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Cleavage of Survivin by Granzyme M Triggers Degradation of the Survivin-X-linked Inhibitor of Apoptosis Protein (XIAP) Complex to Free Caspase Activity Leading to Cytolysis of Target Tumor Cells*

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Granzyme M (GzmM) is a chymotrypsin-like serine protease that preferentially cuts its substrates after Met or Leu. GzmM is constitutively expressed in activated innate effector natural killer (NK) cells. GzmM-induced cell death is consistent with the kinetics of cytotoxicity of NK cells. These suggest that GzmM may play an important role in innate immunity. Our previous work demonstrated that GzmM induces caspase-dependent apoptosis. However, it is unknown about how GzmM causes caspase activation. Here, we showed that the inhibitor of the apoptosis gene family member Survivin is a physiological substrate for GzmM. GzmM hydrolyzes Survivin at Leu-138 to remove the last four C-terminal residues. The truncated form (sur-TF) is more rapidly hydrolyzed through proteasome-mediated degradation. In addition, Survivin is in complex with X-linked inhibitor of apoptosis protein (XIAP) to inhibit caspase activation as an endogenous inhibitor. Survivin cleavage by GzmM abolishes the stability of the Survivin-XIAP complex and enhances XIAP hydrolysis, which amplifies caspase-9 and 3 activation of target tumor cells. The noncleavable L138A Survivin overexpression can significantly inhibit GzmM-mediated XIAP degradation, caspase activation, and GzmM- and NK cell-induced cytotoxicity. Moreover, Survivin silencing promotes XIAP degradation and enhances GzmM-induced caspase activation as well as GzmM- and NK cell-induced cytolysis of target tumor cells.

Granzyme M (GzmM)[2] is a chymotrypsin-like serine protease that preferentially cuts its substrates after Met or Leu (1). GzmM is constitutively expressed in activated innate effector NK cells but is not detected in CD4 or CD8 T cells even after activation (2, 3). Our work and the work by Smyth and coworkers (4, 5) previously demonstrated that GzmM-induced cell death is consistent with the kinetics of cytotoxicity of NK cells. These findings suggest that GzmM may play an important role in innate immunity. Our recent work elucidated the substrate binding activity and catalytic mechanisms of human GzmM based on structural analysis of human GzmM, the inactive D86N-GzmM mutant bound with a peptide substrate and human GzmM in complex with a catalytic product (6). We further demonstrated Asp-86 and His-41 in the catalytic triad contribute more to enzymatic activity than the attack residue Ser-182. D86N-GzmM mutant is an ideal and catalytically inactive enzyme for functional study.

We previously showed that GzmM induces caspase-dependent apoptosis with DNA fragmentation through direct cleavage of the inhibitor of caspase-activated DNase (5). However, it is unclear how GzmM causes caspase activation. GzmM induces mitochondrial swelling and loss of mitochondrial transmembrane potential (7). GzmM also initiates release of cytochrome *c* and accumulation of reactive oxygen species (ROS). GzmM directly degrades an ROS antagonist TRAP1 to promote ROS generation.

Survivin is the smallest member of the inhibitor of apoptosis (IAP) gene family that is involved in protecting cells from apoptosis, control of cell division, and cellular adaptation to an unfavorable environment (8, 9). IAP family proteins confer protection from caspase-initiated apoptosis as their name indicates. Overexpression of Survivin in various cellular systems is clearly associated with inhibition of cell death, whereas abrogation of Survivin function or expression leads to spontaneous cell death or promotes the effect of other apoptotic stimuli (10). Like most other IAP members, Survivin does not directly associate with or inhibit caspases (11). The cytoprotective function of Survivin depends on its association with other cofactors, such as the hepatitis B X-interacting protein, Smac, and XIAP (12–14). Dohi *et al.* (15) reported that cyclic AMP-dependent protein kinase A phosphorylates cytosolic Survivin at Ser-20. This phosphorylation disrupts the association of Survivin with XIAP that abolishes XIAP stability and accelerates staurosporine-induced cell death.

In this study, we found that Survivin is a physiological substrate of GzmM. GzmM cleaves Survivin after Leu-138, and

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[2] The abbreviations used are: GzmM, granzyme M; NK, natural killer cell; XIAP, X-linked inhibitor of apoptosis protein; Smac, second mitochondrial activator of caspases; CHX, cycloheximide; IAP, inhibitor of apoptosis protein; Ad, adenovirus; HA, hemagglutinin; shRNA, short hairpin RNA; GST, glutathione *S*-transferase; Z, benzyloxycarbonyl; fmk, fluoromethyl ketone; WT, wild type; AIP, aryl hydrocarbon receptor-interacting protein; ROS, reactive oxygen species; rSurvivin, recombinant Survivin; BIR, baculovirus IAP repeat; PI, propidium iodide; AFC, 7-amino-4-trifluoromethyl coumarin.

Survivin cleavage abolishes the stability of the Survivin-XIAP complex to trigger XIAP degradation that amplifies caspase-9 and -3 activation. The noncleavable L138A Survivin overexpression can significantly inhibit GzmM-mediated XIAP degradation and caspase activation. HeLa cells overexpressing L138A Survivin apparently suppress GzmM- and NK cell-induced cytotoxicity. Moreover, Survivin silencing promotes XIAP degradation and enhances GzmM-induced caspase activation as well as GzmM- and NK cell-induced cytolysis of target tumor cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—All of the cell lines are from American Type Culture Collection. Human embryonic kidney epithelial 293A (HEK293A) and HeLa cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Invitrogen), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Jurkat cells were cultured in RPMI 1640 medium. All of the stable HeLa transfectants were cultured in Dulbecco's modified Eagle's medium with 500 μg/ml G418. The caspase inhibitor Z-VAD was purchased from Calbiochem. Antibodies to Survivin, Smac, HA tag, and protein A/G-agarose were obtained from Santa Cruz Biotechnology. Antibodies to XIAP, caspase-9, and caspase-3 were purchased from Cell Signaling Technology (Beverly, MA). Antibodies to FLAG and β-actin, MG132, cycloheximide (CHX), and *o*-nitrophenyl β-D-galactopyranoside were from Sigma. Polyclonal antibody against GzmM was generated in our laboratory.

Plasmid Construction—Wild type (WT) Survivin cDNA and its mutants with a point mutation at amino acid residue 138 (L138A) or 141 (M141A) were amplified from FLAG-pcDNA3-Survivin and cloned into pcDNA3.1 with a C-terminal HA tag or pET26b with a C-terminal His_6 tag. The FLAG-tagged truncated version of Survivin (sur-TF) was also constructed into pcDNA3.1 vector. Survivin cDNA was inserted into pGEX-6P-1 vector to generate GST-Survivin protein. All the constructs were confirmed by sequencing analysis.

GST Pulldown Assay—Recombinant D86N-GzmM was incubated with GST or GST-Survivin bound to glutathione-Sepharose 4B beads in 0.5 ml of binding buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.3 mM dithiothreitol, 0.5% Nonidet P-40, and protease inhibitor mixture) at 4 °C overnight. The beads were subsequently washed four times with the binding buffer. The bound proteins were eluted in 20 μl of 1× SDS-PAGE sample buffer and analyzed by immunoblotting.

Immunoprecipitation and Immunoblotting—For immunoprecipitation assays, HEK293A cells were transfected with the indicated plasmids. Plasmid-transfected cells were washed twice with phosphate-buffered saline and lysed in IP lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, and protease inhibitor mixture). Supernatants were incubated with D86N-GzmM in 0.5 ml of IP lysis buffer for 4 h and immunoprecipitated with mouse IgG or anti-FLAG antibody at 4 °C overnight. The immunoprecipitated proteins were eluted from the protein A/G-agarose by boiling for 10 min in 1× SDS-PAGE sample buffer and immunoblotted with the indicated antibodies.

