Cleaving the oxidative repair protein Ape1 enhances cell death mediated by granzyme A

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The cytolytic T lymphocyte protease granzyme A (GzmA) initiates a caspase-independent cell death pathway. Here we report that the rate-limiting enzyme of DNA base excision repair, apurinic endonuclease-1 (Ape1), which is also known as redox factor-1 (Ref-1), binds to GzmA and is contained in the SET complex, a macromolecular complex of 270–420 kDa that is associated with the endoplasmic reticulum and is targeted by GzmA during cell-mediated death. GzmA cleaves Ape1 after Lys31 and destroys its known oxidative repair functions. In so doing, GzmA may block cellular repair and force apoptosis. In support of this, cells with silenced Ape1 expression are more sensitive, whereas cells overexpressing noncleavable Ape1 are more resistant, to GzmA-mediated death.

Cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells are the key immune effectors that eradicate infected cells or tumors. To destroy these targets, CTLs and NK cells mostly use the granule exocytosis pathway, which releases perforin (PFP) and granzymes (Gzm) from cytolytic granules into the immunological synapse formed with the target[1]. GzmA and GzmB, the most abundant granzymes, are delivered to the target cell cytosol through PFP and independently induce cell death.

The tryptase GzmA activates cell death through a caspase-independent mechanism. GzmA causes characteristic features of apoptosis, including membrane blebbing, loss of mitochondrial transmembrane potential, nuclear fragmentation and chromatin condensation; however, instead of the usual apoptotic double-stranded oligonucleosomal DNA fragmentation, GzmA causes a distinctive form of DNA damage—single-stranded DNA nicking[2–4].

GzmB induces apoptosis through caspase-dependent and caspase-independent mechanisms. GzmB is unique among serine proteases because, like cysteine protease caspases, it cleaves after aspartic acid residues. In fact, when GzmB bypasses caspase activation to initiate apoptosis, it does so by directly cleaving known caspase substrates. The caspase-independent granzyme-activated pathways of cell death provide a failsafe way to eliminate viruses and tumors that can evade caspase-mediated apoptosis by overexpressing inhibitors of apoptosis or proteins of the Bcl-2 family.

Affinity chromatography with the proteolytically inactive S184A mutant of GzmA (S-AGzmA) was used previously to identify potential substrates of GzmA[5]. The cytoplasmic SET complex (270–420 kDa) binds to immobilized S-AGzmA and is a principal target of GzmA[6]. The SET complex, whose function in cells is unknown, contains two previously identified GzmA substrates, the nucleosome assembly protein SET and the DNA-bending protein HMG2, as well as the tumor suppressor protein pp32[4,6]. Despite its presumed nuclear function, the SET complex is associated mostly with the endoplasmic reticulum (ER)[6].

Cleavage by GzmA destroys SET nucleosome assembly activity and DNA binding and bending by HMG2. Treating cells with GzmA and PFP causes SET complex proteins to translocate to the nucleus within minutes (Z.F. *et al.*, manuscript in preparation). The SET complex also reconstitutes GzmA-dependent single-stranded DNA nicking in isolated nuclei[6]. GzmA treatment of the SET complex activates single-stranded nicking in isolated nuclei in the absence of other factors. An unidentified DNase, GzmA-activated DNase (GAAD), in the complex induces the characteristic single-stranded DNA nicks[6].

The size of the SET complex suggests that it contains components in addition to GAAD, HMG2, pp32 and SET. Here we report that Ape1 (also known as APE, APEX, REF-1 and HAP1) is also present in the SET complex and is cleaved and inactivated by GzmA. Ape1 is a multifunctional protein that has DNA binding and endonuclease activity associated with its carboxy terminus and a redox function in its amino-terminal domain[7]. Both of these functions are involved in the early oxidative stress response. Ape1 moves into the nucleus in response to an increase in reactive oxygen species (ROS)[8]. Ape1, the rate-limiting enzyme of the base excision repair (BER) pathway, repairs abasic sites—the most frequent DNA damage in cells caused by oxidation of DNA[9,10]. In addition to repairing DNA, Ape1 also reduces oxidized immediate-early transcription factors, including AP-1 (Fos/Jun), NF-κB and Myb[11,12]. When oxidized, these factors cannot bind to DNA or activate transcription. Ape1 also potently stimulates the DNA binding and transactivation of p53 in response to oxidative stress and proapoptotic signals, by reduction and by additional undefined mechanisms[13].

GzmA cleavage of Ape1 may prevent cellular repair and recovery from the death stimulus. We show here that cells with silenced Ape1 expression are more susceptible, whereas cells that overexpress Ape1

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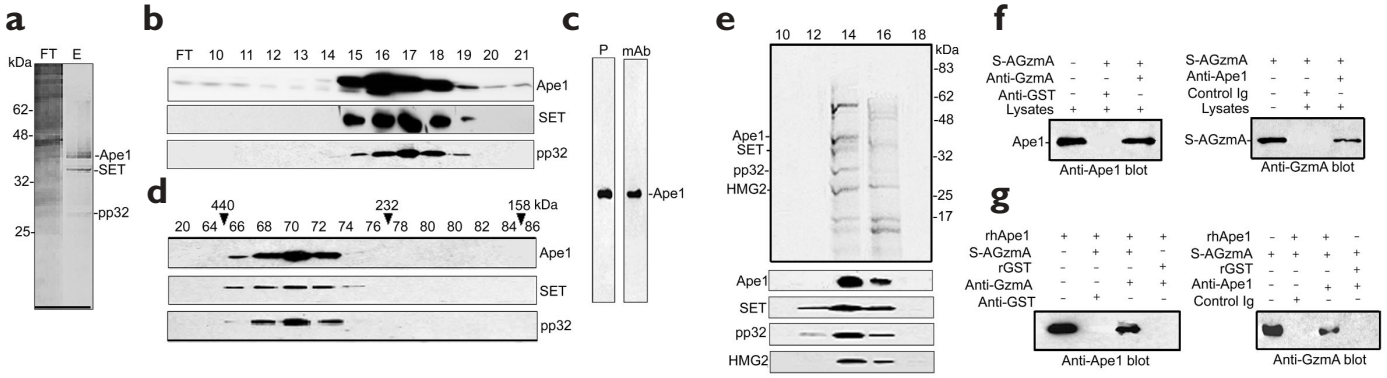

Figure 1. Ape1 co-elutes with the SET complex. (**a**) Coomassie blue staining of the flow-through (FT) and eluate (E) of K562 cytoplasmic lysates applied to immobilized S-AGzmA. The 37-kDa band contained in the eluate was identified by N-terminal sequencing to be Ape1. (**b**) SET, pp32 and Ape1 co-elute from the S-AGzmA column. Fractions were analyzed by immunoblot. (**c**) The Ape1 polyclonal antiserum (P) used in **b** and the Ape1 mAb are highly specific. A single reactive band is present in immunoblots of K562 lysates. (**d,e**) Ape1 copurifies with the SET complex proteins SET, pp32 and HMG2 when the S-AGzmA eluate in **a** is purified sequentially by S400 gel filtration (**d**) and ion exchange Q column (**e**) chromatography. Fractions from the Q column elution were analyzed by silver stain (above) and immunoblot (below). (**f**) Ape1 in K562 cell lysates coprecipitates with S-AGzmA. Cell lysates incubated with S-AGzmA were immunoprecipitated with a mAb to GzmA (left), Ape1 antiserum (right) or a control antibody. Similar results were obtained when purified SET complex was used in place of cell lysates (not shown). (**g**) rhApe1 binds directly to S-AGzmA. rhApe1 and S-AGzmA were co-incubated in PBS for 1 h at 4 °C and then immunoprecipitated as in (**f**).

with a mutated GzmA cleavage site are more resistant, to GzmA-induced cell death.

Results

Ape1 binds to S-AGzmA and is in the SET complex

We identified Ape1 as a possible component of the SET complex by N-terminal sequencing (MPKRGKKGAV) of a Coomassie-stained band of 37 kDa found in the purified SET complex[6] that eluted from an S-AGzmA affinity column (**Fig. 1a**). This was confirmed by probing immunoblots of purified SET complex with Ape1 monoclonal antibody (mAb) or polyclonal antiserum (**Fig. 1b** and data not shown). Both antibodies were highly specific, recognizing a single band of 37 kDa in cell lysates (**Fig. 1c**).

