Livin, a Novel Inhibitor of Apoptosis Protein Family Member*

Gary M. Kasof and Bruce C. Gomes‡

From AstraZeneca Pharmaceuticals, Enabling Sciences and Technology, Wilmington, Delaware 19803

A novel human inhibitor of apoptosis protein (IAP) family member termed Livin was identified, containing a single baculoviral IAP repeat (BIR) domain and a COOH-terminal RING finger domain. The mRNA for livin was not detectable by Northern blot in most normal adult tissues with the exception of the placenta, but was present in developmental tissues and in several cancer cell lines. Highest levels were observed in two melanoma-derived cell lines, G361 and SK-Mel28. Transfection of livin in HeLa cells resulted in protection from apoptosis induced by expression of FADD, Bad, RIP, RIP3, and DR6. Similar to other IAP family members, the anti-apoptotic activity of Livin was dependent on the BIR domain. Livin was also capable of inhibiting DEVD-like caspase activity triggered by tumor necrosis factor-α. In vitro binding studies demonstrated a direct interaction between Livin and the active form of the downstream caspases, caspase-3 and -7, that was dependent on the BIR domain of Livin. In addition, the unprocessed and cleaved forms of caspase-9 co-immuno-precipitated with Livin in vivo, and recombinant Livin could inhibit the activation of caspase-9 induced by Aparf-1, cytochrome c, and dATP. The subcellular distribution of the transfected Livin was analyzed by immuno-fluorescence. Both Livin and Survivin were expressed in the nucleus and in a filamentous pattern throughout the cytoplasm. In contrast to the apoptotic activity, the COOH-terminal RING domain mediated its subcellular localization patterning. Further studies found that transfection of an antisense construct against livin could trigger apoptosis specifically in cell lines expressing livin mRNA. This was associated with an increase in DNA fragmentation and in DEVD-like caspase activity. Thus, disruption of Livin may provide a strategy to induce apoptosis in certain cancer cells.

The inhibitor of apoptosis protein (IAPs) family is characterized by one or more repeats of a highly conserved ~70 amino acid domain termed the baculoviral IAP repeat (BIR) and suppress apoptosis triggered by a wide variety of stimuli, including viral infection, chemotherapeutic drugs, staurosporin, growth factor withdrawal, and by components of the tumor necrosis factor-α (TNF-α)/Fas apoptotic signaling pathways (1–3). While first identified in baculovirus, the IAP family has been conserved evolutionarily from viruses to nematodes, flies, and several mammalian species. There are currently five human IAP family members, c-IAP1, c-IAP2, XIAP, NAIP, and Survivin (4–7). All of the human IAP family members, with the exception of NAIP, have been shown to interact with specific cysteine proteases, or caspases, required for the cleavage of certain proteins involved in the disassembly of the cell during apoptosis (8). The caspases are synthesized as inactive zymogen forms which upon apoptotic stimulation are proteolytically processed in a sequential manner into their active heterotrameric forms. c-IAP1, c-IAP2, XIAP, and Survivin have been reported to bind to and inhibit the active forms of the terminal caspases-3 and -7, but do not interact with caspases-8, which is the most proximal caspase from the TNF-α/Fas receptor (9–12). However, in the case of Survivin, caspase inhibition may be more indirect through association with Cdk4 leading to inhibition of the pro-caspase-3 by p21 (13). c-IAP1, c-IAP2, and XIAP also bind to the c-Jun N-terminal kinase (JNK) pathway, and this pathway plays a role in the inhibition of apoptosis by IAPs (14). Abrogation of caspase activity, a common downstream component of apoptosis, enables IAPs to have widespread anti-apoptotic potential.

