a zinc binding domain known as a RING finger (Lovering *et al.*, 1993), which has also been identified in a number of cellular proteins including the products of the proto-oncogenes c-*cbl* (Blake *et al.*, 1991) and c-*pml* (de The *et al.*, 1991), as well as the recently described family of signal transducing molecules TRAF2 (Rothe *et al.*, 1994; Song and Donner, 1995) and CRAF1/CD40bp (Hu *et al.*, 1994; Cheng *et al.*, 1995; Sato *et al.*, 1995). While RING domains have been found in several DNA binding proteins, they have not been shown to bind DNA, and probably act to mediate protein–protein interactions (Borden *et al.*, 1995). The second highly conserved feature of baculovirus IAP proteins is the presence of amino-terminal repeats of an ~65 amino acid sequence termed a baculovirus IAP repeat (BIR). Both the BIR repeats and RING domains have been shown to be essential in preventing cell death in insect cells (Clem and Miller, 1994b).

To obtain further insight into the role of *iap* in regulating apoptotic cell death, we examined the properties of *iap* in the context of a viral infection of mammalian cells. Baculovirus *iap* is able to prevent cell death induced by infection of mammalian cell lines with Sindbis virus, suggesting that IAP might prevent apoptosis by mimicking the actions of an evolutionarily conserved regulator of the cell death pathway. Consistent with this hypothesis, we have identified cellular homologs to *iap*. The human and *Drosophila iap*-like genes, d*ilp* and h*ilp*, appear to be widely expressed in their species of origin. Like *iap*, human *ilp* was found to protect cells from apoptosis induced both by virus infection and ICE expression. These findings suggest that the ILP proteins represent a new family of cellular factors which are involved in modulating cell survival.

Results

Baculovirus IAP protein can protect mammalian cells from cell death

Previous experiments have demonstrated that overexpression of the *iap* gene from the baculovirus *Orgyia pseudotsugata* nuclear polyhedrosis virus (Op-*iap*) can inhibit cell death induced by baculovirus infection in *Spodoptera frugiperda* cells (Clem *et al.*, 1991; Clem and Miller, 1993, 1994b; Crook *et al.*, 1993; Birnbaum *et al.*, 1994). To determine whether *iap* overexpression can confer protection in mammalian cells, the full-length Op-*iap* gene (Op-*iap*), or a mutant gene containing a premature stop codon (Op-*iap*/stop), were introduced into a Sindbis virus vector and expressed under the control of a duplicated copy of a late viral subgenomic promoter (Hertz and Huang, 1992). The resulting viruses were then tested for their ability to induce cytopathic effects in target cells permissive for Sindbis virus infection (Lustig *et al.*, 1988). Cells infected by Sindbis virus rapidly undergo apoptosis unless a cell survival gene such as *bcl-2* is introduced into the virus (Levine *et al.*, 1993). The viability of baby hamster kidney (BHK) cells (Figure 1A) and N18 mouse neuroblastoma cells (Figure 1B) infected with viruses containing wild-type Op-*iap* was significantly greater than that of cells infected with the viral vector alone or viruses containing Op-*iap*/stop. This protection was similar to that conferred by inclusion of the cell survival gene *bcl-*x_L in the recombinant virus. *bcl-*x_L-mediated protection was also inhibited by the introduction of a premature stop codon. These observations suggest that *iap* inhibits cell death by modulating an evolutionarily conserved step in apoptosis.

Molecular cloning of Drosophila and human homologs of baculovirus iap

The demonstration that Op-*iap* can protect mammalian cells from virus-induced cell death suggested that it may be a viral homolog of an evolutionarily conserved gene(s) involved in the control of programmed cell death. In a search of the nucleotide databases, an open reading frame (ORF) with homology to Op-IAP was identified within a 5307 bp *Drosophila melanogaster* genomic sequence that had been isolated from region 52D of the *Drosophila* genome (Ross *et al.*, 1994). To determine whether this region encodes an IAP-like protein, we used PCR to

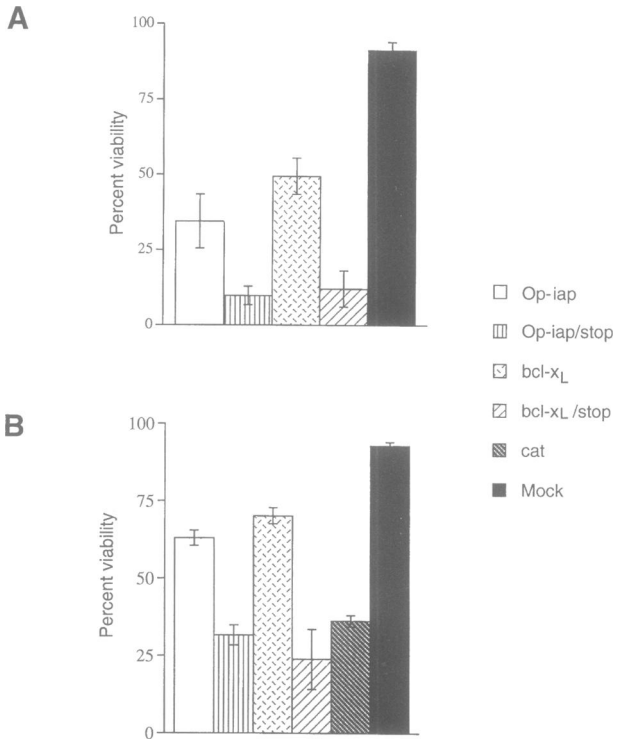

Fig. 1. Baculovirus *iap* can protect cells from apoptosis induced in response to Sindbis virus infection. (**A**) BHK cells were infected with recombinant Sindbis viruses containing Op-*iap*; Op-*iap* containing a premature stop codon (Op-*iap*/stop); *bcl-*x_L; *bcl-*x_L gene containing a premature stop (*bcl-*x_L/stop); or with media alone (mock). Cell viability was assayed 48 h after viral infection, and viability measured by trypan blue exclusion. Data are expressed as mean ± standard error of three or more independent experiments. (**B**) The viability of N18 mouse neuroblastoma cells infected and assayed as above. (cat) refers to infection with a Sindbis virus containing a chloramphenicol acetyltransferase gene.