Measurement of Caspase Activity—Caspase-9 and -3 activity in HeLa cells was measured by using the fluorogenic substrate LEVD-AFC or DEVD-AFC (Calbiochem). Briefly, 2×10^5 HeLa cells were incubated alone or with D86N-GzmM or GzmM plus Ad for 6 h, washed twice with phosphate-buffered saline, and then lysed in 25 μl of lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% Nonidet P-40, 1 mM dithiothreitol, 0.1 mM EDTA). The supernatants were incubated with LEVD-AFC or DEVD-AFC at 37 °C for 2 h and measured by a microplate fluorimeter (ThermoLabsystems). Fluorescence units were converted to picomoles of AFC using a standard curve generated with free AFC. Enzymatic activity from triplicate samples was then analyzed by linear regression and normalized as fold change to the mock-treated cells.

Yeast Two-hybrid Assay—The full-length cDNA of Survivin was fused into the Gal4 DNA activating domain vector (pGADT7-Survivin), and D86N-GzmM cDNA was inserted into the Gal4 DNA binding domain vector (pGBKT7-D86N-GzmM) according to the manufacturer's instructions (Clontech). pGBKT7-D86N-GzmM and pGADT7-Survivin were co-transformed into the yeast stain AH109. All transformants were plated on selective medium lacking tryptophan and leucine and followed by a liquid β-galactosidase assay. Positive and negative controls were also performed in parallel.

Survivin and XIAP Silencing by RNA Interference—The target sequence 5′-GAGCCAAGAACAAAATTGC-3′ for Survivin and 5′-GTGGTAGTCCTGTTTCAGC-3′ for XIAP were synthesized as a short hairpin structure (16) and inserted into pSUPER vector (pSUPER-Survivin-shRNA and pSUPER-XIAP-shRNA). HeLa cells were transfected with pSUPER-Survivin-shRNA (sur-RNAi) or pSUPER-XIAP-shRNA (XIAP-RNAi) and empty pSUPER vector (Vector Laboratories) for 24 h. Cell lysates were immunoblotted with anti-Survivin or anti-XIAP antibody. β-Actin was used as a loading control.

Protein Expression and Purification—Active GzmM and inactive D86N-GzmM mutant (enzymatically inactive GzmM produced by mutating the active site Asp-86 to Asn) proteins were expressed in Rosetta (DE3) strain and refolded from inclusion bodies according to our previous method (6). GST, GST-Survivin, pET26b-Survivin, pET26b-L138A, and pET26b-M141A were expressed in Rosetta (DE3) strain. The GST fusion proteins were purified with the glutathione-Sepharose 4B beads (Amersham Biosciences). The soluble His-tagged WT, L138A and M141A Survivin were purified by nickel-nitrilotriacetic acid columns.

Protein Half-life Detection and Ubiquitination Assay—For the protein half-life assay, HEK293A cells were transfected with FLAG-sur-FL or FLAG-sur-TF for 18 h and then treated with 20 μM cycloheximide or 20 μM MG132. Cell lysates were analyzed with an antibody to FLAG. For the ubiquitination assay, HEK239A cells were co-transfected with FLAG-sur-TF or empty vector and HA-ubiquitin plasmid for 18 h. The cells were treated with 20 μM MG132 for 6 h and subsequently harvested in the IP lysis buffer. The FLAG-sur-TF protein was precipitated with an anti-FLAG antibody and probed with anti-HA antibody for ubiquitination detection. For the GzmM-pro-

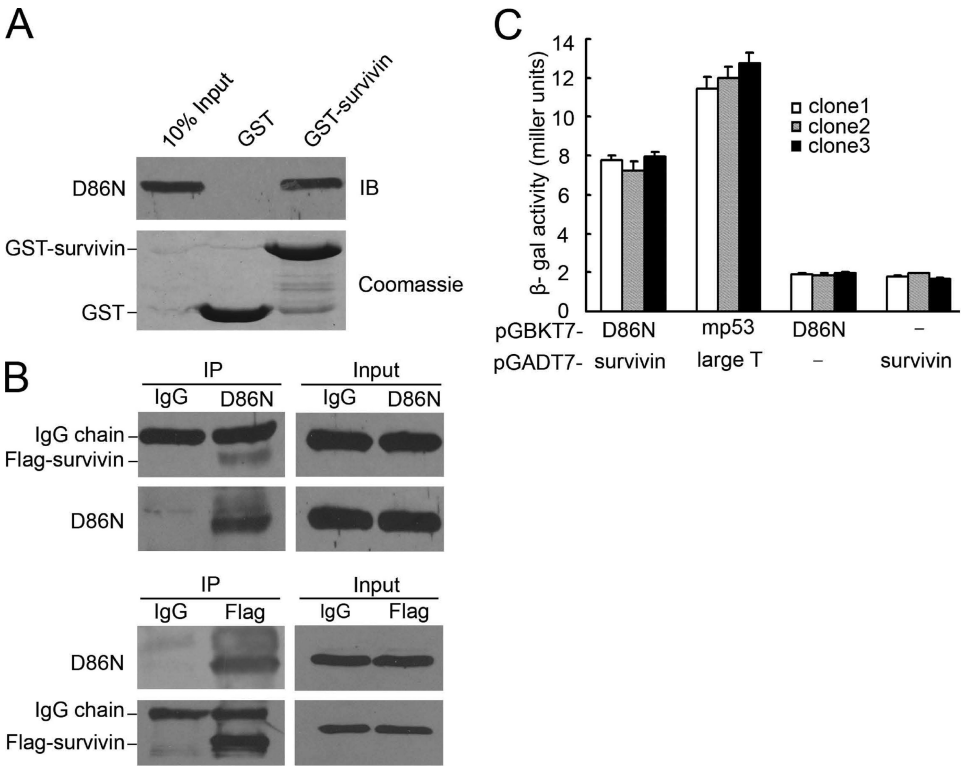


FIGURE 1. **Survivin associates with granzyme M *in vitro* and *in vivo*.** *A,* Survivin binds to D86N-GzmM in GST pulldown assay. Recombinant D86N-GzmM (mutation of the active site aspartic acid 86 to asparagine) was incubated with GST or GST-Survivin, and bound proteins were detected by Western blotting using anti-GzmM antibody (*top panel*). Comparable amounts of GST and GST-Survivin were confirmed by Coomassie Brilliant Blue staining (*bottom panel*). *IB,* immunoblotting. *B,* Survivin coprecipitates with D86N-GzmM by co-immunoprecipitation assay. Cell lysates (1×10^6 eq) from HEK293A cells transfected with pcDNA3-FLAG-Survivin for 24 h were incubated with D86N-GzmM at 4 °C for 4 h and then were immunoprecipitated with mouse IgG or anti-GzmM antibody (*top panel*) or with mouse IgG or anti-FLAG antibody (*bottom panel*). The immunoprecipitates were identified by either anti-FLAG or anti-GzmM antibody. *IP,* immunoprecipitation. *C,* interaction between D86N-GzmM and Survivin was confirmed by the yeast two-hybrid assay. The full-length cDNAs encoding D86N-GzmM and Survivin were inserted into the plasmids for the DNA-binding domain (*pGBKT7*) or DNA activation domain (*pGADT7*) of Gal4, respectively. pGBKT7 or pGBKT7-D86N-GzmM plasmid was co-transformed with pGADT7 or pGADT7-Survivin vector into the AH109 yeast strain. Binding activity was quantified by a liquid β-galactosidase (*β-gal*) assay and shown as mean ± S.D. The yeast strain co-transformed with pGBKT7-murine p53 and pGADT7-SV40 large T-antigen was served as a positive control.

cessed Survivin ubiquitination, 1×10^7 HeLa cells were treated with GzmM plus Ad in the absence or presence of 20 μM MG132 for 6 h, and cell lysates were immunoprecipitated with anti-Survivin antibody followed by immunoblotting with anti-ubiquitin antibody.

Cleavage Assay—3 μM recombinant His-tagged WT, L138A, or M141A Survivin was incubated with different concentrations of GzmM or D86N-GzmM for the indicated time points in 20 μl of cleavage buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$). For the cell-free cleavage assay, HeLa cells, Jurkat cells, or HEK293A cells transfected with the indicated plasmids were lysed in Nonidet P-40 lysis buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride) and subjected to SDS-PAGE. The reactions were terminated in 5× SDS loading buffer for Western blotting or Coomassie Brilliant Blue staining.

