BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

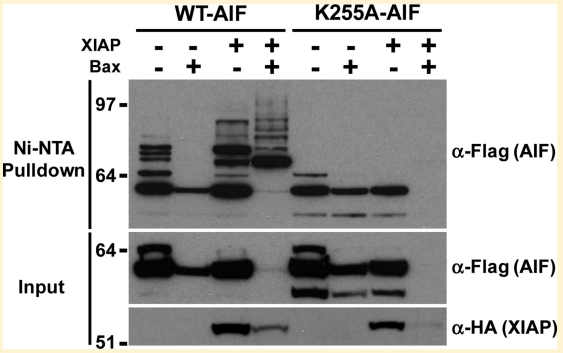
pubs.acs.org/biochemistry

Nondegradative Ubiquitination of Apoptosis Inducing Factor (AIF) by X-Linked Inhibitor of Apoptosis at a Residue Critical for AIF-Mediated Chromatin Degradation

Eric M. Lewis,[†] Amanda S. Wilkinson, Nicole Y. Davis, David A. Horita, and John C. Wilkinson*

Department of Biochemistry, Wake Forest University School of Medicine, Winston-Salem, North Carolina 27157, United States

ABSTRACT: Apoptosis inducing factor (AIF) is a mediator of caspase-independent cell death that is also necessary for mitochondrial energy production. How these seemingly opposite cellular functions of AIF are controlled is poorly understood. X-linked inhibitor of apoptosis (XIAP) is an endogenous inhibitor of caspases that also regulates several caspase-independent signaling pathways. The RING domain of XIAP possesses E3 ubiquitin ligase activity, though the importance of this function to signal regulation remains incompletely defined. XIAP binds and ubiquitinates AIF, and in this study, we determined the functional consequences of XIAP-mediated AIF ubiquitination. Unlike canonical ubiquitination, XIAP-dependent AIF ubiquitination did not lead to proteasomal degradation of AIF. Experiments using ubiquitin mutants demonstrated that the XIAP-dependent ubiquitin linkage was not formed through the commonly used lysine 48, suggesting a noncanonical ubiquitin linkage is employed. Further studies demonstrated that only lysine 255 of AIF was a target of XIAP-dependent ubiquitination. Using recombinant AIF, we determined that mutating lysine 255 of AIF interferes with the ability of AIF not only to bind DNA but also to degrade chromatin in vitro. These data indicate that XIAP regulates the death-inducing activity of AIF through nondegradative ubiquitination, further defining the role of XIAP in controlling AIF and caspase-independent cell death pathways.



Apoptosis inducing factor (AIF) is a mitochondrial flavoprotein that has been implicated as a critical factor in mitochondrial metabolism and energy production but that also participates in the orchestration of certain cell death pathways.[1] Encoded by a nuclear gene, the AIF protein is translocated to the mitochondria where the first 54 amino-terminal residues are cleaved within the matrix. Under healthy cellular conditions, AIF is tethered to the mitochondrial inner membrane, with the majority of the protein present within the inner membrane space.[2] The expression of AIF has been correlated with the expression of complex I in the mitochondrial respiratory chain,[3] and AIF has been shown to support both mitochondrial energy production and organellar structure.[4,5] These activities are performed, at least in part, through the intrinsic NADH oxidase activity of the protein.[5] A critical role for AIF in healthy cells is underscored by multiple in vivo studies characterizing the effects of genetic ablation of AIF. Aif-null mice die early in embryogenesis,[6,7] whereas targeted deletion of AIF in skeletal muscle and brain led to a variety of pathologies attributed to respiratory chain defects[8] and mitochondrial fragmentation.[9]

In contrast to a role in supporting normal mitochondrial activity, AIF has been implicated in the control of a variety of experimental models of cell death[10−14] and is generally considered to be the predominant mediator of caspase-independent cell death. Outer mitochondrial membrane permeabilization following death-inducing cues allows AIF to undergo a second round of cleavage into a death-inducing form (Δ102 or tAIF),[2] a process that is mediated by calpains or cathepsins in what may be a stimulus-dependent manner.[15−18] This proteolysis allows AIF to translocate to the nucleus where it binds DNA and induces chromatin condensation and internucleosomal DNA cleavage.[1] Because AIF does not possess intrinsic nuclease activity, this process involves the recruitment of partner endonucleases such as cyclophilin A or endonuclease G,[19−21] and a recent study has implicated histone H2AX as a critical factor for the assembly of an AIF-mediated DNA degradation complex.[22] While the ability of AIF to translocate and bind DNA during cell death is clear, the mechanisms that may regulate this process are poorly defined, and only a handful of AIF regulators have been reported. Heat shock protein 70 (Hsp70) has been shown to inhibit the nuclear translocation of AIF, thereby blocking AIF-mediated death induction.[23−25] We recently identified X-linked inhibitor of apoptosis (XIAP), a potent inhibitor of caspase-dependent apoptosis, as a binding partner of AIF. Further investigation of this interaction led to the discovery that XIAP-mediated AIF ubiquitination occurs, which could serve as a regulatory point in the control of the life and death functions of AIF.[26]

Received: September 22, 2011
Revised: November 18, 2011
Published: November 21, 2011

XIAP is a highly potent inhibitor of apoptosis, a well-described form of cell death mediated by the caspase family of cysteinyl proteases.[27,28] The best understood mechanism by which XIAP blocks apoptosis is through directly inhibiting the activities of both initiator (caspase-9) and executioner (caspases-3 and -7) caspases with nanomolar affinity.[29−34] However, other potential anti-apoptotic activities have been reported, including control of Smad-mediated transcriptional activation,[35−37] activation of N-terminal c-Jun kinase (JNK) and NF-κB,[38−41] and regulation of intracellular copper levels, through direct regulation of the copper binding protein COMMD1.[42,43] Of note, the potential for XIAP to control these disparate signaling cascades can be functionally uncoupled from its ability to directly inhibit caspases,[44] leading to the possibility of multiple mechanisms by which XIAP can control cell survival.

XIAP contains a RING domain at its extreme carboxyl terminus, which has been shown to possess E3 ubiquitin ligase activity.[45,46] Protein ubiquitination is an ATP-dependent process in which the ubiquitin polypeptide is attached to one or more lysyl residues within the substrate protein.[47] This results from a series of transfer reactions involving three sequential enzymes: an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin ligase that provides substrate specificity. Because ubiquitin itself contains several lysine residues that serve as acceptor sites for further ubiquitination events, multiple forms of both mono- and polyubiquitination have been identified.[48] Among these forms, polyubiquitination in which branching occurs through lysine 48 of ubiquitin (K48) is the best understood.[49] K48-linked polyubiquitination targets ubiquitinated proteins for degradation by the proteasome and thereby serves as a major control mechanism for protein stability and activity.

A diverse range of substrates for the ubiquitin ligase activity of XIAP have been identified, including XIAP itself, the IAP homologue survivin, caspase-9, the XIAP antagonist Smac/DIABLO, and COMMD1.[43,45,50−52] In many cases, substrate ubiquitination by XIAP leads to degradation, serving as an obvious method of signal control through targeted removal of signaling substrates. However, it is interesting to note that several XIAP ubiquitination substrates, such as the MAP kinase kinase MEKK2 and the copper chaperone CCS, appear to undergo nondegradative ubiquitination.[53,54] These observations suggest that the ubiquitin ligase activity of XIAP is multifaceted, with the ability to alter substrate activity through means distinct from proteasomal degradation.

This study aims to determine the nature and consequences of modification of AIF by XIAP. We show that unlike all other known XIAP substrates, which are ubiquitinated by linkages involving either K48 or K63 of the ubiquitin polypeptide, XIAP employs a noncanonical ubiquitin linkage to modify AIF. This linkage occurs at lysine 255 of the AIF polypeptide, a residue that has previously been implicated in the DNA binding activity of AIF. Indeed, mutation of this residue has no effect upon the NADH oxidase activity of AIF but prevented AIF from binding DNA and inducing chromatin degradation. Finally, we show that XIAP is capable of inhibiting AIF-mediated cell death in a manner independent of caspase inhibition. Overall, these data suggest that ubiquitination of AIF by XIAP is a unique regulatory step that blocks AIF death induction while maintaining normal NADH oxidase activity.

EXPERIMENTAL PROCEDURES

Materials. The following reagents were obtained. Protein G-coupled agarose, glutathione agarose, Ni-NTA agarose, glutamax, Dulbecco's modified Eagle's medium (DMEM), and phosphate-buffered saline (PBS) were from Invitrogen. Streptavidin agarose was from Thermo Scientific. Fetal bovine serum (FBS) was from Hyclone. The site-directed mutagenesis kit was from Stratagene. Protease inhibitor tablets were from Roche. Energy regeneration solution (ERS), ubiquitin activating enzyme (E1), UbcH5a (E2), and ubiquitin were from Boston Biochem. All other chemicals were from Sigma. The following antibodies were obtained: anti-XIAP (BD-Transduction Laboratories, 610717), anti-AIF and anti-p53 (BD-Pharmingen, 51-8103KC and 554294, respectively), anti-His and anti-Myc (Cell Signaling, 2366 and 2276, respectively), horseradish peroxidase (HRP)-conjugated anti-Flag and HRP-conjugated anti-HA (Sigma, A8592 and H6533, respectively), and HRP-conjugated anti-mouse and anti-rabbit (Amersham, NA931V and NA934V, respectively).

Cell Culture, Transfections, and Plasmids. HEK 293 and HeLa cells were grown in DMEM containing 10% FBS supplemented with 2 mM glutamax at 37 °C in an atmosphere of 95% air and 5% CO_2. Wild-type MEFs (WT), XIAP-null MEFs (KO), and XIAP-null MEFs reconstituted with control (FG9), wild-type XIAP (XIAP), D148A-XIAP (D148A), or D148A/W310A-XIAP (D148A/W310A) were as described previously.[55] Transfections of HEK 293 cells were performed by the method of calcium phosphate precipitation as described previously,[56] and transfections of HeLa cells were performed using Lipofectamine 2000 using the manufacturer's instructions. Plasmids containing AIF truncation variants (pEBB AIF-Δloop-FLAG, AIF-Δtail-FLAG, AIF-EC-FLAG, AIF-NBD-FLAG, and AIF-FBD-FLAG) were produced by polymerase chain reaction amplification of the individual subdomain cDNAs using pEBB AIF-FLAG as a template. Each fragment was then subcloned into the parental pEBB-FLAG vector. Plasmids encoding AIF point mutants (AIF-K510R/A, AIF-K518R/A, AIF-K255A, AIF-R265A, AIF-K510/518R/A, AIF-K255A/R265A, and AIF-Quad) were produced by site-directed mutagenesis (Stratagene) using the manufacturer's instructions. Sequences for all AIF variants were confirmed by DNA sequencing (Genwiz). Sequences for all primers employed are available upon request. All remaining plasmids used in this study (pEBB AIF-FLAG, AIF-TB, Ub-Δ54-AIF-FLAG, Ub-Δ102-AIF-FLAG, pCW7 His-Myc-Ubiquitin Wt, K6R, K11R, K27R, K29R, K48R, K63R, pEBB HA-XIAP, GST-XIAP, pEBB GST-H467A XIAP, and pCDNA3-Bax) have been reported previously.[26,43,57,58]