The BIR domain forms a novel zinc-fold that is the critical motif for their anti-apoptotic activity and interaction with caspases (15). While many IAPs contain up to three tandem BIR repeats, a single BIR domain is sufficient for caspase interaction and protection from apoptosis (16). Many of the IAP proteins (c-IAP1, c-IAP2, XIAP, as well as viral and insect IAPs) also contain a RING domain near their COOH termini. The role for the RING domain varies depending on the IAP and/or the apoptotic stimulus, but does not appear to be required for the anti-apoptotic activity of human IAPs (10, 16–18). Deletion of the RING domain in c-IAP2 has suggested a critical role in TNF-α-mediated NF-κB activation, thereby providing an additional mechanism for the IAPs anti-apoptotic activity (19). Recent studies have also found that c-IAP1 and XIAP have ubiquitin ligase activity which leads to their degradation during apoptosis and that this activity is dependent on their RING domain (20). However, it is unclear if this is a general feature of the RING domain in other IAP family members.

Several of the IAP family members have been reported to play a role in pathological conditions, particularly neurodegenerative disorders and cancer. For instance, the NAIP gene was originally identified based on its deletion in patients with spinal muscular atrophy, a neurodegenerative disorder characterized by motor neuron depletion through apoptosis (21). The correlation between NAIP, spinal muscular atrophy, and apoptosis suggests that NAIP may be required for the survival of these neurons and that mutations in the NAIP locus contribute to spinal muscular atrophy. In addition, NAIP levels are transiently elevated following ischemia and damage can be inhibited by overexpression of NAIP in vivo (22). Expression of other
IAP family members have been correlated with cancer. For example, XIAP and c-IAP1 are found in most cancer cell lines (23). Survivin is overexpressed in nearly all human tumors and transformed cell lines, but is rarely present in normal adult tissues (4, 11, 24–27). Survivin is induced by angiogenic factors such as vascular endothelial growth factor, fibroblast growth factor, and angiopoietin-1 which may explain its elevated levels in tumors (28, 29). Depletion of Survivin using antisense or dominant negative mutants induces apoptosis implying that Survivin expression contributes to the survival of cancer cells (24, 30–33). Tumors expressing Survivin, as well as other anti-apoptotic signal generally have a poorer prognosis, likely due to their resistance to classical chemotherapy (25).