Loading GzmM with Adenovirus—Stable HeLa transfectants or Jurkat cells were incubated with different concentrations of GzmM or D86N-GzmM plus an optimal concentration of Ad at 37 °C for the indicated times as described previously (5, 17). Briefly, HeLa or Jurkat cells were washed three times in Hanks' buffered saline solution and resuspended in a loading buffer (Hanks'-buffered saline solution with 0.5 mg/ml bovine serum albumin, 1 mM $CaCl_2$, and 1 mM $MgCl_2$). Cells in 50 μl of loading buffer were incubated with different concentrations of GzmM or D86N-GzmM plus an optimal dose of Ad at 37 °C for the indicated times. Cells were incubated for an additional 15 min in 1 mM phenylmethylsulfonyl fluoride before preparing cell lysates or for flow cytometry.

NK Cell-mediated Cytotoxicity Assay—Peripheral blood mononuclear cells from healthy donors were purchased from the Beijing Blood Center and were purified by Ficoll-Hypaque gradient centrifugation separation. Primary NK cells were enriched from peripheral blood mononuclear cells by positive selection using the human NK cell isolation kit according to the manufacturer's instructions (Miltenyi Biotec). The purity of the enriched human NK cells was >90% as determined by flow cytometry. These enriched human primary NK cells were cultured in 200 units of interleukin-2-containing medium for activation. Target cell lysis by NK effector cells was quantitated in a 4-h ^{51}Cr-release assay. Briefly, HeLa cells overexpressing noncleavable mutant (L138A-Survivin) or silencing Survivin (sur-RNAi) or vector control cells were labeled with 50 μCi of ^{51}Cr and plated at 1×10^4 cells/well. NK effector cells were added at various effector/target (E:T) ratios. After a 4-h co-incubation at 37 °C, the supernatant was removed, and radioactivity was measured in a gamma counter. The percent cytotoxicity was determined as follows: percent cytotoxicity = ((sample release − spontaneous release)/(maximum release − spontaneous release)) × 100. Maximum release of target cells was measured following treatment with 1% Triton X-100 detergent.

Flow Cytometry Analysis—GzmM- or D86N-GzmM-treated target cells were stained with fluorescein isothiocyanate-conjugated annexin V and propidium iodide (PI) and analyzed by flow cytometry (FACSCalibur, BD Biosciences).

RESULTS

GzmM Associates with Survivin—We previously demonstrated that GzmM induces caspase-dependent apoptosis with mitochondrial damage, cytochrome *c* release, and caspase activation (7). However, it is not clear how GzmM causes caspase

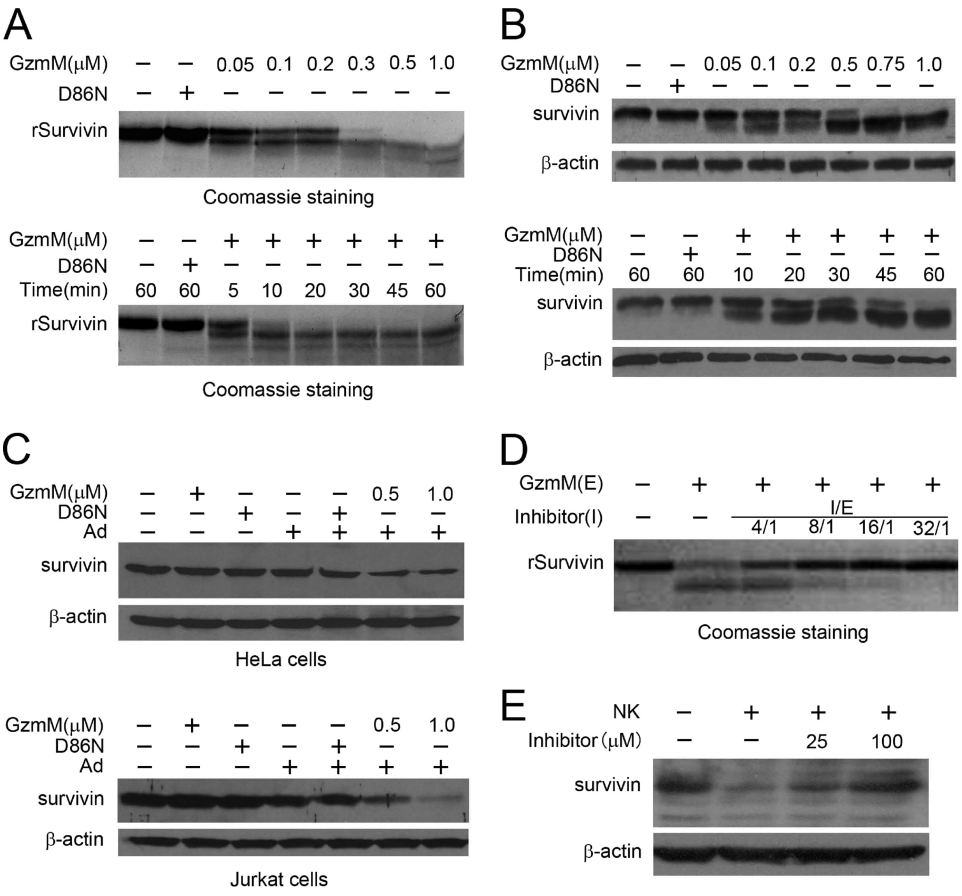


FIGURE 2. **Survivin is a physiological substrate of GzmM.** *A,* Survivin was cleaved by GzmM in a dose- and time-dependent manner. rSurvivin (3 μM) was incubated with different concentrations of GzmM at 37 °C for 60 min or with 0.5 μM GzmM for the indicated times. The reactions were stopped by adding 5× SDS loading buffer and visualized by Coomassie Brilliant Blue staining. *B,* GzmM cleaves native Survivin. HeLa cell lysates (2×10^5 eq) were treated with different doses of GzmM at 37 °C for 60 min or with 1 μM GzmM for the indicated times. The reactions were resolved by SDS-PAGE and immunoblotted with anti-Survivin antibody. β-Actin served as a negative loading control. *C,* Survivin is degraded in GzmM-loaded intact cells. HeLa cells (2×10^5) were treated with the indicated concentrations of GzmM plus Ad at 37 °C for 6 h, and whole cell lysates were probed with anti-Survivin antibody (*top panel*). Similar results were obtained in Jurkat cells (*bottom panel*). β-Actin was used as a negative loading control. *D,* cleavage of rSurvivin is abolished by the GzmM-specific inhibitor Ac-KVPL-CMK. *I/E,* inhibitor:GzmM molar ratio. *E,* GzmM inhibitor Ac-KVPL-CMK can block NK-mediated Survivin proteolysis. HeLa cells were incubated alone or co-incubated with NK cells (E:T ration of 10:1) for 4 h at 37 °C. In the same experiment, HeLa and NK cells were preincubated with the indicated concentrations of GzmM inhibitor before co-incubation. After terminating the reactions, the samples were electrophoresed and immunoblotted with indicated antibodies.

activation. IAP family proteins act as endogenous inhibitors of caspases. They contain the baculovirus IAP repeat (BIR) domain that is essential for the anti-apoptotic effect (18). To explore whether IAP family members are involved in GzmM-mediated caspase activation, we overexpressed several major members of IAP family proteins in HEK293A cells. The cell lysates were treated with active GzmM or the enzymatically inactive GzmM (D86N-GzmM). Among XIAP, Survivin, c-IAP1, or c-IAP2 examined, only Survivin was cleaved by GzmM (data not shown). To determine whether Survivin interacts with GzmM, we utilized GST pulldown assay to detect their *in vitro* association. The recombinant fusion protein of GST with Survivin (GST-Survivin) was able to bind to D86N-GzmM (Fig. 1*A*). The interaction between D86N-GzmM and Survivin was also confirmed by a co-immunoprecipitation assay. D86N-GzmM was preincubated with cell lysates from HEK293A cells transfected with FLAG-Survivin and immunoprecipitated with antibodies against FLAG or GzmM. Survivin could precipitate with D86N-GzmM and vice versa (Fig. 1*B*), although control IgG could not precipitate Survivin or D86N-GzmM. This interaction was further verified by using the Gal4-based yeast two-hybrid system assay. The full-length cDNAs encoding D86N-GzmM and Survivin were inserted into the plasmids for the DNA-binding domain (pGBKT7) or DNA activation domain (pGADT7) of Gal4, respectively. pGBKT7 or pGBKT7-D86N-GzmM plasmid was co-transformed with the pGADT7 or pGADT7-Survivin vector into the AH109 yeast strain. Their binding activity was quantified by a liquid β-galactosidase assay. Survivin was strongly associated with D86N-GzmM in yeast AH109 (Fig. 1*C*). pGBKT7-D86N-GzmM co-transformed with pGADT7 or pGADT7-Survivin with pGBKT7 had background β-galactosidase activity. The yeast strain co-transformed with pGBKT7-murine p53 and pGADT7-SV40 large T-antigen served as a positive control.

