Caspase-Mediated Cleavage Converts Livin from an Antiapoptotic to a Proapoptotic Factor: Implications for Drug-Resistant Melanoma

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ABSTRACT

Inhibitor of apoptosis protein (IAP) is a family of intracellular proteins that plays an essential role in the regulation of apoptosis. Recently, we and others discovered a new member of this family, termed Livin. Many studies have focused on the inhibitory effect of IAPs on caspases. Here, we describe a novel regulatory mechanism by which Livin is cleaved by the caspases. Strikingly, the cleaved Livin, although containing intact baculovirus IAP repeat and RING domains, does not only lose its antiapoptotic function but also gains a proapoptotic effect. The cleavage is site specific at Asp-52 and is restricted to effector caspase-3 and -7. Most importantly, we demonstrate the role of Livin and this regulatory mechanism in the drug resistance of melanoma patients. Using primary cultures derived from melanoma patients, we found a correlation between Livin overexpression, in vitro drug resistance, and the patient's clinical response.

INTRODUCTION

Apoptosis is an active mechanism of cell death controlling the development and homeostasis of multicellular organisms. Tight regulation is required to ensure a delicate balance of life and death. Indeed, loss of apoptosis regulation results in a wide variety of diseases. Excess apoptosis might result in neurodegenerative disorders (1), reperfusion injury after ischemic episodes (2), and immunodeficiency (3). On the other hand, lack of apoptosis is involved in cancer (4) and autoimmune disorders (2). Several gene families are involved in the negative regulation of apoptosis, including the IAPs. The IAP family, which was discovered during the last 5 years, plays a key role in apoptosis regulation and has become increasingly prominent in the field of cancer (5). Thus far, eight human IAPs have been identified: c-IAP1, c-IAP2, NAIP, Survivin, XIAP, Bruce, ILP-2, and Livin. These proteins contain one or more repeats of a highly conserved 70-amino acid domain termed the BIR, located at the NH2 terminus. With the exception of NIAP and Survivin, human IAPs contain a conserved sequence termed RING finger at the COOH terminus. IAPs can block apoptosis mainly through their ability to bind and inhibit specific caspases. Initially, the molecular interaction between IAPs and caspases was thought to be mediated through the BIR domains (6). However, recent crystallographic resolution studies revealed that conserved amino acids in the linker region between BIR1 and BIR2 of XIAP are the most critical for interaction with caspase-3 and -7. Surprisingly, the BIR2 domain itself has almost no direct contact with caspase-3 and -7 (7–9). The linker region preceding BIR2 can inhibit caspases through its ability to sterically hinder the substrate access. Yet, this region alone is not sufficient, and the BIR domain is required to either align or stabilize the structure. The BIR domain also has a regulatory function, because molecules such as SMAC/Diablo and HtrA2, which inhibit IAP function, bind to this region (10). Several reports showed that many proteins containing a RING domain have E3-ubiquitin ligase activity. This activity is important in mediating the transfer of ubiquitin to heterologous substrates as well as to the protein itself, thus targeting them for intracellular degradation (11, 12). Indeed, several IAPs were shown to mediate RING-dependent ubiquitylation of caspases as well as for themselves (13). Yet, the full potential of this function in apoptosis regulation is still unclear.

The essential role that IAPs play in the apoptotic process suggests that their activity must be tightly regulated. Indeed, it was reported that IAPs are regulated at the transcriptional/posttranscriptional levels and by interaction with inhibitory proteins (14). Another important mechanism to negatively regulate IAPs is the ability of certain caspases, such as caspase-3 and -7, to specifically cleave these antiapoptotic proteins. Of the IAP family members, XIAP and cIAP1 were shown to undergo a site-specific cleavage that is mediated by caspases (15, 16).

We and other groups reported on the discovery of a novel IAP member, designated Livin/ML-IAP/KIAP (17–20). Livin contains a single BIR domain at the NH2 terminus as well as a COOH-terminal RING domain. We further demonstrated that Livin encodes two splicing variants, Livin α and β (20). The two proteins are highly similar, except for 18 amino acids located between the BIR and the RING domains, which are present in the α but not the β isof orm. Despite the high similarity, we showed different antiapoptotic properties of the two isoforms.

