ILPIP, a Novel Anti-apoptotic Protein That Enhances XIAP-mediated Activation of JNK1 and Protection against Apoptosis*

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M. Germana Sanna‡, Jean da Silva Correia†, Ying Luo†, Betty Chuang†, Lorien M. Paulson‡, Binh Nguyen†, Quinn L. Deveraux†, and Richard J. Ulevitch‡

From the ‡Department of Immunology, The Scripps Research Institute, La Jolla, California 92037, §Rigel Inc., South San Francisco, California 94080, and ¶The Genomics Institute of the Novartis Research Foundation, South San Francisco, California 94080

We have previously described a new aspect of the Inhibitor of Apoptosis (IAP) family of proteins anti-apoptotic activity that involves the TAK1/JNK1 signal transduction pathway (1, 2). Our findings suggest the existence of a novel mechanism that regulates the anti-apoptotic activity of IAPs that is separate from caspase inhibition but instead involves TAK1-mediated activation of JNK1. In a search for proteins involved in the XIAP/TAK1/JNK1 signaling pathway we isolated by yeast two-hybrid screening a novel X chromosome-linked IAP (XIAP)-interacting protein that we called ILPIP (hILP-Interacting Protein). Whereas ILPIP moderately activates JNK family members when expressed alone, it strongly enhances XIAP-mediated activation of JNK1, JNK2, and JNK3. The expression of a catalytically inactive mutant of TAK1 blocked XIAP/ILPIP synergistic activation of JNK1 thereby implicating TAK1 in this signaling pathway. ILPIP moderately protects against interleukin-1β converting enzyme- or Fas-induced apoptosis and significantly potentiates the anti-apoptotic activity of XIAP. In vivo co-precipitation experiments show that both ILPIP and XIAP interact with TAK1 and tumor necrosis factor receptor-associated factor 6. Finally, expression of ILPIP did not affect the ability of XIAP to inhibit caspase activation, further supporting the idea that XIAP protection against apoptosis is achieved by two separate mechanisms: one requiring JNK1 activation and a second involving caspase inhibition.

Caspases, a family of cysteine proteases, are the most extensively studied activators of apoptosis. Among the anti-apoptotic gene products is the IAP1 (Inhibitor of Apoptosis) family of proteins. Initially discovered in baculovirus, where they were shown to be through direct caspase inhibition. Several of the human IAP family proteins have been reported to directly bind and inhibit specific members of the caspase family (17). XIAP has been shown to participate in the BMP signaling pathway by binding with both the BMP receptor and the adaptor molecule TAB1, which is a co-activator of TAK1, thus linking the BMP receptors to TAB1-TAK1 and therefore participating in the bone morphogenetic protein-signaling pathway involved in mesoderm induction and patterning in early Xenopus embryos (18). XIAP also stimulates NF-κB via the TAK1 signaling pathway (19). Consistent with these findings, we have recently described an alternative mechanism for the IAP anti-apoptotic protection, which is distinct from caspase inhibition and involves activation of the MAPK-JNK1 through the TAB1-TAK1 complex (1, 2, 20). XIAP involvement in signal transduction pathways is still poorly characterized. Few proteins have been shown to interact with XIAP. A recently described XIAP-interacting protein is SMAC/DIABLO, which promotes caspase activation by binding and inhibiting the IAPs (21, 22). XAF1 has also been reported to bind to XIAP and inhibit its anti-apoptotic effect apparently 1 The abbreviations used are: IAP, Inhibitor of Apoptosis; ILPIP, hILP-Interacting Protein; XIAP, X chromosome-linked IAP; JNK, amino-terminal c-Jun kinase; TAK1, Transforming growth factor-β-activated kinase-1; ICE, interleukin-1β converting enzyme; TNF, tumor necrosis factor; TRAF6, TNF receptor-associated factor 6; BMP, bone morphogenetic protein; NF-κB, nuclear factor kappa B; MAPK, mitogen-activated protein kinase; SMAC, mitochondria-derived activator of caspases; DIABLO, direct IAP binding protein with low pI; TAB1/TAB2, TAK1 binding protein; BIR, baculovirus IAP repeats; XAF1, XIAP-associated factor 1; CMV, cytomegalovirus; ERR, extracellular signal-regulated kinase; X-gal, 5-bromo-4-chloro-3-indolyl-D-galactopyranoside; FACS, fluorescence-activated cell sorting; PE, phycoerythrin; GST, glutathione S-transferase; AFC, 7-amino-4-trifluoromethyl coumarin; DEVD, Asp-Glu-Val-Asp; ATF-2, activating transcription factor 2; SPAR, STE20/SPS1-related proline alanine-rich kinase.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AF203697.