amplify a 580 bp fragment from cDNA prepared from *Drosophila* SC2 cells. Sequence analysis of this fragment confirmed that it encoded an in-frame IAP homolog. To obtain full-length cDNAs, a DNA fragment derived from the original PCR product was used to screen a cDNA library prepared from adult *Drosophila* RNA. Several independent cDNAs were isolated, and a single ORF was identified (Figure 2). In the longest cDNA, stop codons in all three reading frames were identified 5′ of a potential initiator methionine. Sequences around the initiator methionine conformed to the consensus initiation codon in *Drosophila* (Cavener and Ray, 1991). Comparison of the *Drosophila ilp* ORF, which we have termed IAP-like protein (dILP), with the baculovirus IAP sequence revealed that the carboxy-terminal ends of both proteins contained a highly conserved RING finger domain (55% amino acid identity over 38 residues).

By comparing *Drosophila ilp* with baculovirus *iap* sequences, highly conserved regions were identified and used to search nucleotide databases for potential mammalian homologs. A 273 bp sequence tag site (STS) which mapped to the q24–25 region of the human X chromosome was identified which contained an 81 bp fragment with a predicted amino acid sequence of greater similarity to the RING finger of dILP and Op-IAP than the RING fingers contained in other known proteins. In order to determine whether this sequence was contained within an ORF which

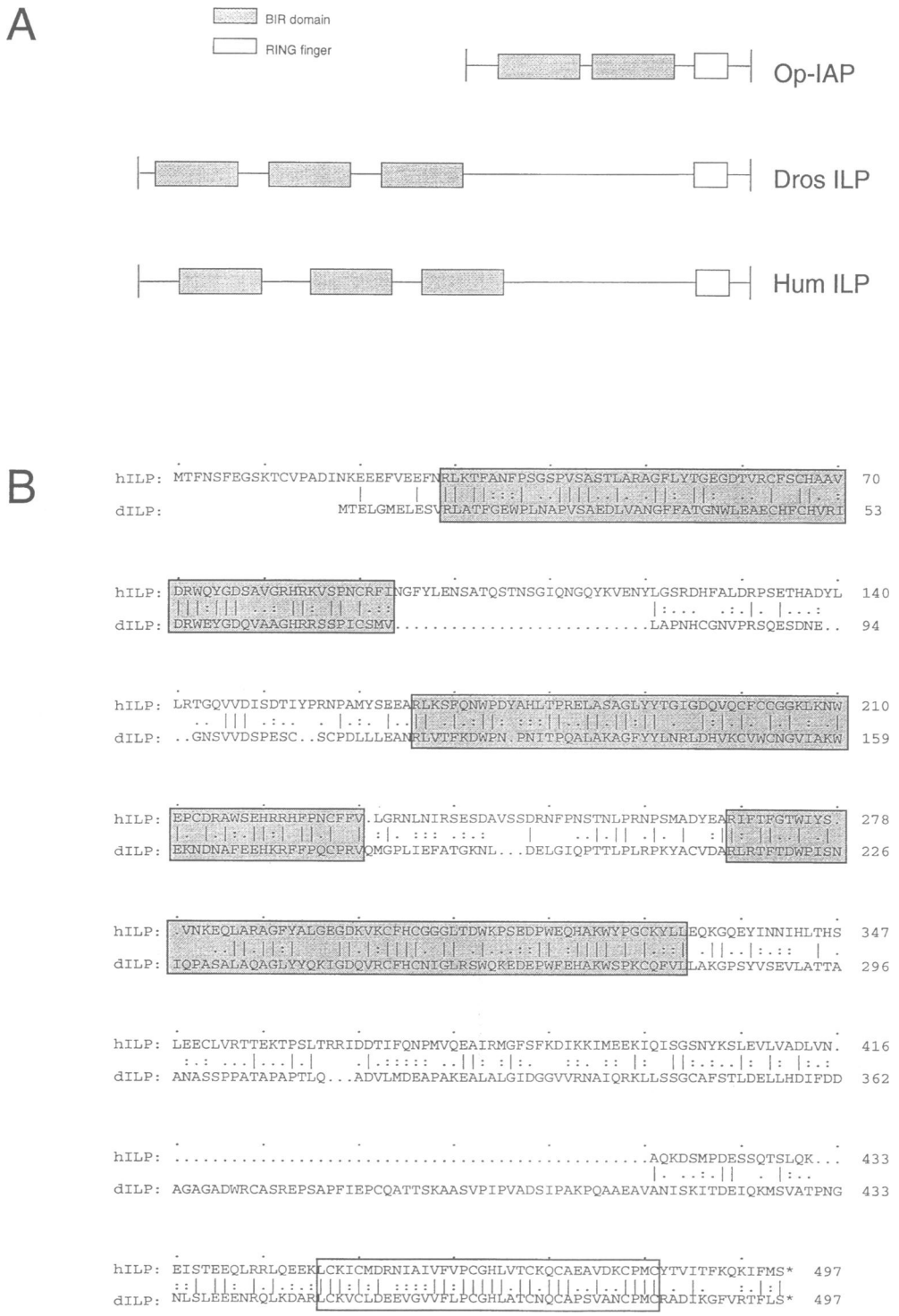

Fig. 2. Predicted amino acid sequences of the *iap*-related genes, d*ilp* and h*ilp*. (**A**) A schematic representation of the organization of Op-IAP, dILP and hILP is described and the localization of the BIR and RING finger domains is shown. (**B**) Sequence alignment of hILP and dILP. Shaded boxes reveal the three BIR repeats, while the open rectangle shows the RING finger domain. Amino acid identities are indicated by a connecting line, the most conservative substitutions are represented by a colon (:) and a period (.) represents moderately conservative substitutions as defined by GCG.

encodes an IAP-like protein, a nested PCR strategy, using oligonucleotide primers to this fragment as well as primers complementary to regions of the cloning sites contained within several cDNA libraries, was designed. PCR products isolated from these libraries, when sequenced, revealed the presence of in-frame BIR repeats upstream of the RING finger domain. cDNA clones encoding the full-length human IAP were isolated subsequently from a human fetal heart cDNA library (Figure 2).

