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Proteolytic cleavage of Livin (ML-IAP) in apoptotic melanoma cells potentially mediated by a non-canonical caspase

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Summary

Background—Several inhibitor of apoptosis proteins (IAPs) are cleaved during apoptosis. Studies of the melanoma-associated IAP (ML-IAP) Livin, using recombinant molecules, have implicated both caspases 3/7 and the serine protease Omi/HtrA2 in its proteolytic cleavage.

Objective—To characterize the apoptotic cleavage of Livin in melanocytic cells, and evaluate the role of known proteases.

Methods—We assessed the capacity of a variety of stimuli to induce Livin cleavage in human melanoma cell lines and normal human melanocytes. The role of caspases and Omi was examined using caspase inhibitors and RNAi, respectively. A potential caspase substrate was further examined by site-directed mutagenesis. Deletion mapping was used to identify the cleavage site.

Results—Livin cleavage was observed in multiple human melanoma cell lines in response to a variety of apoptotic stimuli (UVB, 4-TBP, cisplatin, TNF, Bax), and not affected by the addition of various protease inhibitors or RNAi-mediated silencing of Omi/HtrA2. Livin cleavage induced by 4-TBP, but not UVB or cisplatin, was blocked by the pan-caspase inhibitor zVAD-fmk. Mutation of Asp⁵² to Glu in Livin did not affect cleavage, while either mutation of Asp⁵² to Ala, deletion of Asp⁵², or deletion of the adjacent region (residues 53–61) abrogated cleavage.

Conclusion—Livin cleavage, induced by multiple apoptotic stimuli in melanoma cells, likely occurs in an Omi-independent fashion at residue 52 within its potential caspase substrate (DHVD⁵²). However, relative insensitivity of the apoptotic cleavage to zVAD-fmk, or Asp⁵² to Glu mutation, suggests the involvement of a non-canonical caspase.

Keywords

Livin; Cleavage; Inhibitor of apoptosis (IAP); Apoptosis; Melanoma

1. Introduction

The inhibitor of apoptosis proteins (IAP) are important regulators of apoptosis. Most IAPs are expressed in normal tissues, and some are over-expressed in cancer and mediate apoptosis

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resistance in tumors [1]. First discovered in baculoviruses, IAPs are characterized by one or more conserved baculoviral IAP repeat (BIR) domains [2]. Eight IAPs have been identified in humans: XIAP, cIAP1, cIAP2, NIAP, Apollon, Ts-IAP/ILP-2, Survivin, and most recently Livin/ML-IAP. Many of these molecules can protect cells from a variety of apoptotic stimuli, including ultraviolet radiation and chemotherapeutic drugs [1]. Most IAPs have been shown to function as caspase inhibitors, directly binding caspases via their BIR domain(s) and adjacent sequences resulting in allosteric monomerization of the caspase [3]. Several IAPs possess ubiquitin ligase activity [4], and XIAP [5] and cIAP2 [6] can direct the ubiquitylation of effector caspases. Other IAPs like Survivin may exert additional anti-apoptotic functions independent of caspases [7,8]. Most IAPs are themselves primarily regulated by the mitochondrial proteins Smac [9] and the Omi/HtrA2 protease [10], which contain a conserved tetrapeptide IAP-binding motif and during apoptosis translocate to the cytosol where they disrupt IAP-caspase interactions.

In addition to competitive binding by Smac and Omi, another important negative regulatory mechanism of IAPs is proteolysis. Several IAPs have been shown to be cleaved during apoptosis. Deveraux et al. [11] demonstrated that in Fas-stimulated cells XIAP is cleaved by activated caspases into two distinct fragments. Similarly, Clem et al. [12] reported that cIAP1 was cleaved in apoptotic cells and *in vitro* in a caspase-dependent manner, with the resulting C-terminal peptide exhibiting proapoptotic activity. In addition to caspases, Omi may potentially regulate cleavage of IAPs. First, the cIAPs and XIAP may be cleaved directly by Omi, as suggested by *in vitro* studies [13,14,15]. Second, in some cases activation of Omi may be required for caspase-mediated cleavage [15]. Thus proteolytic digestion of IAPs by caspases or Omi may be important for potentiating apoptosis.

In contrast to other IAPs, ML-IAP (Livin) has a more restricted range of expression and has been identified only in melanoma [16,17] and some lymphoma cell lines [16]. It is expressed as two isoforms [18] and appears to function as a classical IAP, with both caspase-binding and caspase-inhibitory activities [16,17], and dependent on its BIR domain for mediating anti-apoptotic activity [16]. More recent studies, however, suggest that the Livin BIR domain is a relatively weak caspase inhibitor and may exert its anti-apoptotic activity primarily through disrupting interactions of Smac with other IAPs [19]. As seen with the IAP Survivin [20], silencing of Livin in melanoma cells by either anti-sense [17] or RNAi [21] compromises cell viability and precipitates apoptosis. Livin may be an important immune target in melanoma, as T-lymphocytes infiltrating melanoma tumors are reactive to Livin [22,23] and Livin-specific antibodies are present in patient sera [22]. We recently reported that Livin is cleaved in melanoma cells upon apoptotic stimulation [24] and this has been confirmed by others [25]. There are conflicting reports, however, regarding the protease(s) involved. Yang et al. [15] demonstrated that recombinant Omi cleaves various IAPs, including Livin. Nachmias et al. [25], on the other hand, reported that Livin cleavage is mediated by caspases, based on their findings that cleavage was partially blocked by a caspase inhibitor and that recombinant Livin was cleaved by recombinant caspases 3 and 7. It is possible that the Livin proteolysis observed in these studies could be a result of high concentrations of recombinant protease and substrate that may not reflect physiologic conditions *in vivo*. In this study, we further characterize the apoptotic stimuli for induction of Livin cleavage in melanocytic cells and re-evaluate the role of caspases and Omi in Livin cleavage. Multiple apoptotic stimuli induce Livin cleavage, which occurs independent of Omi at Asp⁵² within the internal caspase substrate, yet the cleavage reaction was not affected by mutation (D52E) of this residue and only partially sensitive to caspase inhibition. These results suggest that other proteases, in addition to conventional caspases, are likely involved in Livin cleavage.