Little is known about the antiapoptotic effect of Livin, and virtually nothing is known about its regulatory mechanism. Here, we demonstrate for the first time the regulation mechanism of Livin after apoptotic stimuli. We show that Livin undergoes site-specific cleavage by effector caspase-3 and -7 to produce a large COOH-terminal subunit containing both the BIR and RING domains. Interestingly, we provide evidence that this subunit does not only lose its original antiapoptotic function but rather acts in a paradoxical fashion as a proapoptotic factor that inflicts more cell death. Finally, using primary cell cultures derived from patients with malignant melanoma, we demonstrate in vitro the significance of Livin in the drug-resistance phenotype characteristic of this disease. Furthermore, we show that there is clinical correlation between Livin expression and chemotherapeutic response, suggesting that drugs targeted at this IAP might play a role in the treatment of this fatal disease.

MATERIALS AND METHODS

Cells and Apoptosis Induction. The Jurkat human T-cell leukemia/lymphoma cell line and 721.221 EBV-transformed B-cell line were grown in RPMI 1640. MCF-7 human breast carcinoma cells, 293T human embryonic kidney cells, and the melanoma cell lines LB33 Mel A1 and B1 (a generous gift from P. G. Couli), 1259-mel, 1074-mel, 1106-mel, and 1612-mel (21) were grown in DMEM. Media were supplemented with 10% FCS, 100
units/ml penicillin, 100 μg/ml streptomycin, and 1 mM l-glutamine. Primary melanoma cells were derived and maintained as described (22). To induce apoptosis, cells were treated with anti-CD95 antibody, clone DX2 (R&D Systems, Minneapolis, MN), staurosporine or protein kinase C inhibitor, and the topoisomerase II inhibitor etoposide (Sigma).

Western Blot Analysis and Antibodies. Whole cell lysates were prepared using lysis buffer (20 mM Tris-HCl, 2 mM EDTA, 6 mM β-mercaptoethanol, 1% NP-40, and 0.1% SDS). Protease inhibitors included 1 mM PMSF, Protease Inhibitor Cocktail (Sigma) diluted 1:10, and Complete Inhibitor Cocktail (Roche, Mannheim, Germany) diluted 1:25. About 0.25–1 x 10^6 cells were lysed in a total volume of 100 μl, incubated at 4°C for 20 min with vigorous vortexing. Protein content was assessed by Bradford assay (Bio-Rad, Hercules, CA), according to the manufacturer’s recommendations. For these antibodies, Envision-HRP (DAKO, Copenhagen, Denmark) was used as a secondary antibody for enhanced chemiluminescence reaction. Polyclonal antibodies against either PARP or XIAP (Cell Signaling Technology, Beverly, MA) were diluted according to the manufacturer’s recommendation. Anti-rabbit IgG horseradish peroxidase-linked antibody (Cell Signaling) was used as a secondary antibody. Enhanced chemiluminescence reaction was performed by mixing solution A (6 ml of Tris, 100 mM, pH 8.5; 3.3 μl of H2O2, 30%) with solution B (6 ml of Tris, 100 mM, pH 8.5; 60 μl of Luminol, 250 mM (Sigma)); and 26.6 μl of p-Coumaric acid, 90 mM (Sigma) for 1 min in the dark.

Plasmid Constructs and Cell Transfection. The retrovector pLXSN (Clontech, Palo Alto, CA) that contains the cDNA of either Livin α or β splicing variants was prepared as described previously (20). Cells were infected with the packaged particles and were placed under selection using G418 (Sigma).

Transfection with the pIRES2-EGFP plasmid (Qiagen, Hilden, Germany) that encodes p30-Livin-α or p28-Livin-β, and as positive controls, the full-length wild-type cDNAs of either Livin α or β was made either by electroporation for 721.221 cells (23) or by using PolyFect (Qiagen) for human HeLa cells (24). For production of recombinant Livin, full-length cDNA of either α or β variants were cloned in-frame with the N-terminus His-tag in the plasmid pmQ30 (Qiagen). The primers were Livin-Exp-F 5’-TGGTTGATCCATGGGAGCTTAAAGACA-3’ and Livin-Exp-R 5’-GGCAAGCTTATGGCAAGAGGGTCG-3’, which have the underlined BamHI and HindIII restriction sites, respectively. The plasmids were introduced into Esherichia coli strain BL21(DE3). The His6-tagged proteins were purified on a Nickel column (Pharmacia). Coomassie Blue staining analysis after SDS-PAGE revealed >90% intact protein.