Cell Lysis and Immunoprecipitation. Cell lysates were prepared in either RIPA buffer (PBS containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 1 mM DTT), Triton buffer [25 mM Hepes (pH 7.9), 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 1 mM NaF, 1 mM $NaVO_4$, and 1 mM DTT], or urea lysis buffer [8 M urea, 300 mM NaCl, 0.5% NP-40, 50 mM Na_2HPO_4, 50 mM Tris (pH 8.0), 1 ng/mL aprotinin, and 10 ng/mL leupeptin] (all lysis buffers were supplemented with 1 mM PMSF and one protease inhibitor cocktail tablet per 10 mL prior to use). Lysates used as input controls were normalized for protein content and then separated by SDS−PAGE using 4 to 12% gradient SDS−PAGE gels (Invitrogen). Lysates destined for streptavidin or HA immunoprecipitation (Triton buffer) were incubated for 1 h at

4 °C with either streptavidin agarose or monoclonal antibody HA.11 (Covance, 14945101) bound to protein G agarose. Beads were recovered by centrifugation and washed in Triton buffer, and precipitated proteins were eluted via addition of LDS sample buffer and heating to 95 °C for 5 min. Urea lysates used for Ni-NTA precipitation were subjected to sonication postlysis (Branson Sonifier 250, output control of 2.5, 75% duty cycle, ~25 pulses), normalized for protein content, and then incubated with Ni-NTA agarose at 25 °C for 2 h. Beads were washed three times in urea wash buffer [8 M urea, 300 mM NaCl, 0.5% NP-40, 50 mM Na_2HPO_4, and 50 mM Tris (pH 8.0)] and then once in Triton buffer, and recovered proteins were eluted as described above. All input and precipitated samples were then subjected to SDS−PAGE analysis.

Cell-Free Ubiquitination Reactions. HEK 293 cells were transfected with plasmids encoding full-length AIF-FLAG or Δ54-AIF-FLAG in the presence of plasmids encoding either GST-tagged wild-type or H467A XIAP. Cell lysates were prepared in Triton buffer and incubated for 1 h at 4 °C with glutathione agarose beads. Beads were then washed four times in Triton buffer and resuspended in ubiquitination reaction buffer [40 mM Hepes (pH 7.9), 60 mM potassium acetate, 2 mM DTT, 5 mM $MgCl_2$, and 10% glycerol] containing 1× ERS, E1 activating enzyme (55 ng), E2 conjugating enzyme (100 ng), and ubiquitin (2.5 μg). Reactions were allowed to proceed for 1 h at 30 °C while the mixtures were being shaken (350 rpm) and then stopped by the addition of 3× LDS. Ubiquitinated material was then visualized by SDS−PAGE and immunoblot analysis.

Immunoblot Analysis. SDS−PAGE was followed by a transfer to nitrocellulose membranes (Invitrogen), which were then blocked with 5% milk in TBS containing 0.02−0.2% Tween, followed by incubation with the indicated antibodies for 1 h at room temperature. After being washed, membranes were incubated with HRP-conjugated anti-mouse IgG or anti-rabbit IgG secondary antibodies for 45 min at room temperature and visualized by enhanced chemiluminescence.

Recombinant Protein Production. pET21b(+) plasmids encoding His-tagged Δ102 AIF containing an N-terminal Precision Protease (GE Biosciences) cleavage site were transformed into BL21(DE3) Codon + RIPL (Stratagene) bacteria using heat shock and then grown to an OD_{600} of 1.2 in Terrific Broth at 37 °C in the presence of 100 μg/mL ampicillin and chloramphenicol. Cultures were induced by the addition of 60 μM IPTG and incubated overnight at 37 °C. Bacteria were harvested by centrifugation, resuspended in sonication buffer [PBS (pH 8.0), 1 ng/mL aprotinin, 10 ng/mL leupeptin, 1 mM PMSF, and a PI tablet], and then sonicated for 5 × 3 min (Branson Sonifier 250, output level 7, 30% duty cycle) on ice. Lysates were cleared by centrifugation (12000*g* for 30 min at 4 °C), and supernatants were incubated with Ni-NTA beads rotating for 2 h at 4 °C. Bound Ni-NTA beads were washed twice in PBS (pH 8.0) and once in PBS (pH 6.4), resuspended in elution buffer [PBS (pH 8.0) and 100 mM imidazole], and rotated for 1 h at 4 °C. Eluted protein was harvested using centrifugation (1500*g* for 5 min at 4 °C) and transferred to fresh tubes. Elutions were concentrated to ~800 μL by centrifugation (2500*g* for 5−6 × 20 min at 4 °C) using Vivaspin 15R concentrators (Sartotrius Stedium biotech) and then dialyzed against PBS (pH 8.0) to remove imidazole overnight in a D-Tube Dialyzer Midi tube (3.5 kDa molecular mass cutoff, Novagen). Protein destined for His tag cleavage was incubated with 50 μg/mL Precision Protease during overnight dialysis and then diluted ≥100-fold with 50 mM Tris-HCl (pH 8.0). Diluted protein was subjected to fast performance liquid chromatography, bound to an anion exchange SP-Sepharose column, and then eluted with a gradient increase of 50 mM Tris-HCl (pH 8.0) and 200 mM NaCl. Fractions correlating to the peak absorbance at 452 nm were collected and concentrated as described above. Final untagged protein was dialyzed against PBS (pH 8.0), and protein concentrations were determined for both His-tagged and untagged protein. Samples were diluted in 20% glycerol, aliquoted, and stored at −80 °C until they were needed.

NADH Reduction Assay. Untagged or His_6-tagged Δ102 wild-type or K255A AIF (20 μM) was incubated with 150 mM KH_2PO_4 in the presence of 40 μM NADH in a total volume of 500 μL. The change in absorbance at 320 nm correlating to the enzymatic reduction of NADH was measured every 5 s over 10 min at 25 °C and then plotted as ΔAbs_{320} per minute.

DNA Binding Assay. Untagged or His_6-tagged Δ102 wild-type or K255A AIF (10 mg/mL) was incubated with 50 mM Hepes (pH 7.4) in the presence of 100 ng of ~1.8 kb linear dsDNA for 15 min at 37 °C. Xylene cyanol was added to each sample, and DNA was separated by electrophoresis (120 V for 20−30 min) using a 1% agarose gel followed by visualization through staining with ethidium bromide.

Isolation of Nuclei. Nuclei were isolated from a confluent 10 cm plate of HeLa cells using a nucleus isolation kit (NUC101-1KT, Sigma) per the manufacturer's instructions with modifications. Briefly, cells were washed and harvested using trypsin, transferred to 15 mL conical tubes, and washed five times in cold PBS. Cells were then treated with 1 mL of lysis buffer and vortexed; an additional 7 mL of lysis buffer was added, and the solution was mixed by inversion and placed on ice for 5 min. Samples were centrifuged (500*g* for 5 min at 4 °C), supernatants aspirated, and pellets vortexed. The lysis buffer steps were repeated once more; then 400 μL of kit storage buffer was added, and samples were vortexed and then incubated on ice for 10 min, which allowed equilibration. Clumps of nuclei were broken up by trituration, counted, and stored at −80 °C until they were needed.

Preparation of Cytosolic Extracts. HEK 293 cells transiently transfected as described above were harvested by trypsinization, resuspended in fractionation buffer [250 mM sucrose, 20 mM Hepes (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and one PI tablet/10 mL], and homogenized using a dounce homogenizer (45 strokes, pestle B). The homogenate was cleared of unbroken cells and nuclei by centrifugation at 400*g* followed by centrifugation at 10000*g* to remove mitochondria. The resulting cytosolic extract was collected and employed in chromatin degradation experiments as described below.

Chromatin Degradation Assay. For experiments employing purified proteins, untagged or His_6-tagged Δ102 wild-type or K255A AIF (500 μg/mL) was incubated in 200 μL of CFS buffer [220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH_2PO_4, 0.5 mM EGTA, 2 mM $MgCl_2$, 0.1 mM PMSF, 1 mM DTT, and 10 mM Hepes (pH 7.4)] with 1.25 × 10^5 nuclei for 90 min at 37 °C. For experiments employing cytosolic extracts, 200 μL of each extract (200 μg of total protein) was incubated with nuclei as described above. After incubations, 200 μL of CFS buffer or fractionation buffer containing 4 μg/mL (2×) propidium iodide was added to each sample, and then the samples were analyzed by flow cytometry.

Nuclei containing degraded chromatin were quantified as sub-G_0 PI positive.

Cell Viability following MNNG Treatment. For titration experiments, MEF-derived cells were seeded in six-well plate format (300000 cells per well) and allowed to attach overnight. Cells were then treated with increasing concentrations of MNNG (from 0 to 500 μM) for 20 min at room temperature. Following treatment, cells were washed with PBS, fresh medium was added, and incubation was continued overnight at 37 °C. Cells were then harvested by trypsinization, washed with PBS, and resuspended in PBS containing 2 μg/mL propidium iodide. Cell viability was then determined using a Becton Dickinson FACSCalibur flow cytometer. Experiments employing MEFs restored with XIAP mutants D148A and D148A/W310A were performed at a single concentration of MNNG (500 μM) and the results analyzed as described above.

RESULTS

XIAP-Dependent AIF Ubiquitination Does Not Lead to Proteasomal Degradation. We previously reported that AIF is a target for ubiquitination by the E3 ubiquitin ligase activity of XIAP.[26] The specificity of this modification was subsequently confirmed using a cell-free system in which XIAP and AIF proteins were precipitated from cell lysates and then subjected to ubiquitination reactions employing purified ubiquitin, E1, and E2 enzymes. As shown in Figure 1A, AIF protein that precipitated in complex with wild-type XIAP underwent robust ubiquitination in vitro, suggesting that precipitated XIAP is sufficient to complete the ubiquitination reaction. Furthermore, AIF modification was specific to XIAP activity, because XIAP variant H467A lacking E3 ubiquitin ligase activity[45] failed to mediate AIF ubiquitination despite substantial coprecipitation. We therefore set out to determine the molecular consequences of XIAP-mediated AIF ubiquitination by assessing the extent of AIF degradation, the type of ubiquitin linkage formed, the residue(s) within AIF conjugated by XIAP, and the functional consequences of AIF modification.

