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Two splicing variants of a new inhibitor of apoptosis gene with different biological properties and tissue distribution pattern

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Abstract Using homology searches, we identified a novel human inhibitor of apoptosis (IAP) gene. This gene has two splicing variants that contain open reading frames of 298 and 280 amino acids and both contained a single copy of baculovirus IAP repeat (BIR) and RING domain. We refer here to the longer and shorter variants as Livin α and β, respectively. Semiquantitative reverse transcriptase-polymerase chain reaction demonstrated a tissue-specific and non-correlated expression pattern in both adult and fetal tissues. Both mRNA variants were detected in various transformed cell lines. Despite their very close similarity, the two isoforms have different antiapoptotic properties. Both isoforms have a significant antiapoptotic activity in the Jurkat T cell line after triggering apoptosis via tumor necrosis factor and CD95 receptors. The Livin α but not β protects cells from apoptosis induced by staurosporine, but in contrast, apoptosis initiated by etoposide was blocked only by the β isoform. This difference in biological activities may indicate the presence of critical amino acids outside the BIR and RING domains. These functional and tissue distribution differences of Livin α and β suggest that Livin may play a complex role in the regulation of apoptosis.

Key words: Antiapoptotic gene; Inhibitor of apoptosis; Alternative splicing

1. Introduction

The inhibitors of apoptosis (IAPs) are a novel family of intracellular proteins which suppress apoptosis induced by a variety of stimuli [1,2]. IAPs were first identified in baculoviruses [3]. To date five members of the IAP family of proteins have been identified in humans: HIAP1, HIAP2, XIAP, NIAP, and Survivin [4–7]. These proteins contain one or more repeats of a highly conserved 70 amino acids domain termed the baculovirus IAP repeat (BIR), located at the amino-terminal. With the exception of NIAP and Survivin, human IAPs contain a conserved sequence termed RING finger at the carboxy-terminal. Mammalian IAPs block apoptosis by binding and inhibiting specific intracellular proteases, primarily caspases-3, -7, and -9 [8]. IAPs can also suppress apoptosis through caspase-independent mechanisms, which involve transcription factors such as NF-κB [9,10]. Recent molecular analyses of the IAP genes have shown that at least one BIR domain is required for the suppression of apoptosis [11]. However the need for the RING domain to inhibit apoptosis appears to depend on the cellular context [12]. Site-directed mutagenesis to certain amino acids between the BIR2 and BIR3 domains of XIAP indicated that some of the amino acid residues outside the BIR domain may also play an important role in the antiapoptotic activity [13].

Alternative splicing regulates a number of apoptosis-related genes, such as the caspases, Bcl-2 and death receptor families [14]. This regulation can involve the production of alternative molecules with clearly separable functions. To date, Survivin is the only IAP gene which has splicing variants with different antiapoptotic effects [15,16].

In this report we described a new member of the IAP family, initially identified by computational analysis of the nucleotide sequence databases. We identified two splicing variants for this novel IAP which are almost identical, except for 54 bp that are truncated from the 5′-part of exon 6. In parallel to our studies, three groups have recently described this gene designated Livin, KIAP or ML-IAP [17–19]. In order to avoid possible confusion, we designated the longer and shorter variants as Livin α and β, respectively. Here, we demonstrate a significant difference in the antiapoptotic properties and tissue distribution of these two forms.

2. Materials and methods

2.1. Bioinformatics

To perform a computer-based search for novel expressed sequence tags (ESTs) homologous to IAP, RING domain consensus sequence of 37 amino acids (EQLRRLQEERTCKVCMDREVSIVFIPCGHLVVCKECA) was used as a bait-probe. This consensus sequence was derived from various mammalian IAPs. Tblastn program was used to search the human EST database (www.ncbi.nlm.nih.gov). ESTs matching the query peptide sequence were retrieved and compared with the non-redundant nucleotide database to eliminate known genes. A single EST, from the melanoma cell line MeWo, showing a significant homology but not corresponding to any known gene, was further analyzed. This EST was compared with the sequences in the high throughput genomic sequences database (HTGs) (www.ncbi.nlm.nih.gov). In an attempt to define the BIR coding upstream exons and the full-length cDNA, the corresponding gDNA sequence was compared against the protein sequence database using the program Blastx (www.ncbi.nlm.nih.gov). Prediction of the gene structure was made with the program GENSCAN [20] (genes.mit.edu/GENSCAN). The calculation of the score values for the acceptor splice site was made with the program SpliceView [21] (www.itba.mi.cnr.it/webgene). The protein secondary structure analysis was made with the program GOR IV [22] (www.pbil.ibcp.fr).