Apoptosis Assays. Nuclear morphology was visualized using acridin orange (Sigma) staining as described (25). Apoptotic cells were scored when the nuclei displayed chromatin condensation and/or nuclear fragmentation. The percentage of apoptotic to viable cells was counted by fluorescence microscopy, and 500 cells were scored for each sample.

Flow cytometry analysis of the apoptotic cells was done using two different methods. In the Sub-G1 assay, the cells were harvested, washed with PBS, and fixed using 100% ethanol. After an overnight incubation at −20°C, the cells were rehydrated with PBS for 30 min on ice. Cells were then resuspended in PBS with RNase A (50 μg/ml) and stained with PI (Sigma) at a final concentration of 5 μg/ml. Flow cytometry analysis was performed in an FLS 2000 instrument. Cells transfected with a GFP-containing plasmid were analyzed using Annexin V-Cy5 and PI stain according to the manufacturer’s instructions (Medical and Biological Laboratories Co., Nagoya, Japan). In stably transfected cultures with a rate of GFP-positive cells >95%, all cells were analyzed for apoptosis. In transiently transfected cells, GFP expression was first analyzed (FL-1), and only GFP-positive cells were analyzed for Annexin V-Cy5 stain (FL-4) and PI stain (FL-3).

Caspase Inhibitors and In Vitro Caspase Assay. Cells were incubated with caspase inhibitors for 1–2 h before treatment with the apoptotic stimuli. Pan caspase inhibitor Z-VAD-FMK (R&D Systems, Minneapolis, MN), specific caspase-3 inhibitor benzoyloxycarbonyl-DQMD-FMK at 60 μM, and caspase-6 inhibitor VEID-CHO at 60 μM were used (Calbiochem). Recombinant caspases were purchased from Calbiochem and incubated for 30 min at 37°C with recombinant Livin. According to the manufacturer, the units of each recombinant caspase are defined differently. Caspase-3 and -8 units are defined as the amount of enzyme that will release 1 pmol of pNA from either DEVD-pNA or Ac-IEHD-pNA, respectively, per minute at 30°C. Caspase-6, -7, and -9 units are defined as the amount of enzyme that will release 1 pmol of pNA from either Ac-VEID-pNA, Ac-DEV-D-pNA, or LEHD-pNA, respectively, per hour at 37°C. Caspase-3 and -7 activity was calibrated using caspase activity assay (Calbiochem). Caspase-9 activity was assessed using caspase-9 colorimetric substrate LEHD-pNA (Biovision, San Francisco, CA). Recombinant granzyme B (BioMol, Plymouth Meeting, PA) activity was confirmed with the Granzyme B Activity Assay Kit (BioMol) using Ac-IETD-pNA.

RESULTS

Livin Is Cleaved during the Apoptotic Process. Upon the induction of apoptosis in Melx, a melanoma cell line that expresses high levels of Livin, we observed a specific cleavage of both Livin isoforms. Full-length Livin α and Livin β were detected as approximately 39,000 and 37,000 proteins, respectively. Treatment with staurosporine produced, in a time-dependent manner, detectable fragments of 30,000 and 28,000, termed p30-Livin α and p28-Livin β, respectively (Fig. 1A). No cleavage was detected after 12 h without treatment. This difference in molecular weight represents the differ-
ence between full-length Livin α (39,000) and Livin β (37,000) and suggests a common cleavage site for both isoforms. Concomittantly with the appearance of the cleavage fragments, a marked depletion of the full length of both Livin isoforms was observed (Fig. 1a). The smaller fragment of ~10,000 could not be detected because of the use of a monoclonal antibody that is specific to an epitope located on the large detectable fragments. To correlate between apoptosis induction, caspase activity, and Livin cleavage, the membrane exposed to anti-Livin antibody was reblotted with anti-PARP antibody. This protein is one of the main targets of caspase-3 and serves as a universal marker of apoptosis. In Fig. 1b, the cleavage of full-length PARP (116,000) into 89,000 and 24,000 fragments showed a strong correlation with Livin cleavage. A decrease in PARP was observed after 12 h of treatment, probably because of protein degradation, as a consequence of a high rate of apoptosis.

