Supplementary Material

Supplementary Methods

In Vitro GzmA Cleavage Reaction

Purified proteins were incubated with indicated amounts of Gzms in 20 μl reaction buffer (100 mM TrisHCl, pH 8.0, 50 mM NaCl) at 37°C for indicated times. The cleavage reaction was stopped by adding 5x SDS-loading buffer and boiling at 95°C for 5 min. Samples were analyzed by SDS-PAGE. The N-terminal sequence of the C-terminal cleavage fragment was determined at the Tufts Core Protein Sequencing Facility.

Gzm and Perforin Treatment of Cells

Cells were treated with Gzms and/or PFN as previously described (Beresford et al., 1999) using a sublytic concentration of purified native rat NK cell PFN, determined independently for each experiment as the concentration that induces 5-10% cell necrosis. K562 cells (2×10^5) in 100 μl loading buffer (Hanks' balanced salt solution with 1 mg/ml BSA, 1 mM $CaCl_2$, and 1 mM $MgCl_2$) were incubated for the indicated time at 37°C with the indicated concentration of Gzm with or without PFN. For immunoblotting experiments, cells were then incubated for an additional 15 min in 1 mM PMSF and boiled in 5x SDS loading buffer before SDS-PAGE and immunoblot. Flow cytometry was performed as described (Martinvalet et al., 2005).

Coimmunoprecipitation and Immunoblotting

HeLa cell NP-40 lysate (5×10^6 cell equivalents in 1 ml) was incubated for 2 h at 4°C with 10 μl of Ku70, Ku80 (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), or GST antibody (Clontech, Mountain View, CA, USA), and 10 μg of S-AGzmA and a slurry of washed Protein G beads

(Pharmacia, Piscataway, NJ, USA). After extensive washing with lysis buffer, the Protein G beads were incubated at room temperature for 1 hr with DNase I. After further washing, samples were boiled in SDS-PAGE sample buffer and analyzed by immunoblot probed with His-tag antibody.

Pull down assay

Glutathione Sepharose 4B beads (Pharmacia) were incubated with GST or GST-fusion protein for 30 min at 4^{o}C, washed in PBS and then incubated with S-AGzmA for 3 hr at 4^{o}C with or without dsDNA. After incubation, the beads were pelleted by centrifugation, and washed three times in PBS. The reaction mixtures with DNA were treated with DNase I at room temperature for 1 h, and then washed again. The beads were resuspended in SDS-PAGE sample buffer, and electrophoresis and immunoblotting were performed as above.

Electrophoretic Mobility Shift Assay (EMSA) and Gel Retardation Assay

EMSA was modified from the method of (Song et al., 2003). A mixture of Ku70 and Ku80 (0.1 μg of each) that was either mock treated or preincubated with the indicated amount of GzmA at 37°C for 2 hr in 20 μl TBS (10 mM TrisHCl, pH 7.8, 100 mM NaCl), was added to ^{32}P-labeled ds oligonucleotide (10 pmol, 5'-GGGCCAAGAATCTTAGCAGTTTCGGG-3' in TBS containing 1 mM EDTA, 10 mM $MgCl_2$, 1 mM dithiothreitol, 10% glycerol) and 0.04 μg/ml poly (dI-dC) and incubated at 25°C for 1 hr. Reaction products were electrophoresed through 6% agarose gels in 0.5% TAE (20 mM Tris acetate, 500 μM EDTA) and analyzed by autoradiography. For gel retardation assay, 0.5 μg of Ku70 or Ku80, or both, were mock treated or preincubated with 0.2 μg of GzmA in 20 μl TBS at 37°C for 1 h. Unlabeled dsDNA (a 600 bp PCR fragment of the PFN gene, 2 μg) was added and the mixture incubated at 25°C for 30 min. The reaction mixtures were

electrophoresed through 1% agarose gels and DNA was visualized by ethidium bromide staining.