†To whom correspondence should be addressed: The Scripps Research Institute, 10550 N. Torrey Pines Rd., La Jolla, CA 92037. Tel.: 858-784-8219; Fax: 858-784-8239; E-mail: ulevitch@scripps.edu.

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by triggering the redistribution of XIAP from the cytosol to the nucleus (11). However, none of these interactions has been correlated to investigate their role in the XIAP/TAK1/JNK1 signaling cascade.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Screening and Isolation of ILPIP**

A cDNA fragment encoding human XIAP was amplified by PCR and inserted into the XhoI site of pBD-GAL4 Cam (Stratagene, La Jolla, CA) to generate pBD-GAL4 Cam/XIAP and human fetal brain library (Matchmaker, CLONTECH, Palo Alto, CA) were sequentially transformed with pBD-GAL4 Cam/XIAP and human fetal brain library (Matchmaker, CLONTECH, 10^8 cfu/ml) in pACT using a lithium acetate transformation protocol. Selection was done by growth on synthetic (SD) medium (yeast nitrogen base/dextrose/aminos acids) lacking histidine, uracil, and tryptophan (CLONTECH). Twenty clones exhibiting activation of the lacZ reporter gene were identified among 3 × 10^6 transformants by the β-galactosidase assay. A few clones showing a strong reproducible interaction with XIAP were chosen. Plasmids were isolated from positive yeast colonies by a glass bead phenolchloroform extraction protocol (CLONTECH). Escherichia coli DH5α bacteria cells were transformed with the plasmid harboring the containing the PACT vector were selected on ampicillin-resistant plates. The PACT plasmids were isolated from bacterial restriction-mapped (XhoI), and the sequence of the insert was determined. The partial cDNA two-hybrid clone was designed to use a probe to screen a human liver Uni-ZAP cDNA lambda library (Stratagene) and 5′-rapid amplification of cDNA ends techniques. Phage plaques were isolated and screened to verify the cDNA size by PCR using the T3 and T7 primers. The phagemid Bluescript vector carrying the cDNA of nine individual clones was isolated by in vivo excision from the Uni-ZAP vector according to the manufacturer's instructions (Stratagene). The isolated cDNAs were sequenced, and sequences were analyzed using the GCG Sequence Analysis software package (Madison, WI). The full-length ILPIP gene encoding a 2.4-kb cDNA was subcloned from BanHI-XhoI restriction sites into pcDNA3 vector encoding an HA tag at the N terminus. The sequence contains an initiator methionine, a stop codon, and a poly-adenylation tail and provides evidence for a novel gene, which we have named ILPIP. Northern blot was performed using the partial ILPIP cDNA isolated from the two-hybrid system following standard procedures.

**Plasmids**

Plasmids encoding JNK1, JNK2, JNK3, p38, ERK2, β-gal-ICE, XIAP, TAB1, and ASK1 (KM, lystine 709 to methionine) used in this study have been previously described (1). TAK1 was expressed in pCMV6. TRA6/TRA6A (amino acids 1–287 deleted at the N terminus, therefore expressing only the TRAF domain) were expressed in pcDNA3 vector encoding an HA tag at the N terminus. The sequence contains an initiator methionine, a stop codon, and a poly-adenylation tail and provides evidence for a novel gene, which we have named ILPIP. Northern blot was performed using the partial ILPIP cDNA isolated from the two-hybrid system following standard procedures.

**Transfection and Cell Culture**

Human embryonic kidney cells (293T) were grown at 37 °C in 5% CO2 in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. For transfection, each well of a six-well plate was seeded with 7 × 10^5 cells. Cells were transfected 18 h later using LipofectAMINE Plus reagent (Invitrogen) for 3 h and incubated for 18 h before lysis. MCP7-Fas cells were grown in RPMI 1640 containing 10% fetal bovine serum, 200 μg/ml G418, and 100 μg/ml hygromycin and grown at 37 °C in 5% CO2. For transfection each well of a six-well plate was seeded with 2.5 × 10^5 cells and 24 h after plating were transfected using LipofectAMINE Plus reagent (Invitrogen). 24 h after transfection, cells were treated with anti-Fas antibody (150 ng/ml). After 16 h, cells were fixed and stained as described below.