When the SET complex was further purified by gel filtration and ion exchange chromatography, Ape1 copurified with the other known SET complex proteins[6], SET, pp32 and HMG2, in a macromolecular complex of 270–420 kDa (**Fig. 1d,e**). When S-AGzmA was added to K562 cell lysates or purified SET complex, GzmA and Ape1 antibodies, but not control antibody, coprecipitated S-AGzmA and Ape1 (**Fig. 1f** and data not shown). S-AGzmA coprecipitated specifically with recombinant human Ape1 (rhApe1) when the recombinant proteins were mixed *in vitro* in the absence of other proteins (**Fig. 1g**). Therefore, Ape1 binds directly to S-AGzmA.

Although Ape1, pp32 and SET were present in the same complex, the interaction between Ape1 and either pp32 or SET was weak in the absence of other SET complex proteins. Ape1 or pp32 antibodies coprecipitated the two proteins from purified SET complex or from K562 cell lysates (**Fig. 2a** and data not shown). When the two recombinant proteins were mixed *in vitro*, however, they did not coprecipitate

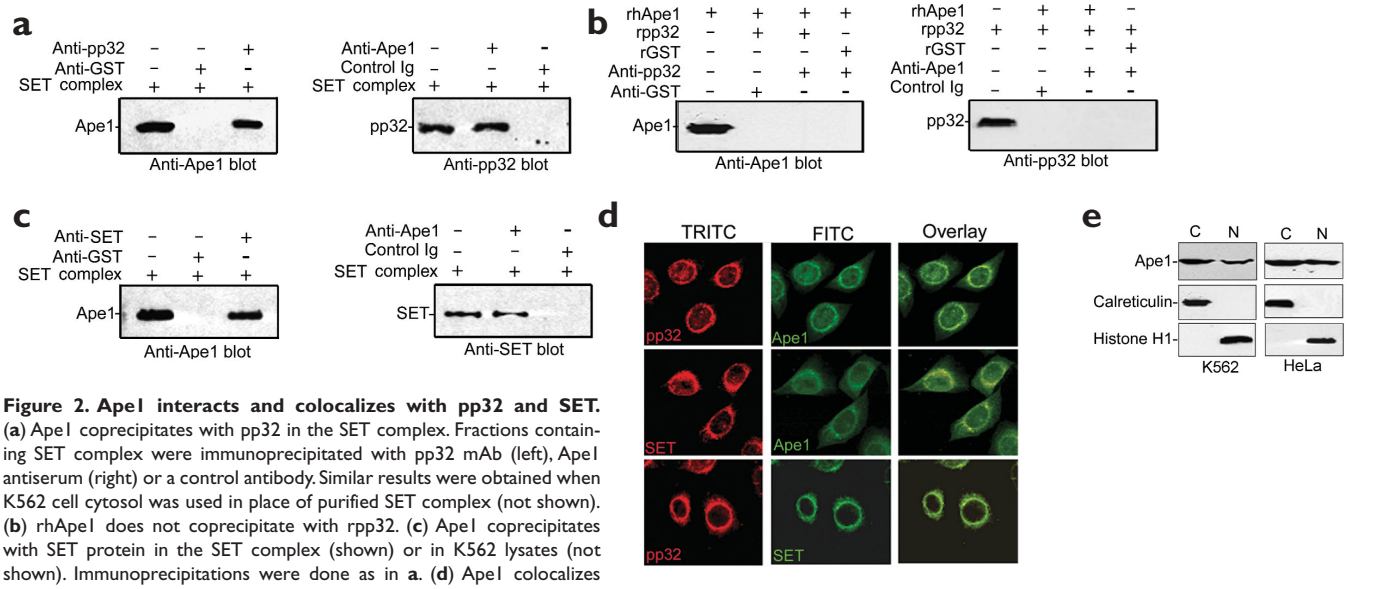

Figure 2. Ape1 interacts and colocalizes with pp32 and SET. (**a**) Ape1 coprecipitates with pp32 in the SET complex. Fractions containing SET complex were immunoprecipitated with pp32 mAb (left), Ape1 antiserum (right) or a control antibody. Similar results were obtained when K562 cell cytosol was used in place of purified SET complex (not shown). (**b**) rhApe1 does not coprecipitate with rpp32. (**c**) Ape1 coprecipitates with SET protein in the SET complex (shown) or in K562 lysates (not shown). Immunoprecipitations were done as in **a**. (**d**) Ape1 colocalizes with pp32 and SET in HeLa cells in a perinuclear distribution. Shown are confocal microscopy images of cells costained for pp32 and Ape1 (top row), costained for SET and Ape1 (middle row), and costained for pp32 and SET (bottom row). TRITC red fluorescence is shown on the left, FITC green fluorescence in the middle, and the merged image on the right. Identical images were obtained with the Ape1 mAb (not shown) and the polyclonal antiserum (shown). (**e**) Ape1 partitions between the cytoplasm (C) and the nucleus (N) in K562 and HeLa cells. NP-40 cell lysates were analyzed by immunoblot, and calreticulin and histone H1 staining verified the cellular fractionation.

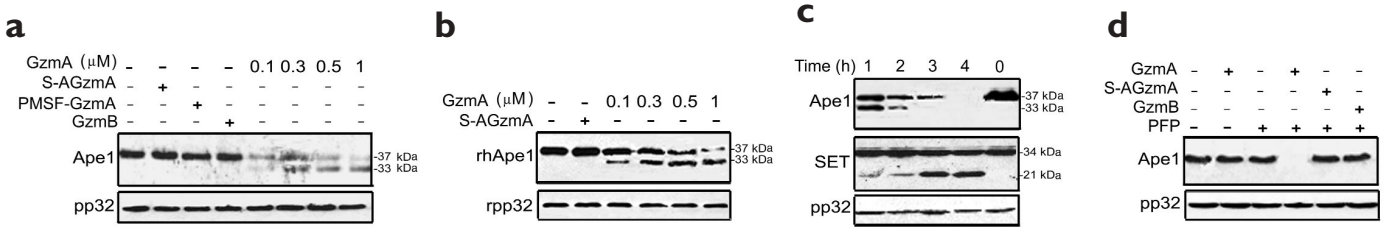

Figure 3. Ape1 is a substrate of GzmA but not GzmB. (**a**,**b**) GzmA, but not enzymatically inactive S-AGzmA or PMSF-treated GzmA or GzmB, cleaves native Ape1 in K562 cell lysates (**a**) or rhApe1 (**b**). K562 cell lysate (2×10^5 cell equivalents), 0.5 μM rhApe1 or 0.5 μM rpp32 was incubated with the indicated concentrations of GzmA or with 0.5 μM S-AGzmA, or PMSF-pretreated GzmA or GzmB, for 2 h at 37 °C, and the products were analyzed by immunoblot. (**c**) Ape1 is degraded in cells treated with PFP and GzmA. K562 cells (2×10^5) were incubated for the indicated times at 37 °C with 2 μM GzmA and sublytic concentrations of PFP. Cells were analyzed by immunoblot for Ape1, SET and pp32. Degradation of both Ape1 and SET is detected within 1 h of loading. The Ape1 cleavage product of 33 kDa, seen *in vitro*, is also visible in GzmA-loaded cells, suggesting that the *in vitro* and *in vivo* cleavage sites are identical. pp32 was unchanged. (**d**) Ape1 is cleaved specifically after treating cells with PFP and GzmA, but not after treatment with comparable concentrations (2 μM) of GzmB or mutant GzmA. Cells were analyzed 4 h after treatment.

(**Fig. 2b**). Similar results were found for the interaction of Ape1 with SET (**Fig. 2c** and data not shown).

Ape1 colocalizes with SET and pp32

Immunoblots of cellular and nuclear fractions of K562 and HeLa cells indicate a predominantly cytoplasmic localization of pp32 and SET[6]; however, localization of pp32 and SET by fluorescence microscopy varies according to the cellular fixation and permeabilization conditions[6]. With harsh conditions (that is, methanol), staining is seen only in the nucleus; with milder conditions, staining is perinuclear. Published studies also report differing results depending on the method and cell type. These differences might be due to denaturation or solubilization of cytoplasmic proteins or disruption of the nuclear membrane during fixation, or to a rapid translocation of SET complex proteins during the time that it takes to complete fixation.