Laser-scanning confocal microscopy

Subconfluent HeLa cells in eight-well chamber slides were incubated with GzmA and/or PFN in loading buffer at 37°C for indicated times before fixation and permeabilization using the Fix-and-Perm kit (Caltag Laboratories, Burlingame, CA, USA). Slides were blocked with blocking buffer (permeabilization buffer containing 1% BSA) and antibodies were diluted in blocking buffer and added for 1 hr at room temperature. For propidium iodide (PI) staining, washed slides were incubated for 10 min with PBS containing 100 µg/ml RNase I and 0.1 µg/ml PI, and then washed again. Samples were mounted with ProLong Antifade mounting medium (Molecular Probes, Eugene, OR, USA) and images were acquired with a Bio-Rad Radiance 2000 laser-scanning confocal microscope by focusing on the central plane of each cell.

Silencing and overexpression of Ku70

Silencing using synthetic 21 nucleotide siRNA duplexes was performed as described (Fan et al., 2003). Synthetic Ku70 siRNAs (Dharmacon Research, Boulder, CO, USA) were as follows:

siRNA#1: sense 5’-GCUCUGCUCAUCAAGUGUCUGdTdT-3’, antisense 5’-CAGACACUUGAUGAGCAGAGCdTdT-3’;

siRNA#2: sense 5’-UCCUUGACUUGAUGCACCUGAdTdT-3’, antisense 5’-UCAGGUGCAUCAAGUCAAGGAdTdT-3’;

siRNA#3: sense 5’-ACGGAUCUGACUACUCACUCAdTdT-3’, antisense 5’-UGAGUGAGUAGUCAGAUCCGUdTdT-3’;

siRNA#4: sense 5’-ACGAAUUCUAGAGCUUGACCAdTdT-3’, antisense 5’-UGGUCAAGCUCUAGAAUUCGUdTdT-3’.

GFP siRNA sequences were previously described (Fan et al., 2003) siRNAs targeting murine Ku70 were purchased from Santa Cruz. Transfected HeLa cells were analyzed 3 days after transfection by immunoblot for protein expression and cells transfected with a mixture of 4 Ku70 siRNAs were used for GzmA and PFN treatment. For overexpression, HeLa cells transiently transfected with either empty vector pcDNA6/V5His or Ku70 expression plasmid pcDNA6/V5HisKu70 subcloned from pBluescript/KS-Ku70, kindly provided by J. W. Shay (Chai et al., 2002), were analyzed 3 days later.

Apoptosis Assays

Cells treated with sublytic PFN and/or GzmA (2 μM) or medium at 37°C for 2 h were fixed with 2% paraformaldehyde in PBS and permeabilized using 0.1% Triton X-100 in PBS. Washed cells were resuspended in TUNEL reaction mixture (Roche, Indianapolis, IN, USA) and incubated for 1 h at 37°C in a humidified atmosphere in the dark, before washing and flow cytometry analysis. Apoptotic cells were quantified by annexin V and propidium iodide (PI) staining. Cells, treated as described for TUNEL assay, were resuspended in 10 mM Hepes pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$ containing annexin V (1 μg/ml) and incubated at room temperature for 10 min. After washing, cells were resuspended in the same buffer containing 2.5 μg/ml PI before analysis using a FACScalibur flow cytometer.

Cytotoxicity Assay

LAK cells were generated from human PBMC by activation with 4 μg/ml phytohemagglutinin (Difco, Detroit, MI, USA) and culture for 7-15 d in RPMI1640 medium containing 10% FCS and 1000 U/ml recombinant IL-2 (Chiron Oncology, Emeryville, CA, USA). Mouse CTL lines were generated from P14 (Pircher et al., 1989) and P14xGzmB-/- (Pham et al., 1996) splenocytes as