**In vitro translation of TAK1 was performed using standard procedures (Promega). XIAP-GST protein was expressed from a pGEX vector (Amersham Biosciences) and flagged as suggested by the manufacturer, and JNK1-HIS protein was purchased from Santa Cruz Biotechnology. Glutathione- or nickel-nitrilotriacetic acid-conjugated beads (from Sigma and Qiagen, respectively) were used to precipitate XIAP-GST and JNK1-HIS. Binding assays were performed in lysate buffer, and TAK1 interaction with XIAP or JNK1 was detected by Western blot using an anti-TAK1 antibody (Santa Cruz Biotechnology).
ILPIP, a New XIAP-interacting Protein

**A**

- Nucleotide and amino acid sequence of ILPIP. ILPIP cDNA was cloned as described in the text. Starting methionines for ILPIP\textsubscript{H9251} and ILPIP\textsubscript{H9252} are highlighted in boldface at amino acid numbers 1 and 139, respectively. The ILPIP\textsubscript{H9251} and ILPIP\textsubscript{H9252} sequences have been submitted to GenBank\textsuperscript{TM} under accession number AY093697.

**B**

- Predicted amino acid homology of ILPIP\textsubscript{H9251} with known proteins. By protein sequence homology, ILPIP is related to serologically defined breast cancer antigen NY-BR-96. Both ILPIP\textsubscript{H9251} and ILPIP\textsubscript{H9252} sequences have been submitted to GenBank\textsuperscript{TM} under accession number AY093697.

**C**

- Protein Kinase Domain

Fig. 1. ILPIP predicted sequences and homologies. 

- A, nucleotide and amino acid sequence of ILPIP. ILPIP cDNA was cloned as described in the text. Starting methionines for ILPIP\textsubscript{H9251} and ILPIP\textsubscript{H9252} are highlighted in **boldface** at amino acid numbers 1 and 139, respectively. The ILPIP\textsubscript{H9251} and ILPIP\textsubscript{H9252} sequences have been submitted to GenBank\textsuperscript{TM} under accession number AY093697.

- B, predicted amino acid homology of ILPIP\textsubscript{H9251} with known proteins. By protein sequence homology, ILPIP is related to serologically defined breast cancer antigen NY-BR-96. Both ILPIP\textsubscript{H9251} and ILPIP\textsubscript{H9252} sequences have been submitted to GenBank\textsuperscript{TM} under accession number AY093697.
ILPIP, a New XIAP-interacting Protein

Researchers studied a novel protein called ILPIP and its interaction with other proteins involved in cellular processes. They observed that ILPIP has a putative protein kinase domain (amino acids 58–369) and encodes a protein of 418 amino acids. The predicted amino acid sequence of ILPIP reveals that it is identical to the putative protein kinase domain of ILPIP.

**RESULTS**

**XIAP Bait Identifies ILPIP in a Two-hybrid Screening**—As a first step in the characterization of the XIAP/JNK1/TAK1/TAB1 interactions, the researchers used a XIAP-Gal4 DNA binding domain fusion construct as bait in the two-hybrid system to screen a human fetal brain cDNA-Gal4 activation domain function library (Matchmaker, CLONTECH). Among the selected primary transformants, positive yeast colonies were individually identified and isolated. Plasmids were isolated from positive yeast colonies and sequenced (Fig. 1A). In the same screening, a shorter isoform of ILPIP was also identified and isolated. Plasmids were isolated from positive yeast colonies and sequenced (Fig. 1B).

**NY-BR-96 exhibit significant homology to SPAK and other Ste20/SPS1-related kinases.** The alignment was generated by the DNAStar megalign program using the ClustalW method. C, schematic representation of the ILPIPα domains. The predicted amino acid sequence of ILPIPα encodes for a protein 418 amino acids long. ILPIPα has a “putative” protein kinase domain (amino acids 58–369). The ATP-binding site is located at the N terminus of the protein. Several putative serine, threonine, or tyrosine-active and phosphorylation sites are shown.