To minimize fixation artifacts, we used the cell fractionation results as the criterion for choosing the microscopy immunostaining protocol. Following this guideline, pp32 and SET are predominantly cytoplasmic and associated with the ER in a perinuclear pattern[6]. Although Ape1 partitioned between the nucleus and the cytoplasm, as assessed by immunoblot and microscopy (**Fig. 2d,e**), cytoplasmic Ape1 concentrated in a perinuclear rim with SET and pp32 and calreticulin (data not shown). When harsher conditions were used in which pp32 and SET were exclusively nuclear, Ape1 staining was also exclusively nuclear (data not shown). These results confirm the colocalization of some, but not all, Ape1 with the SET complex.

GzmA cleaves Ape1

To determine whether Ape1 is a substrate of GzmA, nanomolar concentrations of GzmA were added to either purified SET complex or K562 cell lysates. GzmA cleaved Ape1 from a band of 37 kDa to a band of 33 kDa (**Fig. 3a**). A GzmA-specific, substituted isocoumarin inhibitor[5] and broadly reactive serine protease inhibitors, but not other protease inhibitors (including aprotinin, E-64 and the caspase inhibitors z-DEVD-fmk and z-VAD-fmk; data not shown), blocked cleavage of Ape1. S-AGzmA, and GzmA and GzmB inactivated by phenylmethanesulfonyl fluoride (PMSF), did not cleave Ape1. GzmA also cleaved rhApe1 (**Fig. 3b**). N-terminal sequencing of the rhApe1 fragment (KNDKEAAGEG) indicated cleavage after Lys31 in the sequence KTAAK$^{\downarrow}$KNDK. Cleavage after lysine is consistent with the tryptase activity of GzmA.

Ape1 is degraded in cells treated with PFP and GzmA

To determine whether Ape1 cleavage is physiologically relevant, we treated K562 cells with GzmA and PFP. Ape1 cleavage in the cytosol and nucleus was detectable within 1 h, at the same time as cleavage of SET (**Fig. 3c** and data not shown). As expected, pp32 remained unchanged. In cells treated either with GzmA or PFP alone, or with S-AGzmA or GzmB plus PFP, Ape1 was unchanged (**Fig. 3d**). The GzmA concentration that induced Ape1 cleavage in exposed K562 cells was comparable to that needed to induce cell death, DNA damage, and SET or HMG2 cleavage[2,5,6].

Laser scanning confocal microscopy showed the nuclear translocation and degradation of Ape1 after introducing GzmA into HeLa cells with a sublytic concentration of PFP. With either PFP or GzmA alone, no GzmA was either within or bound to cells (**Fig. 4a**). There were also no changes in chromatin, as assessed by propidium iodide staining, or changes in Ape1 staining (**Fig. 4b**). Twenty minutes after treatment with both GzmA and PFP, some GzmA was visible in a perinuclear pattern, the nuclear chromatin began to condense, and Ape1 had concentrated in the nucleus. The Ape1 remaining in the cytoplasm had moved to a more peripheral location and seemed to be associated with the plasma membrane.

Chromatin condensation was readily apparent within 40 minutes. Cytosolic and nuclear Ape1 staining decreased progressively from 40 minutes to 2 hours. These kinetics were consistent with the immunoblot results (**Fig. 3c**). By 2 hours, nuclear fragmentation and chromatin condensation were pronounced and Ape1 staining was markedly diminished. Cellular immunostaining with the Ape1 mAb gave similar results (data not shown). Thus, GzmA induced apoptotic nuclear morphological changes and chromatin condensation within 1–2 hours. Ape1 degradation occurred with kinetics similar to those observed for other GzmA substrates, such as histones and lamins[14,15].

GzmA disrupts Ape1 redox and BER activity

We next tested whether GzmA cleavage interferes with the oxidative repair functions of Ape1. This endonuclease repairs DNA abasic sites and reduces oxidized transcription factors to enable them to bind DNA and transactivate gene expression. We produced a duplex oligonucleotide apyrimidinic (AP) substrate containing a single abasic site by treating an oligonucleotide containing a single uracil with uracil DNA glycosylase (UDG)[16]. The AP substrate or control untreated DNA was incubated at 4 °C with increasing concentrations of rhApe1 in the presence of EDTA to block the AP endonuclease activity of the enzyme and then analyzed by electromobility shift assay (EMSA). rhApe1 bound to AP substrate, but not to control oligonucleotide, in a dose-dependent manner (**Fig. 5a**).

When rhApe1 was pretreated with GzmA before being added to AP substrate, formation of the Ape1-DNA complex was inhibited in a dose-dependent fashion (**Fig. 5b**). Neither enzymatically inactive S-AGzmA nor recombinant glutathione *S*-transferase (rGST) affected

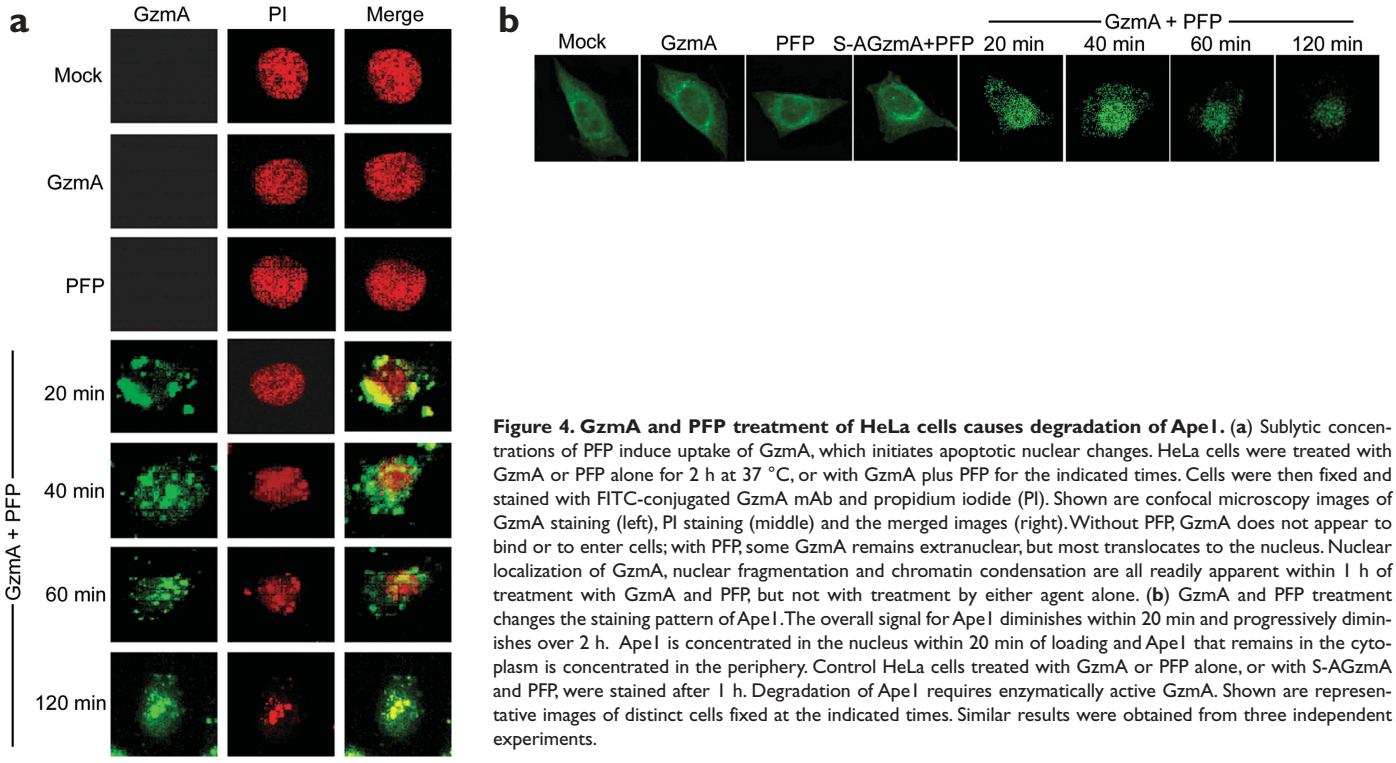

Figure 4. GzmA and PFP treatment of HeLa cells causes degradation of Ape1. (**a**) Sublytic concentrations of PFP induce uptake of GzmA, which initiates apoptotic nuclear changes. HeLa cells were treated with GzmA or PFP alone for 2 h at 37 °C, or with GzmA plus PFP for the indicated times. Cells were then fixed and stained with FITC-conjugated GzmA mAb and propidium iodide (PI). Shown are confocal microscopy images of GzmA staining (left), PI staining (middle) and the merged images (right). Without PFP, GzmA does not appear to bind or to enter cells; with PFP, some GzmA remains extranuclear, but most translocates to the nucleus. Nuclear localization of GzmA, nuclear fragmentation and chromatin condensation are all readily apparent within 1 h of treatment with GzmA and PFP, but not with treatment by either agent alone. (**b**) GzmA and PFP treatment changes the staining pattern of Ape1. The overall signal for Ape1 diminishes within 20 min and progressively diminishes over 2 h. Ape1 is concentrated in the nucleus within 20 min of loading and Ape1 that remains in the cytoplasm is concentrated in the periphery. Control HeLa cells treated with GzmA or PFP alone, or with S-AGzmA and PFP, were stained after 1 h. Degradation of Ape1 requires enzymatically active GzmA. Shown are representative images of distinct cells fixed at the indicated times. Similar results were obtained from three independent experiments.

rhApe1 binding to the AP substrate. When the experiment was repeated in the presence of Mg^{2+} at 37 °C and analyzed for endonuclease activity, GzmA at concentrations as low as 50 nM inhibited AP substrate cleavage (**Fig. 5c**). Because the rhApe1 concentration in this experiment was similar (20 nM), inhibition was probably due to competitive binding rather than to proteolysis.

