Granzyme a Activates an Endoplasmic Reticulum-associated Caspase-independent Nuclease to Induce Single-stranded DNA Nicks

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Granzyme A Activates an Endoplasmic Reticulum-associated Caspase-independent Nuclease to Induce Single-stranded DNA Nicks

Cytotoxic T lymphocytes (CTLs) initiate apoptosis of virally infected cells or transformed tumor cells primarily via the exocytosis of cytolytic granules containing perforin and the granzyme family of serine proteases (reviewed in Ref. 1). Granzyme B (GzmB) activates the caspase pathway of apoptosis, but also induces cell death in the presence of caspase blockade (2–8). Granzyme A (GzmA), the most abundant granzyme which is expressed in all cytolytic T and natural killer cells, activates cell death through a caspase-independent mechanism (7, 9). Although GzmA does not activate oligonucleosomal DNA fragmentation, it causes single-stranded DNA nicking and other characteristic features of apoptosis, including membrane perturbation, chromatin condensation, and loss of mitochondrial inner membrane potential (7, 10). To begin to determine the mechanism of GzmA-induced cell death, we used an inactive form of GzmA to isolate three GzmA-interacting proteins: SET (putative HLA-associated protein II, template-activating factor (TAF)-1β, or I$_{2}$PP2A), pp32 (putative HLA-associated protein I or I$_{1}$PP2A), and hsp27 (11, 12). SET, but not pp32 or hsp27, is a substrate of GzmA in vitro, in CTL attack, and in perforin-facilitated GzmA loading experiments. More recently, we found that during perforin loading or CTL attack, GzmA targets key nuclear proteins (10, 11). GzmA cleaves lamins A, B, and C, disrupting the nuclear lamina (10). It also degrades histone H1 and proteolyzes the tails of the core histones, opening up chromatin to exogenous DNases. (13).

The GzmA substrate SET is ubiquitously expressed and has been identified in both the nucleus and the cytoplasm. It was initially identified as a translocated gene in acute undifferentiated leukemia, in which the sequence for all but the last 6 amino acids is linked to the nucleoporin can (Nup214), which presumably targets the translocated gene product to the nucleus (14). SET is a 277-amino acid 44-kDa acidic protein (calculated pI 3.9–4.1) with a 52-amino acid C-terminal acidic tail (15). The protein, exclusive of its acidic tail, is highly homologous to evolutionarily conserved nucleosome assembly proteins (NAPs) in yeast, plants, and animals (Xenopus, 97% identity; Drosophila, 63% identity; and Arabidopsis, 42% identity). NAP activity requires the acidic tail (16, 17). The homologous protein TAF-1α, which differs only in the N-terminal 24 amino acids, has markedly reduced NAP activity. SET, but not TAF-1α, facilitates transcription, a function that has been attributed to its postulated ability to reverse nucleosome assembly and to increase DNA accessibility (18). SET and other NAP family members physically associate with the p300/cAMP response element-binding protein-binding protein family of transcriptional coactivators and with core histones (19–22). pp32 has both a canonical nuclear localization signal and leucine-rich motifs for binding to CRM1/exportin-1 for nuclear export (30–32). Therefore, pp32 is co-localized with SET from the cytoplasm by two groups (11, 15). Like SET, pp32 is ubiquitously expressed, has a C-terminal acidic tail, and inhibits protein phosphatase 2A (28). Although SET has been implicated as a proto-oncoprotein, pp32 suppresses transformation of rat embryo fibroblasts by a variety of oncogene pairs (29). pp32 has both a canonical nuclear localization signal and leucine-rich motifs for binding to CRM1/exportin-1 for nuclear export (30–32). Therefore, pp32 likely shuttles between the nucleus and cytoplasm. Indeed, as for SET, some reports localize it to the nucleus, and others
identify it in the cytoplasm. Two groups recently isolated an ~150–170-kDa nuclear complex containing SET and its homolog TAF-1α together with pp32 and its homolog APRIL (23, 32). This complex inhibits histone acetylation (23) and binds to HuR, a protein that stabilizes mRNAs containing AU-rich regions in the 3′-untranslated regions of many proto-oncogene and cytokine mRNAs (32).

We show that GzmA destroys the NAP activity of SET. We also found that SET and pp32 are localized in a 270–420-kDa endoplasmic reticulum (ER)-associated complex. In a GzmA-dependent manner, the purified SET complex induces single-stranded DNA nicks in isolated nuclei. In fact, the SET complex contains a 25-kDa nuclease that degrades eukaryotic or plasmid DNA. We postulate that this unidentified SET complex DNase is the caspase-independent GzmA-activated DNase (GAAD).