Survivin Is a Physiological Substrate of GzmM—To determine whether Survivin serves as a direct substrate of GzmM, recombinant Survivin (rSurvivin) was incubated with active GzmM or inactive D86N-GzmM as shown in Fig. 2*A*. GzmM directly cleaves rSurvivin at a concentration as low as 50 nM with Coomassie Brilliant Blue staining (Fig. 2*A*, *top panel*). rSurvivin is mostly degraded to its truncated form at 300 nM. The cleaved band was just about 1 kDa smaller than full-length Survivin. rSurvivin begins to degrade within 5 min with 0.5 μM GzmM treatment (Fig. 2*A*, *bottom panel*). After 10 min, rSurvivin was almost degraded to its truncated form. Inactive D86N-GzmM did not cut Survivin. Thus, Survivin cleavage needs its enzymatic activity of GzmM. To verify whether GzmM is able to cleave native Survivin, HeLa cell lysates were incubated in the presence of GzmM or D86N-GzmM and probed for Survivin. We found GzmM cuts native Survivin in a time- and dose-dependent fashion (Fig. 2*B*). However, inactive D86N-GzmM never degrades Survivin. The cleaved band is similar to rSurvivin cleavage. β-Actin was unchanged as a loading control.

To further determine whether GzmM can cleave Survivin in intact target tumor cells, HeLa cells were treated with GzmM in the presence of delivery agent replication-deficient Ad at 37 °C for 6 h. Survivin is degraded by active GzmM (Fig. 2*C*, *top*

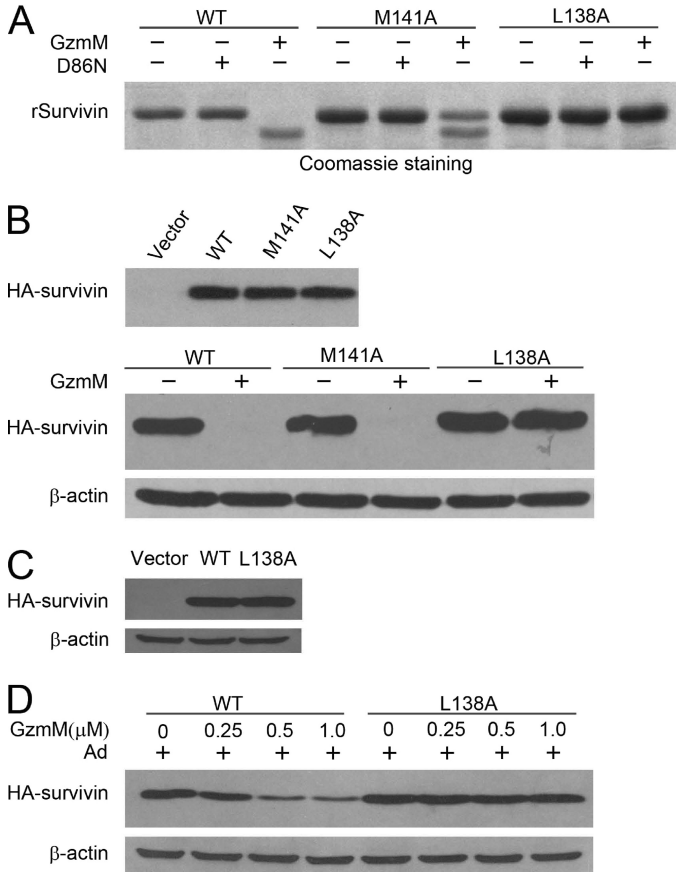


FIGURE 3. **GzmM cuts Survivin after leucine 138.** *A,* cleavage site Leu-138 was identified through site-directed mutagenesis. Recombinant WT or point mutant M141A or L138A Survivin protein was incubated with 0.5 μM GzmM at 37 °C for 20 min. The reactions were stopped by adding 5× SDS loading buffer and analyzed by Coomassie Brilliant Blue staining. Inactive D86N-GzmM was used as a negative control. *B,* L138A-Survivin mutant is not cleaved by GzmM in Leu-138-Survivin overexpressed HEK293A cells. Plasmids of WT, M141A, or L138A Survivin in pcDNA3.1 with C-terminal HA tag were transfected into HEK293A cells, and Survivin was overexpressed by detection of anti-HA antibody (*top panel*). After 24 h of transfection, cell lysates were incubated with 0.5 μM GzmM at 37 °C for 1 h. The reactions were resolved by SDS-PAGE and probed with anti-HA antibody (*bottom panel*). β-Actin was used as a good loading control. *C,* L138A-Survivin was overexpressed in HeLa cells. HeLa cells stably transfected with pcDNA3.1 empty vector (*Vector*) or C-terminally HA-tagged WT Survivin or L138A-Survivin (*L138A*) was screened out. *D,* L138A-Survivin was not degraded in L138A-Survivin overexpressed HeLa cells. HeLa cells stably expressing WT or L138A-Survivin was treated with the indicated concentrations of GzmM plus Ad for 6 h, and the cell lysates were immunoblotted with anti-HA antibody. β-Actin was used as a loading control.