We next investigated whether GzmA interferes with the ability of Ape1 to carry out its other known function, reduction of oxidized early response transcription factors. For this function, Ape1 itself must be in a reduced state[11]. We reduced rhApe1 by dialyzing it against buffer containing dithiothreitol (DTT). Preincubation of nuclear extracts with reduced, but not oxidized, rhApe1 enhanced protein binding and the electrophoretic mobility gel shift of an oligonucleotide encoding an AP-1 binding site for a Fos-Jun heterodimer in a dose-dependent manner (**Fig. 5d**). Neither reduced nor oxidized rGST control protein induced formation of a retarded DNA-protein complex.

In addition, the retarded DNA-protein complex formed in the presence of reduced rhApe1 was specifically blocked with anti-Ape1 but not control antibody (**Fig. 5e**). Binding to the AP-1 site was inhibited by nanomolar concentrations of GzmA in a dose-dependent fashion (**Fig. 5f**). The required GzmA concentration (10 nM GzmA *versus* 1.4 μM rhApe1) suggested that GzmA inactivation of Ape1 redox activity might be mediated through catalytic inactivation of rhApe1. Taken together, these results show that GzmA blocks Ape1 binding to DNA, its AP endonuclease activity and its redox activation of transcription factors such as AP-1.

Silencing Ape1 expression enhances cell death

Because Ape1 repairs oxidative damage, we thought that GzmA cleavage of Ape1 might prevent cellular repair of CTL targets. To determine

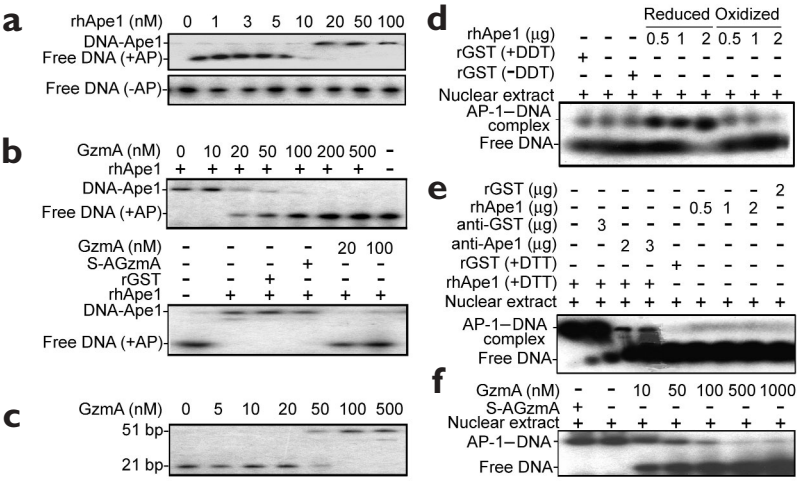

Figure 5. GzmA disrupts the AP endonuclease and redox activities of Ape1. (**a**) Ape1 binds dose-dependently to a double-stranded oligonucleotide containing a single AP site, but not to a control oligonucleotide lacking an AP site (bottom). (**b**) Nanomolar concentrations of GzmA inhibit rhApe1 (20 nM) binding to oligonucleotide DNA containing an AP site, whereas mutant GzmA or rGST control protein has no effect. (**c**) GzmA disrupts Ape1 endonuclease activity. In the presence of Mg^{2+} to activate the endonuclease, increasing concentrations of GzmA inhibit Ape1 cleavage of the 51-bp AP substrate to its expected 21-bp fragment. (**d**) rhApe1 reduced by treatment with DTT enhances the binding of HeLa nuclear extracts to an oligonucleotide containing an AP-1 site, as detected by EMSA. Neither nonreduced rhApe1 nor rGST protein induces binding to the AP-1 site. (**e**) Gel retardation is blocked in the presence of anti-Ape1. (**f**) Active GzmA blocks the Ape1-induced AP-1 DNA-binding activity. HeLa nuclear extracts were preincubated with reduced rhApe1 (1.4 μM) in the presence of the indicated concentrations of GzmA or 1 μM S-AGzmA before adding ^{32}P-labeled AP-1 oligonucleotide. Gel retardation analysis was done as in (**a**).

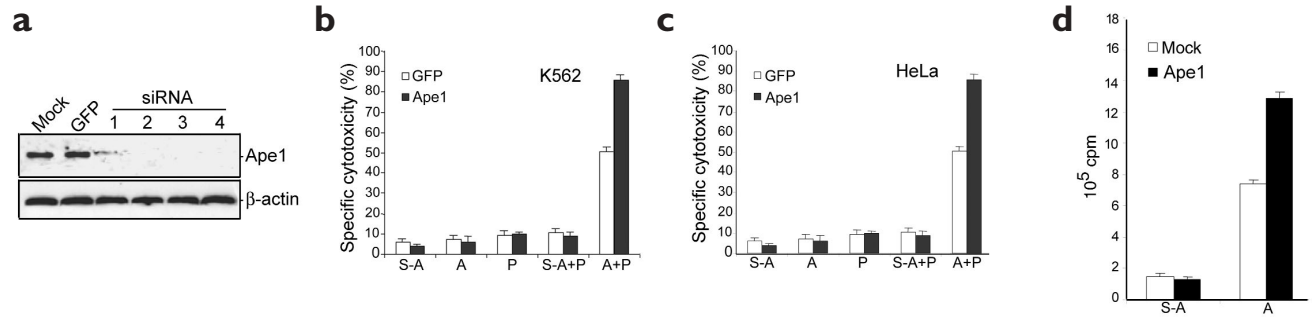

Figure 6. Silencing of Ape1 expression enhances GzmA-mediated cytolysis and DNA nicking. (**a**) Ape1 expression is silenced in HeLa cells after treatment with siRNA duplexes. The four constructs are numbered by their location in the sequence beginning with the most 5′ construct. siRNA 1 blocked Ape1 expression by about 90%, whereas the other constructs blocked all detectable expression of Ape1, as analyzed by immunoblot. siRNAs 3 and 4 were used in the subsequent experiments. The blot was stripped and reprobed with β-actin antibody to verify equal loading. (**b,c**) K562 and HeLa cells in which Ape1 expression has been silenced using Ape1 siRNA 4 are more sensitive than are control cells to GzmA-induced release of ^{51}Cr, measured 4 h after treating cells with GzmA and PFP. Cell death after treatment with inactive S-AGzmA (S-A), GzmA alone (A), PFP alone (P) or S-AGzmA plus PFP (S-A + P) are comparable in the GFP- and Ape1-silenced cells. Data are the means of nine independent experiments for each cell line. (**d**) Ape1 silencing significantly enhances GzmA-mediated DNA damage in isolated K562 nuclei, as measured by radionucleotide incorporation at nicked DNA with Klenow DNA polymerase. Data are the means of three experiments with K562 cells, either mock transfected or transfected with Ape1 siRNA 3 or siRNA 4. Error bars indicate mean ± s.d.

whether Ape1 cleavage is incidental or necessary for GzmA-induced cell death, we silenced Ape1 expression using RNA interference[17]. Four 21-nucleotide short interfering RNA (siRNA) duplexes for Ape1 were separately transfected into K562 and HeLa cells. After 3 days, expression of Ape1 was completely undetectable by immunoblot after transfection with siRNA duplexes 2, 3 or 4, whereas cells transfected with a control siRNA for green fluorescent protein (GFP) showed unchanged expression of Ape1 (**Fig. 6a**). Oligonucleotide siRNA 1 also reduced Ape1 protein by more than 90% as compared with mock transfected cells.