**EXPERIMENTAL PROCEDURES**

**Recombinant Proteins and Perforin**—Recombinant GzmA, S-AGzmA, pro-GzmA (before enterokinase treatment), and GzmB were produced and purified as previously reported (11, 33). Perforin was purified from the rat RNR-16 cell line as described (34). SET cDNA was polymerase chain reaction-amplified with Vent polymerase from a human B cell library (CLONTECH, Palo Alto, CA) and directionally ligated into pET28b (Novagen) using primers containing BamHI and XhoI restriction sites. pp32 was subcloned from a pET3d vector, a kind gift from Z. Damuni (28), into pET30a using BamHI and NcoI restriction sites. GST cDNA was polymerase chain reaction-amplified from pGEM-2(κ) (Amersham Pharmacia Biotech) using primers containing NotI and XhoI restriction sites and inserted into pET30(+) (35). Recombinant pp32, SET (rSET), and GST were expressed with C-terminal His₈ tags in transformed BL21(DE3) cells (Novagen) induced with 1 mM isopropyl-β-D-thiogalactopyranoside (Sigma). Recombinant proteins were purified sequencially over Novagen nickel and Bio-Rad anion-exchange columns.

**Antibodies**—Protein A-purified rabbit anti-N-terminal SET anti-serum was produced against amino acids 3–18 as described (35). Mouse anti-SET monoclonal antibody (supernatant from hybridoma KM1720) was a kind gift from K. Nagata (19). Mouse anti-pp32 monoclonal antibody (RJ1) was derived from fusion of the spleen from a mouse immunized subcutaneously every 3 weeks with 10⁵ cell eq/100 μl of recombinant pp32 in incomplete Freund’s adjuvant following the method previously described (36). The RJ1 antibody was purified from culture supernatants by protein A affinity chromatography. Commercially available antibodies that were used are as follows: mouse monoclonal antibody for marking mitochondria (Calbiochem); rabbit polyclonal antisera to His₈ (Santa Cruz Biotechnology), GST (CLONTECH), and calreticulin and hsp27 (Stressgen Biotech Corp.); fluorescein isothiocyanate- or tetramethylrhodamine B isothiocyanate-conjugated secondary antibodies for 1:1000 dilutions and then blocked using permeabilization buffer with 10% goat serum. Antibodies preincubated with protein A-Sepharose 4B were used as a negative control. Immunofluorescence microscopy was performed using a Bio-Rad Radiance 2000 laser scanning confocal microscopy system. Cells were fixed and permeabilized using the Fix-and-Perm kit (Calgk Labo-ratories, CA) and stained with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) and a mixture of 1 μg/ml propidium iodide and 10 μg/ml 7-aminoactinomycin D. Immunofluorescence micrographs were obtained with a Bio-Rad Radiance 2000 laser scanning confocal microscope and processed with Metamorph imaging software (Universal Imaging Corp.).

**RESULTS**

**Recombinant Granzyme A Cleaves Recombinant SET, but Not pp32**—rSET was produced in E. coli with a C-terminal His₈ tag. A major rSET band migrating at p45 corresponds to the 44-kDa native cellular protein. Both native and recombinant proteins migrate aberrantly under SDS-PAGE since their calculated masses are 32 and 34 kDa, respectively. Recombinant p45 reacted with antibodies to an N-terminal peptide (amino acids 3–16) of SET and to His₈ (data not shown). A minor band at 34 kDa may represent abnormally folded rSET since it reacted with both antibodies as well. rSET, like cellular SET (11), was cleaved by GzmA to a major 25-kDa band (Fig. 1, A and B). Another prominent early cleavage product migrated with apparent molecular mass of 20 kDa.
Fig. 1. Cleavage of rSET in vitro and of native SET during GzmA loading. A, rSET was treated with GzmA for the indicated times and analyzed by Coomassie Blue staining of SDS-polyacrylamide gels. Full-length rSET migrated at 45 kDa, similar to native SET (44 kDa). Major cleavage products at 25, 22, and 20 kDa were analyzed by amino acid sequencing. The 25-kDa product is the N-terminal major fragment. Minor fragments at 22 and 20 kDa begin at Val8 and Glu12, respectively. N-terminal sequences obtained from p22 and p20 are underlined in C. B, shown are the results from mass spectroscopy analysis of the cleavage reaction. The trace of rSET alone (upper panel) shows the singly charged ion at m/z 34,113.5 and the doubly charged ion at m/z 17,061. Cleavage analyzed 3 h after adding GzmA (lower panel) identified two major products migrating at 21,423 and 12,692 Da. A similar trace was obtained at 6 h (not shown). C, shown are the sequences of rSET and native SET (in boldface). Numbering corresponds to that of the native protein. The asterisk indicates the dominant cleavage site, and the plus sign indicates a minor cleavage site identified by mass spectroscopy analysis. Cleavage at Lys34 disrupts a potential bipartite nuclear localization signal (NLS) and removes the acidic tail required for NAP activity, but keeps the domain required for protein phosphatase 2A (PP2A) inhibition intact. aa, amino acids. D, SET was cleaved to a 25-kDa fragment during perforin (P) loading of GzmA (A) into K562 cells. Cell lysates were analyzed by SDS-PAGE, and immunoblots were probed for SET and pp32. SET cleavage was seen only with active GzmA and was complete within 2 h. S-A, S-AGzmA. E, SET was cleaved in K562 cells perforin-loaded with GzmA, but not in cells loaded with GzmB or inactive forms of GzmA. K562 cells were mock-treated (lane 1) or treated with GzmA alone (lane 2), perforin alone (lane 3), or perforin with GzmA (lane 4), S-AGzmA (lane 5), Pro-GzmA (lane 6), or GzmB (lane 7).
With longer incubation or radiolabeling (data not shown), minor bands could be identified at 22, 18, and 12 kDa. SET degradation was completely inhibited by the GzmA-specific inhibitor Ph-HNCONH-CtEToIOC (11) (data not shown). The N-terminal sequence identified p25 as the N-terminal cleavage fragment of SET. The N-terminal sequences of p22 (VSKKE) and p20 (ELNSN) identified these as further degradation products of p25, cleaved after Lys7 and Lys10, respectively (Fig. 1C). As expected, p25, p22, and p20 were not recognized by the anti-His6 antibody (data not shown).