To determine if XIAP-dependent AIF ubiquitination leads to proteasomal degradation, we employed a cell-based approach in which AIF fused to a biotin tag was expressed in cells in the absence and presence of both XIAP and ubiquitin. Protein was then captured using streptavidin-conjugated beads, and precipitated AIF, total AIF, and ubiquitin were visualized by immunoblot analysis. As shown in Figure 1B, XIAP-dependent AIF ubiquitination did not result in a decrease in AIF protein levels, suggesting the ubiquitinated AIF is not degraded. To confirm this observation, we next assessed the fate of AIF following chemical inhibition of the proteasome. Cells were transfected with plasmids encoding AIF and His-tagged ubiquitin in the absence and presence of XIAP and then left untreated or treated with the proteasomal inhibitor MG132 for 5 h. Cell lysates were prepared; ubiquitinated proteins were captured with Ni-NTA-conjugated beads, and the presence of AIF in precipitated material was evaluated by immunoblot analysis. As shown in Figure 1C (top panel), XIAP induced substantial ubiquitination of AIF, but the amount of ubiquitinated material was insensitive to MG132 treatment. As a control for the effectiveness of MG132 in this system, immunoblot analysis was also performed to examine the stabilization of p53. As expected, a substantial increase in the level of ubiquitinated p53 was observed following treatment with MG132 (Figure 1C, bottom panel). These data extend our previous findings and demonstrate that the consequence of

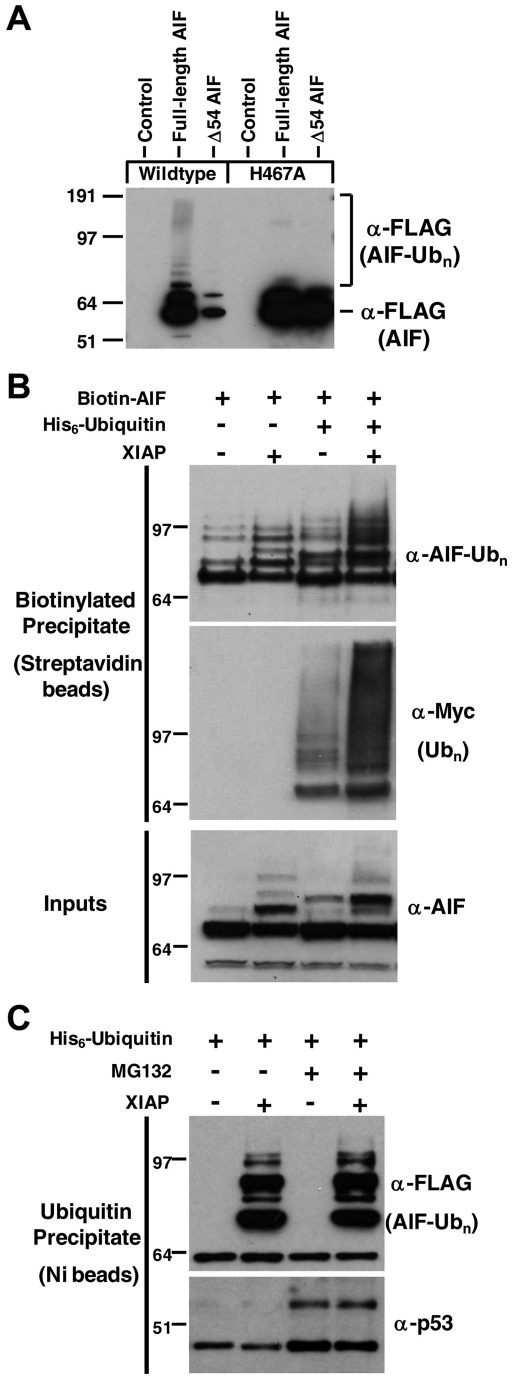


Figure 1. Ubiquitination of AIF by XIAP does not lead to degradation. (A) HEK 293 cells were transfected with plasmids encoding either wild-type or H467A XIAP as a GST fusion along with plasmids encoding either full-length or Δ54 AIF with carboxyl-terminal FLAG tags. Cell lysates were precipitated with glutathione agarose beads, and precipitated material was mixed with purified ubiquitin, E1, and E2 enzymes to initiate in vitro ubiquitination. Samples were then separated by SDS–PAGE and immunoblotted for FLAG (AIF). Laddering indicates ubiquitination of AIF protein. (B) HEK 293 cells were transfected with plasmids encoding biotinylated AIF in the absence and presence of XIAP and His_6-Myc-ubiquitin (His-Ub). Cell lysates were subjected to streptavidin precipitation and immunoblotted for AIF and the Myc epitope for ubiquitin. (C) HeLa cells were transfected with plasmids encoding AIF, His-Ub, and XIAP and then treated for 5 h with DMSO (solvent) or MG132. Cell lysates were incubated with Ni-NTA agarose, and precipitated material was immunoblotted for the FLAG epitope (for AIF) and p53.

dx.doi.org/10.1021/bi201483g | *Biochemistry* 2011, 50, 11084–11096

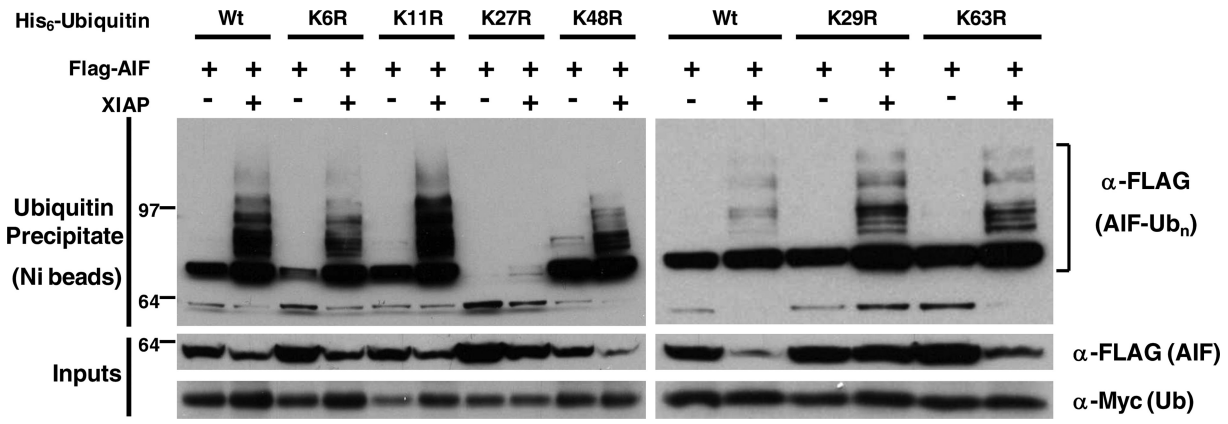


Figure 2. XIAP-dependent ubiquitination of AIF occurs through a noncanonical linkage. HEK 293 cells were transfected with plasmids encoding full-length AIF and the indicated His-Ub mutants in the absence and presence of XIAP. Cells lysates were incubated with Ni-NTA agarose, and precipitated material along with inputs was immunoblotted for FLAG (AIF) and Myc (ubiquitin).

XIAP-mediated AIF ubiquitination is not proteasomal degradation.

AIF Is Ubiquitinated through a Noncanonical Linkage. Degradation by the proteasome is the major cellular consequence of protein ubiquitination through a lysine 48 linkage. Because XIAP-dependent AIF ubiquitination did not result in AIF degradation, the type of XIAP-dependent ubiquitin linkage formed was explored by expressing AIF in the presence of ubiquitin variants in which individual lysine residues were mutated to arginine (K6R, K11R, K27R, K29R, K48R, and K63R, all containing an amino-terminal histidine tag). These mutants allow for the blockade of any polyubiquitin chain formation at the lysine mutation site. Cells were transfected; lysates were prepared, and ubiquitinated proteins were precipitated through binding to Ni-NTA beads. Bound protein was subjected to immunoblot analysis to visualize changes in AIF ubiquitination as a consequence of the ubiquitin variants employed. As shown in Figure 2, the ubiquitin mutants K6R, K11R, K29R, K48R, and K63R were all used as substrates by XIAP to modify AIF. The results employing K48R and K63R are particularly significant, because other targets of the XIAP E3 ligase activity have been suggested to undergo K48 and K63 polyubiquitin linkages. Interestingly, K27R ubiquitin failed to support modification of AIF by XIAP. However, this variant is expressed to lower levels than others tested and has been observed to suppress XIAP modification of substrates that employ K48 linkages (data not shown), suggesting this mutant has nonspecific inhibitory effects. Overall, these data suggest that in comparison to other XIAP targets, AIF is unique in that XIAP employs neither a K48 nor a K63 ubiquitin linkage for modification.

The Loop Region of AIF Is Necessary for Ubiquitination. Having determined that the fate of ubiquitinated AIF was not degradation and that XIAP employed a noncanonical ubiquitin linkage for modification, we next assessed the number and position of residues within AIF that are modified by XIAP. Human AIF contains 41 lysine residues localized throughout the protein. To simplify the search for XIAP-dependent ubiquitin targets, a series of AIF truncations containing specific functional domains (Figure 3A) were produced and tested for binding to and/or modification by XIAP. The design of these AIF variants was based on previous reports defining AIF structural organization.[59,60] Not only do these mutants allow for the more rapid determination of XIAP-dependent ubiquitination sites, but they also allow the elucidation of those regions within AIF that are necessary for association with XIAP. Cells were transfected with either full-length AIF or truncated AIF variants in the presence of HA-tagged XIAP. Cell lysates were then prepared and subjected to immunoprecipitation with HA antibodies. The presence of AIF variants in precipitated complexes was then evaluated by immunoblot analysis. As shown in Figure 3B, XIAP displayed substantial binding to full-length and Δloop AIF, but little to no binding was observed between XIAP and the AIF variants Δtail, enzymatic core (EC), FAD binding domain (FBD), or NADH binding domain (NBD). We hypothesize that for an AIF variant to be ubiquitinated by XIAP, it must first be capable of binding. Thus, the Δloop AIF variant was the focus of subsequent ubiquitination studies, as this variant represents the smallest deletion of AIF that still displayed substantial co-immunoprecipitation with XIAP. Furthermore, the deleted region contains the two potential ubiquitination sites, K510 and K518, which have both been implicated in the DNA binding activity of AIF. On the basis of these observations, the ability of Δloop to undergo XIAP-mediated ubiquitination was tested. Cells expressing full-length or Δloop AIF in the absence or presence of XIAP were subjected to His-ubiquitin, Ni-NTA precipitation, and immunoblot analysis. Figure 3C shows that while AIF-Δloop binds XIAP, it does not become polyubiquitinated, suggesting either that the residues within the deleted region were sites of ubiquitination or that removal of the loop portion of the protein inhibits ubiquitination without removing targeted residues. To explore these possibilities, lysine residues at positions 510 and 518 within the context of full-length AIF were mutated to arginine and tested for the ability to be ubiquitinated by XIAP. As shown in Figure 3D, mutation of K510, K518, or both failed to prevent XIAP-mediated ubiquitination, suggesting that while the AIF-Δloop variant is not modified by XIAP, failure to be modified is not due to the loss of target sites for ubiquitination.