Here, we attempted to identify novel members of the IAP family via homology searches. One gene was identified which we termed *livin* that encodes a protein with a single BIR domain and a COOH-terminal RING domain. Expression of Livin inhibited apoptosis by a number of stimuli, whereas an antisense construct was shown to induce apoptosis. Like its other family members, Livin was capable of binding to caspases and could inhibit the proteolytic processing of caspase-9 in *vitro*. Deletion mutants of Livin (*D*<sub>N</sub>154, *D*<sub>C</sub>86, and 86–154) were generated by PCR and subcloned into the *Kpn*<sub>I</sub>/*Apa*<sub>I</sub> sites within *pcDNA3.1/myc-His* (Invitrogen, Carlsbad, CA). FADD, Bax, and DR6, were subcloned by PCR from Incyte clones 3334311, 2057308, and 2733717 and ligated into pTracer-SV40 (Invitrogen, Carlsbad, CA) at *Eco*<sub>R</sub>/N*ot*<sub>I</sub>, *Eco*<sub>R</sub>V/*Not*<sub>I</sub>, and *Not*<sub>I</sub>/*Spe*<sub>I</sub> sites, respectively. Caspase-9 was prepared by PCR from Incyte clone 3590462 and subcloned into *pcDNA3.1/V5/His-TOPO*. Apaf-1 was cloned into *pcDNA3.1/V5/His-TOPO* from a PCR product generated from a human testis cDNA library (CLONTECH, Palo Alto, CA). The pTracer-SV40-RIP plasmid was prepared by PCR from pMH-RIP (34) and ligated into the *Kpn*<sub>I</sub>/*Not*<sub>I</sub> sites in pTracer-SV40. The constructions of pTracer-SV40-RIP3 and pZeoSV2-Bcl-x<sub>L</sub> were described previously (34). *pcDNA3.1-myc-survivin* was provided by Dr. Kevin Hudson (AstraZeneca Pharmaceuticals, Macclesfield, Cheshire, UK). The nucleotide sequences of all of the clones were confirmed by fluorescent terminator cycle sequencing using *FIG.1*.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—A partial cDNA sequence of *livin* was identified in the proprietary Incyte LifeSeq data base (accession number 1419118). To obtain the full-length construct we screened an adult kidney cDNA library (Life Technologies, Grand Island, NY) with a *livin*-specific probe, 5′-CCTTCTATGACTGGCCGCTGA-3′ using the GeneTrapper<sup>TM</sup> cDNA positive selection system (Life Technologies, Grand Island, NY). The coding sequence of *livin* was PCR cloned into *pcDNA3.1/V5/His-TOPO* (Invitrogen, Carlsbad, CA) in both the sense and antisense orientations. Livin was subsequently subcloned by PCR into the *Bam*<sub>H</sub>I/*Eco*<sub>R</sub> sites in pGEX-6P (Amersham Pharmacia Biotech, Piscataway, NJ) for purification of a glutathione S-transferase fusion protein. Deletion mutants of Livin (*D*<sub>N</sub>154, *D*<sub>C</sub>86, and 86–154) were generated by PCR and subcloned into the *Kpn*<sub>I</sub>/*Not*<sub>I</sub> sites in *pcDNA3.1/myc-His*. FADD, Bax, and DR6, were subcloned by PCR from Incyte clones 3334311, 2057308, and 2733717 and ligated into pTracer-SV40 at *Eco*<sub>R</sub>/N*ot*<sub>I</sub>, *Eco*<sub>R</sub>V/*Not*<sub>I</sub>, and *Not*<sub>I</sub>/*Spe*<sub>I</sub> sites, respectively. Caspase-9 was prepared by PCR from Incyte clone 3590462 and subcloned into *pcDNA3.1/V5/His-TOPO*. Apaf-1 was cloned into *pcDNA3.1/V5/His-TOPO* from a PCR product generated from a human testis cDNA library (CLONTECH, Palo Alto, CA). The pTracer-SV40-RIP plasmid was prepared by PCR from *pcMH-RIP* (34) and ligated into the *Kpn*/*Not* sites in pTracer-SV40. The constructions of pTracer-SV40-RIP3 and pZeoSV2-Bcl-x<sub>L</sub> were described previously (34). *pcDNA3.1-myc-survivin* was provided by Dr. Kevin Hudson (AstraZeneca Pharmaceuticals, Macclesfield, Cheshire, UK). The nucleotide sequences of all of the clones were confirmed by fluorescent terminator cycle sequencing using
an automated 377 DNA sequencer (PerkinElmer Life Sciences and Applied Biosystems, Foster City, CA).

**Production of Recombinant Livin—**pGEX-6P-livin was inoculated overnight in *Escherichia coli* BL21 and subsequently stimulated with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h. The bacterial pellet was resuspended in phosphate-buffered saline plus lysozyme (1 mg/ml) and lysed by sonication. Lysates were incubated with 1% Triton X-100 for 30 min at 4 °C and then centrifuged to remove the insoluble material. Supernatants were added to a glutathione-S-transferase 4B column and the bound protein was eluted with PreScission protease (Amersham Pharmacia Biotech, Piscataway, NJ) thereby cleaving the glutathione S-transferase fusion.

**Maintenance of Cell Lines—**HeLa, G361, SK-Mel29, HMCB, A375, WM115, HT114, and SW480 cell lines (ATCC, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin (100 units/ml)/streptomycin (0.1 mg/ml). The cells were maintained at 37 °C, 5% CO2. Transfections were performed using LipofectAMINE Plus (Life Technologies, Grand Island, NY) according to the manufacturer's specifications.