**ALS2CR2**, a novel gene identified within the ALS2 (juvenile amyotrophic lateral sclerosis) critical region (27). At the protein level, the strongest homologies were found with serologically defined breast cancer antigen NY-BR-96 (~20% identity, 46% homology) and with SPAKs (STE20/SPS1-related proline alanine-rich kinases), a member of the Ste20/SPS1 family of kinases and other Ste20-like kinases (Fig. 1B, up to ~20% overall amino acid identity, ~45% similarity). ILPIP has a putative protein kinase domain (amino acids 58–369, Fig. 1C) with a hypothetical ATP-binding site located at the N terminus of the protein and several serine, threonine, or tyrosine active and phosphorylation sites toward the C terminus (Fig. 1C). In the same screening, a shorter isoform encoding a protein of 280 amino acids was isolated (Fig. 1A). The shorter isoform was named ILPIPβ and originates from a methionine at amino acid 139 of the ILPIPα due to an insertion.

**Caspase Activation in Cytosolic Extracts**

Cytosolic extracts from transfected 293T (100-mm dishes) were prepared essentially as described previously (24), with several modifications (25). Briefly, cells were washed once with ice-cold buffer A and pelleted by centrifugation. Packed cell pellets were suspended in 1–2 volumes of buffer A, incubated on ice for 20 min, and then disrupted by 15–30 passages through a 26-gauge needle. Cell extracts were clarified by centrifugation at 16,000 × g for 10 min, and the resulting supernatants were used for “cell-free” assays. For initiating caspase activation, 10 μl horse heart cytochrome c (Sigma) together with 1 mM dATP was added, and the assays were incubated at 30 °C for 10 min. 1 μl (10 μg of total protein) was measured for caspase activity by monitoring the release of AFC DEVD-containing synthetic peptides using continuously reading instruments as described previously (26). Fluorogenic 7-amino-4-trifluoromethyl coumarin (AFC) caspase substrate (Ac-DEVD-AFC) was purchased from Sigma.

**Fig. 2. Tissue distribution and expression pattern of ILPIP mRNA.** Northern blot analysis of ILPIP. A 32P-radiolabeled DNA fragment of ILPIP was hybridized at high stringency to a nitrocellulose membrane bearing 2 μg of poly(A) RNA/ lane isolated from normal human tissues (CLONTECH). A single 2.4-kb transcript was detected using the ILPIP probe with higher levels of expression in muscle, liver, and heart. ILPIP was also expressed as a 2.4-kb band in human embryonic kidney cells. Glyceraldehyde-3-phosphate dehydrogenase was used to check equal loading of RNA. The size of the transcripts is shown on the left.

**Fig. 3. ILPIP moderately activates JNK family members and strongly enhances the XIAP-mediated activation of JNK1, JNK2, and JNK3.** Effect of XIAP, ILPIPα, or ILPIPβ individual or synergistic expression on JNK kinases activation. 293T cells were transfected with vectors encoding for JNK1, JNK2, or JNK3 (200 ng each) in the absence or presence of increasing concentrations of XIAP, ILPIPα, or ILPIPβ (200 or 800 ng). The amount of transfected DNA was kept constant in each sample by adding control pcDNA3 vector. In vitro kinase assay was performed using ATP-2 as substrate. Kinase activity was quantitated by PhosphorImager analysis and is expressed as fold induction relative to the basal level of phosphorylation of each JNK. UV was used as a positive control for each JNK activation (data not shown for JNK2 and JNK3). Western blots showing expression levels of JNK1, XIAP, ILPIPα, or ILPIPβ are shown for each experiment. Expression of ILPIPα or ILPIPβ corresponds to proteins of 52 or 35 kDa, respectively. Asterisks indicate the presence of an unspecific band that appears when the anti-HA antibody is used.
in the 5' region of the gene (data not shown), which introduces two stop codons before the initial ILPIP methionine. The existence of the ILPIP and ILPIPβ isoforms was confirmed by RT-PCR on RNA extracted from several different cell lines. Two PCR products were obtained, and sequence analysis matched the original sequences for ILPIP and ILPIPβ. Northern blot analysis revealed that ILPIP is expressed in normal tissues as a single 2.4-kb transcript with higher levels of expression in muscle, liver, and heart (Fig. 2). ILPIP was also expressed as a 2.4-kb band in human embryonic kidney cells (293T) (Fig. 2). ILPIP Moderately Activates JNK Family Members and Strongly Enhances the XIAP-mediated Activation of JNK1, JNK2, and JNK3—We have previously shown that XIAP-mediated activation of JNK1 is necessary for its anti-apoptotic (1, 2). Thus we addressed the question whether expression of ILPIP alone or ILPIP and XIAP together would have an effect on MAPK activation. 293T cells were co-transfected with vectors encoding for JNK1, JNK2, or JNK3 in the absence or presence of increasing concentrations of DNA expressing XIAP or ILPIP alone (200, 800 ng) or in combination (200 ng of XIAP and 200 and 800 ng of ILPIP or ILPIPβ). The % apoptosis indicates the number of apoptotic cells among the β-galactosidase-positive cells. Data represent the mean ± S.E. of at least three experiments, each run in duplicate and scored blind. B, effect of XIAP or ILPIPβ individual or synergistic expression on Fas-induced apoptosis. MCF7-Fas cells were co-transfected with control vector pcDNA3 alone or plasmids encoding for XIAP or ILPIPβ alone (200, 800 ng) or in combination (200 ng of XIAP and 800 ng of ILPIPβ). DNA expressing β-galactosidase (200 ng) was also transfected to allow visualization of transfected cells and quantitation of apoptotic cells. Transfected samples were treated for 16 h with anti-Fas antibody (150 ng/ml) and analyzed as described above.