AIF Ubiquitination Occurs at Lysine 255. The ability of AIF to bind DNA is thought to contribute to AIF-dependent cell death. It has also been reported that residues within AIF, specifically lysines 255, 510, and 518 and arginine 265, are necessary for DNA binding.[59] Having ruled out AIF K510 and K518 as ubiquitin targets of XIAP, we further investigated K255 as a possible XIAP-dependent ubiquitination site. Initial polyubiquitin AIF screening was done using double mutants K255A/R265A and K510A/K518A. Using the Ni-NTA precipitation approach, we observed that while the K510A/K518A

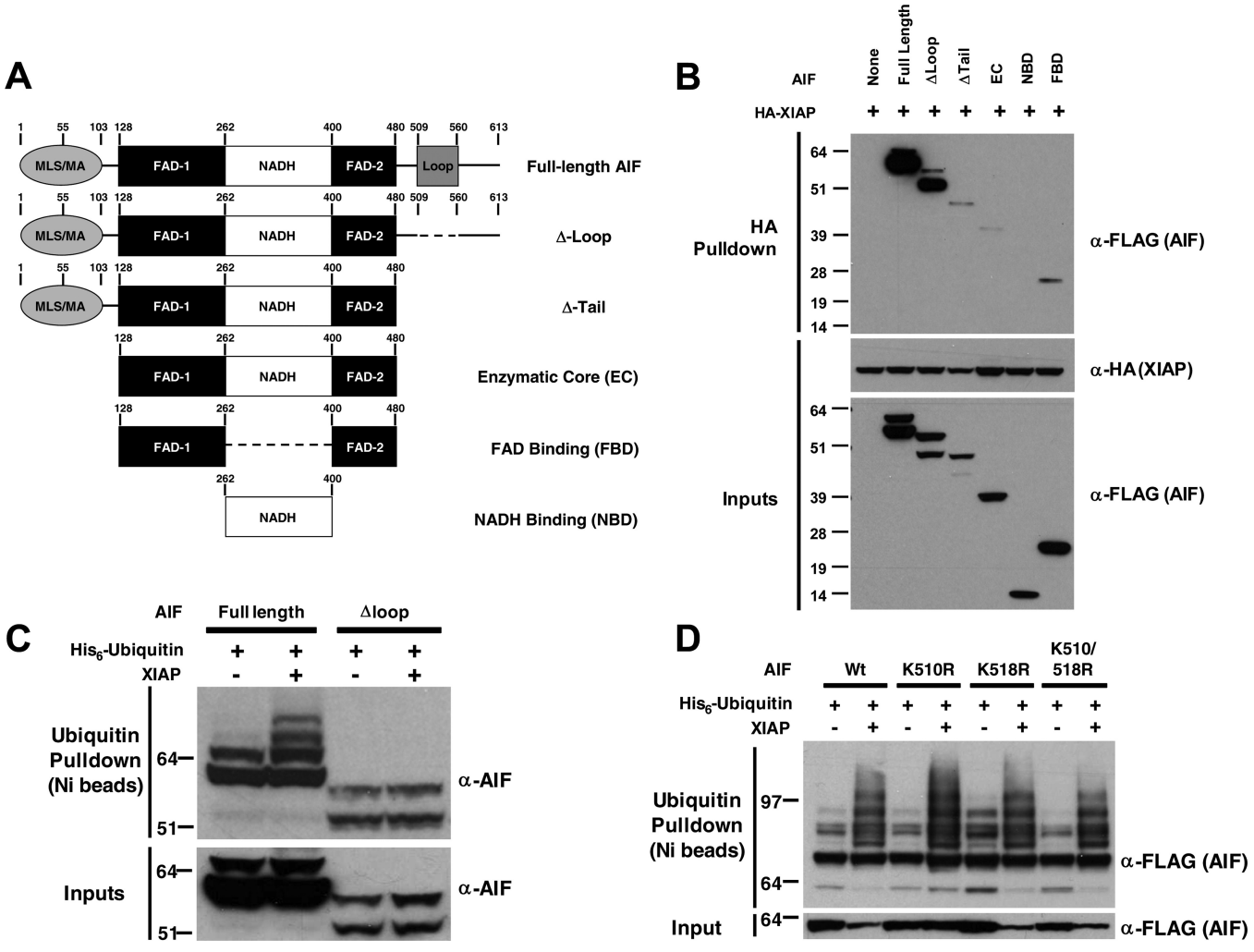


Figure 3. XIAP ubiquitination of AIF is dependent on the loop region but does not involve lysine 510 or 518. (A) Schematic representation of AIF variants employed in this study. MLS/MA, mitochondrial localization signal/mitochondrial anchor domain. (B) HEK 293 cells were transfected with HA-XIAP along with the indicated AIF variants. Cell lysates were precipitated using anti-HA and immunoblotted for FLAG (AIF) and HA (XIAP) epitopes. (C) HeLa cells were transfected with plasmids encoding either wild-type AIF or the AIF-Δloop variant in the absence and presence of XIAP. Cell lysates were then incubated with Ni-NTA beads, and precipitated proteins along with inputs were immunoblotted for HA (AIF). (D) HeLa cells transiently expressing His-Ub and the indicated AIF mutants in the absence or presence of XIAP were subjected to Ni-NTA precipitation with precipitated proteins and inputs immunoblotted for FLAG (AIF).

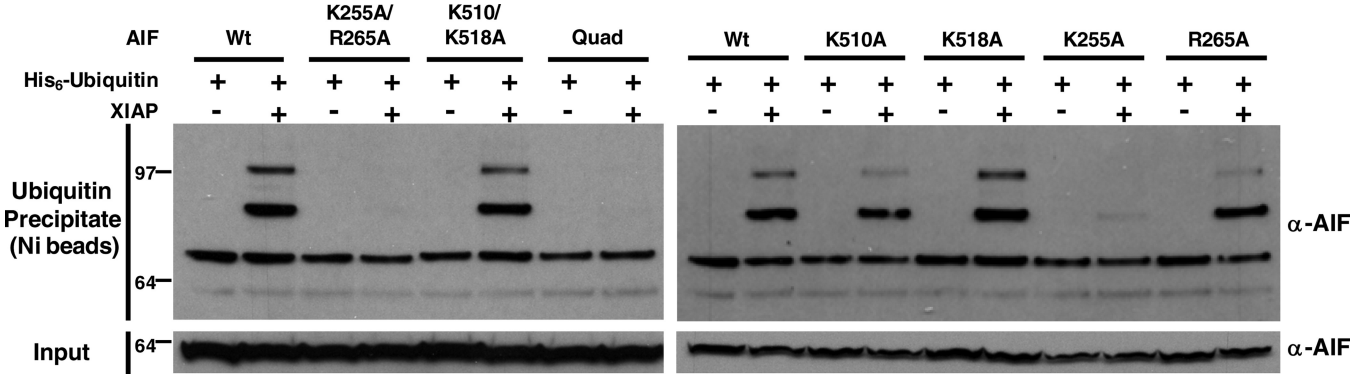


Figure 4. XIAP ubiquitinates AIF at lysine 255, a known DNA binding site. Lysates from HeLa cells transiently expressing His-Ub and the indicated AIF mutants in the absence or presence of XIAP were subjected to Ni-NTA precipitation, and precipitated protein along with inputs were immunoblotted for AIF.

double mutant was ubiquitinated to an extent similar to that of the wild type, the K255A/R265A double mutant and the quadruple K255/R265/K510/K518 mutant both failed to display polyubiquitination in the presence of XIAP (Figure 4), suggesting that K255 is the site of modification. To confirm this observation, we generated the K255A single mutant and tested for

dx.doi.org/10.1021/bi201483g | *Biochemistry* 2011, 50, 11084–11096

modification by XIAP. As expected, this variant displayed complete resistance to XIAP-mediated ubiquitination (Figure 4). Because the blockage of ubiquitination appears to occur only when K255 is mutated, this suggests that XIAP-dependent ubiquitination of AIF is restricted to this single lysine residue. These results taken together with the previous noncanonical polyubiquitin branching data implicate XIAP as a regulator of AIF ubiquitination at a site necessary for the ability of AIF to bind DNA.

Lysine 255 of AIF Is Critical for DNA Binding. Typical signatures of AIF-dependent death are AIF nuclear translocation, DNA binding, and nuclear chromatin degradation. To investigate the role of K255 in these AIF functions, we generated recombinant versions of the death-inducing (Δ102 AIF) forms of either wild-type or K255A AIF. As part of the purification strategy, both AIF variants were produced in the absence and presence of a carboxyl-terminal His_6 tag. Figure 5A shows a Coomassie blue-stained gel indicating the expressed protein at the expected molecular weight (top panel) and immunoblot analysis recognizing the protein as AIF as well as the absence or presence of the His tag (bottom panels). To confirm that the proteins were functional, we tested the ability of these proteins to reduce NADH. Figure 5B shows that all AIF preparations were fully capable of reducing NADH, demonstrating that there was no difference in enzymatic activity between the wild type and the K255A AIF mutant. Functional consequences of K255 mutation were next assessed in a DNA binding assay. Figure 5C shows that while there was little difference in the DNA binding capabilities of wild-type AIF, wild-type His-AIF, and K255A-His AIF, removal of the His tag from the K255A AIF variant resulted in a substantial reduction in the ability of this variant to bind DNA. These results indicate that K255 is absolutely essential for efficient binding of AIF to DNA and notably that a positively charged His tag can artificially compensate for the loss of this residue.

Lysine 255 Is Essential for AIF-Dependent Chromatin Degradation. Previous reports exploring the impact of the K255A mutation upon AIF function addressed the ability of this variant to undergo nuclear translocation and bind DNA in vitro;[59] however, an assessment of chromatin degradation has not been reported. To further explore the impact of K255 substitution in the context of the ability of AIF to induce chromatin degradation, we isolated and then incubated HeLa nuclei with the panel of recombinant AIF proteins. Nuclei were then stained with propidium iodide and analyzed using flow cytometry. As shown in panels A and B of Figure 6, both His-tagged wild-type and K255A AIF were able to degrade chromatin. However, when untagged protein was used, a dramatic ablation of chromatin degradation by the mutant was observed. These data not only correlate well with the DNA binding data but also highlight the critical importance of evaluating AIF variants following removal of the carboxyl-terminal histidine tag.[4] To correlate these findings with the ability of AIF to undergo ubiquitination following an apoptotic stimulus, we assessed the impact of the K255A mutation upon AIF ubiquitination following transfection of cells with the pro-apoptotic Bcl-2 family member Bax. Consistent with previous findings,[26] wild-type AIF exhibited a unique ubiquitination signature following expression of XIAP in cells undergoing apoptotic cell death (Figure 6C, lane 4). Interestingly, despite similar levels of protein expression, the K255A variant failed to display this unique ubiquitin signature (Figure 6C, lane 8), suggesting this AIF variant is insensitive to modification by