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Abbreviations: IAP, inhibitor of apoptosis; BIR, baculovirus IAP repeat; EST, expressed sequence tag; HTG, high throughput genomic sequence; TNF, tumor necrosis factor; STS, staurosporine

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2.2. RNA extraction and cDNA synthesis

The total RNA was extracted by TRI-reagent (Sigma, St. Louis, MO, USA). The RNA was treated with DNase I (Promega, Madison, WI, USA) according to the manufacturer's instructions. First-strand cDNA was prepared using oligo(dT)15 with M-MLV reverse transcriptase (Gibco-BRL, Gaithersburg), as described previously [23]. Reverse transcriptase-polymerase chain reaction (RT-PCR) for the β-actin gene was performed to ensure comparability between cDNAs from different tissues. Primers and amplification conditions were as described [24]. Human fetal and adult multiple tissue cDNA panels were used according to the instructions (Clontech, Palo Alto, CA, USA).

2.3. Isolation and sequence of Livin α and β

Total RNA from a human melanoma cell line (MeWo) was used to obtain Livin α and β cDNA. RT-PCR (35 cycles) was made with the gene-specific primers (FR1: 5′-CTCCCCTCCCAGGGTGTCT-3′ and RV2: 5′-GGGAAAACCCACTTTATTTCTATTC-3′), based on the EST and gDNA sequences. The amplification products were cloned into pCRII-TOPO vector (Invitrogen, Carlsbad) by TOPO-TA cloning kit. The two inserts were sequenced in both directions.

2.4. Semiquantitative RT-PCR

The expression levels of Livin α and β mRNAs were estimated by semiquantitative RT-PCR using the gene-specific primers: FR748: 3′-GTCCCTGCCTCTGGGTAC-5′ and RV1103: 5′-CAGGGAGCC-CACTCTGCA-3′, that distinguish between the two splice variants. PCR (38 cycles) was carried out by denaturation at 95°C for 2 min, annealing at 62°C for 30 s and extension at 72°C for 30 s. Levels of Survivin mRNA were measured by RT-PCR using the gene-specific primers: Surv-FR: 5′-CGACCCCATAGAGGAACATA-3′ and Surv-RV: 5′-GAGAGAAGCAGCCACTGTTAC-3′. The amplification conditions were similar to Livin.

2.5. Construction of viral vectors and gene transduction

The pCRII-TOPO vectors containing the coding sequences of Livin α and β were digested by *Eco*RI. The obtained inserts were subcloned into pLXSN, a M-MuLV-based retroviral vector [25]. The M-MuLV-based retroviral vector (pBabepuro) containing human Bcl-2 [26] was a gift from Dr. M. Bennett (University of Washington, Seattle, WA, USA). The transfer vectors were then packaged by co-transfection with the pCL-Ampho packaging construct in 293 cells [27]. Supernatants containing the viruses were collected after 2 days, filtered and 1 ml of viral supernatant was used to infect 5×10^4 cells in the presence of polybrene 5 μg/ml. Two days after infection, cells were split and placed under selection using either G418 (400 μg/ml) in the case of pLXSN vectors or puromycin (1.5 μg/ml) in the case of pBabepuro vector. After drug selection, pooled colonies were cultured and used for analysis.

Expression of the ectopic Livin α and β was evaluated by RT-PCR using a Livin forward primer (FR748) and a vector-specific reverse primer pLXSN-RV 5′-GGACTTTCCACACCCTAACTGA-3′. The absence of residual contaminating gDNA was demonstrated by the failure to amplify the genomic locus of INFα [23].

2.6. Cells and apoptosis induction

The Jurkat human T cell line was grown in RPMI 1640 with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2. Cells (3×10^5) were plated into 24-well plates for a few hours before adding the apoptotic stimulus. To induce apoptosis, cells were treated with anti-CD95 antibody (0.3 μg/ml) (clone DX2; R&D Systems, Inc.) for 20 h; tumor necrosis factor (TNF)-α (100 ng/ml) (Pepro Tech, UK) together with 40 ng/ml cycloheximide (Sigma) for 12 h; staurosporine (STS) (0.5 μM) (Sigma) for 3 h; etoposide (10 μg/ml) (Sigma) for 18 h.