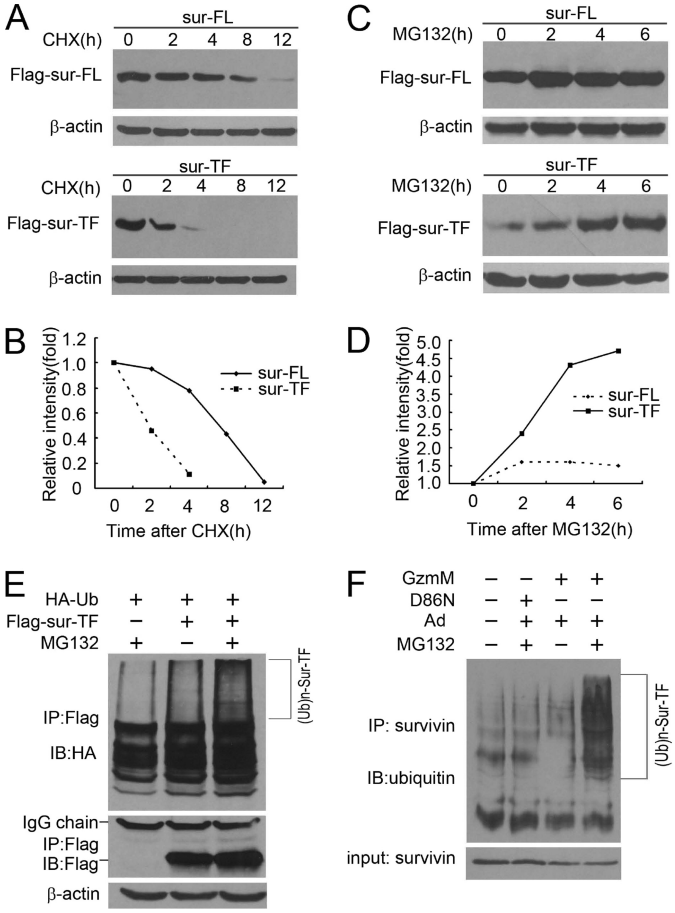


FIGURE 4. **Cleaved form of Survivin is susceptible to hydrolysis through proteasome-mediated degradation.** *A,* truncated form of Survivin is short lived. HEK293A cells were transfected with N-terminally FLAG-tagged full-length Survivin (1–144 amino acids, *sur-FL*) or the truncated form of Survivin (1–138 amino acids, *sur-TF*) for 24 h and then were treated with 20 μg/ml CHX for the indicated times. Protein level of sur-FL or sur-TF was determined by probing for anti-FLAG antibody. β-Actin was used as a loading control. *B,* quantification of the time-dependent expression of sur-FL or sur-TF in *A* was analyzed by densitometry. *C,* proteasome inhibitor MG132 can block Survivin degradation. HEK293A cells were transfected as in *A*, and their lysates were treated with 20 μM MG132 for different time points. *D,* quantification of the time-dependent expression of sur-FL or sur-TF was analyzed as in *B*. *E,* truncated form of Survivin is easily ubiquitinated *in vivo*. FLAG-tagged sur-TF or empty vector was co-transfected with HA-tagged ubiquitin into HEK293A cells. After 24 h of transfection, cells were treated with 20 μM MG132 for 6 h. sur-TF was immunoprecipitated with anti-FLAG antibody. Ubiquitination (*Ub*) of sur-TF was detected with anti-HA antibody. *IP,* immunoprecipitation; *IB,* immunoblotting. *F,* GzmM processed Survivin is ubiquitinated in HeLa cells. Polyubiquitinated Survivin was probed by anti-ubiquitin antibody (*IB*) after precipitation of Survivin with anti-Survivin antibody (*IP*) in the GzmM-loaded HeLa cells with or without pretreatment of the proteasome inhibitor MG132.

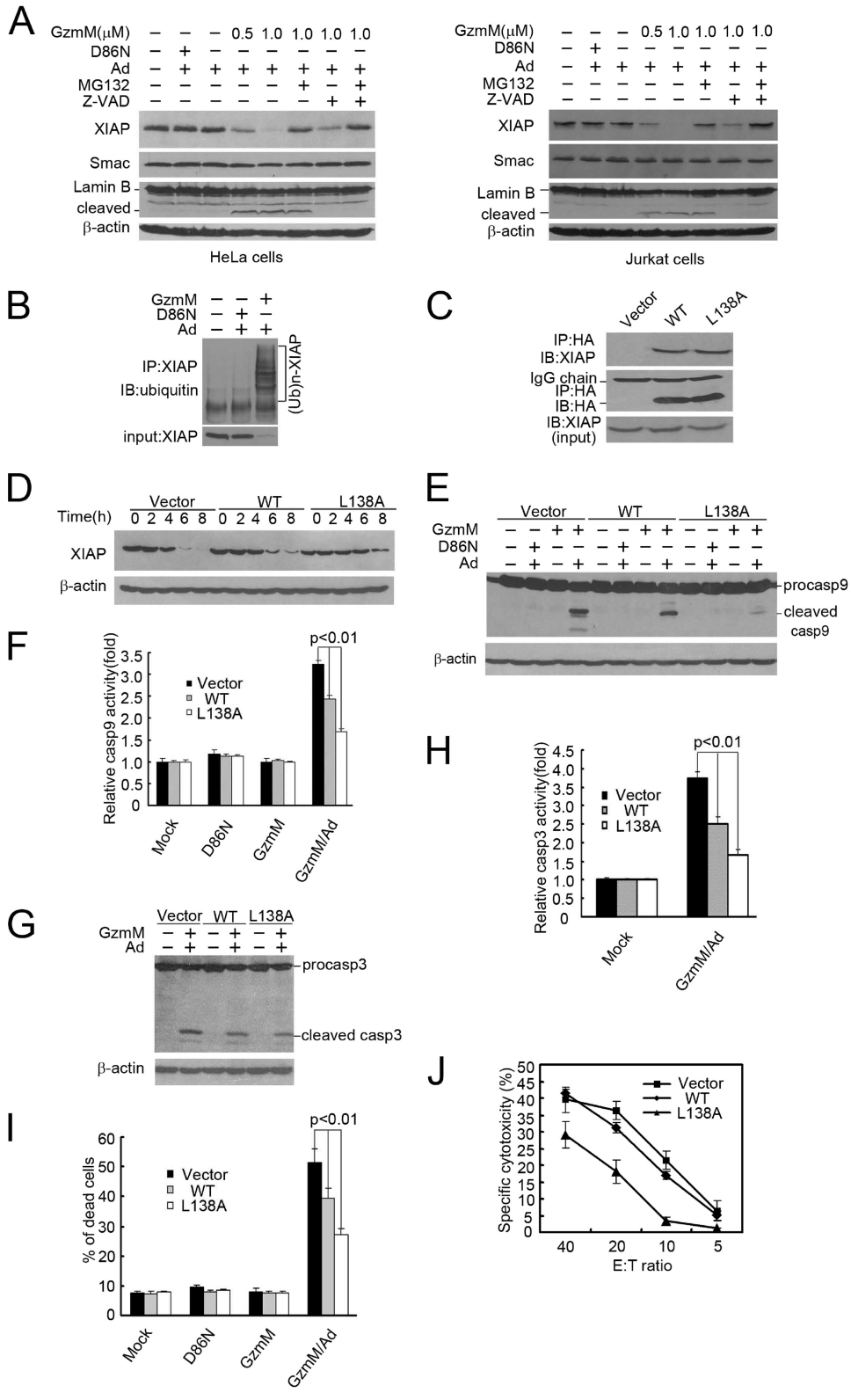
panel). The truncated band was not detected in GzmM-loaded cells, which might be degraded by other proteases. GzmM and Ad alone or D86N-GzmM plus Ad did not cleave Survivin. β-Actin was unchanged as a loading control. Similar results were obtained in Jurkat cells (Fig. 2*C*, *bottom panel*). We previously designed a specific GzmM inhibitor Ac-KVPL-CMK that can specifically block GzmM-mediated cytotoxicity (6). This inhibitor is able to block GzmM-induced rSurvivin cleavage in a dose-dependent manner (Fig. 2*D*). Cleavage of rSurvivin is completely abolished when the molar ratio of inhibitor to GzmM is larger than 32:1. To verify whether this GzmM-specific inhibitor can suppress NK-mediated Survivin proteolysis, HeLa cells and NK cells were pretreated with different doses of the GzmM inhibitor before co-incubation (E:T ratio 10:1) at 37 °C for 4 h. The GzmM inhibitor can suppress NK cell-mediated Survivin cleavage in a dose-dependent fashion (Fig. 2*E*). These results indicate that Survivin is a direct physiological substrate for GzmM.

GzmM Directly Cuts Survivin after Leu-138—The cleaved band of Survivin is about 1 kDa smaller than full-length Survivin, indicating that GzmM cleaves Survivin at its N or C terminus. To determine which terminal of Survivin is cleaved by GzmM, rSurvivin with the C-terminal His_6 tag was incubated with 0.5 μM GzmM for 30 min and probed with anti-His antibody. However, the truncated product was undetectable (data not shown). This indicates the cleavage site of Survivin is

located at the C terminus. Our structural analysis and a peptide-based screening approach have indicated that GzmM cleaves its substrates at Leu or Met in the P1 site (1, 6). Based on the specific cleavage of residues and the size of the cleaved band by GzmM, Met-141 or Leu-138 near the C terminus was potentially predicted to be a cleavage site. To further determine the exact cleavage site, Met-141 or Leu-138 was individually mutated into Ala via site-directed mutagenesis and expressed in *Escherichia coli*. WT Survivin and M141A or L138A mutant were treated with GzmM. WT Survivin or M141A mutant was still processed to produce its truncated form, although the L138A mutant was not degraded by GzmM (Fig. 3*A*). These data imply that Leu-138 is the sole cleavage site for GzmM.