FIG. 4. ILPIP moderately protects against ICE- or Fas-induced apoptosis and significantly potentiates the anti-apoptotic activity of XIAP. A, effect of XIAP, ILPIPα, or ILPIPβ individual or synergistic expression on ICE-induced apoptosis. 293T cells were transfected with plasmids encoding for ICE-β-galactosidase alone (200 ng) or ICE together with increasing concentrations of DNA expressing XIAP or ILPIP alone (200, 800 ng) or in combination (200 ng of XIAP and 200 and 800 ng of ILPIP or ILPIPβ). The % apoptosis indicates the number of apoptotic cells among the β-galactosidase-positive cells. Data represent the mean ± S.E. of at least three experiments, each run in duplicate and scored blind. B, effect of XIAP or ILPIPα individual or synergistic expression on Fas-induced apoptosis. MCF7-Fas cells were co-transfected with control vector pcDNA3 alone or plasmids encoding for XIAP or ILPIP alone (200, 800 ng) or in combination (200 ng of XIAP and 800 ng of ILPIP). DNA expressing β-galactosidase (200 ng) was also transfected to allow visualization of transfected cells and quantitation of apoptotic cells. Transfected samples were treated for 16 h with anti-Fas antibody (150 ng/ml) and analyzed as described above.

FIG. 5. XIAP/ILPIPα synergistic activation of JNK1 is mediated by TAK1. Effects of LacZ, TAK1 (KW), or ASK1 (KM) on XIAP and ILPIPα synergistic activation of JNK1. Plasmids encoding wt JNK1 (100 ng) and increasing amounts of XIAP or ILPIPα alone or in combination (200 and 800 ng) were transfected in 293T cells stably expressing LacZ control gene (A), TAK1 (KW) (B), or ASK1 (KM) (C). In vitro kinase assay was performed on immunoprecipitated JNK1 using ATF-2 as substrate and kinase activity quantitated by PhosphorImager. UV stimulation is also shown. Western blots show equal expression of JNK1. Similarly there was equal expression of XIAP or ILPIPα (data not shown).
to UV activation (Fig. 3A). Cooperative activation of JNK2 and JNK3 was also observed, although to a lesser extent (Fig. 3B). Expression of ILPIP or ILPIP plus XIAP had no effect on p38 or ERK2 activation (data not shown). Therefore ILPIP acts synergistically with XIAP in specifically activating JNK family members.

**ILPIP Moderately Protects against ICE- or Fas-induced Apoptosis and Significantly Potentiates the Anti-apoptotic Activity of XIAP**—XIAP is known to protect against a variety of apoptotic stimuli. Because XIAP and ILPIP synergistically activated JNK1 and XIAP-mediated activation of JNK1 is important for the protection against apoptosis, we investigated whether ILPIP and XIAP also cooperatively protect against apoptosis. 293T cells were transfected with a plasmid encoding for ICE-\(\beta\)-galactosidase in the presence of increasing amounts of XIAP or ILPIP DNA, alone or in combination. A green fluorescent protein-expressing vector was used as negative control. Each apoptotic assay was quantified both with X-gal staining of cells and Annexin V-PE-FACS analysis. The results obtained using the two different techniques were comparable, and therefore only the data from representative experiments performed with X-gal are shown. Expression of ILPIP was able to partially inhibit ICE-induced apoptosis (Fig. 4A), although this effect was not as pronounced as that of XIAP. However, ILPIP remarkably enhanced XIAP protection against ICE-induced apoptosis (up to 90% of viable cells), suggesting that ILPIP and XIAP cooperatively protect against ICE-induced apoptosis. Similar results were obtained in MCF7/Fas cells.