Interestingly, the amino-termini of dILP and hILP are predicted to contain three BIR motifs, while the baculovirus IAP proteins contain only two. One additional difference between the two proteins is that, in the cellular proteins, the BIR domains are separated from the RING

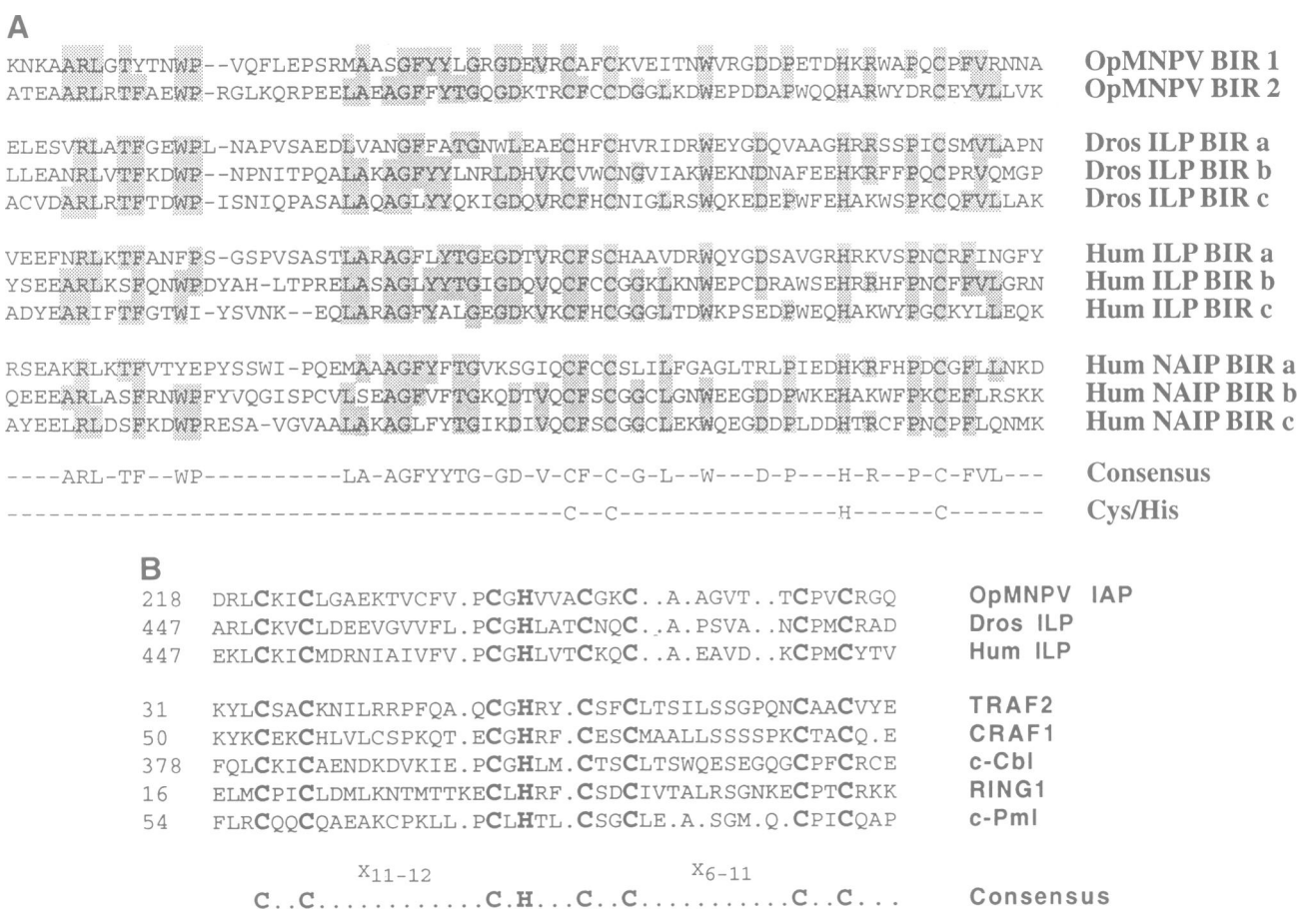

Fig. 3. Viral *iap* and cellular *ilp* genes share two conserved domains: BIR repeats and RING fingers. (**A**) Alignment of the BIR repeats in the IAP protein of *Orgyia pseudotsugata* nuclear polyhedrosis virus (OpMPNPV), *Drosophila* ILP, human ILP and human NAIP. Amino acids that are identical in more than half of these BIR domains are shaded. An amino acid consensus, as well as the location of conserved cysteines and histidines, are indicated below the alignment. (**B**) Alignment of the RING ringer domains between viral IAP, *Drosophila* ILP and human ILP, as well as several well characterized human RING finger domains. The characteristic cysteine and histidine spacing of a RING finger domain is shown as a consensus sequence.

finger by an amphipathic region of 120–170 amino acids. This region is absent in baculovirus IAP proteins.

Properties of cellular IAP-like proteins

The predicted overall structures of dILP and hILP are very similar. Both proteins contain three BIR elements and a highly conserved carboxy-terminal RING finger domain (Figure 3). Both proteins are 497 amino acids in length and the calculated molecular weights of dILP and hILP are 54 and 57 kDa, respectively. Both d*ilp* and h*ilp* appear to be widely expressed at the mRNA level. d*ilp* mRNA was found to be expressed at high levels in larval, embryonic and adult *Drosophila* mRNAs (Figure 4A). Similarly, RNA transcripts for h*ilp* were detected in all tissues and cell lines tested (Figure 4B). Thus, the *ilp* genes appear to be expressed throughout *Drosophila* development and in a wide variety of mammalian tissues.

To characterize the subcellular distribution of hILP, an expression plasmid encoding an epitope-tagged version of hILP was produced. This expression plasmid was transiently transfected into the 293 human embryonic kidney cell line, and the transfected protein was localized by staining with an epitope-specific monoclonal antibody. These experiments revealed that hILP staining could be detected diffusely throughout the cytoplasm of cells (Figure 5). No evidence for accumulation of the protein in proximity to organelles or within the nucleus was seen. Like a number of other evolutionarily conserved regulators of apoptosis, hILP appears to be primarily a cytoplasmic protein.