GzmA was added with PFP into ^{51}Cr-labeled K562 or HeLa cells that were either mock transfected, or transfected with siRNA to GFP or Ape1 (**Fig. 6b,c** and data not shown). In four independent experiments, GzmA plus PFP induced 50 ± 2% specific lysis above the PFP background in control transfected K562 cells, but nearly twice that (86 ± 3%) in K562 cells with silenced Ape1 expression ($P < 0.001$). There were no substantial differences between the background lysis (generally < 10%) induced with GzmA or PFP alone, or with PFP and enzymatically inactive GzmA. Similarly, Ape1-silenced HeLa cells were almost twice as susceptible to GzmA with PFP as were control-transfected cells (specific cytotoxicity above background, 70 ± 2% *versus* 42 ± 1%; $P < 0.001$).

The single-stranded DNA damage induced by GzmA can be detected by Klenow DNA polymerase labeling of nicked DNA in isolated nuclei[2]. To determine whether Ape1 also interferes with GzmA-initiated DNA nicking, cell lysates from mock transfected or Ape1-silenced K562 cells were treated with active or mutant GzmA and analyzed for incorporation of [^{32}P]dATP (**Fig. 6d**). In three independent experiments, the amount of nicking in Ape1-silenced cells was greater than in cells expressing Ape1 (mean 10^6 cpm ± s.d.: 1.29 ± 0.04 in Ape1-silenced cells *versus* 0.74 ± 0.02 × 10^6 in mock transfected cells; $P < 0.001$).

Expression of noncleavable Ape1 reduces cell death

To verify further the biological relevance of cleavage of Ape1, we constructed a GzmA-noncleavable mutant of human Ape1 (mApe1) by mutating the GzmA cleavage site Lys31 to alanine. As expected, mApe1 was not cleaved after incubation for 2 hours at 37 °C with a molar excess of GzmA, whereas rhApe1 was efficiently cleaved (**Fig. 7a** and data not shown). The mutation did not alter the redox and AP endonuclease activities of mApe1, as measured by EMSA and cleavage of an oligonucleotide containing an AP site (**Fig. 7b,c**).

Mutant Ape1 was then expressed from pcDNA3.1 in HeLa cells. Three days after transfection, the amount of Ape1 in lysates from cells transfected with pcDNA3.1-mApe1 was 2.7-fold higher than in lysates from pcDNA3.1-transfected cells, as assessed by densitometric analysis of immunoblots (data not shown). After treatment with GzmA and PFP, Ape1 was no longer detectable in control cells after 4 hours but was still present in cells expressing noncleavable mApe1 (**Fig. 7d,e**). These data confirm that the *in vivo* cleavage site of Ape1 is after Lys31. Cleavage of SET was similar in cells transduced with pcDNA3.1 or pcDNA3.1-mApe1, whereas pp32 was not cleaved in either.

HeLa cells expressing noncleavable mApe1 were less susceptible to cytolysis by GzmA and PFP than were cells transfected with the empty vector (**Fig. 7f**). Whereas 74 ± 3% of control cells were lysed, only 30 ± 2% of cells expressing noncleavable mApe1 were lysed ($P < 0.001$). No appreciable lysis occurred when transfected cells were treated with GzmA or PFP alone or with PFP and inactive S-AGzmA. Therefore, GzmA cleavage of Ape1 is important in ensuring GzmA-induced cell death.

Ape1 redox function has a more protective role

To understand the relative importance of the two known Ape1 functions in protection from GzmA-mediated cell death by noncleavable Ape1, we used polymerase chain reaction (PCR) mutagenesis to express noncleavable variants of Ape1 that lacked either the redox or the AP endonuclease activities of Ape1. Mutation of Cys65 specifically disables protein redox activity and mutation of Asp210 specifically destroys the endonuclease function of Ape1[18–20]. Although Cys65 is not on the surface of the original structural model of Ape1, which raises doubts about its role as the active site for redox function, it is not deeply buried and subsequent modeling has suggested that it may be accessible[21].

These essential residues were mutated to alanine in the L31A mutant of mApe1-pcDNA3.1 to express GzmA-noncleavable variants of Ape1 lacking redox function (mApe1C65A), AP endonuclease activity (mApe1D210A), or both (mApe1CDA). Three days after transfecting HeLa cells with these functionally compromised mApe1-containing vectors, Ape1 expression was 2.5- to 2.7-fold higher in cells transfected with the mApe1 mutants than in control pcDNA3.1-transfected cells (**Fig. 7g**). HeLa cells expressing mApe1 that were treated with PFP and GzmA 3 days after transfection were protected