2. Materials and methods

2.1. Cell culture

Human metastatic melanoma lines YUSAC2 and YUGEN8 are previously described [20]. Human invasive melanoma line WM35 was kindly provided by Meenhard Herlyn (Wistar Institute, Philadelphia, PA) and maintained in MCDB 153 and Leibovitz L-15 medium containing 2% FCS and insulin as described [26]. Normal human melanocytes were obtained from discarded foreskins and cultured as described [24].

2.2. Apoptosis induction and detection

Cells were seeded ($(3-5) \times 10^5$ /well/dish) in 6-well plates or 35-mm dishes the day prior to use. The 4-*tert*-butylphenol (4-TBP), cisplatin, and etoposide (all from Sigma, St. Louis, MO) were prepared and stored as described previously [24]. Recombinant human TRAIL was obtained from EMD Biosciences (La Jolla, CA) and stored at -80°C . Recombinant human TNF- α and cycloheximide (both from Sigma) were stored at -20°C . Ultraviolet-B (UVB, 290–320 nm) was delivered at a rate of $2\text{ J/m}^2/\text{s}$ using FS20T12 bulbs and a Kodacel filter as described previously [24]. Bax was expressed in YUSAC2 cells using a binary adenoviral vector system [27] kindly provided by Bingliang Fang (MD Anderson Cancer Center, Houston, TX). Adenoviruses expressing the GAL4 transactivator (Ad/pGK-GV16) and inducible Bax (Ad/GT-Bax) genes were propagated in 293 cells and purified from cell lysates by ultracentrifugation as described elsewhere [28]. Prior to use, viruses were titrated on YUSAC2 cells and stored at 4°C . Some experiments incorporated zVAD-fmk and zFA-fmk (ICN Biomedicals, Aurora, OH) which were solubilized in DMSO and stored at -20°C . The protease inhibitors AEBSF, E64, EST, leupeptin, pepstatin A, TLCK, TPCK, ALLN, and PD150606 were obtained from EMD Biosciences, and solubilized according to the manufacturer's instructions. Apoptosis kinetics were determined by detecting DNA content in propidium iodide-stained cells by flow cytometry as described previously [24]. Caspase activity was measured in cell lysates using the fluorogenic caspase-3 substrate Ac-DEVD-AMC (Peptides International Inc., Louisville, KY) as previously described [8].

Experiments in HeLa cells involved transient lipid-based transfection of Livin constructs. Briefly, cells were seeded (2×10^5 /well) in 6-well plates and the next day a mixture containing $0.5\text{ }\mu\text{g}$ DNA, $1\text{ }\mu\text{L}$ Lipofectamine-2000 (Invitrogen, Carlsbad, CA) and $200\text{ }\mu\text{L}$ Optimem (Invitrogen) was prepared, and after 30 min this was added to each well and cells were incubated for 6 h in media without antibiotics. The media was then changed, and transfected cells were incubated overnight prior to treatment with apoptotic stimuli and preparation of cell lysates for Western blotting.

2.3. Western blotting

Cell lysates were prepared, electrophoresed on 10 or 15% acrylamide gels, transferred to PVDF membranes, blocked with non-fat milk, reacted sequentially with primary and secondary antibodies, and visualized by enhanced chemiluminescence as described previously [24]. For some experiments, cells were fractionated into mitochondrial and cytosolic components and analyzed separately as described [8]. Sources and use of antibodies to Livin [24], Actin [24], XIAP [24], and VDAC [8] have been described previously. Polyclonal rabbit antiserum against Omi/HtrA2 was kindly provided by Chunying Du (Stowers Institute for Medical Research, Kansas City, MO) and used at a dilution of 1:2000. A monoclonal anti-V5 tag antibody (Invitrogen) was used at a dilution of 1:5000.

2.4. RNA interference

The RNA oligos 5'-CUUCAUCGCAGAUGUGGUGdTdT-3' (sense) and 5'-CACCACAUCUGCGAUGAAGdTdT-3' (anti-sense) corresponding to the human Omi/HtrA2 sequence, and non-specific oligos 5'-AGACAGAAGACAGAUAGGCdTdT-3' (sense) and 5'-GCCUAUCUGUCUUCUGUCdTdT-3' (anti-sense) were synthesized and HPLC-purified by the core facility at the University of Utah. Each pair of oligos was solubilized in RNA interference (RNAi) Suspension Buffer (Qiagen, Valencia, CA) and mixed at a final concentration of 20 μ M, heated at 90 °C for 1 min, incubated at 37 °C for 1 h, and then stored at -20 °C. Cells were seeded in medium without antibiotics (2×10^5 /well) in 6-well plates and the next day a mixture containing 2 μ L RNA duplex, 7 μ L Lipofectamine-2000 and 300 μ L Optimem was prepared, and after 20 min this was added to each well and cells were incubated 48 h in 1.5 mL medium without antibiotics.