described (Martinvalet et al., 2005) Splenocytes were activated with 1 µg/ml LCMV peptide gp33 (KAVYNFATC) and cultured for 8-15 days in medium with 25 IU/ml recombinant IL-2. Cytotoxicity was assayed by ^{51}Cr release assay. For human CTL, ^{51}Cr-labeled autologous B-LCL target cells (10^4) in triplicate microtiter wells were mixed with CTL at the indicated effector:target (E:T) ratio in 200 µl tissue culture medium containing 5 µg/ml concanavalin A and incubated for 4 hr at 37°C and supernatants were counted on a Top Count microplate reader. YT cytotoxicity assay was performed similarly at an E:T ratio of 2:1. For the mouse CTL experiments, MEF target cells were radiolabeled in the presence or absence of 5 µg/ml of gp33 peptide for 1 hr at 37^0C before washing and mixing with effector cells. Percent specific cytotoxicity was defined as ([cpm –spontaneous release]/[total release–spontaneous release]) X 100.

Figure Legends

Suppl Fig 1 GzmA cleaves Ku70 after Arg^{301} in the middle of the β-bridge, which links the N terminal α/β-domain (pink) with the central β-barrel domain (blue) and C terminal α-helical arm (brown). Cylinders indicate α-helices and arrows, β-strands. DNA slides through the center of the indicated ring (adapted from (Walker et al., 2001)).

Suppl Fig 2 Mutant Ku70 produced by changing Arg^{301} to Ala is cleaved as efficiently as wild-type Ku70.

Suppl Fig 3 GzmA cleavage of Ku70 is unaffected by treating K562 cells with the ROS scavenger Tiron.

Suppl Fig 4 Ku70 is specifically cleaved by GzmA. It is not cleaved in K562 cells targeted by the GzmA-deficient NK cell line, YT. ICAD, a GzmB and caspase substrate, is degraded.

Suppl Fig 5 GzmA and PFN treatment of K562 cells, but neither PFN nor GzmA alone, disrupts Ku complex formation, assessed by flow cytometry.

Suppl Fig 6 Ku70 protects against GzmA-induced DNA damage. The figure shows representative flow cytometry plots for TUNEL staining.

Suppl Fig 7 Apoptosis was detected by Annexin V and PI staining 1 hr after adding GzmA and PFN. The figure depicts a representative experiment.

Suppl Fig 8 SET degradation is unaffected by silencing Ku70. The GzmA dose response for SET cleavage is similar in Ku70 siRNA and GFP siRNA treated cells.

References for supplementary methods and figures

Beresford PJ, Xia Z, Greenberg AH, Lieberman J (1999) Granzyme A loading induces rapid cytolysis and a novel form of DNA damage independently of caspase activation. *Immunity* **10:** 585-594

Chai W, Ford LP, Lenertz L, Wright WE, Shay JW (2002) Human Ku70/80 associates physically with telomerase through interaction with hTERT. *J Biol Chem* **277:** 47242-47247

Fan Z, Beresford PJ, Oh DY, Zhang D, Lieberman J (2003) Tumor Suppressor NM23-H1 Is a Granzyme A-Activated DNase during CTL-Mediated Apoptosis, and the Nucleosome Assembly Protein SET Is Its Inhibitor. *Cell* **112:** 659-672

Martinvalet D, Zhu P, Lieberman J (2005) Granzyme A induces caspase-independent mitochondrial damage, a required first step for apoptosis. *Immunity* **22:** 355-370

Pham CT, MacIvor DM, Hug BA, Heusel JW, Ley TJ (1996) Long-range disruption of gene expression by a selectable marker cassette. *Proc Natl Acad Sci U S A* **93:** 13090-13095

Pircher H, Burki K, Lang R, Hengartner H, Zinkernagel RM (1989) Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen. *Nature* **342:** 559-561

Song JY, Lim JW, Kim H, Morio T, Kim KH (2003) Oxidative stress induces nuclear loss of DNA repair proteins Ku70 and Ku80 and apoptosis in pancreatic acinar AR42J cells. *J Biol Chem* **278:** 36676-36687

Walker JR, Corpina RA, Goldberg J (2001) Structure of the Ku heterodimer bound to DNA and its implications for double-strand break repair. *Nature* **412:** 607-614