To further explore the cleavage of each isoform, we used retroviral infection to establish a Jurkat T-cell leukemia/lymphoma cell line and an EBV-transformed B-cell line 721.221, expressing high, stable levels of either Livin α or β. These cells were chosen because of their low levels of endogenous Livin. In our previous work, we showed that both Livin isoforms can protect against anti-CD95/Fas antibody-induced apoptosis (20). Notably, testing other chemotherapeutic agents revealed different antiapoptotic properties of Livin isoforms. Although only Livin α can protect from staurosporine-induced apoptosis, only Livin β can only block etoposide-induced apoptosis. We therefore chose a panel of these three drugs, etoposide, staurosporine, and anti-CD95/Fas ligand, to induce apoptosis in the transfected Jurkat and 721.221 cells. Consistently, both Livin isoforms were cleaved by etoposide- and staurosporine-induced apoptosis, as shown in Fig. 2. Similar results were observed when cells were treated with anti-CD95/Fas antibody (data not shown). We further investigated the correlation between the timing of the cleavage and apoptosis rate. Notably, cleavage was observed as soon as 2 h after the treatment with etoposide, whereas significant levels of apoptosis were detected only...
presence or absence of 20 mM or 200 mM zVAD-FMK, a pan caspase inhibitor, before treatment with staurosporine (0.5 μM) to induce apoptosis. Jurkat cells transfected with Livin α or β were incubated for 2 h with specific caspase-3 inhibitor z-DQMD-FMK at 60 μM and caspase-6 inhibitor VEID-CHO at 60 μM before treatment with etoposide (2.5 μg/ml) for 10 h. c. MCF-7, a breast cancer cell line null for caspase-3, stably transfected with Livin α or β, was treated with staurosporine (2 μM) for 12 h to induce apoptosis. Livin was detected using anti-Livin antibody. 

Livin Cleavage Is Caspase Mediated. It has been shown previously that a large group of proteins undergo specific cleavage by caspases during the apoptotic process. To determine whether Livin cleavage is also mediated by caspases, a pan caspase inhibitor zVAD-FMK was used. Preincubation of Jurkat cells with zVAD-FMK before treatment with etoposide significantly diminished the cleavage of Livin in a dose-dependent manner, indicating that this is a caspase-dependent event (Fig. 3a). Despite a high concentration of zVAD-FMK, complete inhibition of the cleavage was not achieved. This is most probably because of the potent apoptotic induction of staurosporine in Jurkat cells (>90% after 3 h at the concentration used). Nonetheless, we cannot exclude other caspase-independent mechanisms of Livin cleavage, although these are unlikely. The three main effector caspases are caspase-3, -6, and -7. To test whether these caspases cleave Livin in vivo, Jurkat cells were preincubated with specific inhibitors for either caspase-3 or -6 before treatment with etoposide. Caspase-3 inhibitor but not caspase-6 inhibitor was able to diminish the cleavage (Fig. 3b). Similar results were obtained with Livin β (data not shown).

To the best of our knowledge, a specific inhibitor of caspase-7 does not exist. We therefore used the MCF-7 cell line, a breast cancer cell line that lacks caspase-3 but expresses caspase-7. MCF-7 cells were transfected with both Livin isoforms, because endogenous Livin was not detected. Upon induction of apoptosis using staurosporine, a clear cleavage of both isoforms, associated with a decline in the full-length protein, was observed (Fig. 3c), demonstrating that other caspases, aside from caspase-3, are able to cleave Livin in vivo.

Effector but not Initiator Caspases Cleave Livin in Vitro. The above results demonstrate for the first time that Livin α and β can be cleaved after the induction of apoptosis. To investigate directly which caspases are able to cleave Livin, recombinant Livin was generated in bacteria. Purified Livin α and β were incubated with either of the recombinant active effector caspase-3 and -7 or with the initiator caspase-8 or -9. As shown in Fig. 3d, effector caspase-3 and -7 but not the initiator caspase-8 and -9 cleaved Livin β. The most efficient cleavage was observed when caspase-7 was used, resulting in the complete cleavage of the recombinant Livin β (Fig. 3d). Similar results were obtained when recombinant Livin α was used (data not shown). In Fig. 3d, similar activity of caspase-3 and -7 was achieved using an appropriate colorimetric substrate. Treatment of recombinant Livin α and β with caspase-8 had no effect, even when 90 units of this enzyme were used, whereas incubation with high concentration of caspase-9 resulted in weak cleavage, suggesting that caspase-9 might cleave Livin at very low efficiency.