**Purification of RNA and Northern Blotting—**Poly(A) RNA was isolated from human melanoma cell lines (SK-Mel29, HMCB, A375, WM115, and HT114) using the Poly(A) Pure kit (Ambion, Austin, TX) according to the manufacturer's specifications. The RNA was denatured in sample buffer (2.2 M formaldehyde, 50% formamide, 50 mM MOPS (pH 7.0), and 1 mM EDTA) heated at 65 °C for 10 min, electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde, 50 mM MOPS (pH 7.0), and 1 mM EDTA, and then transferred by capillary elution onto Hybond-N nylon filters (Amersham Pharmacia Biotech). In addition, we used commercially available human RNA blots, prepared from adult and fetal tissues and cancer cell lines (CLONTECH, Palo Alto, CA). The blots were hybridized to random primed radiolabeled *livin* (full-length cDNA containing 5'- and 3'-untranslated region) or *actin* (CLONTECH, Palo Alto, CA) incubated in Hybriol-I (Oncor, Gaithersburg, MD) overnight at 42 °C. The blots were then washed with 2 × SSC + 0.05% SDS at room temperature followed by high stringency washing, 0.1 × SSC + 0.1% SDS at 50 °C, and visualized using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**GFP Viability Assays—**Viability was assessed using the green fluorescent protein (GFP) marker produced from pTracer-SV40-derived vectors. Transfected cells were fixed with 2% paraformaldehyde in phosphate-buffered saline, counterstained with 10 μg/ml propidium iodide, 200 μM RNase A, and 0.1% Tween 20 for 30 min at room temperature, and then mounted on microscope slides with Immuno-mount (Shandon, Pittsburgh, PA). A laser scanning cytometer (Compucyte, Cambridge, MA) was used to determine the percent of GFP-positive cells. The propidium iodide staining served as a marker required for staining a PhosphorImager.

**Caspase Activity Assays—**The ability of antisense against *livin* to induce apoptosis was examined using TdT-mediated dUTP nick end labeling (TUNEL) assay. Transfected cells were stained with fluorescein isothiocyanate-conjugated dUTP according to the manufacturer's specifications (Roche Molecular Biochemicals, Indianapolis, IN). The cells were then counterstained with 10 μg/ml propidium iodide and 200 μg/ml RNase A, and collected on microscope slides by a cytospin. The percent of TUNEL-positive cells was evaluated using the laser scanning cytometer as described above for GFP.

**In Vitro Binding Assay—**In vitro binding reactions were performed by combining recombinant active caspase-3 or -7 (PharMingen, San Diego, CA) with [35S]methionine-labeled Survivin or Livin (wild-type and deletion mutants), prepared using the TNT T7 reticulocyte lysate system. Samples were incubated with polyclonal antibodies against either caspase-3 or -7 (PharMingen, San Diego, CA) in 0.5 ml of NETN buffer (20 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, and 0.2% Nonidet P-40) for 1.5 h at 4 °C followed by protein G-Sepharose (Amersham Pharmacia Biotech) for 30 min. All samples were then washed three times in NETN buffer, resolved by SDS-PAGE, and detected using a PhosphorImager.

**Immunoprecipitations and Western Blotting—**With caspase-9 were shown using HeLa cells transiently transfected with either pCDNA3.1/V5/His-TOPO-livin or pCDNA3.1-myc-survivin and treated with TNF-α and cycloheximide. Cell lysates were prepared in 1 ml of RIPA buffer (0.01 M sodium phosphate (pH 7.2), 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS) supplemented with a protease inhibitor mixture tablet (Roche Molecular Biochemicals, Indianapolis, IN) which were spun at 20,000 × g for 30 min at 4 °C. The soluble supernatants were immunoprecipitated using either a monoclonal antibody against V5 (Invitrogen, Carlsbad, CA) followed by protein G-Sepharose or with a monoclonal antibody against Myc conjugated to agarose (Santa Cruz Biotechnology, Santa Cruz, CA). The samples were washed three times in RIPA buffer, boiled in Laemmli buffer, and resolved by SDS-PAGE. The gels were semi-dry blotted onto nitrocellulose and probed with a monoclonal antibody against caspase-9 (PanVera Corp., Madison, WI). Immuno-complexes were detected by enhanced chemiluminescence (ECL) according to the manufacturer's specifications (Amersham Pharmacia Biotech).