2.5. Construction of Livin mutants

The pcDNA3.1V5HisTOPO-Livin construct [17] expressing wild-type full-length human Livin with a C-terminal V5 tag was kindly provided by Gary Kasof (formerly of Astrazeneca, Worcester, MA). Mutation of the potential caspase cleavage site at residue 52 from Asp to Glu (LivD52E) was generated by a site-directed mutagenesis technique based on the Quik-Change system (Stratagene, La Jolla, CA). Briefly, mutagenic primers 5'-GCCTGGGACCACGTGGAAaGGGCAGATCCTGGGCC-3' and 5'-GGCCCAGGATCTGCCCtTCCACGTGGTCCCAGGC-3', containing the D52E mutation (mutated nucleotide in lower case), were annealed to pcDNA3.1V5HisTOPO-Livin. The primers were extended and incorporated into nicked circular strands with *Pfu Ultra* DNA polymerase (Stratagene). The wild-type (methylated) parental strands were digested with *DpnI* prior to transformation. Mutation of residue 52 from Asp to Ala (LivD52A) was similarly prepared, using mutagenic primers 5'-TGCAGAGCCTGGGACCACGTGGccGGGCAGATCCTGGGCCAGCT-3' and 5'-AGCTGGCCCAGGATCTGCCCggCCACGTGGTCCCAGGCTCTGCA-3', containing the D52A mutation (mutated nucleotides in lower case). For construction of Livin deletion mutants, a two-stage PCR-based strategy was employed with 1st round amplification of fragments flanking the deletion and 2nd round amplification of the fragments after joining them into a single full-length segment. The vector pcDNA3.1V5HisTOPO-Livin was used as a template. Primers used for the 2nd round incorporated *NheI* (forward primer) and *EcoRI* (reverse primer) sites at the 5' ends. For the deletion mutants LivLE Δ 52-61, LivLE Δ 62-71 and LivLE Δ 72-82, primers used for 1st round PCR incorporated *XhoI* sites at the 5' ends to facilitate linkage of the fragments. Generation of the *XhoI* site resulted in introduction of a leucine and glutamic acid residue within the deleted region. For the remaining deletion mutants, primers used for 1st round PCR contained overlapping complimentary sequences at the 5' ends to allow hybridization of the fragments. No additional residues were introduced with this approach. After 2nd round PCR, the reconstructed Livin cDNAs containing deletions were excised from agarose gels, cut with *NheI* and *EcoRI*, and ligated into these sites in pBK-CMV (Stratagene). The integrity of all constructs was verified by sequencing, and plasmid DNA was prepared from transformed *E. coli* using a commercial kit (Qiagen).

3. Results

3.1. Multiple apoptotic stimuli/pathways induce Livin cleavage

We previously observed that in YUSAC2 melanoma cells exposed to UVB, the 40-kDa Livin protein was cleaved to yield a 30-kDa fragment [24]. We examined this cleavage phenomenon in additional melanoma lines derived from metastatic (YUGEN8) and locally invasive (WM35) lesions, as well as normal melanocytes. Cells were treated with UVB and Livin levels were monitored over a 48-h period by Western blotting. The polyclonal antibody used recognizes

both the α and β isoforms [18], hence often a “doublet” is visualized. As shown in Fig. 1a, Livin was cleaved in all three melanoma lines over 24–48 h, roughly corresponding to the kinetics of UVB-induced apoptosis seen previously in YUSAC2 and YUGEN8 cells [24]. The presence of a doublet fragment suggests that both Livin isoforms are cleaved. The residual cleavage fragment was not visualized, likely due to rapid degradation or because its small size precluded resolution and detection. By contrast, in UVB-treated melanocytes there was progressive degradation of the full-length molecule by 48 h and a discreet cleavage fragment was not seen (Fig. 1a). We next asked if other apoptotic stimuli could induce Livin cleavage in melanoma cells. As shown in Fig. 1b, Livin cleavage occurred in both YUSAC2 and YUGEN8 cells in response to the drugs 4-TBP, cisplatin and etoposide, comparable to that induced by UVB. Treatment of these cells with staurosporine also resulted in Livin cleavage (not shown). Thus multiple apoptotic stimuli can induce Livin cleavage.

Given that the apoptotic pathways triggered by UVB and these drugs are poorly characterized, we examined whether Livin cleavage occurred in response to stimuli that trigger-specific apoptotic pathways. To assess death receptor signaling, we first attempted to stimulate cells with TRAIL but they were resistant to TRAIL-induced apoptosis and Livin cleavage was not seen (not shown). Since melanoma cells are often resistant to TNF-receptor ligands [29], we assessed Livin cleavage in a cell line sensitive to death receptor signaling. Although Livin expression has been reported in some HeLa cell lines [17], those maintained in our laboratory did not express detectable levels of endogenous Livin (Fig. 1c). Transfection of HeLa cells with a Livin expression construct resulted in marked expression of Livin (Fig. 1c), which was cleaved following stimulation with TNF- α (Fig. 1c) or TRAIL (not shown). Since apoptosis in response to death receptor signaling in some cells may be dependent on activation of the mitochondrial pathway [30], we next asked whether Bax-mediated mitochondrial stimulation could induce Livin cleavage. YUSAC2 cells were infected with an inducible Bax-expressing adenovirus [27], and Bax expression was associated with Livin cleavage (Fig. 1d). Thus direct stimulation of mitochondrial apoptosis is sufficient to induce Livin cleavage in melanoma cells.

We next compared the kinetics of Livin cleavage with that of apoptosis induction by cisplatin and 4-TBP in the melanoma lines. As shown in Fig. 2, cleavage tended to either coincide or precede apoptosis depending on the stimulus and the cell line. For example, in YUSAC2 and WM35 cells treated with cisplatin, apoptosis and cleavage proceeded over a 48-h period (Fig. 2a and c) as observed in UVB-treated cells (Fig. 1a and Ref. [24]). On the other hand, apoptosis lagged behind cleavage in cisplatin-treated YUGEN8 cells (Fig. 2b). In YUSAC2 and YUGEN8 cells treated with 4-TBP, cleavage was generally maximal by 2 h while apoptosis was not complete until 24 h (Fig. 2a, b and d). In 4-TBP-treated WM35 cells, both cleavage and apoptosis had peaked by 2 h (Fig. 2c).