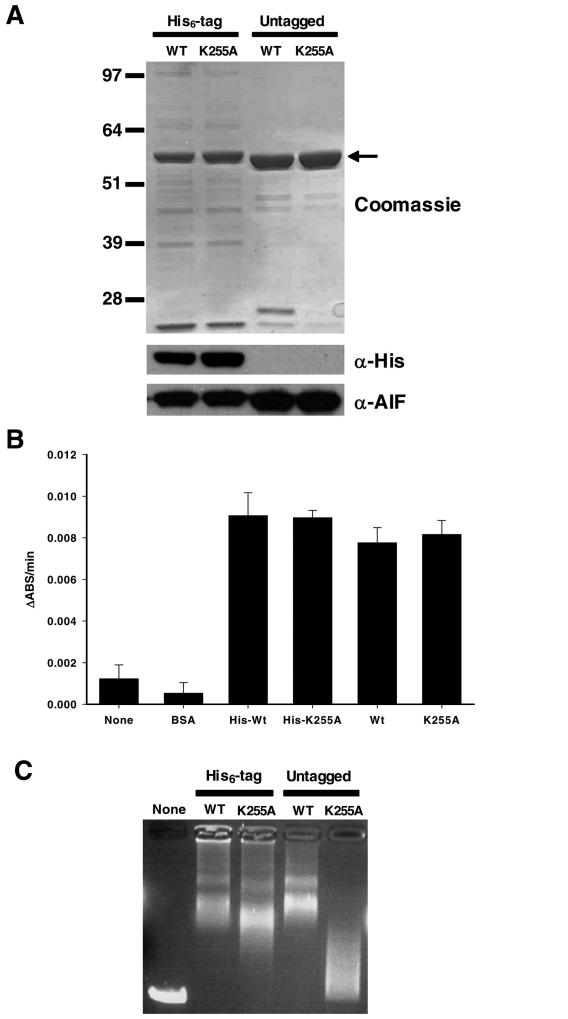


Figure 5. Lysine 255 of AIF is needed for efficient DNA binding. (A) Recombinant His-tagged wild-type and K255A AIF were expressed in BL21 DE5 cells and isolated by Ni-NTA precipitation for the His-tagged protein and continued isolation using SP-Sepharose for the untagged protein. Purified proteins were subjected to SDS–PAGE and stained with Coomassie blue (top panel) or immunoblotted for AIF and His (bottom panels). The arrowhead indicates the position of AIF within the Coomassie-stained gel. (B) Recombinant AIF was incubated with NADH, and reduction of NADH was followed by the change in absorbance at 320 nm per minute (mean ± standard deviation; $n = 3$). (C) Recombinant AIF was incubated with 100 ng of DNA and subjected to electrophoresis using a 1% agarose gel. DNA migration was visualized using ethidium bromide staining.

XIAP following activation of the apoptotic pathway. To directly assess the ability of XIAP to inhibit AIF-mediated DNA degradation, we mixed HeLa nuclei with cytosolic extracts from HEK 293 cells transfected with control, Δ102-AIF, XIAP, or both Δ102-AIF and XIAP expression plasmids, and DNA degradation was assessed as in Figure 6A. As shown in Figure 6D, whereas extracts from control and XIAP-transfected cells failed to induce significant DNA degradation (18.6 and 18.5% degraded nuclei, respectively), extracts from cells transfected with AIF displayed substantial DNA degrading activity (45.6% degraded nuclei). Notably, cotransfection of XIAP along with AIF resulted in the complete inhibition of DNA degradation (21.2% degraded nuclei), suggesting that XIAP directly abrogates the ability of AIF to induce chromatin degradation. Overall, these data, when taken together with the observation

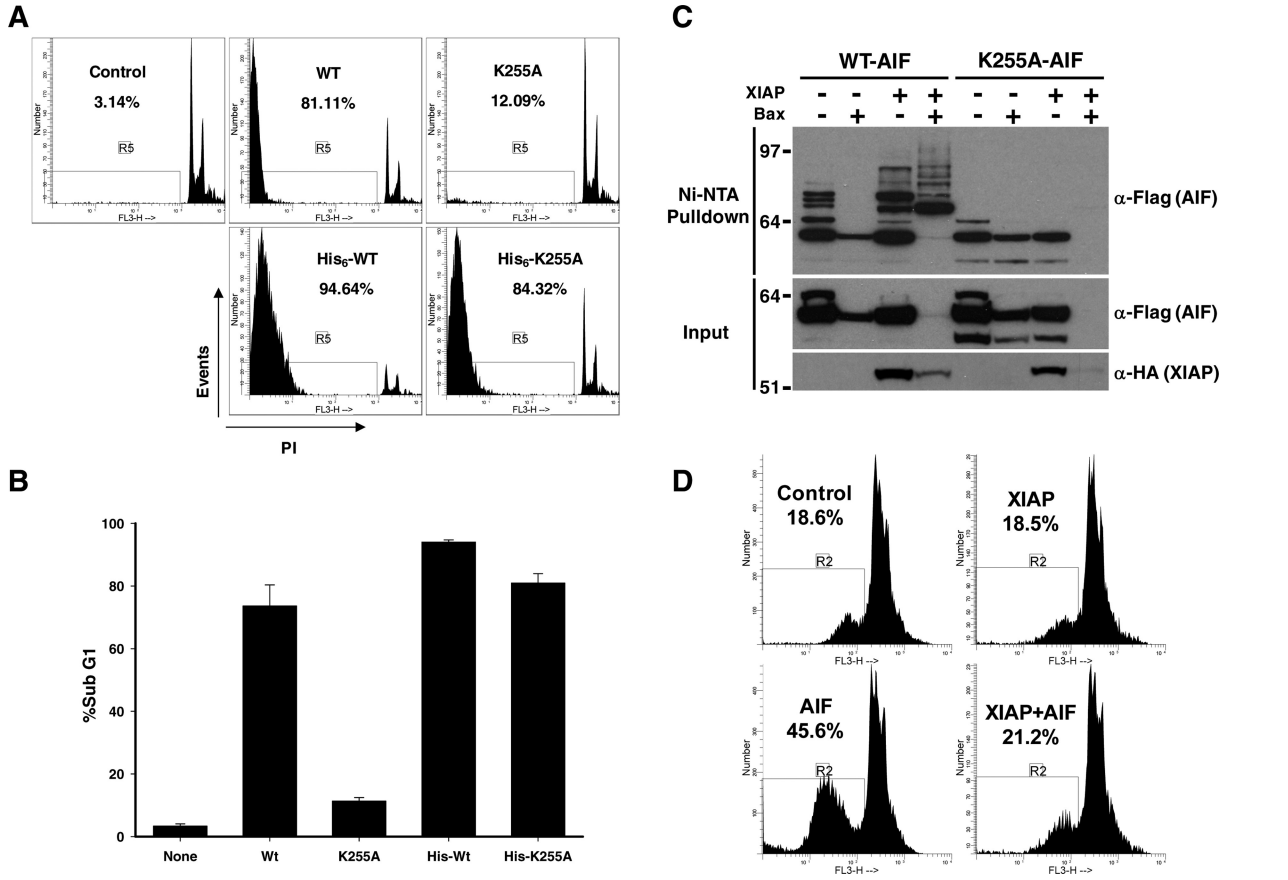


Figure 6. Lysine 255 of AIF is needed for efficient AIF-dependent chromatin degradation and modification in intact cells. (A) HeLa nuclei were incubated with recombinant AIF and then stained with propidium iodide. Chromatin degradation was assessed by flow cytometry and quantified by sub-G1 population gating. (B) Quantification of sub-G1 nuclei (mean ± standard deviation; $n = 3$). (C) HEK 293 cells were transfected with His-tagged ubiquitin and either wild-type AIF (lanes 1−4) or K255A AIF (lanes 5−8) in the absence (lanes 1, 2, 5, and 6) or presence (lanes 3, 4, 7, and 8) of XIAP and in the absence (lanes 1, 3, 5, and 7) or presence (lanes 2, 4, 6, and 8) of Bax. Ubiquitinated material was then precipitated using Ni-NTA beads, and the presence of Flag-tagged proteins (AIF) in precipitated complexes was detected by immunoblot analysis. (D) HEK 293 cells were transiently transfected with control, Δ102-AIF, XIAP, or Δ102-AIF and XIAP expression plasmids; 24 h post-transfection, cytosolic extracts were prepared and incubated with HeLa nuclei. DNA degradation was assessed by flow cytometry as described for panel A.

that XIAP-dependent ubiquitination of AIF occurs in a nondegradative manner at position K255, suggest that XIAP directly affects AIF function by ubiquitinating AIF in a way that blocks the ability of AIF to bind DNA and induce chromatin degradation, effectively inhibiting AIF-dependent cell death.

XIAP Protein Levels Influence AIF-Mediated Cell Death in a Physiological Setting in a Manner Independent of Caspase Inhibition. Treatment of murine embryonic fibroblasts with the DNA alkylating agent MNNG is a well-established model system that depends upon AIF for death induction and has been used to define several features of AIF-mediated cell death.[11,22,61] Therefore, this system was employed to assess the role and significance of XIAP in controlling AIF-mediated death. MEFs derived from wild-type and XIAP knockout mice have been reported, as well as XIAP$^{-/-}$ MEFs in which XIAP expression has been restored by lentiviral infection.[55] Interestingly, these "restored" MEFs exhibit XIAP protein expression levels that are significantly higher (2−3-fold) than those found in wild-type-derived MEFs (Figure 7A, inset). The sensitivity of this panel of cell lines to death was tested over a range of MNNG concentrations from 0 to 500 μM, with viability determined by propidium iodide staining and flow cytometry 24 h after treatment. As shown in Figure 7A, cells lacking XIAP (KO and the control restored line FG9) exhibited greater cell death than wild-type MEFs at all concentrations tested. Moreover, the restored XIAP cell line displayed substantial resistance to MNNG-induced death when compared to wild-type cells. Overall, these data suggest that XIAP plays a direct role in controlling AIF-mediated cell death, with viability correlating directly with XIAP expression levels. A caveat to the experiment shown in Figure 7A is that XIAP may impact cell survival through the inhibition of caspase activity rather than directly affecting AIF-mediated death induction. To determine the role of caspase inhibition for XIAP-mediated protection in this system, additional cell lines in which XIAP expression was restored with XIAP variants D148A (which cannot inhibit caspase-3) and D148A/W310A (which cannot inhibit caspase-3 and -9) were tested for resistance to MNNG. As shown in Figure 7B, cell lines restored with either XIAP variant were as resistant as wild-type MEFs to MNNG treatment, though neither line was as resistant as the restored cells expressing wild-type XIAP. Interestingly, when XIAP protein levels in D148A and D148A/W310A cells were assessed, these cells displayed levels more similar to those of wild-type cells than that of the restored line that expresses wild-type XIAP at higher levels (Figure 7C). Taken together, these data demonstrate that XIAP blocks AIF-mediated cell death in a manner that is independent