No stable C-terminal fragment was detected on electrophoresed samples. We therefore turned to mass spectroscopy to identify the dominant GzmA cleavage site. Serial samples analyzed during GzmA digestion showed loss of 34,113-Da singly charged rSET and a commensurate increase in 21,422 and 12,692-Da species. This indicates cleavage after Lys176 in the sequence QTQNK↓ARSKRQ (predicted 21,417-Da N-terminal and 12,683-Da C-terminal fragments). Cleavage at Lys176 disrupts a possible bipartite nuclear localization signal, KRSSQTQNKASRRK^{181}. Mass spectroscopy also revealed a transient minor fragment of 19,369 Da, corresponding to the sequence QTQNK after cleavage at Lys119.

GzmA did not cleave recombinant pp32 (data not shown), suggesting that pp32 is not a substrate of GzmA. This has been confirmed by lack of pp32 degradation in cells loaded with GzmA or in cells subjected to CTL granule-mediated cytolysis (Fig. 1D and data not shown).

SET Is Cleaved to a 25-kDa N-terminal Fragment after GzmA Loading or CTL Attack—During CTL attack, SET is completely degraded within minutes in a GzmA-specific manner since degradation is completely blocked by the GzmA-specific inhibitor Ph-HNCONH-CtEToIOC (11). SET fragments, however, are not readily visualized on immunoblots of lysed cells unless special care is taken to inhibit proteolysis prior to Nonidet P-40 lysis. To determine whether the in vitro cleavage site is also used in vivo, we loaded GzmA and perforin into K562 cells and added phenylmethylsulfonyl fluoride before lysis. Immunoblots of whole cell lysates showed the appearance of a 25-kDa SET fragment, which reacted with the anti-N-terminal peptide antiserum (Fig. 1D). This finding was confirmed after lymphokine-activated killer cell attack of K562 cells (data not shown). The 25-kDa SET fragment was readily apparent after treatment of K562 cells with GzmA plus perforin, but not with either alone. The cleaved fragment was also not detected after perforin loading of GzmB or inactive forms of GzmA (S-AGzmA or pro-recombinant GzmA) (Fig. 1E). Therefore, the dominant in vitro cleavage site is likely to be dominant in vivo.

rSET Has NAP Activity That Is Disrupted by GzmA Treatment—SET has been shown to bind histones and to have NAP activity (17, 21–23). SET interaction with histones was confirmed by the specific elution of histones from an rSET affinity column (data not shown). We also verified the NAP activity of rSET (Fig. 2A) with the supercoiling assay previously described (17). Topoisomerase-relaxed plasmid was incubated with purified core histones in the presence of increasing concentrations of rSET. Supercoiled plasmid, formed after assembly of histones and DNA into nucleosomes, could be distinguished from relaxed plasmid by its higher mobility after proteinase digestion on agarose gels. In a dose-dependent fashion, rSET facilitated chromatin assembly. The NAP activity of rSET was ATP- and sequence-independent since it could be demonstrated for multiple plasmids with no shared sequence in the absence of ATP (data not shown).