3.2. Role of proteases in Livin cleavage

Given the previous demonstration by Yang et al. [15] that recombinant Omi cleaves Livin, we first examined its potential role in Livin cleavage in melanoma cells. We first confirmed that Omi translocates from mitochondria to cytosol in melanoma cells under experimental conditions in which Livin cleavage had been observed. Following treatment with UVB or 4-TBP, YUSAC2 and YUGEN8 cells were fractionated into mitochondrial and cytosolic components which were separately blotted for Omi. As shown in Fig. 3a, Omi localized to the mitochondria in untreated cells, but was easily detected in the cytosol from both cell lines upon treatment with either UVB or 4-TBP. To examine whether translocated Omi was required for Livin cleavage, we knocked down Omi expression in YUSAC2 cells prior to induction of apoptotic Livin cleavage. Transfection of YUSAC2 cells with siRNA specific for Omi, but not control siRNA, resulted in considerable knockdown of Omi protein at 48 h (Fig. 3b). Reduced levels of Omi, however, did not compromise either 4-TBP- or UVB-induced Livin and XIAP

cleavage (Fig. 3b). Similar results were obtained in YUGEN8 cells (not shown). Thus Omi is not required for Livin cleavage in melanoma cells.

Next, we screened a panel of non-toxic protease inhibitors for the capacity to block Livin cleavage. YUSAC2 cells were pre-incubated with inhibitors of serine (AEBSF), cysteine (E64, EST), trypsin-like (leupeptin, TLCK), chymotrypsin (TPCK) or aspartic (pepstatin A) proteases, or calpain inhibitors (ALLN, PD150606), then stimulated with UVB or 4-TBP. None of these inhibitors at recommended concentrations, however, was capable of blocking cleavage of either Livin or XIAP induced by UVB (Fig. 3c) or 4-TBP (Fig. 3d).

Cleavage of other IAPs such as XIAP [11] and cIAP-1 [12] appears to be mediated by caspases, and Nachmias et al. [25] showed that recombinant Livin was cleaved by recombinant caspases 3 and 7. To assess the role of caspases in Livin cleavage in melanoma cells, YUSAC2 cells were exposed to apoptotic stimuli in the absence or presence of the pan-caspase inhibitor zVAD-fmk. While addition of zVAD-fmk significantly reduced cleavage of XIAP in UVB-treated cells, Livin cleavage was essentially unaffected (Fig. 4a). The concentration of zVAD-fmk used (20 μ M) was sufficient to block caspase activity in UVB-treated YUSAC2 cells (Fig. 4b). In cells treated with 4-TBP, however, the presence of zVAD-fmk conferred only partial protection against Livin cleavage (Fig. 4a). The presence of a weak XIAP cleavage fragment in cells treated with 20 μ M zVAD-fmk (Fig. 4a) further suggests there may be residual caspase activity in cells under these conditions. We next examined Livin cleavage using a higher concentration of zVAD-fmk (50 μ M) sufficient to abrogate XIAP cleavage (Fig. 4c). Under these conditions, cisplatin-induced Livin cleavage was not affected in either YUSAC2 or YUGEN8 cells (Fig. 4c) and 4-TBP-induced cleavage was again only partially blocked (not shown). In the presence of 100 μ M zVAD-fmk, however, 4-TBP-induced cleavage was blocked completely in YUSAC2 cells while UVB-induced cleavage remained largely unaffected (Fig. 4d). It is known that such high concentrations of zVAD-fmk can have non-specific effects [31], so we incorporated the cathepsin B inhibitor zFA-fmk, used by others [32] as a negative control for zVAD-fmk. At a concentration of 100 μ M, zFA-fmk partially blocked XIAP cleavage and 4-TBP-induced Livin cleavage (Fig. 4d), suggesting that at least some of the effects of zVAD-fmk at this concentration can be attributed to caspase inhibition. Thus the dependence of Livin cleavage in melanoma cells on caspases varies somewhat depending on the apoptotic stimulus, as cleavage induced by UVB and cisplatin is largely resistant to caspase inhibition, while that induced by 4-TBP is more sensitive to caspase inhibition.

Previous studies showed that recombinant Livin was cleaved by recombinant caspases 3 and 7, and that mutation of Asp⁵² (D52E) in Livin blocked its cleavage *in vitro* [25]. We prepared the same mutant (LivD52E), transfected it into HeLa cells, and assessed whether it could be cleaved upon apoptotic stimulation. As shown in Fig. 4e, mutation of this potential caspase substrate in Livin did not affect cleavage in 4-TBP- or UVB-treated cells. By contrast, a less conservative substitution at this residue (D52A) prevented cleavage induced by either 4-TBP or UVB (Fig. 4e). Given that conventional caspases cleave at Asp residues and recognition is blocked by Asp $\rightarrow$ Glu substitution [33], these results suggest several possibilities regarding Livin cleavage in our cells. First, cleavage is not caspase-mediated; second, cleavage is mediated by a non-canonical caspase such that recognition is only partially inhibited by zVAD-fmk and not affected by the Asp $\rightarrow$ Glu substitution; or third, cleavage does not occur at Asp⁵².

3.3. Localization of cleavage region and generation of uncleavable Livin mutants

To investigate these possibilities further, we sought to map the cleavage site. The 30-kDa size of the Livin cleavage fragment suggested that the 40-kDa full-length protein was cleaved either at the amino- or carboxy-terminal third of the molecule, or that cleavage was occurring at each end of the molecule to yield a large internal fragment. The incorporation of a carboxy-terminal

V5 tag in our Livin expression construct permitted us to distinguish between these possibilities. Livin-transfected HeLa cells were treated with 4-TBP and cell lysates were blotted with antibodies against Livin and V5. As shown in Fig. 5a, the Livin cleavage fragment was similarly visualized with the Livin- and V5-specific antibodies, confirming the presence of the carboxy-terminal V5 tag in the cleavage fragment, and that cleavage was occurring in the amino-terminal third of the molecule. Thus the putative cleavage region spans approximately 30 amino acids, situated upstream of the BIR domain (Fig. 5b).