2.7. Apoptosis assays

Nuclear morphology was visualized using acridine orange (Sigma) staining as described [28]. Apoptotic cells were scored when the nuclei displayed chromatin condensation and/or nuclear fragmentation. Annexin V binding was determined by incubating the cells with annexin V-FITC and propidium iodide (Sigma) according to the manufacturer's instructions. The percentage of apoptotic to viable cells revealed by the two staining methods was counted by fluorescence microscopy and 500 cells were scored for each sample.

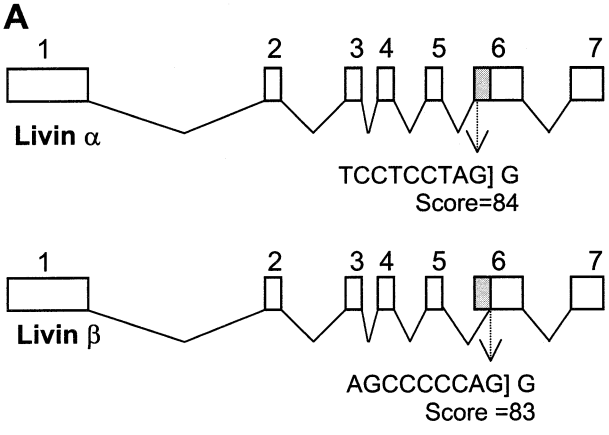
3. Results

3.1. Bioinformatics

Using a RING domain consensus sequences we conducted a homology search of the public EST databases. One EST (AL138387) from the melanoma cell line (MeWo) showed a significant homology to the RING consensus sequence but did not correspond to any previously published gene. Comparing this EST to the HTGs database, a significant similarity was found with two gDNA sequences from chromosome 20 (AL121827 and AL096828). Analysis of the 5′-region of the gDNA sequence showed significant similarity to human skin tumor EST (AA379765). The results of gene prediction analysis revealed the full structure with seven exons spanning 4.6 kb. To clone the full-length cDNA, we performed RT-PCR using mRNA from MeWo cell line. The organization of the Livin gene is depicted schematically in Fig. 1A.

3.2. Livin has two splice variants

Amplification of the entire cDNA sequence of Livin using primers that match the 5′- and 3′-untranslated regions demonstrated the presence of two PCR products. Sequencing various clones of these products revealed the existence of two splice variants. Analyzing the gDNA sequences 5′ to exon 6 revealed canonical acceptor splice site sequences with very similar score values in both variants (Fig. 1A). The shorter variant lacks the first 54 bp of exon 6. The two cDNAs contain open reading frames of 298 and 280 amino acids which were designated Livin α and β, respectively. Conceptual translation indicates that Livin α and β are almost identical proteins that share the amino-terminal BIR and carboxy-terminal



B

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1   MGPKDSAKCLHRGPQPSHWAAGDGPTQERCGPRSLGSPVL
41  GLDTCRAWDHVDGQILGQLRPLTEEEEEEGAGATLSRGPA
81  FPGMGSEELRLASFYDWPLTAEVPPELLAAAGFFHTGHQD
121 KVRCFFCYGGLQSWKRGDDPWTEHAKWFPSCQFLLRSKGR
161 DFVHSVQETHSQLLGSWDPWEEPEDAAPVAPSVPASGYPE
201 LPTPRREVQSESAQEPGGVSPAEAQRAWWVLEPPGARDVE
241 AQLRRLQEERTCKVCLDRAVSIVFVPCGHLVCAECAPGLQ
281 LCPICRAPVRSRVRTFLS
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Fig. 1. (A) Schematic representation of the organization of the two Livin genes. Boxes represent exons, the shaded box at the 5′ of exon 6 represents the 54 bp not present in Livin β. (B) Predicted amino acid sequence of Livin. The BIR and RING domains are underlined. The shaded sequence represents the 18 amino acids lacking in Livin β.

RING domains. However, Livin β lacks 18 amino acids in the BIR–RING inter-linking region (Fig. 1B).