Deletion of the acidic tail (amino acids 226–277) largely abrogates the NAP activity of SET and its ability to facilitate transcription (17). We therefore investigated whether GzmA treatment of rSET disrupts its in vitro NAP activity. Pretreatment of rSET with GzmA inhibited the NAP activity of rSET in a concentration- and time-dependent manner (Fig. 2B). We previously showed that GzmA cuts small peptides from the core histones in the tail regions, which are not required for nucleosome formation (10). After granzyme A treatment, the apparent molecular mass of the core histones shifted by <1–2 kDa. Although removing tail peptides is not expected to interfere with nucleosome formation (38), we needed to verify that the loss of NAP activity was not secondary to GzmA cleavage of the histone tails. When GzmA and SET were added without preincubation to relaxed plasmid and core histones, although the core histones were proteolyzed, no loss of NAP activity was evident. However, preincubation of SET with GzmA for 30 min disrupted the NAP activity. Moreover, recombinant fragments of SET that correspond to the N- and C-terminal fragments after GzmA cleavage also have no NAP activity (17) (data not shown). Therefore, GzmA cleavage abrogates the NAP function of SET.

pp32 and SET Are in a Multimeric Complex—The repeated co-isolation of pp32 and SET suggests that these proteins may be associated within cells (11, 15, 23, 32). Both pp32 and SET eluted with 500 mM NaCl when cytoplasmic lysates were applied to immobilized S-AGzmA. Moreover, both proteins bound strongly to an rSET affinity column, requiring 500 mM NaCl for elution (data not shown). We therefore analyzed the S-AGzmA column eluate by gel filtration chromatography. Both pp32 and SET eluted in the void volume when applied to a Superdex 200 gel filtration column (data not shown), indicating that the SET and pp32 proteins are contained in cytoplasmic complexes >200 kDa in size. We therefore analyzed the S-AGzmA eluate on a Sephacryl 400 column, able to resolve protein complexes between 2 × 10⁴ and 8 × 10⁶ Da. Both pp32 and SET coeluted in a single broad peak between ~270 and 440 kDa (Fig. 3A). The peak contained additional bands on SDS-polyacrylamide gels that remain to be identified (Fig. 3A). The breadth of the peak suggests that the SET complex may be heterogeneous in the cytoplasm and that it contains components additional to...
**Fig. 3. SET and pp32 are in a 270–420-kDa multimeric complex.** A, SET and pp32 were eluted from an S-AGzmA affinity column with 500 mM NaCl. When the S-AGzmA eluate was applied to an S400 gel filtration column, pp32 and SET coeluted in a broad peak in fractions 65–80 at ~270–420 kDa, as determined by the migration of standard proteins and immunoblots for pp32 and SET. Coomassie Blue-stained SDS-polyacrylamide gel and SET and pp32 immunoblots of fractions 35–90 are compared with the S-AGzmA column eluate (S-A) and the flow-through fraction (FT). M, markers. B, coprecipitation of SET and pp32 from K562 cell lysates was dependent on GzmA. Cytosolic lysates were incubated with no antibody or with anti-pp32 or control anti-GST antibody in the presence or absence of S-AGzmA. Samples without antibody were analyzed directly; samples with antibody were treated with protein A-Sepharose, and bound proteins were eluted into SDS sample buffer after extensive washing. The immunoblot was probed with polyclonal antibody to SET. C, rSET bound to S-AGzmA, but recombinant pp32 (rpp32) did not. Samples were analyzed as described for B, except that immunoblots were probed with an antibody to the His6 tag on all the recombinant proteins. D, coprecipitation of rSET and recombinant pp32 was enhanced by S-AGzmA. SET and pp32 were incubated with anti-pp32 or control anti-GST antibody in the presence or absence of S-AGzmA. To assure the specificity of the recombinant pp32-rSET interaction, recombinant GST (rGST) was incubated with rSET and precipitated using anti-GST antibody. The immunoblot was probed with anti-SET antiserum.
those recently identified in a smaller complex (~150–170 kDa) in the nucleus (23, 32).

GzmA Enhances Coprecipitation of SET and pp32—To confirm the association of SET and pp32, we performed coprecipitation experiments (Fig. 3D). SET was not detected in the immunoprecipitate of cytosolic lysates treated with anti-pp32 monoclonal antibody. However, the two proteins coprecipitated when S-AGzmA was added to the lysates. Similar results were found when immunoprecipitation was performed with anti-SET antiserum, followed by probing the immunoblots with anti-pp32 antibody (data not shown). This suggests either that SET and pp32 bind weakly or that the antibodies interfere with complex formation. Adding inactive GzmA probably enhances the pp32-SET interaction through formation of a trimeric complex.