**Western Blots** were performed to confirm that the antisense construct of *livin* could reduce Livin expression, but not Survivin. Transfected HeLa cells were maintained in 100 μM ZVAD-fmk (Enzyme Systems, Livermore, CA) to prevent apoptosis. Cell lysates were prepared in Laemmli buffer and Western blots were performed as described above using antibodies against V5, Survivin (R & D Systems, Minneapolis, MN), and glyceraldehyde-3-phosphate dehydrogenase.
Immunofluorescence—The subcellular localizations of Livin and Survivin were assessed by indirect immunofluorescence from HeLa cells transfected with epitope-tagged expression constructs. Twenty-four hours post-transfection the cells were fixed with 2% paraformaldehyde and then permeabilized with 0.2% Triton X-100 in phosphate-buffered saline. Coverslips were incubated with antibodies against the V5 or Myc (Oncogene Research Products, Cambridge, MA) epitopes for 1 h at 37 °C. Staining was detected using a fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Life Technologies, Grand Island, NY) for 1 h at 37 °C. Cells were visualized by epifluorescence using an Olympus AX70 microscope equipped with a Kodak DCS 520 digital camera (Hitech Instruments, Inc., Edgemont, PA).

RESULTS AND DISCUSSION

Identification and Expression of Livin—A homology search of a proprietary Incyte data base using BLAST, revealed an 840-nucleotide sequence present in a fetal kidney library and predicted to encode a novel protein with a BIR domain and a COOH-terminal RING finger domain (accession number 1419118). To obtain the full-length gene, we used the GeneTrapper™ cDNA positive selection system to screen an adult kidney cDNA library. A low abundant clone was found which contained an in-frame stop codon in the 5′-untranslated region, suggesting it was a full-length gene. This gene, which we termed livin, was 1297 base pairs and predicted to encode a 280-amino acid protein (Fig. 1A; GenBank accession number AF311388). Genomic sequence corresponding to the livin cDNA was found in the EMBL data base (accession number AL121827) and localized on chromosome 20. The overall protein identity of Livin to other IAP family members based on the GAP pairwise sequence alignment (GCG™, Madison, WI) was 24.1% to c-IAP1, 26.1% to c-IAP2, 34.7% to XIAP, 25.5% to NAIP, and 26.3% to Survivin. At a structural level it was similar to Survivin with respect to having just a single BIR domain (Fig. 1B). However, Livin did not have a coiled-coil domain like Survivin, but rather contained a COOH-terminal
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RING domain found in c-IAP1, c-IAP2, and XIAP.

The tissue distribution of livin was studied by Northern blotting with mRNA prepared from human adult and developmental tissues as well as several cancer cell lines. Using the entire cDNA sequence of livin as a probe, three distinct mRNAs were detected with approximate sizes of 1.4, 2.0, and 2.8 kilobases (Fig. 2). The smaller of the transcripts was consistent with the cDNA isolated in our screen. The sizes of the three transcripts were distinct from other IAP family members, suggesting that they were specific for livin. Despite the presence of livin in fetal and adult kidney cDNA libraries, within the normal tissues tested here, livin was found only in placenta and fetal brain. It is likely that livin is expressed transcriptionally in other tissues, but at levels too low to be detected by the Northern blot. Elevated levels of livin were seen in cancer cell lines, particularly in the melanoma cell lines G361 and SK-Mel29, and to a lesser extent, in HeLa (Fig. 2). Interestingly, the segments of the chromosome corresponding to the livin locus display increased copy number in nearly all melanoma cell lines and primary tumors (35). While livin has a narrower distribution than survivin in cancer cells, the general patterns of expression between these genes are similar, with no detectable expression in normal adult tissues and elevated levels in placenta, developing tissues, and cancer cell lines (4, 11).