Next, we generated three mutants covering the putative cleavage region containing internal deletions of approximately 30 nucleotides each (Fig. 5b). These three mutants (LivLEΔ52–61, LivLEΔ62–71, LivLEΔ72–82) were separately transfected into HeLa cells, and then cleavage was assessed by Western analysis following treatment with 4-TBP. As shown in Fig. 5c, the first mutant (LivLEΔ52–61) was resistant to apoptotic cleavage while deletions in the other two mutants did not affect their cleavage. The increased migration of the second mutant (LivLEΔ62–71) relative to the others (Fig. 5c) may be due to loss of a phosphorylated threonine, given that the residues T⁶³EEEEEE constitute a potential phosphorylation site for casein kinase II [34]. Thus residues in the first third of the putative cleavage region were required for Livin cleavage.

Having established that mutation of residue 52 did not compromise cleavage (Fig. 4e), we made additional sub-deletion mutants spanning this region starting at residue 53. These four additional mutants (LivΔ53–55, LivΔ55–57, LivΔ57–59, LivΔ60–62) were evaluated for apoptotic cleavage following transfection into HeLa cells. As shown in Fig. 5d, all four sub-deletion mutants were efficiently cleaved. To ascertain whether the resistance of LivLEΔ52–61 to cleavage was due to introduction of the LE residues or loss of residue 52, the mutants LivΔ52–61 and LivΔ53–61 were respectively prepared (Fig. 5b). Surprisingly, both were found to be resistant to cleavage (Fig. 5e). Confirming the importance of residue 52 for cleavage, additional mutants lacking residue 52 (LivΔ52) or 51–53 (LivΔ51–53) were similarly resistant to cleavage (Fig. 5e). The resistance of LivΔ52 to cleavage was also confirmed with UVB and cisplatin as apoptotic stimuli (Fig. 5f). These results suggest that although residue 52 is required for cleavage, loss of nine adjacent residues (53–61) may sufficiently perturb the structure of this part of the molecule to block protease recognition or cleavage.

4. Discussion

We found Livin cleavage to be a consistent phenomenon associated with apoptosis in melanocytic cells, induced by a variety of apoptotic stimuli. Direct activation of the mitochondrial apoptotic pathway, by over-expression of Bax, was sufficient for induction of Livin cleavage. Interestingly, Livin cleavage may not routinely occur in normal melanocytes upon apoptotic stimulation. We found previously [24] and in the present study that while levels of full-length Livin are reduced in UVB-treated melanocytes, the 30-kDa fragment is not detected. While it is possible that in non-malignant cells Livin may be proteolyzed by an alternate mechanism or the cleaved fragment may be highly unstable, it is more likely that in melanocytes a higher apoptotic threshold may be required for Livin cleavage. We did in fact observe in some experiments the 30-kDa fragment in melanocytes treated with 4-TBP which is a more potent apoptotic stimulus than UVB (Brouha and Grossman, unpublished observation).

In this study, we investigated further the potential role of known proteases in the apoptotic cleavage of Livin. While others have shown that Livin can be cleaved by recombinant Omi [15] or caspases 3/7 [25] *in vitro*, our studies using intact melanoma cells demonstrate that Omi is not required for Livin cleavage, and the role of caspases is not straightforward and may depend on the apoptotic stimulus as discussed below. Although we observed rapid translocation

of Omi during apoptosis from mitochondria to cytosol with kinetics similar to that of Livin cleavage, the extent of cleavage was not diminished under conditions of Omi knockdown. Given that Omi is a serine protease, our findings are consistent with the inability of the serine protease inhibitor AEBSF to block Livin cleavage. We also screened inhibitors of other major classes of proteases, including cathepsins and calpains which have been implicated in apoptosis [35], but none was able to block Livin cleavage *in vivo*.

It would be tempting to speculate that Livin is cleaved by caspases given the presence of an internal potential caspase substrate (DHVD⁵²) [16,17] and the reported caspase-dependent cleavage of other IAPs [11,12]. In support of this notion, Nachmias et al. [25] demonstrated partial inhibition of cleavage in staurosporine-treated Jurkat cells in the presence of 200 μ M zVAD-fmk, and cleavage of Livin (but not a D52E mutant) by recombinant caspases 3/7 *in vitro*. They did not observe Livin cleavage in cells exposed to 20 μ M zVAD-fmk, and a control peptide was not used to control for non-specific effects at the higher concentrations [25]. We found that 20 μ M zVAD-fmk was sufficient to inhibit most of the caspase activity in apoptotic melanoma cells, and a concentration of 50 μ M was sufficient to block XIAP cleavage. While Livin cleavage induced by UVB or cisplatin was not affected by zVAD-fmk concentrations up to 100 μ M, cleavage induced by 4-TBP was only partially inhibited by 20–50 μ M zVAD-fmk and required a zVAD-fmk concentration of 100 μ M for complete inhibition. The control peptide zFA-fmk at concentrations of 100 μ M, however, did have some inhibitory effect on XIAP cleavage, so it would be hazardous to exclude non-specific effects at higher concentrations. Regarding the reported resistance of the Livin D52E mutant to caspase-mediated cleavage, this mutant was not evaluated in transfected cells [25], but we suspect that the cleavage observed in this study (and with that with Omi [15]) may be a consequence of high enzyme-substrate concentrations and susceptibility to cleavage at residue 52 (see below). We found here that LivD52E (but not LivD52A) was cleaved in transfected cells comparable to wild-type Livin, and since mutation of the terminal Asp to Glu in model caspase substrates is known to reduce caspase cleavage by 20,000-fold [33], our data suggest that Livin cleavage is not mediated by conventional (canonical) caspase(s) in cells. Regarding whether caspases are *required* for Livin cleavage, the differential sensitivity to zVAD-fmk in UVB/cisplatin-*versus* 4-TBP-treated cells suggests that the potential role of caspases in Livin cleavage may depend on the apoptotic stimulus. The very high concentrations of zVAD-fmk required to block 4-TBP-induced cleavage of Livin (compared to XIAP) suggest that if Livin cleavage is mediated by caspase, it is relatively insensitive to caspase inhibition. Alternatively, activation of the putative protease may be dependent upon upstream caspase activation, which could vary with apoptotic stimulus.