The cytosolic pp32-SET complex contains additional proteins, which might influence the interaction between pp32 and SET. We therefore assayed recombinant proteins, rSET, but not recombinant pp32, coprecipitated with S-AGzmA (Fig. 3C) (11). rSET weakly coprecipitated with recombinant pp32 in the absence of GzmA (Fig. 3D). However, the association between the recombinant proteins was enhanced by the addition of S-AGzmA, as it was for unpurified proteins in the cytosolic lysates. In addition, the converse immunoprecipitation with polyclonal antiserum to rSET pulled down a small amount of recombinant pp32, which was enhanced in the presence of S-AGzmA (data not shown). Therefore, GzmA enhances a weak interaction between pp32 and SET that does not require participation of additional proteins. However, other proteins in the SET complex may contribute to the integrity of the complex in intact cells.

SET and pp32 Localize Primarily to the ER—SET and pp32 were isolated from the cytoplasm by two groups (11, 15). However, SET has been found in cell nuclei in immunohistochemistry studies (19, 35), and its NAP function suggests that SET should spend time in the nucleus. Two recent studies found that pp32 and SET and their respective homologs APRIL and TAF-1α are associated in nuclei (23, 32). pp32 has a canonical nuclear localization signal (KKRK) at positions 236–239 and three leucine-rich repeats (spanning amino acids 65–123), which may be nuclear export signals for interaction with CRM1 (30–32). Therefore, SET and pp32 may shuttle between nuclear and cytoplasmic compartments. We therefore used laser scanning confocal microscopy to identify the subcellular localization of pp32 and SET in adherent non-confluent HeLa and COS cells (Fig. 4A). In most cells, pp32 and SET colocalized and were concentrated in a perinuclear region of the cytoplasm. An occasional cell (<1% of cells) showed nuclear staining of both pp32 and SET (data not shown). In cells with nuclear localization, staining was diffusely nucleoplasmic, avoiding the nucleolus. Co-staining with antibodies to proteins that localize to the ER and mitochondria showed that pp32 and SET colocalized with calreticulin and BiP (data not shown) in the ER, but not with hsp60 in mitochondria (data not shown) or the Calbiochem mitochondrial marker.

Immunofluorescent staining (Fig. 4) was performed under mild fixation and permeabilization conditions using the kit from Caltag Laboratories. When harsher conditions were used, the nuclear/cytoplasmic distribution of SET changed (data not shown). Fixation with Caltag reagent A or 4% paraformaldehyde gave identical results. However, in cells treated with 1% SDS or methanol, SET stained primarily in the nucleus. The use of different conditions for preparing slides may explain the apparent reported discrepancies in subcellular localization of pp32 and SET. To sort out fixation artifacts, nuclear and cytoplasmic fractions of K562 cells were isolated and analyzed by immunoblotting (Fig. 4B). The vast majority of SET (and pp32 (data not shown)) was in the cytosolic fraction. Overexposure of the immunoblot revealed a faint nuclear band. When K562 cells were loaded with perforin and GzmA, within 10 min, the cytosolic p44 SET band began to disappear, and the p25 fragment appeared in the cytoplasmic fraction. Therefore, GzmA cleaves SET in the cytoplasm and does not induce its nuclear translocation. To visualize better the small amount of nuclear SET, isolated nuclei were treated with GzmA. GzmA also cleaved nuclear SET, but not pp32 (Fig. 4C).

Reconstitution of Single-stranded DNA Damage in Isolated Nuclei by GzmA-treated SET Complex—Single-stranded DNA damage can be detected within 2–4 h after perforin loading cells with GzmA (7) (data not shown). These DNA breaks could be identified by Klenow labeling of nicked ends with 32P, but not by in situ terminal deoxynucleotidyltransferase labeling (data not shown), and could be visualized on denaturing (but not native) agarose gels. When isolated nuclei were incubated with GzmA, no DNA damage occurred. However, in the presence of a small amount of cytosol from Nonidet P-40-lysed K562
Granzyme A Activates a SET Complex DNase

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**DNA-PAGE analysis** identifies nucleases after electrophoresis through polyacrylamide gels impregnated with calf thymus DNA by the appearance of EtBr-negative bands after protein renaturation. An EtBr-negative band was visible at ~25 kDa when the purified SET complex, but not irrelevant fractions, was analyzed. Thus, the SET complex contains a 25-kDa nuclease that degrades eukaryotic DNA (Fig. 5D). Because the DNase has the same apparent molecular mass as GzmA-cleaved SET, it was important to determine whether cleaved SET is the DNase. However, rSET, recombinant pp32, and GzmA-cleaved rSET have no DNase activity. Therefore, the SET complex GAAD is distinct from pp32 and SET. DNA degradation of plasmid and eukaryotic DNAs by GAAD required Mg²⁺, but not Ca²⁺ (data not shown).