**Fig. 6. XIAP and ILPIP interact with TAK1 and TRAF6.** A, *in vivo* interaction of XIAP or ILPIP with TAK1. Vectors encoding XIAP-FLAG or ILPIP-FLAG were co-transfected with wt TAK1-HA or AKT-HA in 293T cells. Cell extracts were immunoprecipitated using anti-FLAG antibody-conjugated beads. Co-precipitated TAK1 or AKT was detected by Western blot analysis with an anti-HA antibody. Cell extracts were directly subjected to immunoblot analysis to check for protein expression. B, interaction of XIAP or ILPIP with TRAF6. Vectors encoding XIAP-FLAG or ILPIP-FLAG were co-transfected with wt TRAF6-Myc or deletion mutant TRAF6-Myc (TRAF6\(\Delta\)-Myc) in 293T cells. Cell extracts were immunoprecipitated using anti-Myc antibody. Co-precipitated XIAP or ILPIP was detected by Western blot analysis with an anti-FLAG antibody. Vectors encoding for XIAP-Myc and ILPIP-FLAG were also transfected in 293T cells to check for co-precipitation between XIAP and ILPIP. Cell extracts were immunoprecipitated using anti-Myc antibody, and co-precipitated ILPIP was detected by Western blot analysis using an anti-HA antibody. Cell extracts were subjected to immunoblot analysis to check protein expression. C, *in vitro* interaction of XIAP with ILPIP. GST-XIAP or GST recombinant proteins were incubated with glutathione-conjugated beads, and *in vitro* translated ILPIP protein was added. Co-precipitation of ILPIP was detected by Western blot using an anti-HA antibody. Input proteins were detected by Western blot using anti-GST antibodies.
when apoptosis was induced by treating the cells for 18 h with anti-Fas antibody (Fig. 4B). ILPIPα partially protected against Fas-induced apoptosis and significantly potentiated the protective effect of XIAP.

**XIAP and ILPIPα Synergistic Activation of JNK1 Involves TAK1**—We have previously reported that XIAP-mediated activation of JNK1 involves the MAP3 kinase TAK1. To investigate if the synergistic effect of ILPIP on XIAP-mediated JNK1 activation was also dependent on TAK1, 293T stably transfected cells expressing either LacZ control vector or catalytically inactive TAK1 (TAK1 (KW)) were transfected with vectors encoding for XIAP or ILPIPα, alone or in combination, and JNK1 activation was determined. 293T cells stably expressing a catalytically inactive mutant of ASK1 (ASK1 (KM)) were also used as a control of specificity. XIAP-, ILPIPα-, or XIAP/ILPIPα-mediated JNK1 activation was inhibited in the presence of TAK1 (KW) (Fig. 5B), whereas LacZ or ASK1 (KM) had no effect (Fig. 5A and C). These data suggest that activation of JNK1 by ILPIPα or XIAP and the synergistic activation of JNK by ILPIP and XIAP are dependent upon TAK1.

**XIAP and ILPIP Interact with TAK1 and TRAF6**—Because TAK1 appears to transmit the signal between the XIAP, ILPIPα, and JNK1, we investigated the possibility that XIAP and ILPIPα would physically interact with TAK1. In an *in vivo* binding assay, vectors encoding XIAP-FLAG or ILPIPα-FLAG were co-transfected with wt TAK1-HA or AKT-HA as a control of specificity in 293T cells. Cell extracts were immunoprecipitated using anti-FLAG antibody and analyzed by Western blot with an anti-HA antibody (Fig. 6A). Cell extracts were also directly subjected to immunoblot analysis to check for protein expression. TAK1 was found to co-precipitate with XIAP and ILPIPα, suggesting that an interaction exists between these proteins. Results were confirmed by reverse co-precipitation using anti-HA antibody (data not shown).