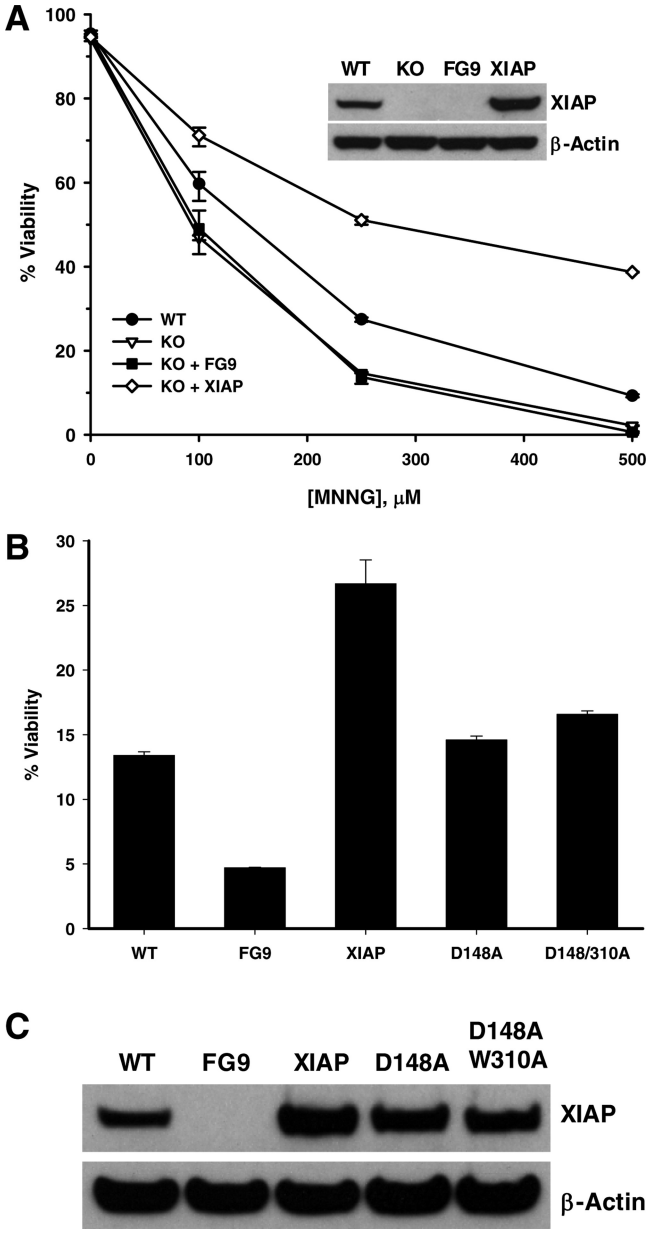


Figure 7. XIAP prevents AIF-mediated cell death. (A) Wild-type (WT), XIAP-null (KO), or XIAP-null MEFs restored with either control (FG9) or XIAP lentivirus (XIAP) were treated with increasing concentrations of MNNG (0−500 μM), and viability was assessed by propidium iodide staining and flow cytometry (mean ± standard deviation; n = 3). The inset shows immunoblot analysis of XIAP expression in cells employed in panel A; equivalent loading was confirmed by immunoblotting for β-actin. (B) WT, FG9, XIAP, D148A, and D148A/W310A MEFs were treated with MNNG (500 μM), and viability was assessed as described for panel A. (C) Immunoblot analysis of cells used in panel B demonstrating XIAP levels. Equal loading was confirmed by immunoblotting for β-actin.

of caspase inhibition and that directly correlates with XIAP protein levels.

DISCUSSION

It has been more than a decade since the identification of AIF as a mediator of cell death, yet the precise molecular role of AIF in various cell death pathways has remained unclear and, at times, even controversial.[62−64] While the death-inducing activity of AIF was originally identified in the context of apoptotic conditions,[1] substantial evidence suggests that in classic, caspase-dependent cell death AIF plays a minor role in completing the death program.[62,65] In contrast, under conditions in which caspase activation is attenuated, AIF appears to be a substantial regulator of death induction[12−14,66] and is generally considered to be a primary mediator of caspase-independent cell death.[67,68] While a unifying model for describing the molecular details of AIF-mediated death remains elusive, several potential points of regulation have emerged. First, AIF must escape from mitochondria. Multiple factors may control this process, including regulators of membrane integrity (such as members of the Bcl-2 family)[17,61,65] and proteases that affect membrane anchorage of AIF (cathepsins and/or calpains).[15,17] Second, AIF must translocate from the cytosol to the nucleus. Several factors have been identified that either promote (cyclophilin A)[19,21] or abrogate (Hsp70)[23,24] this process. Finally, once it reaches the nucleus, AIF must bind DNA and recruit factors that conduct disassembly of chromatin, such as H2AX, CypA, and/or EndoG.[19,20,22] Interestingly, all of these steps may be controlled, at least in part, by changes in cellular redox status, and it has been demonstrated that redox-dependent conformational changes in AIF structure affect the sensitivity to proteolysis, nuclear translocation, and DNA binding.[4,69]

In this study, we present evidence delineating an additional mechanism by which the death-inducing activity of AIF may be controlled, nondegradative ubiquitination induced by XIAP. XIAP employs a noncanonical ubiquitin linkage to modify AIF, and this modification occurs at a single position within the AIF polypeptide. Moreover, this position, K255, is absolutely essential for the ability of AIF to bind DNA and induce chromatin degradation. These data have several implications for the broadening ability of XIAP to control multiple cell death pathways by distinct mechanisms, the diverse consequences of protein ubiquitination, and the mechanistic control of AIF-mediated death induction.

It is well established that XIAP can block caspase-dependent apoptosis through the steric inhibition of the caspase active site upon binding.[32,70] However, a role for XIAP-dependent ubiquitination as part of the mechanism of caspase inhibition has been reported,[71] and it is possible that such an activity may contribute to the regulation of multiple XIAP binding proteins. Indeed, several disparate factors, including Smac/DIABLO,[52] PTEN,[72] COMMD1,[43] CCS,[54] MEKK2,[53] and AIF,[26] have been shown to be substrates for the E3 ligase activity of XIAP. Interestingly, among these substrates, both CCS and MEKK2 have emerged as targets that are not induced to undergo degradation following modification by XIAP. On the basis of studies presented here, AIF may now be included in this group, with the added distinction of being the only known XIAP target that is not modified by either a K48 or a K63 ubiquitin linkage. Further, the ability of XIAP to modify AIF suggests that through its E3 ubiquitin ligase activity XIAP regulates cell death paradigms extending beyond classic caspase-dependent apoptosis. XIAP may now be considered a regulator of both caspase-dependent and caspase-independent cell death.

Ubiquitination has been shown to take on many forms, each with distinct consequences for the targeted protein. Polyubiquitin chain branching through lysine 48 of the ubiquitin molecule acts as a well-established mediator of proteasomal degradation.[49] Other linkages through lysines 6, 11, 27, 29, and 63 of ubiquitin can lead to multiple different cellular

consequences.[73] Studies presented here definitively show that AIF is not degraded following ubiquitination. What, then, is the fate of the modified molecule? Several possibilities can be envisioned. First, modification may affect the ability of AIF to associate with protein cofactors. Indeed, our previous studies suggested that the ability of XIAP itself to bind AIF was attenuated following ubiquitination, in that the most heavily modified AIF variant (Δ102-AIF) demonstrated a reduced capacity to coprecipitate with XIAP. Other AIF binding proteins, such as CypA, Hsp70, and H2AX, may be similarly affected, and the ability of AIF to regulate these targets, or in turn be regulated itself, may be altered. Second, AIF ubiquitination may affect the ability of the protein to reach the nucleus following release from mitochondria. This could occur as a consequence of altered binding to cofactors necessary for translocation (such as CypA, as described above) or an alternative activity of ubiquitination that prevents AIF from reaching the nuclear space. Finally, and perhaps most likely, by targeting a residue critical not only for DNA binding[59] but also for induction of chromatin degradation (Figure 6), XIAP abrogates the ability of AIF to bind DNA and induce chromatinolysis. Modification at K255 alters a positively charged side chain to include the comparatively bulky and hydrophobic ubiquitin moiety, which can block DNA binding both by steric hindrance and through the substantial increase in molecular hydrophobicity.

In principle, this final possibility could be addressed by the inclusion of recombinant XIAP protein (along with purified E1 and E2 ubiquitination enzymes) with AIF in our in vitro chromatin degradation assays. Interestingly, while we have been able to recapitulate AIF ubiquitination in vitro using a purified XIAP−AIF complex that precipitated from mammalian cells (Figure 1A), this reaction is not mediated by bacterially made recombinant XIAP, even though this protein retains the ability to inhibit caspases in vitro (E. M. Lewis and J. C. Wilkinson, unpublished observations and ref 44). Moreover, the same is true when attempting to mediate the in vitro ubiquitination of other XIAP substrates such as Smac/DIABLO and COMMD1 (J. C. Wilkinson and E. Burstein, unpublished observations). The molecular explanation for this lack of activity is unclear but may indicate that additional factors are required for XIAP ubiquitin ligase activity, and the XIAP-mediated ubiquitination in the context of an intact cell involves regulatory steps that cannot be recapitulated in vitro. A consequence of these observations is the inability to test, using purified proteins, the direct impact of XIAP-mediated ubiquitination on the ability of AIF to condense chromatin within purified nuclei. Nevertheless, in light of previous studies demonstrating the importance of K255 as a critical residue for AIF−DNA binding,[59,74] and data presented here showing not only that mutation of K255 prevents chromatin degradation but also that this activity is restored by the positively charged histidine tag, inhibition of death-inducing activity is the likely consequence of XIAP-mediated ubiquitination of AIF. Indeed, our experiments demonstrating the ability of XIAP to prevent AIF-containing cytosolic extracts from degrading nuclei as well as our data demonstrating the ability of XIAP to prevent AIF-mediated death in a caspase-independent fashion strongly support this conclusion.

In summary, data presented in this study highlight new molecular properties by which XIAP-mediated ubiquitination can attenuate the death-inducing activity of AIF in a nondegradative manner. We have characterized the nature of the ubiquitin linkage and identified the position within AIF that is modified by XIAP. To the best of our knowledge, this is the first report of an enzymatic mechanism whereby the death-inducing activity of AIF may be impaired. We have further demonstrated the ability of XIAP to block AIF-mediated cell death and shown that this effect is independent of the caspase inhibitory activity of XIAP. As XIAP is currently a clinical target in the development of new cancer therapies, it will be important to add this mechanism to the spectrum of activities by which XIAP promotes cellular survival.

AUTHOR INFORMATION

Corresponding Author

*Department of Biochemistry, Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157. Telephone: (336) 716-5722. Fax: (336) 716-7671. E-mail: jcwilkin@wfubmc.edu.

Present Address

†Department of Chemistry and Physics, High Point University, High Point, NC 27262.

Funding

This work was supported by Grant W81XWH-08-1-0045 (to J.C.W.) from the Department of Defense Prostate Cancer Research Program, Grant RSG-09-166-01-CCG (to J.C.W.) from the American Cancer Society, and Grant R01 A1064609 from the National Institutes of Health (to D.A.H.).

ACKNOWLEDGMENTS

We thank Drs. D. Lyles, E. Burstein, and G. Maine for critical discussions and reading of the manuscript. We also thank E. Rolfe and A. Hoyt for technical assistance.

ABBREVIATIONS

AIF, apoptosis inducing factor; CCS, copper chaperone for SOD1; COMMD1, copper metabolism (Murr1) domain containing 1; CypA, cyclophilin A; EndoG, endonuclease G; H2AX, histone-2 AX; Hsp70, heat shock protein 70; JNK, c-jun N-terminal kinase; MEF, mouse embryonic fibroblast; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; PTEN, phosphatase and tensin homologue; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Smac/DIABLO, second mitochondrial activator of caspases/direct inhibitor of apoptosis-binding protein with low pI.