Anti-apoptotic Activity of Livin—While the BIR motif can confer an anti-apoptotic signal via caspase interactions, some BIR motifs are not as effective at suppressing apoptosis, and there are BIR-containing proteins with no apparent anti-apoptotic function (16, 18, 36). Therefore, not all BIR domain-containing proteins may be defined properly as an IAP. The potential anti-apoptotic activity of Livin was investigated with respect to several pro-apoptotic signals acting at different points within the apoptotic process, including DR6, FADD, RIP, RIP3, and Bax. DR6 is a member of the TNF receptor family and thus is at the most upstream level of the apoptotic process (37). FADD (38), RIP (39), and RIP3 (40, 41) are adapter proteins within the TNF-α/Fas pathway. Bax is a potent inducer of apoptosis but may not have a direct role in TNF-α/Fas pathway (42). HeLa cells were transfected with these apoptotic genes in the pTracer vector containing a GFP marker for accessing viability. Transfection with any of the pro-apoptotic genes led to roughly a 90% reduction in viability, as compared with empty pTracer vector (Fig. 3A). Co-transfection of the apoptotic genes with either livin or survivin pro-

Fig. 5. Livin inhibits TNF-α-triggered DEVD-like caspase activity and binds to active caspase-3 and -7 in vitro. HeLa cells (2 × 10⁶) were transfected with 1 μg of pcDNA3.1/V5/His-TOPO-livin, pZeoSV2-Bcl-x₁, or empty pcDNA3.1 control. Twenty hours post-transfection the cells were treated with TNF-α (1000 units/ml) and cycloheximide (30 μg/ml) for 5 h (hatched) or left untreated (solid). DEVD-APC hydrolysis produced from cytosolic extracts was monitored over a 1-h period at 37 °C and quantified in relative fluorescence units (RFU). Error bars represent standard error of the mean, n = 3. B, equal amounts of recombinant activated caspase-3 or -7 (1 μg) were incubated with [35S]methionine-labeled Survivin, Livin, or the deletion mutants, ΔC86 (NH₂ terminus) and 86–154 (BIR domain), which were prepared in vitro using the TNT T7 reticulocyte lysate system. Samples were immunoprecipitated with antibodies against the respective caspases and then resolved by SDS-PAGE and visualized by autoradiography. Aliquots of the in vitro translated (IVT) proteins (2 μl) were also analyzed in order to determine their relative abundance.
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Livin Inhibits Caspase Activity and Binds to Caspase-3, -7, and -9—Several IAP family members including XIAP, c-IAP1, and c-IAP2 directly bind to pro-caspase-9 and prevent its processing (14). An association has also been observed between pro-caspase-9 and a cleaved product of XIAP containing its BIR3 and RING domains, and therefore presumably has a similar structure to Livin (44). Possible interactions between Livin and/or Survivin with caspase-9 were tested in HeLa cells transfected with these IAP family members. The cells were subsequently treated with TNF-α to evaluate interactions with activated caspase-9, or left untreated to examine the unprocessed form. Cell lysates were immunoprecipitated with antibodies against the epitope tags on Livin (V5) and Survivin (Myc) and then probed by Western blot for caspase-9. In the absence of TNF-α, Livin, but not Survivin, immunoprecipitated with the unprocessed 45-kDa form of caspase-9 (Fig. 4A). Upon stimulation with TNF-α, an increase in binding was observed between Livin and the 35-kDa cleaved form of caspase-9. Both Survivin and Livin were expressed in the lysates (Fig. 4B). These data demonstrate an association between Livin and pro-caspase-9 and a partially activated form of caspase-9 containing the NH2-terminal prodomain. No interaction of Livin was seen with the fully active 12- and 20-kDa subunits of caspase-9 using the Biacore, suggesting that the prodomain is required for the interaction.

The effect of Livin on caspase-9 processing was tested in vitro. Incubation of procaspase-9 with Apaf-1, cytochrome c, and dATP can induce the processing of caspase-9, which can be inhibited by XIAP, c-IAP1, and c-IAP2 (14). Here, we found that addition of recombinant Livin directly inhibited caspase-9 processing as seen by a decrease in the 35-kDa fragment (Fig. 4C). Thus, Livin appears to act on caspase-9 in a similar manner to XIAP, c-IAP1, and c-IAP2 but distinct from Survivin.