Both dILP and hILP were found to contain three copies of the BIR motifs originally identified at the amino-terminus of viral *iap* genes. In addition to the viral *iap* genes, one other gene has been reported to contain BIR repeats, the human neural apoptosis inhibitory protein (NAIP) gene (Roy *et al.*, 1995). *NAIP* is partially deleted in the majority of individuals with spinal muscular atrophy. Sequence comparisons between d*ilp*, h*ilp* and *NAIP* revealed a previously unrecognized third copy of a BIR repeat at the amino-terminus of NAIP. Thus NAIP, like dILP and hILP, contains three copies of the BIR motif at its amino-terminus. Alignment of the BIR repeats from viral IAP, dILP, hILP and NAIP reveal that each BIR motif within these proteins is almost equally related to the BIR domains within the same protein and to the BIR domains within the other proteins (Figure 3A). A consensus amino acid sequence for a BIR domain is discernible from this alignment. It also appears that the carboxy-terminal half of a BIR domain has a primary amino acid sequence suggestive of a zinc finger with a $CX_2CX_{16}HX_6C$ spacing.

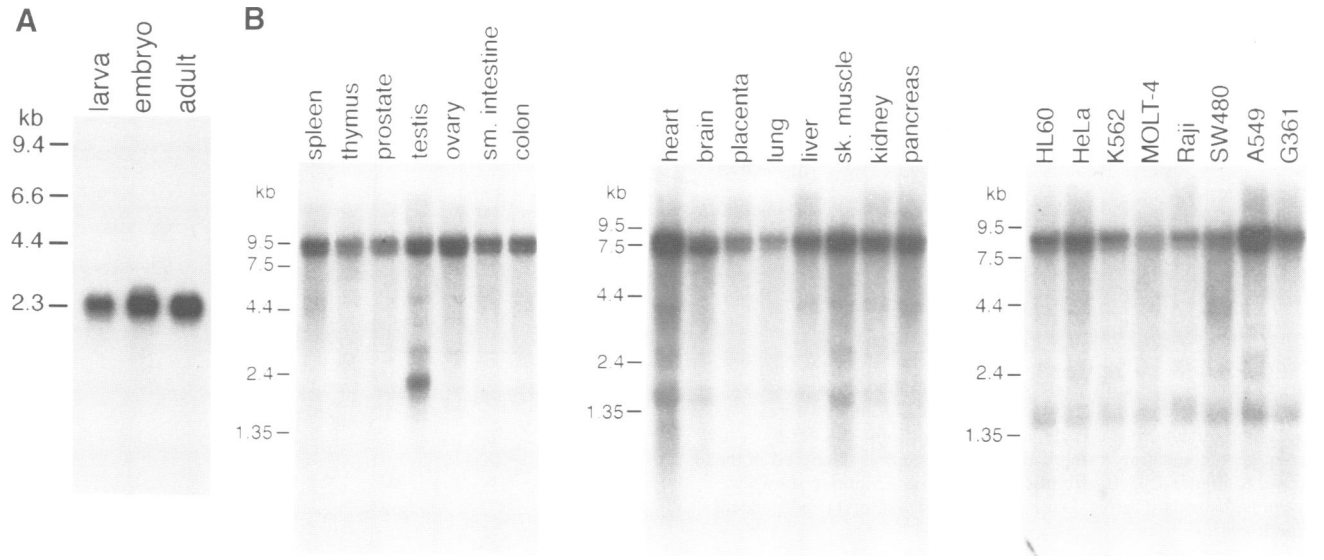

Fig. 4. Expression of d*ilp* and h*ilp* mRNA. (**A**) Northern blots containing 2 μg of poly(A)$^+$ RNA isolated from larva, embryo and adult *Drosophila* were separated by formaldehyde gel electrophoresis, and resulting Northern blots were probed with a probe specific for d*ilp*. A single mRNA species of 2.3 kb was identified. (**B**) Northern blots containing 2 μg of poly(A)$^+$ RNA from a variety of human tissues as well as cell lines were probed with a DNA segment specific for h*ilp*. h*ilp* was ubiquitously expressed in all tissues and cell lines identified. Major mRNA species between 8.5 and 9.5 kb were identified in all tissues. In the testis an additional 2.2 kb mRNA species was identified.

The carboxy-terminal domains of Op-IAP, dILP and hILP each encode highly related RING finger domains. Such a domain is lacking from NAIP. Although each of the *iap*-related genes contain cysteine and histidine residues spaced at intervals which are characteristic of a RING finger domain, the RING fingers of Op-IAP, dILP and hILP are more closely related to each other than they are to RING finger domains contained within a variety of other proteins (Figure 3B). This degree of evolutionary conservation suggests that d*ilp* and h*ilp* may be true cellular homologs of the viral *iap* genes. However, Southern blot analysis revealed the presence of several h*ilp*-related sequences within the human genome (data not shown). Consistent with this, a search of the expressed sequence tag (EST) databases revealed the presence of at least five additional ESTs which encode proteins containing BIR domains and/or IAP-like RING finger domains. Thus, h*ilp* may be only one of several human genes related to the viral *iap* genes.

hilp can protect transfected cells from apoptosis

Given the striking similarity in overall organization and primary amino acid sequence between viral IAP and the cellular IAP proteins, we have investigated the ability of h*ilp* to protect cells from apoptosis. Recombinant Sindbis viruses were generated containing the h*ilp* gene or an antisense control, and these viruses were assayed for their cytopathic effects on BHK cells (Figure 6). Following infection, the viability of cells infected with the *ilp*-expressing virus was significantly higher than the viability of cells infected with the control virus. These data suggest that, like Op-IAP, hILP can suppress cell death induced in response to viral infection.