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TABLE I

| Substrate   | GzmA cleavage sites | Sequence | Reference |
|-------------|---------------------|----------|-----------|
| pIL-18      | Arg<sup>10</sup>    | DAPVR    | SLNCT     | 43        |
| Thrombin receptor | Arg<sup>11</sup>    | TLDPR    | SFLRR     | 44        |
| Histone H1  | Lys<sup>85</sup>    | KLGLK    | SLVSK     | 13        |
| Histone H2b | Lys<sup>12</sup>    | APAPK    | KGSKK     | 13        |
| SET         | Lys<sup>176</sup>   | QTQKN    | ASRKR     | 10        |
| Lamin B     | Arg<sup>13</sup>    | TVTSR    | ASSR      | 10        |

Granzyme A activates a SET Complex DNase

Asymmetrical DNA breaks in some types of cells subjected to CTL-mediated lysis (42). Therefore, the relative contributions of caspase-activated DNase- and GAAD-mediated DNA damage may vary between target cells.

Until recently, only interleukin-1β and the thrombin receptor had been clearly shown to be physiologically relevant GzmA substrates (43, 44). Lamins and histones have recently been added to the list (10, 13). Cleavage sites for six GzmA substrates have now been identified, all cleaved at nanomolar concentrations, with cleavage verified by in vivo experiments (Table I). These showed that Lys and Arg are both equally good at the P1 position. Although GzmA seemed to prefer a basic residue at the P5 site, and possibly Ser at some of the other residues distal to the cleavage site, there was no obvious sequence preference N-terminal to the cleavage site. Therefore, the tertiary structure near the substrate-binding site, rather than a short linear peptide sequence, is important for GzmA substrate specificity. Interestingly, P<sup>γ</sup> sites are also important for GzmB substrate and inhibitor binding (45).

The SET complex comes off as a broad peak, which migrates with an apparent mass of 270–420 kDa on gel filtration columns. SET and pp32 are mostly associated with the ER in a variety of cultured cells (K562, HL-60, HeLa, and COS). In the rare cell with nuclear staining, the signal is completely nuclear, and both SET and pp32 colocalize in the nucleoplasm (data not shown). This further supports the contention that these proteins are together in a complex. What mediates nuclear translocation of the complex remains to be explored. The combined molecular masses of SET and pp32 (44 and 33 kDa, respectively) leave room in the SET complex for a number of other components. One of these is GAAD. GAAD can be further separated by chromatography from pp32 and SET. Moreover, neither pp32 nor SET has DNase activity, even after treatment with GzmA. Of note, when recombinant pp32 or SET is expressed in bacteria, a bacterial DNase binds to it, but can be purified from it (data not shown). This suggests that GAAD may have a bacterial homolog.

GzmA may activate GAAD by any of several, not necessarily exclusive, mechanisms. GzmA might (1) facilitate the nuclear translocation of GAAD or its access to DNA, (2) inactivate a protein DNase inhibitor within the SET complex by proteolysis, (3) activate a precursor nuclease within the SET complex by proteolysis, (4) induce modification of the phosphorylation of the SET complex, and/or (5) reconfigure the proteins in the SET complex. Both SET and pp32 have been shown to inhibit protein phosphatase 2A, which suggests that this pathway may be regulated by phosphorylation. Because SET is a GzmA substrate, an attractive and economical explanation for GzmA activation of DNA damage would be that SET is an important component of the DNase inhibitor. However, neither recombinant pp32 nor rSET, alone or in combination, inhibits the in vitro DNase activity (data not shown), making this hypothesis less likely.

GzmA concentrates in the nucleus during CTL-mediated cell death. How GzmA gets into the nucleus is unknown, but it is an ATP-independent process (46). The molecular mass of the covalently linked homodimer (55 kDa) places it above the threshold for passive diffusion into the nucleus. Although GzmA is highly basic (calculated pI 9.1), it does not contain any stretches of basic amino acids that comprise recognized nuclear localization signals. Adding GzmA to isolated nuclei in the absence of any cytosolic component induces lamin and histone cleavage (10, 13). Therefore, the nuclear translocation of GzmA does not require the SET complex. However, disruption of the nuclear lamina may facilitate entry of GAAD into the nucleus or facilitate DNA accessibility.

GzmA degrades histone H1 and cleaves the core histones in isolated nuclei (10). Adding rSET with GzmA to the isolated nuclei does not enhance GzmA-induced histone degradation or the ability of GzmA to enhance DNA degradation by exogenous nuclease in isolated nuclei. These results suggest that GzmA does not use the NAP activity of SET to increase chromatin accessibility. In fact, GzmA cleavage of SET abrogates its NAP activity, and the cleaved p25 SET fragment does not translocate to the nucleus. Therefore, GzmA induces a loss of SET function.