Incubation of Livin α and β with granzyme B, a caspase-like protease with a broad spectrum of substrates including effector...
Caspase-3 and -7, whereas Livin D52 protein, after the 18 amino acids that distinguish between isoforms (black). The mutation sites are marked by arrows: D52 and D238. cDNAs encoding full-length Livin α and β or mutants Livin D52→E and D238→E were translated in vitro in reticulocyte lysate. The products of the translation reaction were incubated for 30 min. with caspase-3 (60 units), caspase-6 (2 units), caspase-7 (1 unit), and caspase-9 (1 unit). Reactions were then resolved on a gel and analyzed with anti-Livin antibody. a.a. amino acids.

Mapping the Cleavage Site. The observed molecular weight of the cleaved fragments suggests that the Livin cleavage site resides somewhere near the NH2-terminal or at the COOH-terminal of the protein, after the 18 amino acids that distinguish between α and β isoforms. Amino acid sequence analysis for candidate tetrapeptides that can be potential caspase substrates at both regions revealed the presence of two possible sites, DHVD52→G at the NH2 terminus and GARD238→V at the COOH terminus (Fig. 4a). The sequence located around aspartic acid 52 showed a high degree of similarity with the consensus substrate sequence for caspase-3 and -7 (26, 27). We therefore prepared constructs of both Livin isoforms in which either aspartic acid 52 or aspartic acid 238 was replaced by glutamic acid, termed Livin D52→E and Livin D238→E, respectively. Livin constructs carrying these mutations and the wild-type sequence were translated in vitro and incubated with purified active caspases. Livin D238→E α and β underwent cleavage similar to the wild-type protein by caspase-3 and -7, whereas Livin D52→E α and β were not cleaved under these conditions (Fig. 4b). In this experiment, caspase-6 did not cleave either isoform, confirming our results with caspase-6 inhibitor in vivo (Fig. 3b).

Livin Cleavage Produce a Proapoptotic Subunit. 721.221 cells can be easily transfected with various constructs (23). The functional relevance of Livin cleavage was therefore tested by generating 721.221 cells stably transfected with either full-length Livin α and β, as well as the COOH-terminal cleavage subunits, which were cloned in pRES-EGFP plasmid. Our previous work showed that Livin α is able to protect from apoptosis induced by anti-CD95/Fas antibody in Jurkat cells (20). Similarly, 721.221 cells expressing Livin α showed a lower rate of apoptosis, after anti-CD95/Fas treatment, as compared with wild-type 721.221 cells (Fig. 5a). Surprisingly, after anti-CD95/Fas treatment, cells expressing p30-Livin α showed a marked increase in GFP-positive cells in comparison with wild-type 721.221 cells (Fig. 5a). This indicates that the cleavage of Livin not only eliminates its antiapoptotic activity but also produces a subunit with a marked proapoptotic effect. The existence of the proteins in the transfected cells was verified by Western blot analysis (Fig. 5b). Jurkat cells, infected with Livin α, were resistant to anti-CD95/Fas, whereas 721.221 cells, electroporated with Livin α, showed only moderate ability to protect from this apoptotic stimulus. This ability was consistent in numerous experiments. Differences in the efficiency of expressing exogenous Livin might explain the variation in the protection levels. Two attempts to generate 721.221 cells that stably express p28-Livin β did not produce stable clones, although GFP-positive cells appeared early during the course of G418 selection. A possible explanation might be a strong proapoptotic activity of this subunit that leads to an early death of the 721.221 cells. Transient transfection was therefore used to assay for p28-Livin β function and to further confirm the above results. pIRES-EGFP plasmids that contain either full-length Livin α or β, as well as p30-Livin α, p28-Livin β, and no insert were transiently transfected to 293T cells. Cells were harvested 24 h after transfection. Apoptosis was determined by Annexin V/PI stain using flow cytometry to analyze only GFP-positive cells. A significantly higher rate of apoptosis was seen in the cells transfected with either p30-Livin α or p28-Livin β in comparison with cells transfected with the full-length proteins or an empty vector (Fig. 5c). In addition, although a marked increase in GFP-positive cells was observed in cells transfected with the full-length cDNAs or the empty vector, cells transfected with cDNAs that encode for the subunits showed a much lower rate of...
GFP-positive cells. The existence of the proteins in the transfected cells was verified by Western blot analysis (Fig. 5d).