It has been previously reported that TAK1, TRAF6, TAB1, and TAB2 associate in a complex (28–30). Therefore we addressed the question whether XIAP and ILPIPα would also bind to TRAF6, TAB1, and TAB2. Vectors encoding TRAF6-Myc or a deletion mutant of TRAF6 (TRAF6Δ-Myc) were co-transfected with ILPIPα-FLAG or XIAP-FLAG in 293T cells. Cell extracts were immunoprecipitated using anti-Myc antibody and analyzed by Western blot with an anti-FLAG antibody (Fig. 6B). TRAF6 was found to co-precipitate with XIAP and ILPIPα therefore suggesting that an interaction exists between these proteins. Interestingly, TRAF6Δ also co-precipitate with XIAP and ILPIPα indicating that the interactions occur through the TRAF domain of TRAF6. Cell extracts were also directly subjected to immunoblot analysis to confirm expression of the respective proteins. Results were confirmed by reverse co-precipitation using anti-FLAG antibody (data not shown). As a control of specificity, TRAF2 was also assayed for coprecipitation with ILPIP and found to be negative (data not shown).

To determine if the adaptor molecules TAB1 and TAB2 were also part of this complex, vectors encoding TAB1 or TAB2 were co-transfected with XIAP-HA ILPIPα-HA in 293T cells. Cell extracts were immunoprecipitated using anti-TAB1 or anti-TAB2 antibody and analyzed by Western blot with an anti-HA antibody. Co-precipitation of XIAP with TAB1 was confirmed as previously published (18). Interestingly, binding between XIAP and TAB2 was also detected. Surprisingly, ILPIPα did not interact either with TAB1 or TAB2 suggesting that ILPIPα interaction with TAB1 and TAB2 is achieved through XIAP and TAK1 (Fig. 6C).

Because ILPIPα was cloned as an XIAP-interacting protein in a yeast two-hybrid screening, we also addressed the question whether XIAP and ILPIPα were able to co-precipitate in an *in vitro* binding assay. Vectors encoding ILPIPα-FLAG and XIAP-Myc were co-transfected in 293T cells, and cell extracts were immunoprecipitated using anti-Myc antibody and analyzed by Western blot with an anti-FLAG antibody. Surprisingly, no interaction was detected (Fig. 6B). Similar results were obtained using HA-tagged-XIAP thus excluding the possibility that the lack of interaction observed could have been due to the low expression of XIAP-Myc. This result may be explained by the fact that the interaction between XIAP and ILPIPα is transient and unstable and thus undetectable with this method.

To determine if ILPIP could directly interact with XIAP, we used *in vitro* translated ILPIPα protein and recombinant GST-XIAP in *in vitro* binding assays. In these studies, GST protein was also used as negative control. XIAP-GST or GST proteins were incubated with glutathione-conjugated beads. ILPIPα was added to the reactions, and bound proteins were separated on SDS gels and analyzed by Western blots using an anti-HA antibody. ILPIPα was found to associate with XIAP but not with GST (Fig. 6D). Thus, these data are consistent with those observed in our yeast two-hybrid studies, which suggested that XIAP and ILPIP interact directly. The totality of these results supports the idea that XIAP, ILPIPα, TAK1, TRAF6, TAB1, and TAB2 are likely to co-exist in a complex.

**ILPIPα Does Not Affect XIAP Inhibition of Caspase Activation**—XIAP has been reported to be a strong inhibitor of caspase activity (25, 31). However, we have previously shown that XIAP protection against apoptosis requires JNK1 and TAK1 activation and does not affect the ability of XIAP to inhibit caspase activity. Because ILPIPα dramatically enhances the XIAP-mediated activation of JNKs, passes through TAK1, and significantly potentiates the anti-apoptotic activity of XIAP against ICE- or Fas-induced apoptosis, we investigated whether expression of ILPIPα would influence the ability of XIAP to inhibit caspase activity.

293T cells were transfected with control vector or vectors encoding XIAP or ILPIPα, alone or in combination, and the effect on caspase activity was detected by measuring cleavage
of short fluorogenic peptides (24, 25) in a cell-free system where exogenously added cytochrome c induces proteolytic activation of caspase-9 and subsequently caspase-3 in cytosolic extracts. As expected, expression of XIAP strongly suppressed cytochrome c-induced caspase activation, whereas expression of ILPIP alone had no effect. Co-expression of ILPIP with XIAP did not appear to inhibit nor enhance the ability of XIAP to block caspase activation (Fig. 7). To rule out the possibility that the effect of ILPIP on XIAP inhibition of caspases activity was too subtle to be detected when caspase activity is near completely inhibited by XIAP, we diluted the XIAP and XIAP/ILPIP containing extracts 10-fold with the control extracts. Under these conditions cytochrome c-mediated activation of caspases is significantly increased above background, however, ILPIP did not affect the inhibition mediated by XIAP. Combined, these data suggest that ILPIP cooperatively enhances XIAP protection against apoptosis by a mechanism that is independent of inhibition of caspase activity.