Given the relative insensitivity of Livin cleavage (compared to XIAP cleavage) to zVAD-fmk and the inability of the D52E mutation to block cleavage in our hands, we were somewhat surprised that deletion of Asp⁵² within the potential caspase consensus site (DHVD⁵²) was sufficient to abrogate cleavage. While it is theoretically possible that loss of a residue could perturb conformational structure and preclude protease recognition of an *adjacent* cleavage site, this result strongly suggests that Livin is cleaved at Asp⁵². Our finding of loss of cleavage upon mutation of Asp⁵² to Ala, but not Glu, is consistent with this model and suggests that this residue is important both for protease recognition and cleavage. Since mutation of this residue to Glu within the potential caspase consensus site should abolish canonical caspase recognition [33], as noted above, it is unlikely that proteolysis is mediated by a canonical effector caspase. Rather, our results taken together suggest that Livin cleavage may involve an alternate proteolytic pathway mediated by a non-canonical caspase. Interestingly, in *Drosophila*, the IAP molecule DIAP1 is cleaved at an Asp residue by effector caspase but at a Glu residue by the apical caspase Dronc [36]. If homologue(s) of Dronc exist in mammalian cells capable of cleaving at Glu residues, such non-canonical caspase(s) might be a good candidate for the protease involved in Livin cleavage.

Deletion of residues (53–61) adjacent to Asp⁵² had a similar effect to deletion of Asp⁵², suggesting that this region may also be important for protease recognition. Interestingly, recognition of general structure rather than specific sequences is a consistent feature of Omi, as analysis of Omi-mediated cleavage fragments of cIAP-1 revealed three internal cleavage sites which lacked conservation at the level of amino acid sequences [15]. The three-dimensional structure of Livin, to our knowledge, has not been reported. To assess the relative topology of this region in the molecule and how deletion of these residues might affect Livin structure, we utilized a secondary structure prediction program (<http://www.embl-heidelberg.de/predictprotein>). Consistent with our results, residues 52–61 in Livin are predicted to reside within a large surface loop, where they would be accessible to protease. This site may be particularly susceptible to proteolysis, perhaps explaining the cleavage of Livin observed *in vitro* with recombinant caspases [25] and Omi [15]. Although deletion of these residues is not predicted to alter the secondary structure of this loop or the remainder of the molecule significantly, cleavage in this region may alter the structure of the neighboring BIR domain or its ability to allosterically inhibit caspases, thereby reducing anti-apoptotic activity of the Livin molecule.

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Abbreviations

4-TBP	4- <i>tert</i> -butylphenol
BIR	baculoviral IAP repeat
IAP	inhibitor of apoptosis protein
RNAi	RNA interference
UVB	ultraviolet-B

References

1. Deveraux QL, Reed JC. IAP family proteins—suppressors of apoptosis. *Genes Develop* 1999;13:239–52. [PubMed: 9990849]
2. Crook NE, Clem RJ, Miller LK. An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. *J Virol* 1993;67:2168–74. [PubMed: 8445726]
3. Salvesen GS, Duckett CS. IAP proteins: blocking the road to death's door. *Nat Rev Mol Cell Biol* 2002;3:401–10. [PubMed: 12042762]
4. Yang Y, Fang S, Jensen JP, Weissman AM, Ashwell JD. Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. *Science* 2000;288:874–7. [PubMed: 10797013]
5. Suzuki Y, Nakabayashi Y, Takahashi R. Ubiquitin-protein ligase activity of X-linked inhibitor of apoptosis protein promotes proteasomal degradation of caspase-3 and enhances its anti-apoptotic effect in Fas-induced cell death. *Proc Natl Acad Sci USA* 2001;98:8662–7. [PubMed: 11447297]