3.3. Tissue distribution and expression pattern

The tissue distribution was first studied by Northern blotting using a probe complementary to the 3′-untranslated region. The result was a very faint band of 1.4 kb in the placenta (data not shown). To distinguish between the two variants, a pair of primers which flank the truncated region of exon 6 was used to investigate various fetal and adult tissues (Fig. 2A), as well as several transformed cell lines (Fig. 2B). Livin α was not detected in any of the tested fetal tissues. On the other hand, relatively high levels of Livin β were detected in fetal kidney and lower levels in heart and spleen. In adult tissues, elevated levels of both Livin α and β were detected in heart, placenta, lung, spleen and ovary. Low levels of only Livin α were detected in brain, skeletal muscle, and peripheral blood lymphocytes. Adult kidney expressed low levels of only Livin β. These results indicate tissue-specific expression and splicing of Livin α and β mRNAs.

When comparing Livin β with Survivin, fetal tissues show more restricted expression of Livin β. In adult tissues, Livin and Survivin were detected in a variety of tissues, however, no correlation was found between the expression of these two genes.

Fig. 2B shows the results of Livin α and β expression in various cell lines derived from different hematopoietic and solid malignancies. High levels of Livin α and β transcripts were detected in melanoma cell line (MeWo), colon carcinoma cell lines (HT29, HT29MTX), and prostate carcinoma cell line DU-145. The rest of the cell lines showed a relatively lower level of both mRNAs. In comparison, no detectable levels of the two transcripts were found in the MDA-MB-231 breast carcinoma and HT1080 fibrosarcoma cell lines.

3.4. Differential antiapoptotic activity of Livin α and β

To test the antiapoptotic activity of Livin α and β, Jurkat cells that stably overexpress ectopic Livin α, Livin β and Bcl-2 were generated. Expression of the ectopic genes was evaluated and compared to the endogenous gene by RT-PCR (Fig. 2C). Three classes of stimuli were used to induce apoptosis in these cells. The first class includes TNF-α and CD95 antibody, which resemble the inducers of cell surface death receptors. While STS, that represents the second class, induces the release of cytochrome *c* [29]. Etoposide represents the third class that causes cell apoptosis after damaging the DNA [30]. Fig. 3 shows that overexpression of both Livin α and β blocks apoptosis induced by TNF-α and anti-CD95, whereas Bcl-2 has a relatively weaker protective effect. When apoptosis was stimulated by STS, Bcl-2 provided the maximal protective