To verify independently the ability of Op-IAP and hILP to prevent programmed cell death, the effect of these genes on ICE-induced death was examined. It has been demonstrated previously that mammalian cells can be induced to undergo apoptosis in response to transient

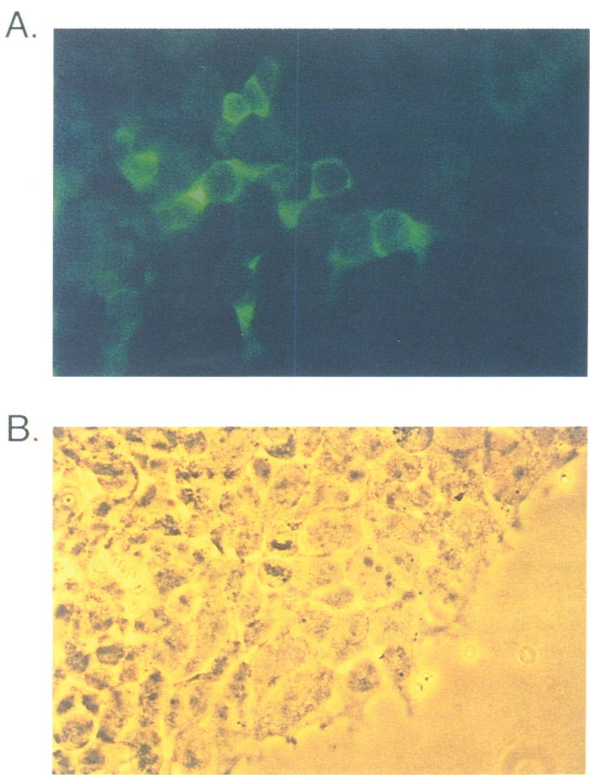

Fig. 5. hILP is expressed in the cytoplasm. The human embryonic kidney cell line, 293, was transiently transfected with a full-length cDNA for h*ilp*, which had been epitope tagged at the N-terminus with a Myc-specific epitope. Expression of hILP in transiently transfected cells was identified subsequently by staining of the cells with an anti-Myc-specific antibody. The figure depicts the same field of view under (**A**) epifluorescence and (**B**) phase-contrast microscopy. The specificity of the resulting immunofluorescence for the transfected gene was demonstrated by the absence of fluorescence staining of cells that were mock-transfected (data not shown).

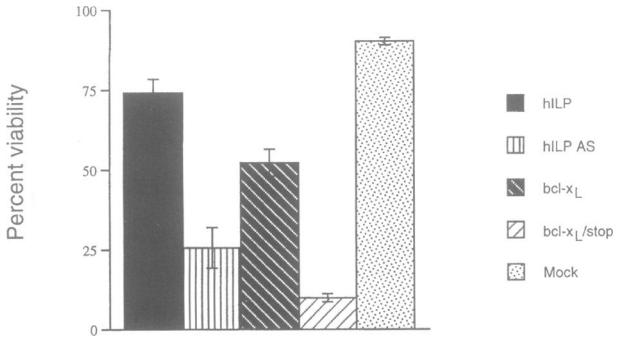

Fig. 6. Protection of cells from Sindbis virus-induced apoptosis by h*ilp*. BHK cells were infected with recombinant Sindbis viruses containing human *ilp* (h*ilp*); an antisense h*ilp* (h*ilp* AS), *bcl-x*$_L$ or a *bcl-x*$_L$ gene containing a stop codon (*bcl-x*$_L$/stop) as described in the legend to Figure 1. Cell viabilities 48 h post-infection are shown (mean and standard error). Data are representative of 11 independent experiments.

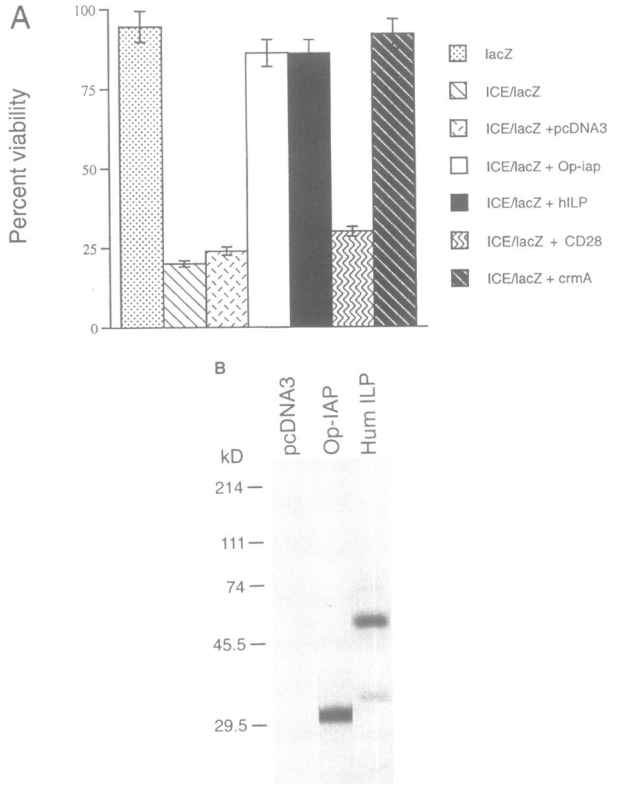

Fig. 7. Both *iap* and h*ilp* protect cells from ICE-induced apoptosis. (**A**) Ten thousand 293 cells were transfected with plasmids encoding either a *lacZ* gene or a pro-ICE/*lacZ* fusion gene. In each case indicated, cells were co-transfected either with a control plasmid or plasmids expressing CrmA, Op-IAP or hILP. Data are expressed as mean and standard deviation of triplicate cultures and are representative of four independent experiments. (**B**) *In vitro* translation of cDNAs used for transfection. The indicated plasmids were translated in rabbit reticulocytes containing [^{35}S]methionine, and the products were resolved by SDS–PAGE and fluorography.