REFERENCES

(1) Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Snow, B. E., Brothers, G. M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D. R., Aebersold, R., Siderovski, D. P., Penninger, J. M., and Kroemer, G. (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature 397*, 441−446.

(2) Otera, H., Ohsakaya, S., Nagaura, Z., Ishihara, N., and Mihara, K. (2005) Export of mitochondrial AIF in response to proapoptotic stimuli depends on processing at the intermembrane space. *EMBO J. 24*, 1375−1386.

(3) Vahsen, N., Cande, C., Briere, J. J., Benit, P., Joza, N., Larochette, N., Mastroberardino, P. G., Pequignot, M. O., Casares, N., Lazar, V., Feraud, O., Debili, N., Wissing, S., Engelhardt, S., Madeo, F., Piacentini, M., Penninger, J. M., Schagger, H., Rustin, P., and Kroemer, G. (2004) AIF deficiency compromises oxidative phosphorylation. *EMBO J. 23*, 4679−4689.

(4) Churbanova, I. Y., and Sevrioukova, I. F. (2008) Redox-dependent changes in molecular properties of mitochondrial apoptosis-inducing factor. *J. Biol. Chem. 283*, 5622−5631.
(5) Miramar, M. D., Costantini, P., Ravagnan, L., Saraiva, L. M., Haouzi, D., Brothers, G., Penninger, J. M., Peleato, M. L., Kroemer, G., and Susin, S. A. (2001) NADH oxidase activity of mitochondrial apoptosis-inducing factor. *J. Biol. Chem. 276*, 16391−16398.
(6) Joza, N., Susin, S. A., Daugas, E., Stanford, W. L., Cho, S. K., Li, C. Y., Sasaki, T., Elia, A. J., Cheng, H. Y., Ravagnan, L., Ferri, K. F., Zamzami, N., Wakeham, A., Hakem, R., Yoshida, H., Kong, Y. Y., Mak, T. W., Zuniga-Pflucker, J. C., Kroemer, G., and Penninger, J. M. (2001) Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. *Nature 410*, 549−554.
(7) Brown, D., Yu, B. D., Joza, N., Benit, P., Meneses, J., Firpo, M., Rustin, P., Penninger, J. M., and Martin, G. R. (2006) Loss of Aif function causes cell death in the mouse embryo, but the temporal progression of patterning is normal. *Proc. Natl. Acad. Sci. U.S.A. 103*, 9918−9923.
(8) Joza, N., Oudit, G. Y., Brown, D., Benit, P., Kassiri, Z., Vahsen, N., Benoit, L., Patel, M. M., Nowikovsky, K., Vassault, A., Backx, P. H., Wada, T., Kroemer, G., Rustin, P., and Penninger, J. M. (2005) Muscle-specific loss of apoptosis-inducing factor leads to mitochondrial dysfunction, skeletal muscle atrophy, and dilated cardiomyopathy. *Mol. Cell. Biol. 25*, 10261−10272.
(9) Cheung, E. C., Joza, N., Steenaart, N. A., McClellan, K. A., Neuspiel, M., McNamara, S., MacLaurin, J. G., Rippstein, P., Park, D. S., Shore, G. C., McBride, H. M., Penninger, J. M., and Slack, R. S. (2006) Dissociating the dual roles of apoptosis-inducing factor in maintaining mitochondrial structure and apoptosis. *EMBO J. 25*, 4061−4073.
(10) Yuste, V. J., Sanchez-Lopez, I., Sole, C., Moubarak, R. S., Bayascas, J. R., Dolcet, X., Encinas, M., Susin, S. A., and Comella, J. X. (2005) The contribution of apoptosis-inducing factor, caspase-activated DNase, and inhibitor of caspase-activated DNase to the nuclear phenotype and DNA degradation during apoptosis. *J. Biol. Chem. 280*, 35670−35683.
(11) Yu, S. W., Wang, H., Poitras, M. F., Coombs, C., Bowers, W. J., Federoff, H. J., Poirier, G. G., Dawson, T. M., and Dawson, V. L. (2002) Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. *Science 297*, 259−263.
(12) Wang, H., Yu, S. W., Koh, D. W., Lew, J., Coombs, C., Bowers, W., Federoff, H. J., Poirier, G. G., Dawson, T. M., and Dawson, V. L. (2004) Apoptosis-inducing factor substitutes for caspase executioners in NMDA-triggered excitotoxic neuronal death. *J. Neurosci. 24*, 10963−10973.
(13) Liu, T., Brouha, B., and Grossman, D. (2004) Rapid induction of mitochondrial events and caspase-independent apoptosis in Survivin-targeted melanoma cells. *Oncogene 23*, 39−48.
(14) Cregan, S. P., Fortin, A., MacLaurin, J. G., Callaghan, S. M., Cecconi, F., Yu, S. W., Dawson, T. M., Dawson, V. L., Park, D. S., Kroemer, G., and Slack, R. S. (2002) Apoptosis-inducing factor is involved in the regulation of caspase-independent neuronal cell death. *J. Cell Biol. 158*, 507−517.
(15) Polster, B. M., Basanez, G., Etxebarria, A., Hardwick, J. M., and Nicholls, D. G. (2005) Calpain I induces cleavage and release of apoptosis-inducing factor from isolated mitochondria. *J. Biol. Chem. 280*, 6447−6454.
(16) Yuste, V. J., Moubarak, R. S., Delettre, C., Bras, M., Sancho, P., Robert, N., d'Alayer, J., and Susin, S. A. (2005) Cysteine protease inhibition prevents mitochondrial apoptosis-inducing factor (AIF) release. *Cell Death Differ. 12*, 1445−1448.
(17) Bidere, N., Lorenzo, H. K., Carmona, S., Laforge, M., Harper, F., Dumont, C., and Senik, A. (2003) Cathepsin D triggers Bax activation, resulting in selective apoptosis-inducing factor (AIF) relocation in T lymphocytes entering the early commitment phase to apoptosis. *J. Biol. Chem. 278*, 31401−31411.
(18) Cao, G., Xing, J., Xiao, X., Liou, A. K., Gao, Y., Yin, X. M., Clark, R. S., Graham, S. H., and Chen, J. (2007) Critical role of calpain I in mitochondrial release of apoptosis-inducing factor in ischemic neuronal injury. *J. Neurosci. 27*, 9278−9293.
(19) Cande, C., Vahsen, N., Kouranti, I., Schmitt, E., Daugas, E., Spahr, C., Luban, J., Kroemer, R. T., Giordanetto, F., Garrido, C., Penninger, J. M., and Kroemer, G. (2004) AIF and cyclophilin A cooperate in apoptosis-associated chromatinolysis. *Oncogene 23*, 1514−1521.
(20) Wang, X., Yang, C., Chai, J., Shi, Y., and Xue, D. (2002) Mechanisms of AIF-mediated apoptotic DNA degradation in *Caenorhabditis elegans*. *Science 298*, 1587−1592.
(21) Zhu, C., Wang, X., Deinum, J., Huang, Z., Gao, J., Modjtahedi, N., Neagu, M. R., Nilsson, M., Eriksson, P. S., Hagberg, H., Luban, J., Kroemer, G., and Blomgren, K. (2007) Cyclophilin A participates in the nuclear translocation of apoptosis-inducing factor in neurons after cerebral hypoxia-ischemia. *J. Exp. Med. 204*, 1741−1748.
(22) Artus, C., Boujrad, H., Bouharrour, A., Brunelle, M. N., Hoos, S., Yuste, V. J., Lenormand, P., Rousselle, J. C., Namane, A., England, P., Lorenzo, H. K., and Susin, S. A. (2010) AIF promotes chromatinolysis and caspase-independent programmed necrosis by interacting with histone H2AX. *EMBO J. 29*, 1585−1599.
(23) Ravagnan, L., Gurbuxani, S., Susin, S. A., Maisse, C., Daugas, E., Zamzami, N., Mak, T., Jaattela, M., Penninger, J. M., Garrido, C., and Kroemer, G. (2001) Heat-shock protein 70 antagonizes apoptosis-inducing factor. *Nat. Cell Biol. 3*, 839−843.
(24) Gurbuxani, S., Schmitt, E., Cande, C., Parcellier, A., Hammann, A., Daugas, E., Kouranti, I., Spahr, C., Pance, A., Kroemer, G., and Garrido, C. (2003) Heat shock protein 70 binding inhibits the nuclear import of apoptosis-inducing factor. *Oncogene 22*, 6669−6678.
(25) Schmitt, E., Parcellier, A., Gurbuxani, S., Cande, C., Hammann, A., Morales, M. C., Hunt, C. R., Dix, D. J., Kroemer, R. T., Giordanetto, F., Jaattela, M., Penninger, J. M., Pance, A., Kroemer, G., and Garrido, C. (2003) Chemosensitization by a non-apoptogenic heat shock protein 70-binding apoptosis-inducing factor mutant. *Cancer Res. 63*, 8233−8240.
(26) Wilkinson, J. C., Wilkinson, A. S., Galban, S., Csomos, R. A., and Duckett, C. S. (2008) Apoptosis-inducing factor is a target for ubiquitination through interaction with XIAP. *Mol. Cell. Biol. 28*, 237−247.
(27) Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972) Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer 26*, 239−257.
(28) Thornberry, N. A., and Lazebnik, Y. (1998) Caspases: Enemies within. *Science 281*, 1312−1316.
(29) Shiozaki, E. N., Chai, J., Rigotti, D. J., Riedl, S. J., Li, P., Srinivasula, S. M., Alnemri, E. S., Fairman, R., and Shi, Y. (2003) Mechanism of XIAP-mediated inhibition of caspase-9. *Mol. Cell 11*, 519−527.
(30) Sun, C., Cai, M., Meadows, R. P., Xu, N., Gunasekera, A. H., Herrmann, J., Wu, J. C., and Fesik, S. W. (2000) NMR structure and mutagenesis of the third Bir domain of the inhibitor of apoptosis protein XIAP. *J. Biol. Chem. 275*, 33777−33781.
(31) Huang, Y., Park, Y. C., Rich, R. L., Segal, D., Myszka, D. G., and Wu, H. (2001) Structural Basis of Caspase Inhibition by XIAP. Differential Roles of the Linker versus the BIR Domain. *Cell 104*, 781−790.
(32) Riedl, S. J., Renatus, M., Schwarzenbacher, R., Zhou, Q., Sun, C., Fesik, S. W., Liddington, R. C., and Salvesen, G. S. (2001) Structural basis for the inhibition of caspase-3 by XIAP. *Cell 104*, 791−800.
(33) Chai, J., Shiozaki, E., Srinivasula, S. M., Wu, Q., Dataa, P., Alnemri, E. S., and Shi, Y. (2001) Structural basis of caspase-7 inhibition by XIAP. *Cell 104*, 769−780.
(34) Eckelman, B. P., Salvesen, G. S., and Scott, F. L. (2006) Human inhibitor of apoptosis proteins: Why XIAP is the black sheep of the family. *EMBO Rep. 7*, 988−994.
(35) Birkey Reffey, S., Wurthner, J. U., Parks, W. T., Roberts, A. B., and Duckett, C. S. (2001) X-linked inhibitor of apoptosis protein functions as a cofactor in transforming growth factor-β signaling. *J. Biol. Chem. 276*, 26542−26549.