The normal role of the SET complex is unclear, although recent work (21–23) provides some intriguing hints that it may be involved in cellular transformation via transcriptional activation. Identification of the other components of the complex may help to delineate its function in non-apoptotic cells. The SET gene, encoding all but the last 6 amino acids, was first identified in a translocation with a nucleoprin in undifferentiated leukemia. Clearly, changing the subcellular localization of the SET protein, known to interact with MLL (47), another gene frequently involved in leukemia translocations, has a profound effect on cell transformation. Another component of the complex, pp32, is a tumor suppressor (29). Because SET binds to both p300/cAMP response element-binding protein-binding protein transcriptional coactivator proteins (22) and histones (17), SET may link the transcription-activating complex and chromatin, facilitating transcription by unwinding chromatin. Histone binding of SET also inhibits histone acetylation, which is important for regulating transcription (23). A recent report also suggests that a complex containing SET and pp32 in the nucleus associates with HuR, a protein that stabilizes mRNAs with AU-rich sites in the 3′ untranslated region (32). One could imagine that toward the end of transcription, HuR binds to both the SET complex and the nascent mRNA; and that after mRNA splicing, they are transported together to the ER. This would explain the cellular distribution of the SET complex.

If the normal role of the SET complex is to promote transcription and/or mRNA stability, why then would the SET complex contain a DNase? A DNase within the complex might be involved in transcription-related DNA repair. The NAP activity of SET may then serve to reassemble repaired DNA into chromatin. In fact, we have provisionally identified a transcription-related DNA repair enzyme in the SET complex. The high degree of conservation of SET throughout eukaryotic evolution suggests that its function predates the development of CTLs and acquired immunity in invertebrates. The CTL protease likely hijacks and disables the normal function of the SET complex, whatever it may be, and subverts a DNA repair pathway to destroy DNA.