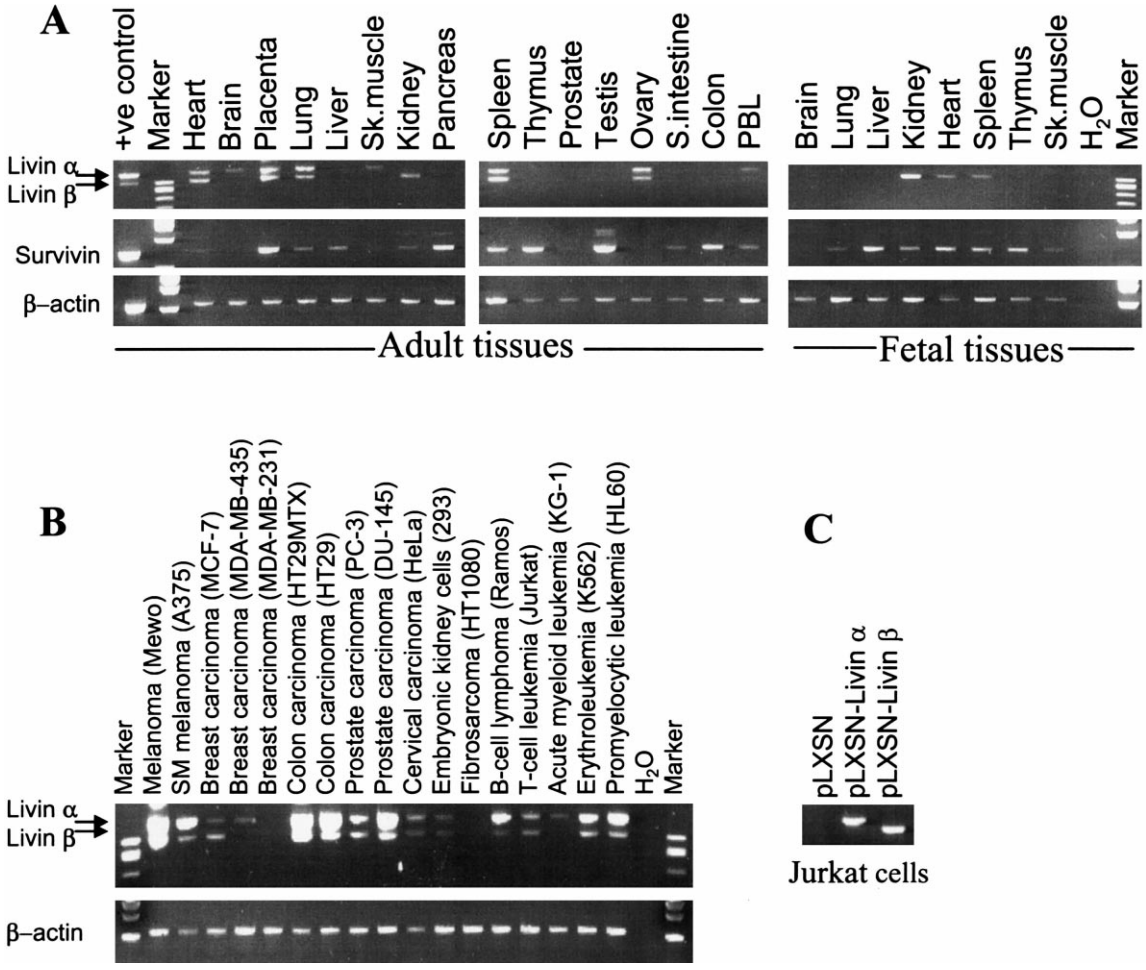

Fig. 2. Tissue distribution of the two Livin variants. Specific RT-PCR was performed on mRNA derived from adult and fetal tissues (A), as well as several transformed cell lines (B). (C) RT-PCR performed on the retroviral infected Jurkat cells to evaluate the expression of the ectopic gene.

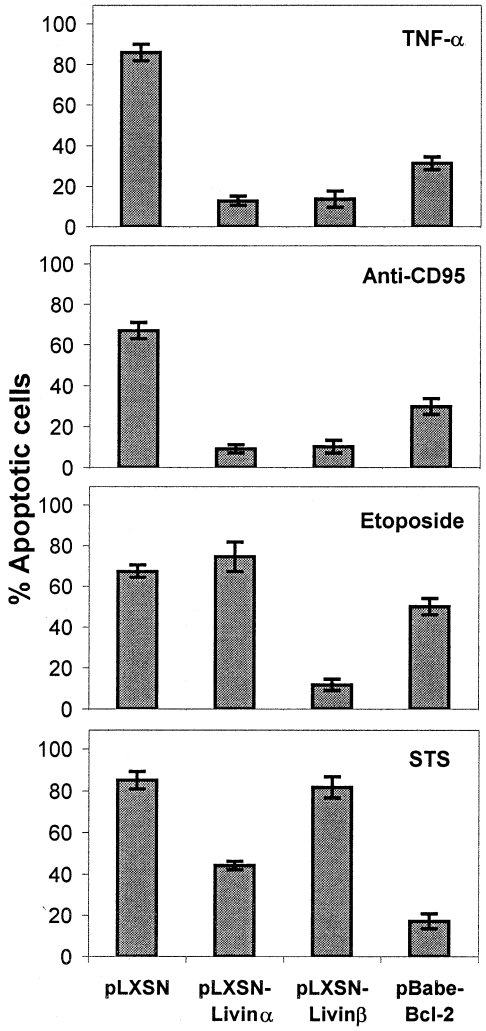

Fig. 3. Differential antiapoptotic effect of Livin α and β in response to four different apoptotic stimuli. The Jurkat cells were infected with M-MuLV-based retroviruses that harbor the expression vector: pLXSN-Livin α, pLXSN-Livin β, or pBabepuro-Bcl-2 (a positive control). The negative control was empty pLXSN. The bars indicate the percent of apoptotic cells (mean ± S.E.M.) of three independent experiments.

effect. On the other hand Livin α but not β shows an intermediate antiapoptotic effect. Unexpectedly, Livin α was shown to have a slight proapoptotic effect when the cells were treated with etoposide, whereas Livin β was seen to have a strong protective effect.