6. Huang H, Joazeiro CA, Bonfoco E, et al. The inhibitor of apoptosis, cIAP2, functions as a ubiquitin-protein ligase and promotes in vitro monoubiquitination of caspases 3 and 7. *J Biol Chem* 2000;275:26661–4. [PubMed: 10862606]
7. Shankar SL, Mani S, O'Guin KN, et al. Survivin inhibition induces human neural tumor cell death through caspase-independent and -dependent pathways. *J Neurochem* 2001;79:426–36. [PubMed: 11677271]
8. Liu T, Brouha B, Grossman D. Rapid induction of mitochondrial events and caspase-independent apoptosis in Survivin-targeted melanoma cells. *Oncogene* 2004;23:39–48. [PubMed: 14712209]
9. Du C, Fang M, Li Y, Li L, Wang X. Smac, a mitochondrial protein that promotes cytochrome *c*-dependent caspase activation by eliminating IAP inhibition. *Cell* 2000;102:33–42. [PubMed: 10929711]
10. Verhagen AM, Silke J, Ekert PG, et al. HtrA2 promotes cell death through its serine protease activity and its ability to antagonize inhibitor of apoptosis proteins. *J Biol Chem* 2002;277:445–54. [PubMed: 11604410]
11. Deveraux QL, Leo E, Stennicke HR, et al. Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. *Embo J* 1999;18:5242–51. [PubMed: 10508158]
12. Clem RJ, Sheu TT, Richter BW, et al. c-IAP1 is cleaved by caspases to produce a proapoptotic C-terminal fragment. *J Biol Chem* 2001;276:7602–8. [PubMed: 11106668]
13. Jin S, Kalkum M, Overholtzer M, et al. CIAP1 and the serine protease HTRA2 are involved in a novel p53-dependent apoptosis pathway in mammals. *Genes Develop* 2003;17:359–67. [PubMed: 12569127]
14. Srinivasula SM, Gupta S, Datta P, et al. Inhibitor of apoptosis proteins are substrates for the mitochondrial serine protease Omi/HtrA2. *J Biol Chem* 2003;278:31469–72. [PubMed: 12835328]
15. Yang QH, Church-Hajduk R, Ren J, Newton ML, Du C. Omi/HtrA2 catalytic cleavage of inhibitor of apoptosis (IAP) irreversibly inactivates IAPs and facilitates caspase activity in apoptosis. *Genes Develop* 2003;17:1487–96. [PubMed: 12815069]
16. Vucic D, Stennicke HR, Pisabarro MT, Salvesen GS, Dixit VM. ML-IAP, a novel inhibitor of apoptosis that is preferentially expressed in human melanomas. *Curr Biol* 2000;10:1359–66. [PubMed: 11084335]
17. Kasof GM, Gomes BC. Livin, a novel inhibitor of apoptosis protein family member. *J Biol Chem* 2001;276:3238–46. [PubMed: 11024045]
18. Ashhab Y, Alian A, Polliack A, Panet A, Ben Yehuda D. Two splicing variants of a new inhibitor of apoptosis gene with different biological properties and tissue distribution pattern. *FEBS Lett* 2001;495:56–60. [PubMed: 11322947]
19. Vucic D, Franklin MC, Wallweber HJ, et al. Engineering ML-IAP to produce an extraordinarily potent caspase 9 inhibitor: implications for Smac-dependent anti-apoptotic activity of ML-IAP. *Biochem J* 2005;385:11–20. [PubMed: 15485396]
20. Grossman D, McNiff JM, Li F, Altieri DC. Expression and targeting of the apoptosis inhibitor, survivin, in human melanoma. *J Invest Dermatol* 1999;113:1081.
21. Crnkovic-Mertens I, Hoppe-Seyler F, Butz K. Induction of apoptosis in tumor cells by siRNA-mediated silencing of the livin/ML-IAP/KIAP gene. *Oncogene* 2003;22:8330–6. [PubMed: 14614456]
22. Schmollinger JC, Vonderheide RH, Hoar KM, et al. Melanoma inhibitor of apoptosis protein (ML-IAP) is a target for immune-mediated tumor destruction. *Proc Natl Acad Sci USA* 2003;100:3398–403. [PubMed: 12626761]
23. Andersen MH, Reker S, Becker JC, Thor Straten P. The melanoma inhibitor of apoptosis protein: a target for spontaneous cytotoxic T cell responses. *J Invest Dermatol* 2004;122:392–9. [PubMed: 15009721]
24. Bowen AR, Hanks AN, Allen SM, et al. Apoptosis regulators and responses in human melanocytic and keratinocytic cells. *J Invest Dermatol* 2003;120:48–55. [PubMed: 12535197]
25. Nachmias B, Ashhab Y, Bucholtz V, et al. Caspase-mediated cleavage converts Livin from an antiapoptotic to a proapoptotic factor: implications for drug-resistant melanoma. *Cancer Res* 2003;63:6340–9. [PubMed: 14559822]

26. Satyamoorthy K, Li G, Gerrero MR, et al. Constitutive mitogen-activated protein kinase activation in melanoma is mediated by both BRAF mutations and autocrine growth factor stimulation. *Cancer Res* 2003;63:756–9. [PubMed: 12591721]
27. Kagawa S, Pearson SA, Ji L, et al. A binary adenoviral vector system for expressing high levels of the proapoptotic gene bax. *Gene Ther* 2000;7:75–9. [PubMed: 10680019]
28. Mesri M, Wall NR, Li J, Kim RW, Altieri DC. Cancer gene therapy using a survivin mutant adenovirus. *J Clin Invest* 2001;108:981–90. [PubMed: 11581299]
29. Grossman D, Altieri DC. Drug resistance in melanoma: mechanisms, apoptosis, and new potential therapeutic targets. *Cancer Metastasis Rev* 2001;20:3–11. [PubMed: 11831644]
30. Wang S, El-Deiry WS. TRAIL and apoptosis induction by TNF-family death receptors. *Oncogene* 2003;22:8628–33. [PubMed: 14634624]
31. Schotte P, Declercq W, Van Huffel S, Vandenabeele P, Beyaert R. Non-specific effects of methyl ketone peptide inhibitors of caspases. *FEBS Lett* 1999;442:117–21. [PubMed: 9923616]
32. Gregoli PA, Bondurant MC. Function of caspases in regulating apoptosis caused by erythropoietin deprivation in erythroid progenitors. *J Cell Physiol* 1999;178:133–43. [PubMed: 10048577]
33. Stennicke HR, Renatus M, Meldal M, Salvesen GS. Internally quenched fluorescent peptide substrates disclose the subsite preferences of human caspases 1, 3, 6, 7 and 8. *Biochem J* 2000;350(Pt 2):563–8. [PubMed: 10947972]
34. Meggio F, Marchiori F, Borin G, Chessa G, Pinna LA. Synthetic peptides including acidic clusters as substrates and inhibitors of rat liver casein kinase TS (type-2). *J Biol Chem* 1984;259:14576–9. [PubMed: 6594339]
35. Goll DE, Thompson VF, Li H, Wei W, Cong J. The calpain system. *Physiol Rev* 2003;83:731–801. [PubMed: 12843408]
36. Muro I, Means JC, Clem RJ. Cleavage of the apoptosis inhibitor DIAP1 by the apical caspase DRONC in both normal and apoptotic *Drosophila* cells. *J Biol Chem* 2005;280:18683–8. [PubMed: 15774476]

Fig. 1.

Multiple apoptotic stimuli induce Livin cleavage. (a) Metastatic (YUSAC2, YUGEN8) and locally invasive (WM35) melanoma lines and normal human melanocytes (MEL) were untreated (0 h) or treated with 960 J/m² UVB, and then cultured for 24 or 48 h as indicated. Cell lysates were subjected to Western blotting using antibodies against Livin and Actin as shown. Migration of the 30-kDa cleaved fragment is indicated by the arrow. (b) YUSAC2 and YUGEN8 cells were untreated (0 h) or 0.9 mM 4-TBP, 90 μ M cisplatin, or 50 μ M etoposide for 1–48 h as indicated. Cell lysates were blotted for Livin. The cleaved fragment is indicated by arrows. (c) HeLa cells were untransfected or transfected with pcDNA3.1V5-HisTOPO-Livin as indicated. Following transfection, cells were incubated in media or media containing TNF- α (10 ng/mL) and cycloheximide (CHX, 10 μ g/mL) as indicated for 24 h. Cell lysates were blotted for Livin (cleaved fragment indicated by arrow) and Actin. (d) YUSAC2 cells were infected with adenoviruses expressing GAL4-inducible Bax (B), GAL4 (G), or both (B/G) as indicated for 24 h. Cell lysates were blotted for Livin and Bax as shown. The cleaved fragment is indicated by an arrow.