To confirm the cleavage site *in vivo*, WT Survivin, M141A, or L138A mutant was subcloned into the pcDNA3.1 vector with a C-terminal HA tag and transfected into HEK293A cells. Their expressions were detected by immunoblotting with anti-HA antibody (Fig. 3*B*, *top panel*). HEK293A lysates with expression of WT Survivin, M141A, or L138A mutant were treated with GzmM and assessed with an antibody against HA. Only the L138A mutant completely prevented the cleavage by GzmM (Fig. 3*B*, *bottom panel*). In contrast, WT Survivin or M141A mutant was completely degraded by GzmM. β-Actin was unchanged as a control. To further verify L138A as the cleavage site in a more physiological condition, HeLa cells with stable expression of WT Survivin or L138A mutant were screened out (Fig. 3*C*). HeLa cells with stable expression of WT Survivin or L138A mutant were treated with GzmM plus Ad for 6 h. We found the WT Survivin was still degraded by GzmM in a dose-dependent pattern, although L138A Survivin was completely resistant to GzmM cleavage (Fig. 3*D*). These results indicate Leu-138 is the cleavage site for GzmM *in vitro* and *in vivo*.

Survivin Cleavage Triggers Its Hydrolysis through Proteasome-mediated Degradation—A recent report showed that the aryl hydrocarbon receptor-interacting protein (AIP) can stabilize Survivin in cells and elevate a cellular anti-apoptotic threshold through direct association with the last residue of Survivin (19). So, we hypothesized that removal of the last four residues of Survivin by GzmM may destroy the stability of Survivin in cells. To test this hypothesis, HEK293A cells were transfected with



N-terminally FLAG-tagged WT Survivin (sur-FL) or the truncated form (sur-TF), and their half-life was determined through protein translation inhibition with CHX treatment. Because of rapid degradation of sur-TF, these experiments were pretreated with MG132 prior to CHX inhibition. We found that sur-TF was more rapidly degraded than that of sur-FL (Fig. 4*A*). sur-TF had a remarkably short half-life of less than 2 h (Fig. 4*B*), and the half-life of sur-FL was longer than 6 h.

Proteasome inhibitor MG132 treatment blocked sur-TF or sur-FL degradation (Fig. 4*C*). Densitometric quantification of protein bands revealed that 6 h of treatment with MG132 resulted in a 5-fold increase of sur-TF protein levels, whereas sur-FL only elevated slightly (1.5-fold) (Fig. 4*D*). These results suggest that sur-TF may be degraded through proteasome-mediated degradation. To determine whether sur-TF is modified by addition of ubiquitin, HEK293A cells were co-transfected with sur-TF and HA-tagged ubiquitin for 18 h and treated with the proteasome inhibitor MG132 for 6 h. sur-TF was precipitated with anti-FLAG antibody followed by immunoblotting with anti-HA antibody. MG132 treatment substantially increased polyubiquitinated sur-TF (Fig. 4*E*). Moreover, GzmM-processed Survivin is ubiquitinated in GzmM-loaded HeLa cells (Fig. 4*F*). Polyubiquitinated Survivin was detectable in the GzmM-treated HeLa cells with the proteasome inhibitor MG132. Inactive D86N-GzmM had no effect. These results suggest that Survivin cleavage by GzmM can trigger proteasome-mediated degradation of Survivin.

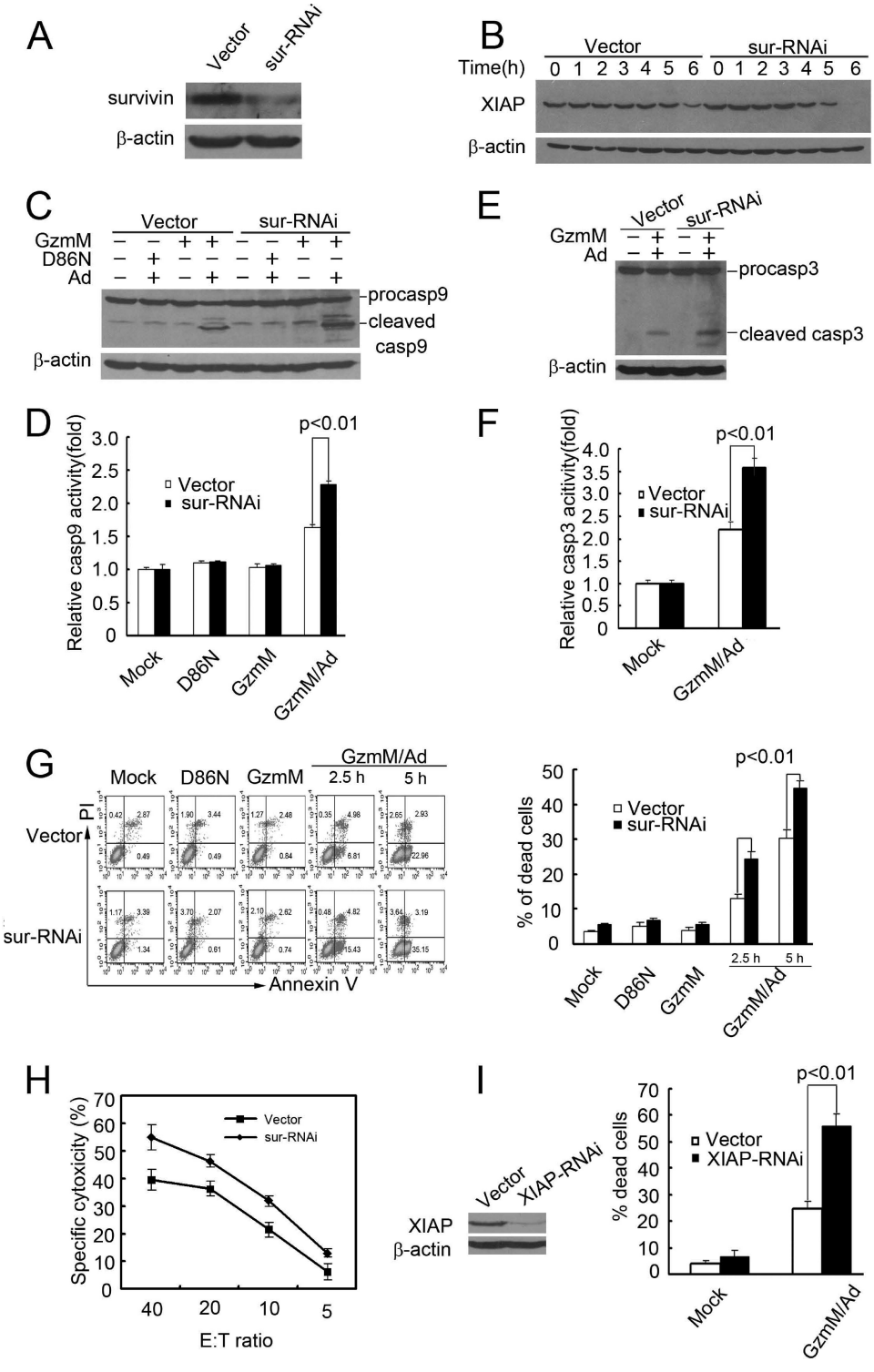
Survivin Cleavage by GzmM Accelerates XIAP Degradation to Promote Caspase Activation—A recent study showed that Survivin can promote XIAP stability against proteasomal degradation and inhibit caspase activity through assembly of the Survivin-XIAP complex (14). To assess whether Survivin cleavage initiates XIAP degradation, HeLa cells were treated with GzmM plus Ad. XIAP was degraded in a dose-dependent manner, whereas Smac was not affected (Fig. 5*A*, *left panel*). The proteasome inhibitor MG132 could block XIAP degradation. The caspase inhibitor Z-VAD modestly prevented XIAP degradation. We chose caspase substrate Lamin B to confirm the efficacy of Z-VAD-fmk (20), because we verified that Lamin B was not degraded by GzmM. Z-VAD-fmk was able to block caspase-mediated Lamin B degradation in GzmM-treated HeLa or Jurkat cells, which demonstrated the inhibitory activity of Z-VAD-fmk. β-Actin was unchanged as a good loading control. Similar results were observed in Jurkat cells (Fig. 5*A*, *right panel*). We observed ubiquitination of XIAP in GzmM-loaded HeLa cells. XIAP was precipitated with anti-XIAP antibody in the GzmM-loaded HeLa cells and probed by anti-ubiquitin antibody. We found that endogenous XIAP was ubiquitinated after treatment with GzmM plus Ad (Fig. 5*B*). These results suggest that GzmM induces XIAP hydrolysis mainly through proteasome-mediated degradation. This raised a possibility that Survivin cleavage may abolish the XIAP-Survivin complex to hydrolyze XIAP through proteasome-dependent degradation. To explore this possibility, we first confirmed the association of Survivin with XIAP in HeLa cells (Fig. 5*C*). L138A mutant can bind to XIAP as the WT Survivin does. It indicates that L138A mutant possesses normal function as the WT Survivin. HeLa cells stably expressing control vector, WT, or L138A Survivin were treated with GzmM plus Ad for the indicated times, and XIAP degradation was monitored (Fig. 5*D*). L138A Survivin overexpression could significantly inhibit XIAP degradation. Control HeLa cells showed more rapid XIAP hydrolysis. β-Actin was unchanged as a good loading control.