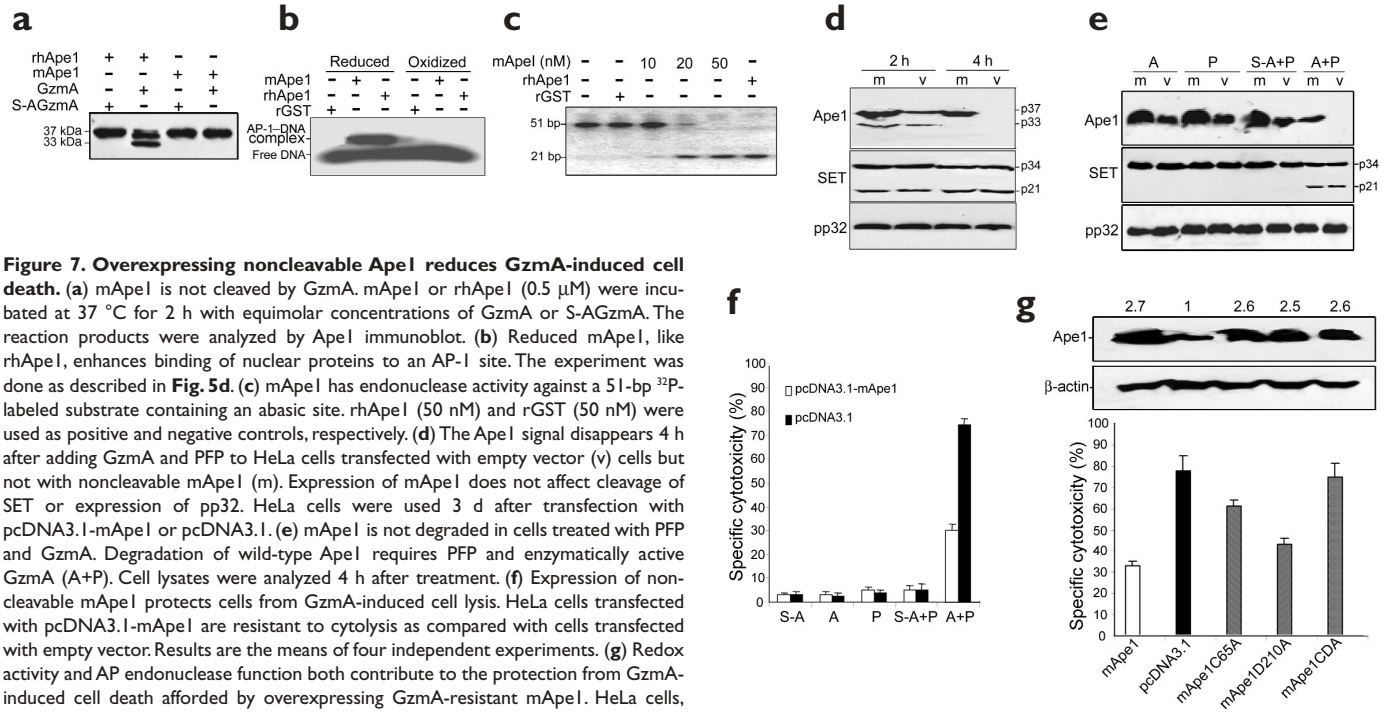

Figure 7. Overexpressing noncleavable Ape1 reduces GzmA-induced cell death. (**a**) mApe1 is not cleaved by GzmA. mApe1 or rhApe1 (0.5 μM) were incubated at 37 °C for 2 h with equimolar concentrations of GzmA or S-AGzmA. The reaction products were analyzed by Ape1 immunoblot. (**b**) Reduced mApe1, like rhApe1, enhances binding of nuclear proteins to an AP-1 site. The experiment was done as described in **Fig. 5d**. (**c**) mApe1 has endonuclease activity against a 51-bp ^{32}P-labeled substrate containing an abasic site. rhApe1 (50 nM) and rGST (50 nM) were used as positive and negative controls, respectively. (**d**) The Ape1 signal disappears 4 h after adding GzmA and PFP to HeLa cells transfected with empty vector (v) cells but not with noncleavable mApe1 (m). Expression of mApe1 does not affect cleavage of SET or expression of pp32. HeLa cells were used 3 d after transfection with pcDNA3.1-mApe1 or pcDNA3.1. (**e**) mApe1 is not degraded in cells treated with PFP and GzmA. Degradation of wild-type Ape1 requires PFP and enzymatically active GzmA (A+P). Cell lysates were analyzed 4 h after treatment. (**f**) Expression of noncleavable mApe1 protects cells from GzmA-induced cell lysis. HeLa cells transfected with pcDNA3.1-mApe1 are resistant to cytolysis as compared with cells transfected with empty vector. Results are the means of four independent experiments. (**g**) Redox activity and AP endonuclease function both contribute to the protection from GzmA-induced cell death afforded by overexpressing GzmA-resistant mApe1. HeLa cells, transfected to overexpress mApe1, empty pcDNA3.1 vector or GzmA-resistant mApe1 defective in redox function (mApe1C65A), BER activity (mApe1D210A) or both (mApe1CDA), were tested for lysis by GzmA and PFP. Blots show expression of the mutant forms of Ape1 and β-actin; the numbers above quantify expression of Ape1 protein relative to β-actin, as determined by immunoblot densitometry. Specific cytolysis of the transfected cells after treatment with GzmA and PFP in two independent experiments is shown below. Error bars indicate mean ± s.d.

from cell death: only 33 ± 2% of cells overexpressing noncleavable mApe1 were lysed as compared with 78 ± 7% of cells transfected with empty vector. Cells transfected with mApe1CDA, in which both redox and AP endonuclease activities were disabled, were as sensitive to GzmA-induced lysis as cells transfected with empty vector (75 ± 6% *versus* 78 ± 7%, difference not significant). Thus, no additional, unknown functional domains in Ape1 contribute to protection from GzmA-mediated cell death.

Cells transfected with noncleavable mutants in which either the redox or the AP endonuclease activity was impaired had intermediate sensitivity to GzmA-mediated cytolysis, but cells expressing the mutant protein that retained redox activity were less vulnerable to cell death. Whereas only 43 ± 3% of cells expressing the mApe1D210A AP endonuclease mutant with redox function were lysed by GzmA, 61 ± 4% of cells expressing the mApe1C65A redox mutant with intact AP endonuclease function were lysed ($P < 0.001$). The difference in GzmA susceptibility for either of these mutants and the GzmA susceptibility of cells transfected with noncleavable mApe1 or empty vector was statistically significant (redox mutant *versus* mApe1, $P < 0.001$, or vector, $P < 0.01$; AP endonuclease mutant *versus* mApe1, $P < 0.01$, or vector $P < 0.001$). Thus, both Ape1 functions helped to protect GzmA-targeted cells from induced cell death when Ape1 was not destroyed by GzmA cleavage; however, the redox function of Ape1 provided more protection from GzmA-mediated cell damage.

Discussion

GzmA induces a caspase-independent cell death pathway that has all the features of apoptosis except that the resultant DNA damage is single-stranded[2]. We previously identified GzmA substrates SET and HMG2 in a multimeric ER-associated complex[4–6]. In isolated nuclei in the presence of GzmA, the SET complex reconstitutes the unique DNA damage that is induced by GzmA[6]. The normal function of the SET complex is, however, unknown.

We have shown here that Ape1, a principal component of the BER pathway, is also associated with the SET complex. Ape1 was coimmunoprecipitated with the SET complex proteins pp32 and SET, and a substantial proportion of Ape1 colocalized with SET and pp32 to associate with the ER. Ape1 rapidly translocated to the nucleus after GzmA loading with PFP. We found that Ape1 is a substrate of GzmA and is cleaved after Lys31. Cleavage by GzmA destroys the known functions of Ape1 in repairing abasic sites and reducing redox sensitive transcription factors to facilitate their binding to DNA. These Ape1 functions are likely to be important in the repair response to GzmA-induced damage, because we found that cells with silenced Ape1 expression are more prone to GzmA-induced cell death, whereas cells expressing noncleavable mApe1 are less susceptible to GzmA-induced death. From the results of our studies of cells expressing GzmA-resistant mutants of Ape1 that lack redox or BER function, we conclude that the redox function of Ape1 would interfere with GzmA-mediated cell death if Ape1 were not destroyed by GzmA cleavage.

Ape1 repairs both oxidative damage to DNA and oxidative changes to transcription factors[9,12]. Therefore, Ape1 may be important in the cellular repair response to oxidative changes triggered during apoptosis. Ape1 is the rate-limiting enzyme for the repair of DNA damage induced by ROS[22,23]. Ape1 rapidly translocates from the cytoplasm to the nucleus in response to ROS[8] and moves into the nucleus after GzmA loading, which induces loss of mitochondrial transmembrane potential and generation of ROS (D. Martinvalet and J.L., unpublished data). Nuclear translocation is probably central to the efficient repair functions of Ape1.

Many of the redox-sensitive transcription factors participate in the early response that enables a cell to survive potentially apoptotic signals; therefore, destroying the function of Ape1 during CTL attack is likely to inhibit repair and to guarantee that the targeted cell is killed. Our experiments in cells with silenced Ape1 expression or expressing GzmA-resistant Ape1 support this hypothesis. They suggest that disabling Ape1 is not incidental but helps to ensure that a cell cannot repair the damage initiated by GzmA and reverse the death program to survive. Ape1 cleavage by GzmA may serve a similar function to cleavage of poly(ADP-ribose) polymerase (PARP) during caspase-mediated apoptosis. PARP is an important participant in BER: as the sensor of single-stranded DNA damage, PARP initiates the DNA repair response in cooperation with p53[24,25]. Caspase cleavage of PARP, like GzmA cleavage of Ape1, blocks DNA repair during apoptosis.

Previous studies have hinted that Ape1 may be involved in resistance to apoptosis. Overexpression of Ape1 confers resistance to apoptosis induced by chemotherapeutic drugs, radiation, hypoxia and tumor necrosis factor[26–29]. Many tumors overexpress Ape1, which may help them to resist apoptosis[29–31]. In addition, expression of Ape1 protein is reduced during apoptosis induced by ischemia in rat neurons and by differentiating agents in leukemia cells[32]. Other apoptotic pathways might also target Ape1; however, Ape1 is not a substrate of GzmB, the other principal CTL granzyme, and has no obvious caspase cleavage sites.

Our immunoblot and fluorescence microscopy analysis showed that Ape1 localizes both to the nucleus and to a perinuclear cytoplasmic region, as determined by costaining with the ER-resident protein calreticulin. Most reports have localized Ape1 to the nucleus, but a few reports have suggested that in some cells, particularly those with high metabolic or proliferative rates, Ape1 is cytoplasmic[30,33]. In fact, Ape1 associates with ribosomes in some motor neurons[33], which is consistent with our observation of ER-associated Ape1. Although its AP endonuclease activity suggests that Ape1 should be localized in the nucleus, the redox activity could operate in either the cytoplasm, where many of the relevant transcription factors are sequestered until needed, or the nucleus.

Ape1 that is associated with the ER might also provide a regulated reserve for activating the BER pathway at times of oxidative stress. This is consistent with the observation that Ape1 translocates in response to ROS[8]. Staining for Ape1 (and for the other SET complex proteins[6]) is highly sensitive to fixation conditions. Preliminary studies suggest that the SET complex rapidly translocates to the nucleus during CTL attack (Z.F. *et al.*, manuscript in preparation), but the signals that mediate its movement in cells remain to be determined. Because fixation disrupts mitochondrial function, it may lead to an increase in ROS. If ROS accumulate before fixation is complete and Ape1 nuclear translocation is rapid enough, then Ape1 might translocate to the nucleus during fixation under some, but not all, conditions. This might explain the apparent discrepancies in the cellular localization of Ape1 in previous studies. The cellular location of the SET complex might also vary between cell types or depend on the activation state of the cell.