**DISCUSSION**

XIAP has been shown to protect against a wide spectrum of apoptotic triggers. A suggested mechanism of IAP apoptotic suppression appears to be through direct caspase inhibition, in fact several of the human IAP family proteins have been reported to directly bind and inhibit specific members of the caspase family. Our findings suggest the existence of an alternative mechanism regulating the anti-apoptotic activity of XIAP that is separate from caspase inhibition and involves the TAB1/TAB2/TAK1/JNK signaling cascade. In an attempt to characterize the pathway connecting XIAP to JNK1 we performed a yeast two-hybrid screening and isolated ILPIP, a novel XIAP-interacting protein. The characterization of ILPIP functional properties highlighted some interesting features. First, ILPIP expression slightly activates JNK and strongly enhances JNK1 activation when co-expressed with XIAP. Activation of MAPKs has been reported to regulate the activities of many transcription factors and regulatory molecules and is required for the regulation of inflammatory responses, cell proliferation, and apoptosis (32). In particular, the involvement of the JNK family in apoptotic cell death has been most actively studied. JNK activation is observed in apoptosis induced by a variety of stimuli in different cell types. However, the consequence of its activation has been contradictory, resulting in protection from apoptosis in some cases and induction of apoptosis in others (32–36). Despite these apparent contradictions, there is a growing consensus that correlation between activation of JNK protection or induction of apoptosis is stimulus-and/or cell type-dependent (37–40). With this in mind, we investigated whether XIAP/ILPIP synergistic activation of JNK1 was correlated with the ability of XIAP to protect against apoptosis. Interestingly, ILPIP moderately protects against ICE- or Fas-induced apoptosis and significantly potentiates the anti-apoptotic activity of XIAP therefore further supporting the idea that XIAP-mediated activation of JNK1 promotes cell survival.

XIAP and ILPIP synergistic activation of JNK1 involves TAK1 as demonstrated by inhibition of JNK1 activity using a catalytically inactive form of TAK1. It has been previously reported that XIAP participates in the BMP signaling pathway by binding with the BMP receptor, the adapter molecule TAB1 (2, 18), and with TAK1 (2). The findings that ILPIP also co-precipitates with TAK1, that both XIAP and ILPIP bind to TRAF6, and that XIAP also binds to TAB2, further support the idea that these molecules behave in a functional complex.

Surprisingly, we were unable to detect association between XIAP and ILPIP in cells, suggesting that such an interaction may be transient. That could be explained by the possibility of ILPIP being a kinase, as predicted by the homology with serine/threonine kinases and by the presence of a putative kinase domain. This possibility is currently under investigation in our laboratory. A direct interaction between XIAP and ILPIP was demonstrated in an *in vitro* binding assay thus supporting the original interaction showed by the yeast two-hybrid system.

Finally, expression of ILPIP did not affect XIAP inhibition of caspase activation further supporting the idea that XIAP protection against apoptosis is achieved by two separate mechanisms: one requiring JNK1 activation and a second involving caspase inhibition. The interaction between XIAP, ILPIP, and TRAF6 may also suggest that XIAP might be involved in the IL-1 inflammatory response, which TRAF6 has been previously shown to regulate (41).

XIAP has been reported to interact with other proteins all of which act as negative regulators. Among these is SMAC/DIABLO, which is a nuclear-encoded, mitochondrially localized protein that is released in response to apoptotic stimuli and acts as a negative regulator of XIAP anti-apoptotic function (21, 22). Similarly Omi/HtrA2, a serine protease localized in the mitochondria, inhibits the protective effect of XIAP by binding to its BIR-3 domain (42). A third negative regulator of XIAP is XAF1, which is thought to exert its effect through sequestering XIAP in the nucleus (43). Importantly, we show here that ILPIP is the first protein able to potentiate the anti-apoptotic effect of XIAP instead of antagonizing it.

Taken together our results describe a novel XIAP-interacting protein that acts as a co-factor enhancing XIAP-mediated activation of JNK1 and the caspase-independent protection of XIAP against apoptosis.

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M. Germana Sanna, Jean da Silva Correia, Ying Luo, Betty Chuang, Lorien M. Paulson, Binh Nguyen, Quinn L. Deveraux and Richard J. Ulevitch

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