transfection of an expression plasmid containing the pro-ICE protease fused to the *Escherichia coli lacZ* gene (Miura *et al.*, 1993). This system provides a reproducible method for assessing the role of individual genes in protecting cells from apoptotic cell death. The 293 cell line was transiently transfected with either the ICE/*lacZ* plasmid, pβactM10Z, which contains pro-ICE fused to *lacZ*, or the *lacZ* plasmid, pactβgal′, which contains only the *lacZ* gene (Miura *et al.*, 1993; Wang *et al.*, 1994), in combination with various control and test plasmids. Transient transfection of the ICE/*lacZ* plasmid resulted in programmed cell death of over half the cells within 18 h of transfection (Figure 7A). Cell death of the transfected cells was readily discernible following the addition of X-gal solution to develop the color reaction (Figure 8). Viable blue cells are flat, well-spread cells with easily discernible nuclei. In contrast, apoptotic cells are smaller, round cells with condensed and often misshapen nuclei. As has been reported (Miura *et al.*, 1993; Wang *et al.*, 1994), cell death induced by ICE/*lacZ* was inhibited by co-transfection with a plasmid containing CrmA (Figures 7 and 8). In contrast, co-transfection of ICE/*lacZ* with a control expression plasmid encoding an irrelevant gene, the CD28 surface antigen, had no effect on the overall cell death. To test the ability of h*ilp* to protect cells from transfection of the pro-ICE construct, the h*ilp* gene was cloned into the pcDNA3 expression plasmid. *In vitro* transcription and translation verified that the resulting plasmid was capable of producing a full-length hILP protein (Figure 7B). Co-transfection of either the h*ilp* or Op-*iap* plasmid protected ICE/*lacZ*-transfected cells nearly as well as co-transfection of the *crm*A gene. These data demonstrate that the viral *iap* gene's ability to protect mammalian cells from cell death is not restricted to apoptosis induced by viral infection, and also demonstrates that both the human *ilp* and viral *iap* genes can protect cells from apoptotic stimuli.

Discussion

For viruses to complete their life cycle successfully, they must perpetuate the survival of the infected host cell. Viruses from unrelated families have been shown to encode genes that can block cellular apoptosis at either of two distinct steps in the apoptotic pathway. Herpesviruses (Henderson *et al.*, 1993) and adenoviruses (Chiou *et al.*, 1994) have been shown to encode homologs of *bcl-2* that can protect cells from programmed cell death. In contrast, poxviruses encoding *crmA* can inhibit programmed cell death that occurs as a result of activation of ICE-related proteases (Miura *et al.*, 1993; Gagliardini *et al.*, 1994; Komiyama *et al.*, 1994; Wang *et al.*, 1994; Los *et al.*, 1995; Tewari *et al.*, 1995). A similar step appears to be blocked by the *p35* gene isolated from baculoviruses (Bump *et al.*, 1995; Xue and Horvitz, 1995). It now appears that another family of apoptotic suppressors has been identified through their homology with the baculovirus *iap* gene. Our data demonstrate that baculovirus *iap* genes can inhibit programmed cell death in mammalian cells. Consistent with the possibility that baculovirus *iap* genes inhibit cell death by mimicking cellular proteins critical in the control of cell survival, we have identified *iap*-like genes in both *Drosophila* and humans. Thus, proteins related to IAP are evolutionarily conserved components of eukaryotic cells. The existence of additional cDNAs encoding genes with BIR and/or RING fingers related to h*ilp* suggest that h*ilp* may be one of a family of genes related to *iap* in the mammalian genome. Both

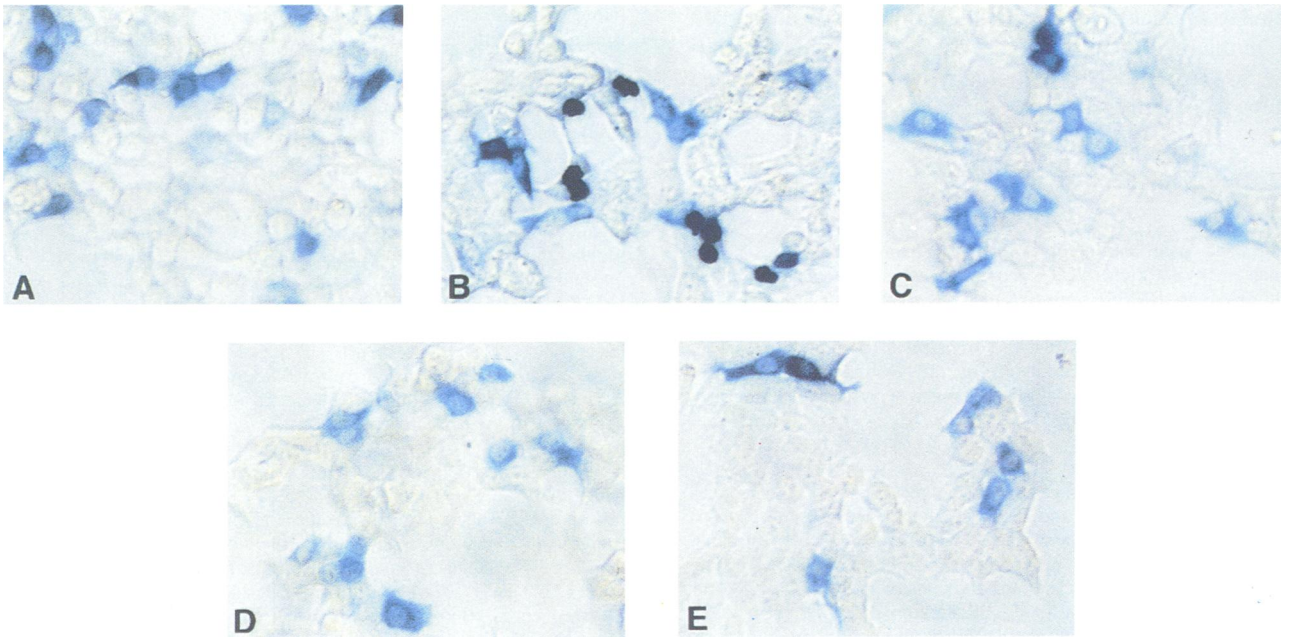

Fig. 8. X-gal staining of 293 cells co-transfected with ICE/*lacZ* and *iap*-related plasmids. Photomicrographs of cells transfected with: (**A**) *lacZ* plus control plasmid; (**B**) pro-ICE/*lacZ* plus control plasmid; (**C**) pro-ICE/*lacZ* plus *crmA* plasmid; (**D**) pro-ICE/*lacZ* plus Op-*iap* plasmid; (**E**) pro-ICE/*lacZ* plus human *ilp* plasmid. Transfections and staining assays were performed as described in the legend to Figure 7.

iap and h*ilp* were able to promote the survival of cells transiently transfected with an expression plasmid encoding the pro-ICE protease. Thus, both *iap* and h*ilp* appear to regulate an evolutionarily conserved step in the induction of cellular apoptosis.