XIAP selectively binds and inhibits caspase-3, caspase-7, and caspase-9 as a caspase inhibitor (8). We postulated that accelerated XIAP degradation induced by Survivin cleavage may amplify caspase activation. We demonstrated that GzmM treatment resulted in caspase-9 activation *in vivo* (Fig. 5*E*). Overexpression of WT Survivin could slightly inhibit caspase-9 activation. L138A Survivin overexpression was able to obviously suppress caspase-9 activation. Caspase-9 activity was determined by using the fluorogenic substrate LEVD-AFC in the above treated HeLa cells and is shown in Fig. 5*F*. These data further confirmed that L138A Survivin overexpression significantly suppresses caspase-9 activity. Inactive D86N-GzmM plus Ad had no effect on caspase-9 activation. These results are representative of three separate experiments. Caspase-3 was detected by using Western blotting and the fluorogenic substrate DEVD-AFC assay in the above treated HeLa cells. Caspase-3 was activated in GzmM-loaded HeLa cells (Fig. 5*G*). WT Survivin overexpression could significantly suppress

FIGURE 5. **Survivin cleavage by GzmM accelerates XIAP degradation to facilitate caspase activation.** *A,* XIAP is degraded in GzmM-loaded target cells, and its degradation is blocked by MG-132. HeLa cells were incubated with the indicated concentrations of GzmM in the presence of proteasome inhibitor MG132 or pan-caspase inhibitor Z-VAD for 6 h. Treated cell lysates were resolved by SDS-PAGE and probed with the indicated antibodies (*left panel*). Similar results were obtained in Jurkat cells (*right panel*). *B,* XIAP is ubiquitinated (*Ub*) after treatment with GzmM plus Ad. Polyubiquitinated XIAP was probed by anti-ubiquitin antibody (immunoblotted (*IB*)) after precipitation of XIAP with anti-XIAP antibody (immunoprecipitated (*IP*)) in the GzmM-loaded HeLa cells. *C,* noncleavable form of Survivin (L138A-sur) can bind to XIAP. Cell lysates from HeLa cells stably expressing empty vector, WT, or L138A Survivin were immunoprecipitated with anti-HA antibody and immunoblotted with anti-HA or anti-XIAP antibody, respectively. *D,* overexpression of noncleavable L138A Survivin attenuates GzmM-mediated XIAP degradation. HeLa cells stably expressing empty vector, WT, or L138A Survivin were incubated with 0.5 μM GzmM plus Ad at 37 °C for the indicated times and probed for XIAP. β-Actin was used as a loading control. *E,* overexpression of noncleavable L138A Survivin is able to suppress caspase-9 (*casp9*) activation. HeLa cells stably expressing empty vector, WT, or L138A Survivin were treated with 0.5 μM GzmM plus Ad at 37 °C for 6 h and probed for caspase-9. *procasp9*, procaspase-9. β-Actin was used as a loading control. *F,* caspase-9 activity was examined by using the fluorogenic substrate LEVD-AFC in above treated HeLa cells. The data are representative of three separate experiments and shown as mean ± S.D. *G,* overexpression of noncleavable L138A Survivin can also inhibit caspase-3 activation. HeLa cells stably expressing empty vector, WT, or L138A Survivin were treated with 0.5 μM GzmM plus Ad at 37 °C for 6 h and probed for caspase-3. β-Actin was used as a loading control. *H,* caspase-3 activity was examined by using the fluorogenic substrate DEVD-AFC in above treated HeLa cells. The data are representative of three separate experiments and shown as means ± S.D. *I* and *J,* overexpression of noncleavable Survivin is resistant to GzmM- and NK cell-induced apoptosis. HeLa cells stably transfected with empty vector, WT, or L138A Survivin were incubated with buffer (*Mock*), D86N-GzmM plus Ad, 1 μM GzmM, or 1 μM GzmM plus Ad at 37 °C for 6 h and followed by staining with annexin V with PI for flow cytometry. Total death cells, including annexin V and PI single-positive as well as double-positive cells, were shown as means ± S.D. (*I*). ^{51}Cr-labeled HeLa cells transfected with empty vector, WT, or L138A Survivin were incubated with interleukin-2-activated NK cells with different E:T ratios for the standard ^{51}Cr-release assay. Data are representative of three independent experiments as shown in means ± S.D. (*J*).

caspase-3 activation (Fig. 5, *G* and *H*). Overexpression of the L138A-Survivin could further attenuate caspase-3 activation. We next assessed the effect of Survivin cleavage on GzmM-mediated cytolysis. HeLa cells stably expressing empty vector, WT, or L138A Survivin were treated with GzmM with Ad at 37 °C for 6 h followed by measurement with annexin V/PI staining. HeLa cells overexpressing noncleavable L138A Survivin were more resistant to GzmM-induced cytotoxicity compared with the cells expressing empty vector or WT Survivin (Fig. 5*I*). Active GzmM or inactive D86N-GzmM alone caused background cell death around 10%. These data are representative of at least three separate experiments. Furthermore, HeLa cells overexpressing the noncleavable mutant L138A-Survivin are resistant to NK cell-mediated cytolysis as shown in the Fig. 5*J*.

Survivin Silencing Enhances XIAP Degradation and GzmM-induced Cytotoxicity—We designed shRNA sequences of Survivin and constructed them into pSUPER plasmid to knock down Survivin expression. Survivin was silenced in HeLa cells by transfection with pSUPER-Survivin-shRNA (Fig. 6*A*). Survivin-silenced HeLa cells showed more rapid hydrolysis of XIAP protein after GzmM plus Ad treatment compared with HeLa cells expressing pSUPER control vector (Fig. 6*B*). XIAP was almost undetectable in Survivin-silenced HeLa cells within 6 h. β-Actin was unchanged as a loading control. GzmM caused more activation of caspase-9 and -3 in the Survivin-silenced HeLa cells compared with the pSUPER control cells (Fig. 6, *C–F*). Inactive D86N-GzmM with Ad did not activate caspase-9 and -3 in the Survivin-silenced HeLa cells or the pSUPER control cells. β-Actin was unchanged as a loading control. We next wanted to determine whether Survivin silencing can enhance GzmM-mediated cytolysis. Survivin-silenced HeLa cells were treated with GzmM plus Ad and stained with annexin V/PI for flow cytometry. HeLa cells with silenced Survivin expression were much more sensitive to GzmM-induced lysis than the pSUPER control cells undergoing apoptosis (Fig. 6*G*). GzmM and Ad alone or D86N-GzmM plus Ad just got background death. To further determine whether Survivin knockdown enhances NK cell attack, ^{51}Cr-labeled Survivin-silenced or empty vector control HeLa cells were incubated with interleukin-2-activated NK cells with different E:T ratios for the standard ^{51}Cr-release assay. We found Survivin silencing accelerates NK cell-mediated cytolysis (Fig. 6*H*). To confirm the resistance of XIAP to GzmM-mediated cytotoxicity, we knocked down XIAP expression in HeLa



cells by pSUPER-XIAP-shRNA vector (Fig. 6*I*, *left panel*). We observed that XIAP knockdown rendered HeLa cells more sensitive to GzmM-mediated apoptosis (Fig. 6*I*, *right panel*).