(36) Itoh, S., Thorikay, M., Kowanetz, M., Moustakas, A., Itoh, F., Heldin, C. H., and ten Dijke, P. (2003) Elucidation of Smad requirement in transforming growth factor-β type I receptor-induced responses. *J. Biol. Chem. 278*, 3751−3761.
(37) Yamaguchi, K., Nagai, S., Ninomiya-Tsuji, J., Nishita, M., Tamai, K., Irie, K., Ueno, N., Nishida, E., Shibuya, H., and Matsumoto, K. (1999) XIAP, a cellular member of the inhibitor of apoptosis protein family, links the receptors to TAB1-TAK1 in the BMP signaling pathway. *EMBO J. 18*, 179−187.
(38) Sanna, M. G., da Silva Correia, J., Ducrey, O., Lee, J., Nomoto, K., Schrantz, N., Deveraux, Q. L., and Ulevitch, R. J. (2002) IAP suppression of apoptosis involves distinct mechanisms: The TAK1/JNK1 signaling cascade and caspase inhibition. *Mol. Cell. Biol. 22*, 1754−1766.
(39) Sanna, M. G., Da Silva Correia, J., Luo, Y., Chuang, B., Paulson, L. M., Nguyen, B., Deveraux, Q. L., and Ulevitch, R. J. (2002) ILPIP, a novel anti-apoptotic protein that enhances XIAP-mediated activation of JNK1 and protection against apoptosis. *J. Biol. Chem. 277*, 30454−30462.
(40) Hofer-Warbinek, R., Schmid, J. A., Stehlik, C., Binder, B. R., Lipp, J., and de Martin, R. (2000) Activation of NF-κB by XIAP, the X chromosome-linked inhibitor of apoptosis, in endothelial cells involves TAK1. *J. Biol. Chem. 275*, 22064−22068.
(41) Levkau, B., Garton, K. J., Ferri, N., Kloke, K., Nofer, J. R., Baba, H. A., Raines, E. W., and Breithardt, G. (2001) XIAP induces cell-cycle arrest and activates nuclear factor-κB: New survival pathways disabled by caspase-mediated cleavage during apoptosis of human endothelial cells. *Circ. Res. 88*, 282−290.
(42) Mufti, A. R., Burstein, E., Csomos, R. A., Graf, P. C., Wilkinson, J. C., Dick, R. D., Challa, M., Son, J. K., Bratton, S. B., Su, G. L., Brewer, G. J., Jakob, U., and Duckett, C. S. (2006) XIAP is a copper binding protein deregulated in Wilson's disease and other copper toxicosis disorders. *Mol. Cell 21*, 775−785.
(43) Burstein, E., Ganesh, L., Dick, R. D., van De Sluis, B., Wilkinson, J. C., Lewis, J., Klomp, L. W. J., Wijmenga, C., Brewer, G. J., Nabel, G. J., and Duckett, C. S. (2004) A novel role for XIAP in copper homeostasis through regulation of MURR1. *EMBO J. 23*, 244−254.
(44) Lewis, J., Burstein, E., Birkey Reffey, S., Bratton, S. B., Roberts, A. B., and Duckett, C. S. (2004) Uncoupling of the signaling and caspase-inhibitory properties of XIAP. *J. Biol. Chem. 279*, 9023−9029.
(45) Yang, Y., Fang, S., Jensen, J. P., Weissman, A. M., and Ashwell, J. D. (2000) Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. *Science 288*, 874−877.
(46) Galban, S., and Duckett, C. S. (2010) XIAP as a ubiquitin ligase in cellular signaling. *Cell Death Differ. 17*, 54−60.
(47) Kerscher, O., Felberbaum, R., and Hochstrasser, M. (2006) Modification of proteins by ubiquitin and ubiquitin-like proteins. *Annu. Rev. Cell Dev. Biol. 22*, 159−180.
(48) Fushman, D., and Walker, O. (2010) Exploring the linkage dependence of polyubiquitin conformations using molecular modeling. *J. Mol. Biol. 395*, 803−814.
(49) Pickart, C. M., and Fushman, D. (2004) Polyubiquitin chains: Polymeric protein signals. *Curr. Opin. Chem. Biol. 8*, 610−616.
(50) Arora, V., Cheung, H. H., Plenchette, S., Micali, O. C., Liston, P., and Korneluk, R. G. (2007) Degradation of survivin by the X-linked inhibitor of apoptosis (XIAP)-XAF1 complex. *J. Biol. Chem. 282*, 26202−26209.
(51) Morizane, Y., Honda, R., Fukami, K., and Yasuda, H. (2005) X-linked inhibitor of apoptosis functions as ubiquitin ligase toward mature caspase-9 and cytosolic Smac/DIABLO. *J. Biochem. 137*, 125−132.
(52) MacFarlane, M., Merrison, W., Bratton, S. B., and Cohen, G. M. (2002) Proteasome-mediated degradation of Smac during apoptosis: XIAP promotes Smac ubiquitination in vitro. *J. Biol. Chem. 277*, 36611−36616.
(53) Winsauer, G., Resch, U., Hofer-Warbinek, R., Schichl, Y. M., and de Martin, R. (2008) XIAP regulates bi-phasic NF-κB induction involving physical interaction and ubiquitination of MEKK2. *Cell. Signalling 20*, 2107−2112.
(54) Brady, G. F., Galban, S., Liu, X., Basrur, V., Gitlin, J. D., Elenitoba-Johnson, K. S., Wilson, T. E., and Duckett, C. S. (2010) Regulation of the copper chaperone CCS by XIAP-mediated ubiquitination. *Mol. Cell. Biol. 30*, 1923−1936.
(55) Rumble, J. M., Bertrand, M. J., Csomos, R. A., Wright, C. W., Albert, L., Mak, T. W., Barker, P. A., and Duckett, C. S. (2008) Apoptotic sensitivity of murine IAP-deficient cells. *Biochem. J. 415*, 21−25.
(56) Duckett, C. S., Nava, V. E., Gedrich, R. W., Clem, R. J., Van Dongen, J. L., Gilfillan, M. C., Shiels, H., Hardwick, J. M., and Thompson, C. B. (1996) A conserved family of cellular genes related to the baculovirus *iap* gene and encoding apoptosis inhibitors. *EMBO J. 15*, 2685−2694.
(57) Burstein, E., Hoberg, J. E., Wilkinson, A. S., Rumble, J. M., Csomos, R. A., Komarck, C. M., Maine, G. N., Wilkinson, J. C., Mayo, M. W., and Duckett, C. S. (2005) COMMD proteins: A novel family of structural and functional homologs of MURR1. *J. Biol. Chem. 280*, 22222−22232.
(58) Wilkinson, J. C., Wilkinson, A. S., Scott, F. L., Csomos, R. A., Salvesen, G. S., and Duckett, C. S. (2004) Neutralization of Smac/Diablo by IAPs: A caspase-independent mechanism for apoptotic inhibition. *J. Biol. Chem. 279*, 51082−51090.
(59) Ye, H., Cande, C., Stephanou, N. C., Jiang, S., Gurbuxani, S., Larochette, N., Daugas, E., Garrido, C., Kroemer, G., and Wu, H. (2002) DNA binding is required for the apoptogenic action of apoptosis inducing factor. *Nat. Struct. Biol. 9*, 680−684.
(60) Mate, M. J., Ortiz-Lombardia, M., Boitel, B., Haouz, A., Tello, D., Susin, S. A., Penninger, J., Kroemer, G., and Alzari, P. M. (2002) The crystal structure of the mouse apoptosis-inducing factor AIF. *Nat. Struct. Biol. 9*, 442−446.
(61) Moubarak, R. S., Yuste, V. J., Artus, C., Bouharrour, A., Greer, P. A., Menissier-de Murcia, J., and Susin, S. A. (2007) Sequential activation of poly(ADP-ribose) polymerase 1, calpains, and Bax is essential in apoptosis-inducing factor-mediated programmed necrosis. *Mol. Cell. Biol. 27*, 4844−4862.
(62) Arnoult, D., Karbowski, M., and Youle, R. J. (2003) Caspase inhibition prevents the mitochondrial release of apoptosis-inducing factor. *Cell Death Differ. 10*, 845−849.
(63) Cande, C., Vahsen, N., Garrido, C., and Kroemer, G. (2004) Apoptosis-inducing factor (AIF): Caspase-independent after all. *Cell Death Differ. 11*, 591−595.
(64) Modjtahedi, N., Giordanetto, F., Madeo, F., and Kroemer, G. (2006) Apoptosis-inducing factor: Vital and lethal. *Trends Cell Biol. 16*, 264−272.
(65) Munoz-Pinedo, C., Guio-Carrion, A., Goldstein, J. C., Fitzgerald, P., Newmeyer, D. D., and Green, D. R. (2006) Different mitochondrial intermembrane space proteins are released during apoptosis in a manner that is coordinately initiated but can vary in duration. *Proc. Natl. Acad. Sci. U.S.A. 103*, 11573−11578.
(66) Vega-Manriquez, X., Lopez-Vidal, Y., Moran, J., Adams, L. G., and Gutierrez-Pabello, J. A. (2007) Apoptosis-inducing factor participation in bovine macrophage *Mycobacterium bovis*-induced caspase-independent cell death. *Infect. Immun. 75*, 1223−1228.
(67) Cregan, S. P., Dawson, V. L., and Slack, R. S. (2004) Role of AIF in caspase-dependent and caspase-independent cell death. *Oncogene 23*, 2785−2796.
(68) Lorenzo, H. K., and Susin, S. A. (2004) Mitochondrial effectors in caspase-independent cell death. *FEBS Lett. 557*, 14−20.
(69) Sevrioukova, I. F. (2009) Redox-linked conformational dynamics in apoptosis-inducing factor. *J. Mol. Biol. 390*, 924−938.
(70) Deveraux, Q. L., Takahashi, R., Salvesen, G. S., and Reed, J. C. (1997) X-linked IAP is a direct inhibitor of cell-death proteases. *Nature 388*, 300−304.
(71) Suzuki, Y., Nakabayashi, Y., and Takahashi, R. (2001) 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. U.S.A. 98*, 8662−8667.
(72) Van Themsche, C., Leblanc, V., Parent, S., and Asselin, E. (2009) X-linked inhibitor of apoptosis protein (XIAP) regulates PTEN ubiquitination, content, and compartmentalization. *J. Biol. Chem. 284*, 20462−20466.
(73) Xu, P., Duong, D. M., Seyfried, N. T., Cheng, D., Xie, Y., Robert, J., Rush, J., Hochstrasser, M., Finley, D., and Peng, J. (2009) Quantitative proteomics reveals the function of unconventional ubiquitin chains in proteasomal degradation. *Cell 137*, 133−145.
(74) Urbano, A., Lakshmanan, U., Choo, P. H., Kwan, J. C., Ng, P. Y., Guo, K., Dhakshinamoorthy, S., and Porter, A. (2005) AIF suppresses chemical stress-induced apoptosis and maintains the transformed state of tumor cells. *EMBO J. 24*, 2815−2826.