The redox activity of Ape1 has been localized through mutational analysis to the N-terminal 127 residues, whereas the AP endonuclease activity is located in a non-overlapping segment encompassing residues 162–318 (ref. 19). The N-terminal 35 amino acids contain a putative bipartite nuclear localization signal and may be involved in nuclear localization. GzmA inhibited both the endonuclease activity and the transcription factor redox activity of Ape1. The concentrations of GzmA that blocked Ape1 activities suggest that GzmA blocks redox function by catalytic cleavage of Ape1, whereas inhibition of DNA repair occurs by competitive inhibition of binding. It is surprising that the redox activity is catalytically blocked by cleavage after Lys31, because deletion of the first 35 residues does not impair redox activity[20]. Two mechanisms could explain how GzmA cleavage of Ape1 destroyed its known redox and endonuclease activities, even though the functional domains were not disrupted. First, in experiments with GzmA-treated Ape1, active GzmA remained in the assay mixtures; therefore, the p33 fragment of cleaved Ape1 might have remained associated with GzmA, thereby masking the Ape1 functional motifs or inducing conformational changes that interfere with function. Second, GzmA might further degrade Ape1 during the assay, because Ape1 was degraded completely with prolonged incubation in all experimental conditions (data not shown).

The normal function of the SET complex is unknown. Does the association of Ape1 with this complex provide any hints? The ER-associated complex of 270–420 kDa contains four known evolutionarily conserved proteins, SET, pp32, HMG2 and Ape1, that have been localized to both the ER and the nucleus, as well as GAAD, a Mg^{2+}-dependent exonuclease that has yet to be identified but can be separated from Ape1 by chromatography (data not shown). Ape1 has been reported to have weak exonuclease activity, but this could be due to a contaminant. In the nucleus, a smaller complex of about 150 kDa, containing SET and pp32 and/or their homologs, binds to CBP/p300 and histones, inhibits histone acetylation and demethylation, and may provide a link between transcriptional activation and chromatin remodeling[34–38]. The larger ER-associated complex is now known to include, in addition to SET and pp32, the DNA bending protein HMG2 (ref. 4) and Ape1, a BER endonuclease and transcription factor activator.

A unifying hypothesis would be that the SET complex is involved in modifying the histone code to open up chromatin, in unwinding chromatin from histones during transcription, in checking and repairing any damaged abasic sites, in activating transcription, and in reassembling repaired DNA into nucleosomes. The possible role of chromatin structure in the BER pathway has not been examined. The above hypothesis implies that other components of the BER pathway, such as DNA glycosylases, may also be present in the SET complex. The elusive GAAD might be normally involved in DNA repair, perhaps in concert with Ape1. Alternatively, the SET complex may be multifunctional, and trying to understand how all of the components fit into one function may be overly simplistic. Identifying the other components of this complex will undoubtedly help to elucidate its functions.

Methods

Cell lines and antibodies. We grew K562 and HeLa cells (ATCC, Manassas, VA) in RPMI1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 2 mM HEPES, 100 units/ml of penicillin, 100 mg/ml of streptomycin and 50 μM β-mercaptoethanol. Mouse monoclonal anti-pp32 (RJ1) and rabbit antiserum to SET peptide 3–16 were produced as described[6,39]. Mouse monoclonal anti-SET (KM1720) was a gift of K. Nagata[40]. Mouse polyclonal anti-GzmA was generated by immunizing mice with 10 μg of GzmA in complete Freund's adjuvant and boosting three times with GzmA in incomplete Freund's adjuvant. We also used the following antibodies: a mAb to Ape1 (Transduction Laboratories, Lexington, KY), rabbit polyclonal antibodies to Ape1 (Santa Cruz Biotechnologies, Santa Cruz, CA), anti-calreticulin and anti–histone H1 (Stressgen Biotech Corporation, Victoria BC, Canada), anti-GST (Clontech, Palo Alto, CA), fluorescein isothiocyanate (FITC)-conjugated mAb CB9 to GzmA (PharMingen, San Diego, CA), mouse β-actin mAb (Sigma-Aldrich, St. Louis, MO), horseradish peroxidase (HRP)-conjugated sheep anti–mouse IgG and HRP-conjugated monkey anti–rabbit IgG (Amersham, Piscataway, NJ), FITC-conjugated goat anti–rabbit IgG (Zymed, South San Francisco, CA), and tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti–mouse IgG (Molecular Probes, Eugene, OR). Unless indicated otherwise, the data shown were obtained with the Ape1 polyclonal antibody.

Recombinant proteins and purified PFP. Recombinant GzmA, S-AGzmA and GzmB were produced and purified as described[5,41]. We purified PFP from the rat RNK-16 cell line and used it at a sublytic concentration as described[42]. Recombinant pp32 (rpp32) was expressed from pET-30a, recombinant SET (rSET) from pET-26b, and rGST from pET-30b (Novagen, Madison, WI) as described[14]. The cDNA for Ape1 inserted into pET-14b was a gift of I. Hickson (University of Oxford, UK). Recombinant proteins with $(His)_6$ tags

were purified sequentially over Novagen nickel and BioRad (Richmond, CA) anion exchange columns.

Cytoplasmic SET complex isolation. K562 cell lysates (10^{10} cell equivalents, obtained from the National Cell Culture Center with support from the National Center for Research Resources, lysed in Nonidet P-40 (NP-40) lysis buffer) were loaded onto an immobilized S-AGzmA column and eluted with 500 mM NaCl in 50 mM Tris-HCl (pH 7.5) as described[5]. The concentrated desalted eluate was applied in Tris-buffered saline (TBS) to an S400 gel filtration column (Amersham). We pooled fractions that contained SET and pp32 according to SDS-PAGE and immunoblot analysis[6]. For some experiments, the SET complex was further purified on a Q column (Pharmacia, Piscataway, NJ) and eluted with 800 mM NaCl in 50 mM Tris-HCl (pH 7.5).

Co-immunoprecipitation and immunoblot. Antibodies were preincubated with protein A–Sepharose (Pharmacia) for 1 h at 4 °C. We washed the antibody-coated beads twice in PBS before adding them to recombinant proteins (50 μg/ml) or cytosolic lysates (5×10^6 cell equivalents in 20 μl of NP-40 lysis buffer) that had been preincubated for 2 h at 4°C with buffer or 50 μg/ml of S-AGzmA. After overnight shaking at 4 °C, the beads were washed extensively in 1% NP-40 plus 0.1% SDS in PBS, and boiled in 2 × SDS loading buffer before being resolved by electrophoresis on SDS-PAGE gels. We analyzed S400 column fractions, or nuclear and cytoplasmic fractions similarly by SDS-PAGE and then transferred the proteins to nitrocellulose before probing with the indicated antibodies as described.

Laser scanning confocal microscopy. Subconfluent HeLa cells, grown in eight-well chamber slides coated with rat collagen I (Becton Dickinson Labware, Bedford, MA), were fixed and permeabilized using the Fix-and-Perm kit (Caltag Laboratories, Burlingame, CA) according to the manufacturer's instructions, and then blocked using permeabilization buffer with 10% goat serum. All antibodies were diluted in permeabilization buffer with 10% goat serum and incubated with the samples at 20 °C; washes between steps were done with PBS. Samples were incubated with primary antibodies for 2 h and then species-specific FITC- or TRITC-conjugated secondary antibodies for 1 h.

For GzmA and PFP loading experiments, cells grown on slides were incubated with medium, GzmA and/or PFP in loading buffer (Hanks' buffered saline solution (HBSS) with 1 mg/ml of bovine serum albumin (BSA), 1 mM $CaCl_2$ and 1 mM $MgCl_2$) at 37 °C for the indicated times and washed with PBS containing 1 mM PMSF before being fixed and permeabilized as above. Fixed and permeabilized cells were stained for Ape1 as above or were incubated with FITC-conjugated CB9 antibody for 1 h at 20 °C, and incubated for 10 min in PBS containing 0.5 μg/ml of propidium iodide and 100 μg/ml of RNase I. We mounted samples using ProLong Antifade mounting medium (Molecular Probes) and left them to dry overnight. Images were acquired with a BioRad Radiance 2000 laser scanning confocal microscope by focusing on the central plane of each cell.