The effect of Livin on DEVD-like caspase activity was tested in HeLa cells treated with TNF-α. TNF-α-induced apoptosis involves a complex signaling response that includes activation of caspase-8 at the receptor level leading to the processing of downstream caspases including caspase-3 and -7 (45). Caspase activity was measured using the fluorescent substrate DEVD-AFC, used to specifically detect DEVD-cleaving caspases (caspase-3, -6, -7, -8, and -10). Cell lysates were prepared from HeLa cells transfected with livin, the anti-apoptotic gene bcl-xL, or an empty vector which were subsequently treated with TNF-α and cycloheximide. Treatment with TNF-α and cycloheximide for 5 h led to a 7-fold increase in DEVD-AFC cleavage (Fig. 5A). However, cells transfected with livin exhibited reduced caspase activity by 15–20%, consistent with the transfection efficiency of these cells. Comparable levels of caspase inhibition were observed upon transfection with bcl-xL. These results demonstrate the ability of Livin to inhibit DEVD-like caspase activity in vitro, although Livin may not directly inhibit these caspases and it is possible that this suppression is simply the result of abrogation of caspase-9 processing upstream of caspase-3.

Next, we tested whether Livin and Survivin could bind to the active forms of caspase-3 and -7. Interactions between Survivin and caspases have been controversial. Initial studies showed that survivin prepared from whole cell lysates could bind to caspase-3 and -7 and also inhibit DEVD-like caspase activity (11), and there was one report that recombinant survivin could directly inhibit the activity of caspase-3 (12). While subsequent studies confirmed that Survivin was capable of binding to...
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caspase-3 directly, in whole cell preparations Survivin bound preferentially to Cdk4 (13). Also, in a recent analysis of the x-ray crystal structure of Survivin, the authors found no direct interaction or inhibition of caspase-3 (46). In our experiments, 35S-labeled Livin and Survivin were prepared by in vitro transcription/translation and were incubated with recombinant active caspase-3 and -7 followed by caspase specific antibodies. Both caspase-3 and -7 interacted with Livin and Survivin, but not with the deletion mutant ΔC89 used as a negative control (Fig. 5B). The BIR domain of Livin was sufficient for caspase binding in agreement with its anti-apoptotic activity (Fig. 5B). A Biacore was used to determine the kinetics of the interaction between Livin and the active forms of caspase-3, and -7. Relatively potent affinity constants were observed for both of the caspases (24.6 pm for caspase-3 and 5.1 pm, for caspase-7).

While we found that Livin and Survivin could interact with caspases-3 and -7, we have yet to find any direct effect on inhibiting the activity of these caspase using the DEVD-AFC substrate (using up to a 1000-fold molar excess of IAP). Thus, there was an apparent discrepancy between the relatively strong binding between Livin and caspases-3 and -7 yet a lack of any effect on enzyme activity. In fact, other reports of IAP inhibition of caspase activity have been reported only when using a large (more than 5000) molar excess of IAP to caspase (10). Perhaps, in some cases either complexes with other unidentified proteins and/or post-translational modifications could enhance the inhibitory effects of Livin and Survivin on caspase activity.

**Subcellular Localization of Livin**—Differential subcellular localization of the IAP family members have implied distinct roles in apoptosis regulation (18, 32). Staining for Livin was performed using antibodies against the V5 epitope tag in transfected HeLa cells. Livin was observed predominantly in the nucleus and in a filamentous pattern throughout the cytoplasm (Fig. 6). Furthermore, transfected Survivin, which was stained using antibodies against the Myc epitope, was observed in the same pattern as Livin (Fig. 6). Previous studies have shown that endogenous Survivin translocates to the nucleus during apoptosis and interacts with the cell cycle regulator Cdk4 (13). Survivin may also associate with microtubules of the mitotic spindle and regulate apoptosis during cell cycle progression (32). While the staining patterns observed here may be obscured due to the overexpression of the proteins, it does suggest that Livin and Survivin have similar subcellular localization. If the staining is an accurate reflection of endogenous Livin, it seems possible that Livin could play a role in preventing caspase cleavage of both cytoskeletal and nuclear proteins during apoptosis and interacts with the cell cycle regulator Cdk4 (13).