Resistance to Chemotherapy Is in Direct Correlation with Livin Expression in Primary Culture Melanoma Cells. The strong expression of Livin in many melanoma cell lines and its ability to protect from chemotherapy in these cell lines (17, 20) prompted us to further explore the clinical significance of Livin expression and its cleavage in melanoma. We initially examined several melanoma cell lines for the expression of Livin, XIAP, and Survivin. Cell lines termed 1259-mel, 1074-mel, 1106-mel, and 1612-mel were derived from patients after treatment and showed relatively similar levels of expression of Livin, XIAP, and Survivin (Fig. 6a). LB33 A1 and B1, two metastatic melanoma cell lines, were derived from the same patient before and after vaccination with autologous melanoma cells (28, 29). The primary Mel A1 expressed the HLA class I molecules A24, A28, B13, B44, Cw6, and Cw7. The patient was vaccinated repeatedly with autologous melanoma cells and achieved remission. Four years later when she relapsed, another cell line was generated, designated Mel B1. These cells showed no expression of the original HLA class I molecules except for A24. Strikingly, we found that the expression of Livin was only detected in Mel B1 and not in Mel A1, whereas both cell lines expressed XIAP and Survivin (Fig. 6a). Furthermore, Mel B1 cells expressing high levels of Livin were completely resistant to etoposide, whereas Mel A1 rapidly underwent apoptosis, as determined by nuclear morphology (Fig. 6b). To further confirm these results, at the indicated times after treatment cells were harvested, total protein was normalized, and Western blot analysis was performed, using anti-Livin antibody. In accordance with the results above, a marked Livin cleavage was not detected because of a very low rate of apoptosis in Mel B1 (Fig. 6c). The membrane was then stripped and exposed to anti-PARP antibody. PARP was detected in all samples, confirming similar protein content. Notably, a marked cleavage of PARP, as a marker of apoptosis, was only detected in Mel A1 (Fig. 6d).

Primary cultures of 27 melanoma patients were next analyzed for Livin, XIAP, and Survivin expression. The protein content of whole cell extracts was normalized by Bradford assay, and Western blot analysis was performed. Livin protein was detected in 10 of the 27 samples. XIAP and Survivin, on the other hand, were detected in almost all tested samples. Among the samples positive for Livin, a marked difference in the levels of Livin was observed. In Fig. 7a, which represents a panel of 19 patients, melanoma samples 55182, 5556, and 55112 showed relatively high levels of both Livin isoforms, whereas others such as 5524 expressed only moderate levels. In contrast, a similar level of expression of XIAP and Survivin was found. To exclude variations in Livin expression, in three independent experiments cells were synchronized, and Western blot was performed with consistent results. To determine whether Livin expression renders the melanoma cells resistant to chemotherapy, we selected six melanoma samples according to their Livin expression. Samples 5556 and 55112 express high levels of Livin; 5524 and 55164 express intermediate levels, whereas no expression was ob-

Fig. 5. Cleavage of Livin eliminates its antiapoptotic effect and produces a proapoptotic subunit. 721.221 cells stably expressing either Livin α or p30 Livin α and wild-type 721.221 cells were treated with 0.1 μg/ml anti-CD95/Fas antibody for 18 h. Nuclear morphology was visualized using acridine orange staining. Apoptotic cells were scored when the nuclei displayed chromatin condensation and/or nuclear fragmentation. Bars, SD. b, Western blot analysis confirming the expression of the appropriate proteins. c, 293T cells were transiently transfected with the indicated plasmids. Twenty-four h after transfection, cells were harvested. By using flow cytometry, GFP-positive cells were analyzed for apoptosis using Annexin-V/PI stain. Bars, SD. d, Western blot analysis of transiently transfected 293T cells 24 h after transfection.
served in samples 5530 and 5533 (Fig. 7a). Cells were plated in 6-well plates 24 h before exposure to etoposide (15 μg/ml). After 24 and 48 h, cells were harvested. The percentage of apoptosis was determined in each sample by nuclear morphology (Fig. 7b). Remarkably, a direct correlation was observed between the rate of apoptosis and Livin expression level. Samples with high Livin expression, 5556 and 55112, were completely resistant to etoposide at the indicated concentrations and times, whereas moderate resistance (15–25%) was observed in samples 5524 and 55164. Finally, melanoma samples 5533 and 5530 showed a marked apoptosis rate, reaching up to 55%. Interestingly, XIAP and Survivin expression was not in correlation with resistance to etoposide.