Despite the ability of h*ilp* and *iap* to prevent cell death in cells transfected with a pro-ICE expression plasmid, the molecular mechanism by which *iap* and related genes prevent programmed cell death remains unclear. For the ICE protease to be enzymatically active, the 45 kDa pro-ICE precursor peptide must be processed into two subunits, p20 and p10 (Thornberry *et al.*, 1992). *In vivo* this cleavage can be carried out by active ICE itself or other members of the ICE protease family (Tewari *et al.*, 1995). Since this assay involves the expression of a pro-form of the ICE protease, cell death is dependent on not only the action of the ICE protease, but also on factors that modulate the activation of pro-ICE to active ICE. Neither IAP nor hILP has obvious homology to previously described viral ICE inhibitors, such as p35 or CrmA. If *iap*-related genes inhibit active ICE protease, they appear to do so by a novel mechanism. Alternatively, the *iap*-related genes may regulate an independently controlled step in apoptosis.

Like *iap* and related genes, the *bcl-2* family comprises both cellular and viral genes which serve to modulate apoptosis (Oltvai and Korsmeyer, 1994). Although the precise molecular mechanisms by which *bcl-2* protects cells from undergoing programmed cell death remains unclear, h*ilp* does not appear to have any homology to members of the *bcl-2* family. Furthermore, based on our immunolocalization, IAP seems to be localized diffusely within the cytoplasm rather than expressed in association with specific cellular organelles as is Bcl-2. The epitope-tagged h*ilp* expression vector has been tested in ICE death assays and is protective at levels close to those of unmodified h*ilp* (data not shown). The ORF of *iap* and h*ilp* does not appear to encode a membrane-spanning domain or other cellular localization sequences. Thus, *iap* and its related cellular homologs appear to define a novel apoptosis suppressor gene family.

Given the close structural and functional relationship between Op-IAP and hILP, it is possible that the baculovirus *iap* gene may have been derived originally from a cellular gene product just as viral oncogenes are thought to be derived from cellular proto-oncogenes (reviewed in Bishop, 1991). Genes that regulate apoptosis may be incorporated into viral genomes to sustain host cell viability, in much the same way as viral genomes have incorporated genes that control cell proliferation to enhance viral replication. Our data suggest that viral acquisition of an *iap*-related gene can promote host cell survival. This suggests that loss of *iap*-related genes may also cause cell death in mammalian cells that express these genes. Consistent with this hypothesis, mutations in the *NAIP* gene are thought to contribute to spinal muscular atrophy (Roy *et al.*, 1995). This neurodegenerative syndrome results from the inappropriate death of motor neurons. In over two-thirds of patients with the severe form of spinal muscular atrophy, there are deletions in the first two coding exons of *NAIP*. These deletions result in the loss of all of the first BIR and most of the second BIR domains. Thus it appears that the BIR domains are essential to the function of NAIP in maintaining motor neuron survival. Similarly, these domains are required for the ability of IAP to prevent apoptosis (Clem and Miller, 1994b). Thus the BIR motif probably plays a critical role in the ability of *iap*-related genes to modulate cell survival. The conservation of cysteine–histidine spacing within this repeat suggests that this domain may be a novel form of zinc finger.

In conclusion, our results demonstrate that the insect virus survival gene *iap* can promote cell survival in mammalian cells. The identification of cellular genes

related to *iap* defines a novel and evolutionarily conserved step in the regulation of cell survival, and reveals the viral gene as a functional homolog of a highly conserved cellular gene family.

Materials and methods

Cell culture

N18 (mouse neuroblastoma), BHK (baby hamster kidney) and 293 (human embryonic kidney) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, and maintained at 37°C in 5% CO_2. The 293 cells were transfected by calcium phosphate as previously described (Perkins *et al.*, 1994) Staining of cells for β-galactosidase activity and viability analysis was performed as described previously (Miura *et al.*, 1993).

Recombinant viruses

The Sindbis virus vector was generated from plasmids TE12 (nucleotides 1–10 381; 11 382–13 637) and TZJSINC (nucleotides 10 382–11 485) (Lustig *et al.*, 1988; Hertz and Huang, 1992) with a *Bst*EII restriction site inserted at the junction between nucleotide 11 485 of TZJSINC and nucleotide 11 382 of TE12. The resulting construct contains a cDNA of the Sindbis virus genome with a duplicated copy of a viral subgenomic promoter and a unique *Bst*EII restriction site inserted between the viral coding sequences and the 3′-untranslated/regulatory sequences of the Sindbis virus genome. The baculovirus Op-*iap* gene (Birnbaum *et al.*, 1994), the human *bcl-*x_L cDNA (Boise *et al.*, 1993) and the bacterial chloramphenicol acetyltransferase gene (CAT) (Gorman *et al.*, 1982) gene were cloned into the virus vector as PCR products with flanking *Bst*EII sites. To generate the Op-*iap*/stop and *bcl-*x_L/stop viruses, a nonsense oligonucleotide containing stop codons in all three reading frames was inserted after codon 8 (*Asc*I site) in the wild-type Op-*iap* virus construct or after codon 78 (*Sma*I site) in the wild-type *bcl-*x_L virus construct.

Viral RNA was transcribed *in vitro* from ~400 ng of linearized plasmid DNA using SP6 RNA polymerase. Ten μl of the 25 μl total transcription reaction was transfected into BHK cells with 30 μl of lipofectin (Gibco/BRL) according to the manufacturer's instructions. The culture medium containing infectious recombinant virus was collected 24 h after transfection and stored at –80°C. Virus titers were determined according to established protocols. Cells were infected with recombinant viruses at a multiplicity of infection of 5 p.f.u./cell in culture medium containing 1% serum for 1 h. After infection, cells were returned to medium containing 10% serum, and cell viability was determined ~48 h later by trypan blue exclusion.