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**Error bars** indicate standard error of the mean, n = 3.
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ing apoptosis and perhaps is regulated by cell cycle proteins similar to Survivin.

To determine the critical motifs required for the localization of Livin, HeLa cells were transfected with the deletion mutants ΔC86, 86–154, and ΔN154 each containing a Myc epitope tag used for staining. The truncated proteins ΔC86 and 86–154 produced aberrant diffuse staining (not shown). However, ΔN154, which contains the ~35 amino acid RING domain, was found in the same filamentous pattern as the wild-type protein (Fig. 6). These results suggest that the COOH-terminal region of Livin, and perhaps more specifically the RING domain, provides a sufficient signal for its proper subcellular distribution.

While the function of RING domains have been largely enigmatic, they are often associated with mediating multiprotein complexes and in some cases co-precipitate with cytoskeletal proteins (47). Taking the functional and localization studies of Livin together, one can propose a modular model whereby the BIR domain mediates its anti-apoptotic activity and interaction with caspases, while the RING domain provides proper cellular localization. Interestingly, other IAP family members that have RING domains do not localize in the filamentous pattern seen here (18). This disparity suggests that the function of the RING domain, like the BIR domain, is not absolutely conserved but rather may depend on either the particular IAP and/or cell type in which it is expressed.

Antisense to Livin Induces Apoptosis—Since depletion of Survivin using antisense was shown to produce defects in cell division as well as increased caspase activation and apoptosis (24, 30–33), we were interested in testing whether similar effects could be seen for Livin. An antisense construct was designed to include the entire coding region of livin cloned in the antisense orientation. There was no open reading frame in the antisense orientation of livin and therefore the construct did not result in the expression of new proteins. To verify that the antisense construct could reduce Livin expression, we compared the levels of overexpressed Livin protein in cells transfected with antisense to that of a control vector. To prevent apoptosis the cells were maintained in the presence of the general caspase inhibitor ZVAD-fmk. In both HeLa (Fig. 7A) and G361 (not shown) cells, transfection with the antisense construct abrogated the expression of Livin seen with the V5 antibody. In contrast, the antisense did not reduce the levels of Survivin (Fig. 7A), suggesting that the construct has a specific effect on Livin, and not on related IAP family members.

The effect of the antisense construct on cell viability was analyzed by co-transfection with an empty pTracer vector producing the GFP marker. Transfections were performed in HeLa and G361 cells, which normally express livin, as well as SW480 cells, which do not have detectable levels of livin mRNA (Fig. 2). In HeLa or G361 cells the antisense decreased the expression of Livin with the V5 antibody. In contrast, the antisense did not reduce the levels of Survivin (Fig. 7A), suggesting that the construct has a specific effect on Livin, and not on related IAP family members.

To extend these results, apoptosis was evaluated in more detail by measuring DNA fragmentation and caspase activation. DNA fragmentation was assessed using the TUNEL assay. Transfection of the antisense construct into G361 cells triggered an ~8% increase in TUNEL staining (Fig. 7C). Typically, the transfection efficiency in these cells was 15–20% suggesting that about half of the transfected cells were TUNEL-positive. To test whether this correlates with an increase in caspase activity, cytosolic extracts of the transfected cells were incubated with the fluorescent substrate, DEVD-AFC. Transfections with the antisense triggered nearly a 3-fold increase in DEVD-AFC cleavage (Fig. 7D). Presumably, this increase in caspase activity is a consequence of diminished livin-caspase interaction.

These studies, along with those of Survivin, demonstrate significant roles for these IAP family members in maintaining viability. While the antisense to livin may act on only certain cell lines, it is interesting that at least in HeLa cells, apoptosis resulted from antisense to either livin or survivin. On the other hand, antisense against xiap had no effect on the viability of HeLa cells (data not shown). Furthermore, expression of survivin could not rescue from apoptosis triggered by livin antisense and vice versa. This suggests that Livin and Survivin are not redundant, with distinct and important roles in regulating apoptosis.

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