4. Discussion

The recent growth in biological databases and the rapid development of novel computational methods have facilitated the identification of many new genes. Here, we report such an example, in which we used the nucleotide sequence databases to identify and clone a novel IAP gene. We used the amino acid consensus sequence of the RING domain of known IAPs and found an EST from the melanoma cell line MeWo. Instead of using laborious methods, such as library screening or rapid amplification of cDNA ends (RACE), we utilized a computer-based approach to obtain the full-length cDNA. This was achieved by searching for the corresponding gDNA sequence and analyzing it by online available programs. Our subsequent analysis of the amplified products showed the presence of two splicing variants for this gene. The short variant appears to lack the first 54 bp of exon 6. Three groups recently reported the cloning of this gene [17–19]. Lin et al. [18] identified the longer variant while Kasof and Gomes [17] identified the shorter variant, which they designated KIAP and Livin, respectively. It is interesting to note how each group has identified only one variant. Using amino acid consensus sequences of the BIR domain to search the Incyte EST database, both groups found the EST clone 1419118 to be expressed in human fetal kidney. Full-length KIAP (α isoform) was cloned by 5′- and 3′-RACE, performed on fetal kidney cDNA, while the full-length Livin (β isoform) was cloned after screening the adult kidney cDNA library. Our results show the expression of Livin β but not α in fetal and adult kidney. However Lin et al. were able to obtain Livin α from fetal kidney. These findings could be explained by the fact that two rounds of RACE amplification were performed which allowed the identification of low abundant transcripts.

Detection of Livin β in several fetal tissues indicates that it may play a physiological role during fetal development. Consistent with our Northern blot results as well as others [17–19], it was evident, using RT-PCR, that expression of both variants is most prominent in the placenta. The simultaneous expression of both variants in certain tissues could be due to similar regulatory mechanisms in these specific tissues. The relatively high expression of both variants in MeWo, HT29 and HT29MTX cell lines could also explain the marked apoptosis-resistance of these cells [31,32]. Although Livin α and β were detected in different cell lines and in several clinical samples from hematopoietic malignancies (results not shown), a larger study is needed to determine their diagnostic potential.

IAPs are important regulators of apoptosis. They block apoptosis triggered by a broad spectrum of stimuli, including ligands of the cell surface death receptors, cytochrome *c* and chemotherapeutic agents [1]. In the death receptor pathway, IAPs inhibit the effectors caspase-3 and caspase-7 [33]. In the cytochrome *c*-dependent pathway they exert their antiapoptotic effect either through interfering with procaspase-9 or via direct inhibition of active caspase-9 [33]. In this study, we tested the antiapoptotic effect of Livin α and β in the Jurkat T cell. Our results as well as those of Vucic et al. [19] show that both isoforms can block apoptosis induced by TNF-α or CD95 antibody. The BIR domain, that exists in both isoforms, is most probably the region responsible for this effect. In the case of XIAP, conserved amino acid residues outside the BIR domain were found to be critical for inhibiting caspase-3 irrespective whether they were located N- or C-terminal to the BIR domain [13]. These residues are Leu and Asp separated by six amino acids. In both isoforms, three copies of this pattern were found, one inside the BIR domain (L131–D138), whereas L42–D49 and L154–D161 were N- and C-terminal to it, respectively. Although further experimental work is still required to clarify the functional importance of these residues, the D138 was already reported to be a critical residue [19].

Significant antiapoptotic differences between Livin α and β were found when cells were treated with either cytochrome *c* releasing (STS) or a DNA damaging agent (etoposide). The

two stimuli function through different apoptosis pathways [34]. The differential antiapoptotic properties illustrated in our results are most probably due to the 18 amino acids missing from Livin β in the region separating the BIR and RING domains. Secondary structure analysis predicted an α-helix in that particular 18 amino acids region. Taken together with our results, these analyses suggest that interaction with the different regulatory molecules from both apoptosis pathways may possibly be mediated through the α-helix domain. In addition, the sequence differences between the two isoforms might also be important in determining the intracellular location of the corresponding proteins.

Our results demonstrate that although Livin α and β are splicing variants of the same gene, they have different antiapoptotic properties and expression patterns. The previously published works described either of the two splice variants and they examined its tissue distribution using Northern analysis which does not distinguish between the two variants. In our work we use specific RT-PCR which can determine which variant is being expressed from each tissue examined. Comparison between the two variants and other IAPs revealed a unique tissue distribution pattern of Livin α and β, which suggests a non-redundant and specialized biological function for each isoform. Our work is unique since it describes for the first time the differential antiapoptotic effect of each variant. These findings indicate that alternative splicing might play an important role in the fine-tuning of Livin biological function. Elucidation of the mechanisms that regulate the expression of the two isoforms may therefore provide further insights into the role of this new IAP in a variety of physiologic and pathological conditions.

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