To demonstrate the relevance of Livin cleavage in primary melanoma, three samples: 55112 and 5524 expressing high and moderate levels of Livin, respectively, and 5530 with no detectable Livin were lysed, normalized for total protein, and analyzed by Western blot analysis (Fig. 7c). Accordingly, sample 55112, which was completely resistant to etoposide, showed no cleavage of Livin, sample 5524 showed moderate cleavage, appearing only 48 h after treatment, and no Livin was detected in the melanoma sample 5530 (Fig. 7c). Marked differences were observed in the time course of Livin cleavage in the melanoma cells in comparison with Jurkat and 721.221 cells (48 h versus 4 h, respectively). These differences are in direct correlation with chemosensitivity. The clinical data regarding these 27 patients revealed that 15 patients received chemotherapy either as the only treatment or before vaccination. Seven patients had disease progression while on chemotherapy, 5 of which had intermediate (5574 and 5524) or high (55182, 55112, and 5556) expression of Livin. Among the 8 chemoresponsive patients, 1 patient had a low level of Livin (5584), and the other 7 had no Livin expression (P = 0.02, Fischer exact test). Seventeen patients died; 8 of these had intermediate to high levels of Livin (median survival, 22.5 ± 16 months), and 1 patient is alive with disease. Among the 9 patients with no evidence of disease (median time of follow-up, 36 ± 21 months), 7 were Livin negative. Although the number of patients we studied is relatively small, the correlation between Livin expression and response to chemotherapy is statistically significant. Further research will clarify the role of Livin and its interactions with other apoptosis regulators in the chemoresistance phenotype of melanoma.

DISCUSSION

A network of regulatory proteins controls the apoptotic process. The role of the IAP family appears to be more crucial and diverse than was initially thought. Through direct molecular interactions, IAPs can modulate the function of several cellular proteins. In this context, caspases are among the most prominent targets of IAPs. Recent studies revealed that whereas IAPs interact with and inhibit caspases, their own function is concomitantly modulated by these caspases (15, 16).

Recently, we and others described the identification of a new member of the IAP family designated Livin (17–20). We showed that Livin has two isoforms with different antiapoptotic properties and tissue distribution patterns (20). The mechanism by which Livin is regulated is still unknown. In the present work, we present several novel and important findings. Our studies demonstrate, for the first time, that the cleavage of Livin is part of the apoptotic process. Livin cleavage appeared early after the apoptotic stimuli and before significant levels of apoptosis were detected, suggesting that Livin cleavage is an early key regulator that controls the progression of apoptosis. After apoptotic stimuli both Livin isoforms, Livin α and β, undergo a specific proteolytic cleavage that trims the 52 amino acids at the NH2 terminus, producing COOH-terminal subunits of approximately...
We further analyzed the involvement of cell death initiator caspase-8 and -9 and effector caspase-3, -6, and -7 in the cleavage process using caspase inhibitors \textit{in vivo} and recombinant caspases \textit{in vitro}. We showed that the effector caspase-3 and -7, but not initiator caspase-8 and -9, cleave Livin. On the basis of the analysis of the amino acid sequence of Livin and the introduction of substitution mutations D52E and D238E, we demonstrate that the Livin cleavage site is DHVD52G. In light of these results, the specificity of caspase-3 and -7 was not surprising because the Livin cleavage site has high homology with the consensus target sequences for these caspases (26, 27). In contrast to general peptidases, caspases cleave their targets at specific sites after aspartic acid. Therefore, caspase-mediated cleavage of several cellular proteins serves as a mechanism to produce subunits with modulated or new functions rather than totally abolishing their effect. The presence of the intact BIR and RING domains in the COOH-terminal subunits of Livin for a relatively long time after the induction of apoptosis indicates an apoptosis-regulatory function of these subunits. Indeed, experiments performed with 721.221, which stably expressed the cleaved subunit of Livin/H9251, p30-Livin/H9251, revealed that p30-Livin not only loses its antiapoptotic effect but also gained significant proapoptotic activity. Despite repeated attempts, we were unable to generate 721.221 cells that stably expressed p28-Livin. However, transient transfection experiments revealed that both Livin subunits have proapoptotic activity in 293T cells. The p28-Livin subunit showed a slightly more potent proapoptotic effect in these cells. It is possible that in 721.221 cells this activity of p28-Livin precluded the generation of stable clones. Additional experiments are required to determine the difference in proapoptotic activity between Livin isoforms.