DNA and protein sequence analysis

The *Drosophila ilp* gene was identified originally in the GenBank/EMBL nucleotide databases (accession no. M96581) by a search for potential *iap* homologs to Op-*iap* using the TFASTA sequence comparison program (Genetics Computer Group, University of Wisconsin). Having characterized full-length cDNAs containing the *Drosophila ilp* gene (see below), the predicted amino acid sequence of this gene was used to search the human STS and EST DNA databases. This search revealed that an 81 bp sequence within a 273 bp STS sequence (accession no. L24579) potentially encoded an *iap*-like RING finger domain, and this sequence was used to identify full-length cDNA clones as described below.

Additional DNA and protein sequence analysis was performed using the FASTA, BLAST, WORDSEARCH, PEPTIDESORT and BESTFIT programs contained within the GCG package. DNA sequencing was facilitated using the sequence project management programs contained within the DNASTAR package (DNASTAR, Madison, WI). Additional searches were performed at the National Center for Biotechnology Information Internet site.

Molecular cloning of Drosophila and human ilp

PCR was performed under standard conditions using Amplitaq DNA polymerase (Chiron) to amplify a ~580 bp fragment from cDNA prepared from Schneider SC2 *Drosophila* cells, using the oligonucleotide primers 5′-ATGGCCCCTGAATGCCCCAGTTTCCGCGGAGGATCTG-3′ and 5′-CATCACGCCGCAGGCTCTGGCAAAGGCAGGTTTC-3′, which are specific to a region predicted to encompass a BIR element as judged by the computer analysis described above. This fragment was subcloned into PCRscript (Stratagene) and subsequently used as a probe to isolate full-length clones from a λgt10 adult *Drosophila* cDNA library (Clontech).

Fragments containing a segment of the human *ilp* gene were isolated with a nested PCR approach using plasmid DNA from a human B cell two-hybrid cDNA library (Clontech) as a template. An initial PCR reaction was performed for 30 cycles under standard conditions using Amplitaq DNA polymerase with the human STS-specific primer 5′-AGTAATGACTGTGTAGCACATGGCACAC-3′ and the library-specific primer 5′-GCGTATAACGCGTTTGGAATCACTACAGGGATG-3′. One μl from this reaction was used as a template for a second 30 cycle PCR reaction with the STS-specific nested primer 5′-TCAACTGCTTCAGCACATTGTTTTACAAGTGAC-3′ and the library-specific nested primer 5′-TTTAATACCACTACAATGGATGATGTATATAAC-3′. The resulting ~600 bp fragment was subcloned into PCRscript, and subsequently used as a probe to isolate full-length clones from a human fetal heart λgt10 cDNA library (Clontech).

Full-length clones containing the *Drosophila* and human *ilp* genes were isolated from cDNA libraries essentially following the manufacturer's recommendations (Clontech). Insert DNA from phage clones was excised with *Eco*RI, subcloned into Bluescript II SK+ (Stratagene) and sequenced by the dideoxy method using Sequenase (United States Biochemical) using the modifications described by Hsiao (1991). Both the *Drosophila* and human *ilp* sequences have been deposited in GenBank with accession nos U32373 and U32974, respectively. The additional human *iap*-related EST sequences referred to in this paper have the following GenBank accession nos: R07927, R57975, R19628, T96284 and T16094.

Northern blot analysis

The same ^{32}P-labeled DNA fragments described above were used to probe Northern blots. Human Multiple Tissue Northern (MTN) blots were probed at high stringency following the supplier's instructions (Clontech). Embryonic, larval and adult poly(A)$^+$ RNA from *D.melanogaster* (Clontech) was used to prepare a Northern blot as described previously (June *et al.*, 1987), which was probed at high stringency.

Immunofluorescence studies

293 cells (5×10^4) were plated directly onto Lab-Tek chamber slides (Nunc), transfected with 200 ng of plasmid DNA by calcium phosphate and maintained for 48 h. All subsequent procedures were performed at room temperature. Cells were washed once with 0.5 ml of phosphate-buffered saline (PBS) and fixed in 2% paraformaldehyde (0.4 ml) for 10 min. Fixed cells were washed once in PBS containing 0.03% saponin (0.5 ml), and permeabilized in PBS containing 0.1% saponin for 15 min. Blocking was performed by incubation in the same solution containing 20% goat serum for 15 min, followed by addition of 0.4 ml of a solution containing 10 μg/ml of anti-Myc monoclonal antibody (clone 9E10, Pharmingen), 1% bovine serum albumin (BSA), 0.01% sodium azide and 0.1% saponin in PBS for 30 min. Slides were washed twice in PBS/0.03% saponin, and incubated with secondary antibody (FITC-conjugated goat anti-mouse, 1/50 dilution, Sigma) for 30 min in the same PBS/BSA/azide/saponin solution. Cells were washed twice in PBS/0.03% saponin and visualized by epifluorescence microscopy using a Leitz DM-RB microscope with a Nikon 6006 camera.

Expression vectors

The human *ilp* gene and the *crmA* gene were subcloned into the pcDNA3 mammalian expression vector (Invitrogen). To construct the epitope-tagged human *ilp* construct, site-directed mutagenesis was performed on human *ilp* in Bluescript SK+ to add the 9E10 Myc epitope (EQLISEEDL) to the predicted amino-terminus of the protein. This modified sequence subsequently was cloned into pcDNA3. The Op-*iap* gene was subcloned into a modified Rous sarcoma virus expression vector (Duckett *et al.*, 1993). The construction of the pactβgal′ and pβactM10Z plasmids has been described previously (Miura *et al.*, 1993).

In vitro translation

Aliquots (1 μg) of the relevant *iap*-related cDNAs were used to program TNT rabbit reticulocyte lysates (Promega) for coupled transcription–translation reactions according to the manufacturer's instructions. Aliquots were resolved on 9.5% SDS–polyacrylamide gels, fluorographed using Enhance (DuPont) and products visualized by autoradiography.

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