DISCUSSION

NK cells act as the first line of defense against virus-infected or transformed tumor cells (21, 22). Unlike GzmA or GzmB, GzmM is not expressed in CD4 or CD8 T cells either constitutively or post-activation (23). However, GzmM is constitutively and abundantly expressed in the innate effector NK cells as is Perforin. A recent study showed that the human NK cell line KHYG-1 with high expression of GzmM has a great capacity to kill tumor cells (24), although GzmA and GzmB are not detectable in this cell line. This implies that GzmM may play an important role in NK cell-mediated cytotoxicity in innate immunity. In our previous reports, we demonstrated that GzmM may promote target cell death in a manner similar to GzmB by activating caspases (5). However, it is still unclear how GzmM induces caspase activation. In addition, we also found that GzmM induces mitochondrial damage, loss of mitochondrial transmembrane potential, liberation of cytochrome *c,* and generation of ROS partially through proteolytic inactivation of heat shock protein 75 (7). Ley and co-workers (25) previously reported that GzmB-deficient NK and LAK cells both display a severe defect in their ability to rapidly induce apoptosis in the sensitive target cell line YAC-1. They concluded that LAK cells require GzmB for the rapid induction of DNA fragmentation of target cells. Through prolonged incubation, GzmB-deficient LAK cells were able to achieve considerable DNA damage. These data suggest GzmM may mediate late DNA fragmentation in the YAC-1 cells. However, we demonstrated that GzmM-induced rapid caspase-dependent apoptosis is characterized by DNA fragmentation in other tumor cell lines such as HeLa and Jurkat cells (5, 7).

Caspases are initially synthesized as precursor zymogens without enzymatic activity. The zymogens are processed by an upstream protease to produce a mature enzyme with full enzymatic activity (26, 27). Caspase activation is meticulously modulated in a normal cell. The IAP gene family members are the BIR-containing proteins. The BIR domains are required for the anti-apoptotic function through direct binding and inhibiting caspases (18). To assess whether IAP family proteins involve GzmM-induced caspase activation, the four IAP family members XIAP, Survivin, c-IAP1, and c-IAP2 were determined with GzmM treatment. We found only Survivin is cleaved by GzmM in cell lysates (data not shown).

Survivin was originally identified as the smallest IAP family member, which contains two dimer interface domains at the N terminus, a central BIR domain, and an extended C-terminal α-helical coiled-coil domain (28). Survivin can counteract apoptosis *in vitro* and in transgenic animals (10). The cleavage site of Survivin by GzmM locates at Leu-138. This site is highly conserved in mouse, rat, pig, and bovine, suggesting that Survivin may be a conserved target of GzmM in these animals. Kang and Altieri (19) showed that AIP could stabilize Survivin, and a Survivin mutant lacking only the last Asp-142 residue failed to bind the AIP. We found the half-life of truncated sur-TF is much shorter than sur-FL in our study. Therefore, cleavage of Survivin by GzmM possibly results in separation of AIP-Survivin complex and thus makes the sur-TF fragment more susceptible to proteasomal degradation. In Fig. 4*E*, the smear and higher molecular weight of ubiquitinated sur-TF are detectable using proteasome inhibitor MG132, implying the susceptibility of these ubiquitinated versions to degradation. Analogous situations can be found in other proteins, such as cyclin E. Caspase-mediated cleavage of cyclin E followed by enhanced proteasome-dependent degradation has been shown to be critical for genotoxic stress-induced apoptosis of tumor cells of hematopoietic origin (29).

A recent report demonstrated that Survivin and XIAP form a complex, which exhibits increased stability during cell death and synergistic inhibition of caspase activation (14). Consistent with this result, we found that the Survivin-XIAP complex exists in HeLa cells. GzmM-mediated cleavage followed by enhanced proteasome-mediated depletion of Survivin abolishes its ability to protect XIAP from proteasome-mediated degradation. In the process of GzmM-induced tumor cell death, stable overexpression of a noncleavable L138A Survivin antagonizes XIAP degradation and caspase activation, and silencing Survivin expression promotes XIAP degradation and caspase activation. These results suggest Survivin cleavage is crucial to mitigate the anti-apoptotic threshold, which possibly antagonizes GzmM-induced cell death.

FIGURE 6. **Silencing Survivin expression accelerates XIAP degradation to free caspase activity and enhances GzmM-induced cytolysis.** *A,* Survivin was silenced in HeLa cells by transfection with pSUPER-Survivin-shRNA. HeLa cells were transfected with pSUPER-Survivin-shRNA (*sur-RNAi*) or pSUPER vector (*Vector*) for 24 h. Cell lysates were immunoblotted with anti-Survivin antibody. β-Actin was used as a negative loading control. *B,* Survivin silencing accelerates GzmM-triggered XIAP degradation. Survivin-silenced HeLa cells were incubated with 0.5 μM GzmM plus Ad at 37 °C for the indicated times and probed for XIAP. β-Actin was used as a negative loading control. *C,* Survivin silencing increases caspase-9 activation. Survivin-silenced HeLa cells were treated with 0.5 μM GzmM plus Ad at 37 °C for 5 h and probed for caspase-9. β-Actin was used as a negative loading control. *D,* caspase-9 activity was analyzed by using the fluorogenic substrate LEVD-AFC in above treated HeLa cells. The data are representative of three separate experiments and shown as means ± S.D. *E,* Survivin silencing enhances GzmM-induced caspase-3 (*casp3*) activation. Survivin-silenced HeLa cells were treated with 0.5 μM GzmM plus Ad at 37 °C for 5 h and probed for caspase-3. β-Actin was unchanged as a negative loading control. *procasp3*, procaspase-3. *F,* caspase-3 activity was determined by using the fluorogenic substrate DEVD-AFC in above treated HeLa cells. The data are representative of three separate experiments and shown as mean ± S.D. *G* and *H,* Survivin silencing is susceptible to GzmM-and NK cell-induced cytolysis. Survivin silenced or control HeLa cells were treated with 0.5 μM D86N-GzmM or GzmM alone or GzmM with Ad for the indicated time points and stained with annexin V with PI followed by flow cytometry. The *left panel* is representative of at least three separate experiments. Total death cells, including annexin V and PI single-positive as well as double-positive cells, are shown as mean ± S.D. (*right panel*) (*G*). ^{51}Cr-labeled Survivin silenced or empty vector control HeLa cells were incubated with interleukin-2-activated NK cells with different effector/target (E:T) ratios for the standard ^{51}Cr-release assay. Data are representative of three independent experiments as shown in means ± S.D. (*H*). *I,* XIAP knockdown sensitizes cells to GzmM-mediated cytolysis. XIAP was silenced in HeLa cells by transfection with pSUPER-XIAP-shRNA (*XIAP-RNAi*) for 24 h (*left panel*). XIAP silenced or vector control HeLa cells were treated with 0.5 μM GzmM plus Ad for 5 h and stained with annexin V with PI for flow cytometry. Total dead cells were calculated as above (*right panel*).

In summary, we presented evidence that Survivin acts as an important physiological substrate of GzmM, and Survivin cleavage abolishes its ability to preserve XIAP stability against proteasomal destruction. Therefore, loss of Survivin-XIAP complex in cancer cells lowers the apoptotic threshold, thereby enhancing the cytotoxic effect of GzmM. Taken together, our findings shed some light on the molecular mechanistic insight into GzmM-induced tumor cell death.

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