Fig. 2.

Kinetics of apoptosis and Livin cleavage. (a) YUSAC2 cells were untreated (0 h) or treated with 90 μ M cisplatin or 0.9 mM 4-TBP, and then cultured for 24 or 48 h as indicated. Percent apoptosis was determined by flow cytometric analysis of DNA content. Separate groups of cells were lysed, and cell lysates were blotted for Livin. Migration of the 30-kDa cleaved fragment is indicated by the arrow. Similar experiments were performed with (b) YUGEN8 and (c) WM35 cells.

Fig. 3.

Omi and other proteases not required for Livin cleavage. (a) YUSAC2 and YUGEN8 cells were untreated or treated with 960 J/m² UVB for 24 h or 0.9 mM 4-TBP for 2 h as indicated, and then mitochondrial (M) and cytosolic (C) fractions were prepared and blotted with antibodies against Omi, VDAC, and Actin as shown. The VDAC and Actin panels represent positive controls for the mitochondrial and cytosolic fractions, respectively. (b) YUSAC2 cells were transfected with 25 nM RNAi specific for Omi (O) or a control sequence (C). After 48 h, cells were treated with UVB or 4-TBP as indicated, then lysed and blotted with antibodies against Livin, XIAP, Omi, and Actin as shown. The cleavage fragments are indicated by an arrow. YUSAC2 cells were untreated (lane 1) or treated with (c) 960 J/m² UVB for 48 h or (d)

0.9 mM 4-TBP for 2 h following 30 min pre-incubation with serine protease inhibitor AEBSF (0.1 mM, lane 3), cysteine protease inhibitors E64 (1 μ M, lane 4) and EST (10 μ M, lane 5), trypsin-like protease inhibitors leupeptin (10 μ M, lane 6) and TLCK (10 μ M, lane 8), chymotrypsin inhibitor TPCK (10 μ M, lane 9), aspartic protease inhibitor pepstatin A (1 μ M, lane 7), or calpain inhibitors ALLN (10 μ M, lane 10) and PD150606 (10 μ M, lane 11). For UVB-treated cells, protease inhibitors were freshly added after 24 h. Cell lysates were blotted with antibodies against Livin and XIAP as shown. Migration of cleaved fragments is indicated by arrows.

Fig. 4.

Role of caspases in Livin cleavage. (a) YUSAC2 cells were untreated or exposed to 960 J/m² UVB or 0.9 mM 4-TBP in the absence or presence of 20 μ M zVAD-fmk, and then cultured for 4–48 h as indicated. Cell lysates were blotted with antibodies against Livin and XIAP as shown. Migration of cleaved fragments is indicated by arrows. (b) YUSAC2 cells were treated as in (a) in the absence (open bars) or presence (filled bars) of 20 μ M zVAD-fmk, then cell lysates were prepared and assayed for caspase activity using the fluorogenic caspase-3 substrate Ac-DEVD-AMC. (c) YUSAC2 and YUGEN8 cells were untreated or treated with 90 mM cisplatin in the absence or presence of 50 μ M zVAD-fmk, and then cultured for 24 or 48 h as indicated. Cell lysates were blotted with antibodies against Livin and XIAP as shown. Migration of cleaved fragments is indicated by arrows. (d) YUSAC2 cells were untreated or exposed to 960 J/m² UVB or 0.9 mM 4-TBP in the absence or presence of 100 μ M zVAD-fmk or zFA-fmk, and then cultured for 2–48 h as indicated. Cell lysates were blotted with antibodies against Livin and XIAP as shown. Migration of cleaved fragments is indicated by arrows. (e) HeLa cells were transfected overnight with wild-type Livin, or mutants LivD52E and LivD52A as indicated. Cells were untreated or stimulated with 0.9 mM 4-TBP or 960 J/m² UVB, then after 2 or 48 h, respectively, lysates were blotted for Livin. The cleavage fragment is indicated by the arrow.

Fig. 5.

Mapping of Livin cleavage site and generation of uncleavable mutants. (a) HeLa cells were transfected with empty vector (pcDNA3) or a Livin expression construct containing a C-terminal V5 tag as indicated. After 24 h, cell lysates were prepared and blotted with antibodies against Livin, V5, or Actin. The cleaved Livin fragment is indicated by arrows. (b) Schematics of full-length wild-type Livin and large deletion mutants constructed for this study. Numbers correspond to amino acid residues in the wild-type Livin sequence. Construction of the first three deletion mutants resulted in addition of leucine (L) and glutamic acid (E) residues as indicated. (c-e) HeLa cells were transfected overnight with individual Livin mutants, and then incubated in the absence or presence of 0.9 mM 4-TBP as indicated. After 2 h, cell lysates were prepared and blotted for Livin. The cleaved Livin fragment is indicated by arrows. (f) HeLa cells were transfected overnight with Livin mutants Liv Δ 52 or Liv Δ 53-55, and then incubated in medium or treated with 960 J/m² UVB or 90 μ M cisplatin (CP) for 48 h as indicated. Cell lysates were prepared and blotted for Livin. The cleaved Livin fragment is indicated by the arrow. Some degradation of Liv Δ 52 was seen in CP-treated cells, but there was no definitive cleavage fragment of the appropriate size (as seen in apoptotic cells transfected with Liv Δ 53-55, arrow).