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REFERENCES

1. Henkart, P. A. (1994) Immunity 1, 343–346
2. Shi, L., Kraut, R. P., Aebersold, R., and Greenberg, A. H. (1992) J. Exp. Med. 175, 553–556
3. Darmon, A. J., Nicholson, D. W., and Bleackley, R. C. (1995) Nature 377, 446–448
4. Shi, L., Chen, G., MacDonald, G., Bergeron, L., Li, H., Miura, M., Rotello, R. J., Miller, D. R., Li, P., Seshadri, T., Yuan, J., and Greenberg, A. H. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 11062–11067
5. Quan, L. T., Tewari, M. O., Rourke, K., Dixit, V., Snipas, S. J., Pouirier, G. G., Roy, C., Pickup, D. J., and Salvesen, G. S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1972–1976
6. Sarin, A., Williams, M. S., Alexander-Miller, M. A., Berzofsky, J. A., Zacharchuk, C. M., and Henkart, P. A. (1997) Immunity 6, 209–215
7. Beresford, P. J., Xia, Z., Greenberg, A. H., and Lieberman, J. (1999) Immunity 10, 585–594
8. Thomas, D. A., Du, C., Xu, M., Wang, X., and Ley, T. J. (2000) Immunity 12, 621–632
9. Shresta, S., Graubert, T. A., Thomas, D. A., Raptis, S. Z., and Ley, T. J. (1999) Immunity 10, 595–605
10. Zhang, D., Beresford, P. J., Greenberg, A. H., and Lieberman, J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 5746–5751
11. Beresford, P. J., Kam, C. M., Powers, J. C., and Lieberman, J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9285–9290
12. Beresford, P. J., Jujo, M., Friedman, R. S., Yoon, M. J., and Lieberman, J. (1998) J. Immunol. 161, 161–167
13. Zhang, D., Pasternack, M. S., Beresford, P. J., Wagner, L., Greenberg, A. H., and Lieberman, J. (2001) J. Biol. Chem. 276, 3683–3690
14. von Lindern, M., van Baal, S., Wiegant, J., Baap, A., Hagemeyer, A., and Grosveld, G. (1992) Mol. Cell. Biol. 12, 3346–3355
15. Vassen, M., Barnikol-Watanabe, S., Getz, H., Awni, L. A., Cole, T., Zimmermann, B., Kratzin, H. D., and Hilschmann, N. (1994) Biol. Hoppe-Seyler 375, 113–126
16. Fuji-Nakata, T., Ishimi, Y., Okuda, A., and Kikuchi, A. (1992) J. Biol. Chem. 267, 20890–20896
17. Kawase, H., Okuwaki, M., Miyaji, M., Ohba, R., Handa, H., Ishimi, Y., Fuji-Nakata, T., Kikuchi, A., and Nagata, K. (1996) Gene Cells I, 1045–1056
18. Matsumoto, K., Okuwaki, M., Kawase, H., Handa, H., Hanaoka, F., and Nagata, K. (1995) Mol. Cell. Biol. 15, 9645–9650
19. Nagata, K., Saito, S., Okuwaki, M., Kawase, H., Furuya, A., Kusano, A., Hanai, N., Okuda, A., and Kikuchi, A. (1998) Exp. Cell Res. 240, 274–281
20. Okuwaki, M., and Nagata, K. (1998) J. Biol. Chem. 273, 34511–34518
21. Matsumoto, K., Nagata, K., Okuwaki, M., and Tsujimoto, M. (1999) FEBS Lett. 463, 285–288
22. Shikama, N., Chan, H. M., Kristie-Demonaco, M., Smith, L., Lee, C. W., Cairns, W., and La Thangue, N. B. (2000) Mol. Cell. Biol. 20, 8933–8943
23. Hao, S., McNamara, P., Heo, S., Turner, A., Lane, W. S., and Chakravartti, D. (2001) Cell 104, 119–130
24. Li, M., Makkine, A., and Damuni, Z. (1996) J. Biol. Chem. 271, 11059–11062
25. Santoro, M. F., Annand, R. R., Robertson, M. M., Peng, Y. W., Brady, M. J., Mankovich, J. A., Hackett, M. C., Ghayur, T., Walter, G., Wong, W. W., and Siegel, D. A. (1996) J. Biol. Chem. 273, 13119–13128
26. Mills, J. C., Lee, V. M., and Pittman, R. N. (1998) J. Cell Sci. 111, 625–636
27. Saito, S., Miyaji-Yamaguchi, M., Shimoyama, T., and Nagata, K. (1999) Biochem. Biophys. Res. Commun. 259, 471–475
28. Li, M., Makkine, A., and Damuni, Z. (1996) Biochemistry 35, 6998–7002
29. Chen, T. H., Brody, J. B., Romansev, F. E., Yu, J. G., Kayler, A. E., Voneiff, E., Kuhajda, F. P., and Pasternack, G. R. (1996) Mol. Biol. Cell 7, 2045–2056
30. Fukushima, M., Asano, S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M., and Nishida, E. (1997) Nature 390, 308–311
31. Fornerod, M., Ohno, M., Yoshida, M., and Mattaj, I. W. (1997) Cell 90, 1051–1060
32. Brennan, C. M., Galeouzi, I. E., and Steitz, J. A. (2000) J. Cell Biol. 151, 1–14
33. Xia, Z., Kam, C. M., Huang, C., Powers, J. C., Mandle, R. J., Stevens, R. L., and Lieberman, J. (1998) Biochem. Biophys. Res. Commun. 243, 384–389
34. Shi, L., Kam, C. M., Powers, J. C., Aebersold, R., and Greenberg, A. H. (1992) J. Exp. Med. 176, 1521–1529
35. Adachi, Y., Pavlakis, G. N., and Copeland, T. D. (1984) J. Biol. Chem. 269, 2258–2262
36. Kohler, G., and Milstein, C. (1975) Nature 256, 495–497
37. Roseenthal, A. L., and Lack, S. A. (1977) Anal. Biochem. 80, 76–90
38. Allan, J., Harborne, N., Rau, D. C., and Gould, H. (1982) J. Cell Biol. 93, 285–297
39. Enari, M., Sakahira, H., Yokohama, H., Okawa, K., Iwamatsu, A., and Nagata, S. (1998) Nature 391, 43–50
40. Oberhammer, F., Wilson, J. W., Dive, C., Morris, I. D., Hickman, J. A., Wakeling, A. E., Walker, P. R., and Sikorska, M. (1993) EMBO J. 12, 3679–3684
41. Nakajima, H., Park, H. L., and Henkart, P. A. (1995) J. Exp. Med. 181, 1037–1046
42. Sellins, K. S., and Cohen, J. J. (1991) J. Immunol. 147, 795–803
43. Immler, M., Hertig, S., MacDonald, H. R., Sadoul, R., Becherer, J. D., Proudfoot, A., Solari, R., and Tschopp, J. (1995) J. Exp. Med. 181, 1917–1922
44. Suidan, H. S., Bouvier, J., Schaerer, E., Stone, S. R., Monard, D., and Tschopp, J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8112–8116
45. Sun, J., Whisstock, J. C., Harriott, P., Walker, B., Novak, A. P., Thomas, P. E., Smith, A. L., and Bird, P. I. (2001) J. Biol. Chem. 276, 15377–15384
46. Bink, E. J., Trapani, J. A., and Jans, D. A. (1999) Immunol. Cell Biol. 77, 206–215
47. Adler, H. T., Nallaneth, F. S., Walter, G., and Tkachuk, D. C. (1997) J. Biol. Chem. 272, 28497–28414