Granzyme *in vitro* cleavage assay. Recombinant hApe1 (0.5 μM) or K562 cell lysates (2×10^5 cell equivalents) were incubated for the indicated times at 37 °C with indicated concentrations of GzmA, S-AGzmA, or PMSF-treated GzmA or GzmB in 20 μl of 50 mM Tris-HCl (pH 7.5), 1 mM $CaCl_2$ and 1 mM $MgCl_2$. We resolved the reaction products on 15% SDS-PAGE gels and transferred them to nitrocellulose for immunoblotting.

Granzyme loading with PFP. K562 cells (2×10^5) in 100 μl of loading buffer were incubated for indicated times at 37 °C with GzmA or GzmB and sublytic concentrations of PFP. Cells were then incubated for an additional 15 min in 1 mM PMSF before being lysed in 20 μl of 0.5% NP-40 lysis buffer containing PMSF. Nuclear pellets were washed twice in NP-40 lysis buffer and extracted in 20 μl of 1% NP-40 lysis buffer containing PMSF. Samples were boiled in 5 × SDS loading buffer before SDS-PAGE and immunoblot analysis.

DNA binding and AP endonuclease assays. Oligonucleotides of 51 bp containing a single uracil at position 22 (22U) and the complementary oligonucleotide (22C) were synthesized and purified by Operon Technologies (Alameda, CA): 22U, 5′-CTTGCATGCCTGCAGGTCGAUTCTAGAGATCCCCGGGTACCGAGCTCGA-3′; 22C, 5′-CGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGC-3′. To generate double-stranded AP substrate, 100 pmol of 22U was 5′ end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). The labeled 22U was annealed with unlabeled complementary 22C. A single AP site was produced by incubating 2 pmol of labeled double-stranded oligonucleotide with 0.1 unit of UDG (New England Biolabs) in 20 μl of 50 mM HEPES (pH 7.8), 1 mM EDTA, 5 mM DTT for 5 min at 37 °C. We used the reaction mixture directly for the binding and AP endonuclease assays.

For binding, 20 nM rhApe1 was pretreated with GzmA for 1 h at 37 °C and then incubated for 10 min at 4 °C in binding buffer (50 mM Tris-HCl (pH 8.4), 1 mM EDTA and 0.2 μg/ml of BSA) containing 2 pmol oligonucleotide substrate. For the AP endonuclease assay, 20 nM GzmA-pretreated rhApe1 was added to Ape1 reaction buffer (10mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 μg/ml of BSA, 0.2 mM DTT) containing 2 pmol of oligonucleotide substrate and incubated for 2 min at 4 °C and then 10 min at 37 °C. We resolved the reaction products by electrophoresis on 6% nondenaturing PAGE gels at 4 °C for 2 h in 6 mM Tris-HCl (pH 7.8), 5 mM sodium acetate and 1 mM EDTA. Radioactive bands in dried gels were visualized by autoradiography.

AP-1 site binding assay. An oligonucleotide encoding an AP-1–binding site derived from the mouse collagenase promoter (5′-AGTGGTGACTCACT-3′) was labeled with [γ-^{32}P]ATP using T4 kinase as described[43]. Nuclear extracts were prepared from nuclear pellets of HeLa cells lysed with 0.5% NP-40 lysis buffer and dissolved in 20 mM Tris-HCl (pH 7.9), 20% glycerol, 0.1 M KCl and 0.2 mM EDTA. We prepared reduced rhApe1 or rGST by dialyzing recombinant proteins into 50 mM sodium phosphate (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 5% glycerol and 5 mM DTT. Oxidized rhAPE or rGST were dialyzed into the same buffer without DTT. HeLa nuclear extract (5 μg) was preincubated with the indicated amount of rhApe1 or 1 μg of rGST, or 1 μg of reduced rhApe1 with the indicated concentrations of GzmA in 20 μl for 60 min at 37 °C before adding ^{32}P-labeled oligonucleotide in 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, 5% sucrose, 2 μg/ml of BSA and 5 mM DTT for a further 30 min at 20 °C. The mixtures were resolved by electrophoresis on 6% nondenaturing PAGE gels as above.

Ape1 silencing by RNA interference. We prepared siRNA duplexes for Ape1 from synthetic 21-nucleotide RNAs (Dharmacon Research, Lafayette, CO) and numbered them in order from the 5′ end of the coding sequence as follows: siRNA 1 (sense, 5′-AAUGUGGAUGGGCUUCGAGCC-3′; antisense, 5′-CUCGAAGCCCAUCCACAUUCC-3′); siRNA 2 (sense, 5′-CUUCAGGAGCUGCCUGGACUC-3′; antisense, 5′- GUCCAGGCAGCUCCUGAAGUU-3′); siRNA 3 (sense, 5′- GUCUGGUACGACUGGAGU ACC-3′; antisense, 5′-UACUCCAGUCGUACCAGACCU-3′); siRNA 4 (5′- AUUACUGCAGGCUGUGCCACU-3′; antisense, 5′-UGGCACAGCCUGCAGUAAUUC-3′). Control duplexes for GFP were prepared with sense (5′-GGCUACGUCCAGGAGCGCAC-3′) and antisense (5′-UGCGCUCCUGGACGUAGCCUU-3′) strand oligonucleotides. siRNAs were deprotected according to the manufacturer's instructions and annealed to form duplex siRNA as described[17]. HeLa or K562 cells (2×10^5 cells/well) were plated into six-well plates in 10% fetal bovine serum (FBS) in Dulbecco's modified Eagle's medium (DMEM) at 37 °C for 12 h before being transfected with siRNA (50 nM final concentration) or control medium in DMEM using Oligofectamine (Life Technologies, Carlsbad, CA) at 37 °C for 6 h with gentle rocking. An equal volume of 20% FBS in DMEM was added and cells were incubated overnight, washed and incubated at 37 °C for an additional 3 d before use. For immunoblotting, washed cells were collected into 5 × SDS loading buffer. For cytotoxicity assays, silenced or control transfected cells were radiolabeled with ^{51}Cr for 1 h and washed before loading with granzyme and PFP as described[2]. After 4 h, ^{51}Cr release in the supernatant of pelleted cells was counted on a Packard Topcount. We calculated specific cytotoxicity using the formula [(sample release) − (spontaneous release)] / [(total release) − (spontaneous release)] × 100.

DNA nicking assay. The Klenow fragment of DNA polymerase was used to label DNA breaks as described[2]. siRNA silenced and mock treated cells (2×10^5) were washed twice with HBSS and resuspended in 40 μl of HBSS plus 1 mg/ml of BSA, 2 mM $MgCl_2$ and 2 mM $CaCl_2$ before being lysed in an equal volume of NP-40 lysis buffer. GzmA or S-AGzmA (3 μg in 20 μl of 140 mM NaCl, 10 mM HEPES (pH 7.2) and 1 mM EGTA) was added, and the treated lysates were incubated at 37 °C for 4 h. Washed nuclear pellets were then incubated at 37 °C for 1 h with 5 units of Klenow (New England Biolabs) and 10 μCi of [^{32}P]dATP (PerkinElmer Life Sciences, Boston, MA) in 10 μl of NP-40 lysis buffer. Radiolabeled nuclei, pelleted by centrifugation for 5 min at 2,580*g*, were washed twice in 5 ml of NP-40 lysis buffer and counted after adding scintillation fluid (PerkinElmer Life Sciences).

Expression of noncleavable forms of Ape1. Human Ape1 was mutated by PCR mutagenesis to replace the GzmA cleavage site Lys31 to alanine using human Ape1 primers containing *Bam*HI and *Xho*I restriction sites: 5′-ATGGGATCCATGCCGAAGCGTGGGA-3′; 5′-ATGCTCGAGTTACAGTGCTAGGTA-3′. PCR mutagenesis was used similarly to mutate Cys65 to Ala and/or Asp210 to Ala in the mApe1 sequence. The mApe1 sequence was cloned into pET26b(+) and confirmed by dideoxynucleotide sequencing. The mutant protein (mApe1) was expressed and purified as for rhApe1 as described above. For overexpression of mApe1 and its inactive variants, mApe1 sequences were cloned into pcDNA3.1(+) and verified by DNA sequencing. HeLa cells (2×10^5) were seeded in six-well plates and transfected with 1 μg of pcDNA3.1-mApe1 or pcDNA3.1 empty vector using 4 μl of Plus solution and 5 μg of Lipofectamine (Life Technologies). After growth for 36–72 h in 10% FBS in DMEM at 37 °C, cells were collected for immunoblotting. Three days after transfection, cells were analyzed as described above for specific cytotoxicity after treating with GzmA and PFP.

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Competing interests statement

The authors declare that they have no competing interests.

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