Two other IAPs, \textit{i.e.}, XIAP and cIAP-1, were shown to be targets for caspase-mediated cleavage (15, 16). In the case of XIAP, the cleavage is located between the BIR2 and BIR3 domains. The COOH-terminal fragment that contains the BIR3-RING domains, which resembles the Livin COOH-terminal fragment, retains its antiapoptotic activity. On the other hand, cleavage of cIAP1 produces a proapoptotic COOH-terminal fragment that has only the spacer-RING domain. The proapoptotic activity of cIAP1 fragment, which does not contain BIR, is not surprising because RING domains of other baculoviral and mammalian IAPs were able to induce apoptosis when they were expressed without their BIR domains (30).

In contrast to XIAP and cIAP, our results show the first example of an IAP cleavage product that acts as a proapoptotic factor, although it contains BIR domain. A possible explanation for this unique behavior is that an additional, as yet undetermined, motif at the first 52 amino acids of Livin can modulate the antiapoptotic effect of the BIR domain. The absence of this motif might enhance the E3-ubiquitin

![Fig. 7. Livin expression in primary melanoma cells mediates etoposide resistance.](image-url)
ligase activity of the RING domain that in turn targets other antiapoptotic proteins for proteasome-mediated degradation. The subunit might also act as a pseudosubstrate, hindering the activity of other IAP family members. We are currently in the process of exploring these possibilities.

Our novel findings prompted us to determine the relevance of Livin expression and cleavage in human malignancies. A possible role for Livin in melanoma has been suspected in light of very high expression levels in most melanoma cell lines tested by us as well as by others (17, 20). Malignant melanoma has an increasing incidence with a high mortality rate because of the chemoresistant phenotype of most tumors. Many biological mechanisms have been implicated in the drug resistance of melanoma (31, 32). Little is known about the role of the IAP family proteins in this disease. Recently, the overexpression of Survivin was reported in malignant and invasive melanoma. Furthermore, antisense treatment against Survivin induced spontaneous apoptosis in melanoma in vitro (33) and in vivo (34). Interestingly, etoposide-resistant melanoma cells showed decreased caspase activation, but this was not in correlation with Survivin expression (31).

In this work, we demonstrate the important role of the endogenous Livin in the chemoresistance of melanoma cells. We tested primary cultures of melanoma cells for the expression of Livin, XIAP, and Survivin. XIAP and Survivin were widely expressed in most of the melanoma samples tested. Livin, on the other hand, was expressed, at variable levels, in 10 of the 27 melanoma samples. Direct correlation between resistance to etoposide-induced apoptosis and Livin expression was observed in vitro. In contrast, expression of XIAP and Survivin was not correlated with the drug-resistance phenotype. The ability of exogenously expressed Livin β to inhibit etoposide-induced apoptosis was demonstrated by us previously (20). Similar experiments showed the ability of exogenous Livin β to protect Jurkat against various other chemotherapeutic agents including daunorubicin, fludarabine, and 1-β-D-arabinofuranosylcytosine.5 The clinical data of the patients from whom the cell lines were established support the in vitro correlation between chemotheraphy resistance and Livin expression. Five of the 7 patients who did not respond to chemotherapy had intermediate to high levels of Livin expression, whereas among the responding patients, only 1 of 8 expressed Livin at a low level. These differences between responders and nonresponders were found to be statistically significant.

The fact that the relapsed melanoma cells Mel-B1 had different HLA class I molecules and high expression of Livin might indicate that under the selective pressure of antitumor CTL response, Livin expression contributes to the survival of this malignant clone against the immune response. The results in primary cultures combined with the clinical data of the patients from whom the cells were derived suggest an essential role of Livin in the drug resistance of melanoma cells. Considering the fact that metastatic melanoma is still a fatal disease, our findings might open the way for a new modality of treatment. In patients with melanoma expressing high levels of Livin, anti-Livin agents might have an impact on the management of the disease.

In summary, Livin can inhibit initiator caspase-9 (19), but caspase-9 and -8 cannot cleave Livin. Thus, Livin is able to interfere with the apoptotic process immediately at the starting point. The situation changes, however, once a sufficient apoptotic signal is received, the effector caspases such as -3, -6, and -7 are now activated, and the cell is committed to apoptosis. Caspase-3 and -7 are also inhibited by Livin (19), but at the same time, as we show here, they are able to cleave Livin and convert it from an antiapoptotic agent to a proapoptotic agent. These results, taking together, demonstrate the versatile nature of Livin in the apoptotic cascade.

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