

Interleukin-1 β -converting Enzyme-like Protease Cleaves DNA-dependent Protein Kinase in Cytotoxic T Cell Killing

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Summary

Cytotoxic T cells (CTL) represent the major defense mechanism against the spread of virus infection. It is believed that the pore-forming protein, perforin, facilitates the entry of a series of serine proteases (particularly granzyme B) into the target cell which ultimately leads to DNA fragmentation and apoptosis. We demonstrate here that during CTL-mediated cytolysis the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), an enzyme implicated in the repair of double strand breaks in DNA, is specifically cleaved by an interleukin (IL)-1 β -converting enzyme (ICE)-like protease. A serine protease inhibitor, 3,4-dichloroisocoumarin (DCI), which is known to block granzyme B activity, inhibited CTL-induced apoptosis and prevented the degradation of DNA-PKcs in cells but failed to prevent the degradation of purified DNA-PKcs by CTL extracts. However, Tyr-Val-Ala-Asp-CH₂Cl (YVAD-CMK) and other cysteine protease inhibitors prevented the degradation of purified DNA-PKcs by CTL extracts. Furthermore, incubation of DNA-PKcs with granzyme B did not produce the same cleavage pattern observed in cells undergoing apoptosis and when this substrate was incubated with either CTL extracts or the ICE-like protease, CPP32. Sequence analysis revealed that the cleavage site in DNA-PKcs during CTL killing was the same as that when this substrate was exposed to CPP32. This study demonstrates for the first time that the cleavage of DNA-PKcs in this intact cell system is exclusively due to an ICE-like protease.

While CTLs can kill their targets by several mechanisms, cell death and DNA fragmentation may be achieved by a combination of the actions of perforin and serine proteases (granzymes) present in their lysosome-like cytoplasmic granules (1, 2). Granule exocytosis delivers perforin, a pore-forming protein to the surface of the target cell, causing membrane damage and ultimately cell death (3). Perforin alone does not cause DNA fragmentation or apoptosis (4), however the granzymes, particularly granzyme B (fragmentin-2), have been implicated in these processes (3, 5–7). Granzyme B is only expressed at high levels in activated CTL, lymphokine-activated killers (LAK), and natural killer (NK) cells (7–9). Inhibition of NK cell-induced cytolysis by inhibitors of serine proteases, such as isocoumarin, provides evidence that granzyme B or a related pro-

tease participates in the apoptotic process (10, 11). Furthermore, the generation of mice with a homozygous null mutation in the granzyme B gene demonstrates that this enzyme plays a critical, non-redundant role in the rapid induction of DNA fragmentation and apoptosis in CTL targets (9). Two recent reports provide evidence that this role is probably indirect and is achieved by cleaving and activating an IL-1 β -converting enzyme (ICE)¹-like protease (12, 13).

Although the degradation of DNA into oligonucleoso-

¹Abbreviations used in this paper: DCI, 3,4-dichloroisocoumarin; ICE, interleukin-1 β -converting enzyme; PARK, poly (ADP-ribose) polymerase; TLCK, tosyl-l-lysine chloromethyl ketone.

mal sized fragments has been shown to be a characteristic of apoptosis for some time (14), and while a number of candidate endonucleases have been reported (15–18), the factor responsible for DNA fragmentation remains elusive. An intriguing observation stems from the report that CTL granule proteinases can induce the internal disintegration pathway in target cells (19, 20). One such proteinase, granzyme B, when it gains access to the target cell through perforin, causes fragmentation in DNA. The identity of the target proteins of granzyme B that trigger apoptosis remains unclear, but a recent report has shown that purified enzyme cleaves and activates CPP32 (12), the precursor of an ICE-like protease that cleaves Poly (ADP-ribose) polymerase (PARP) during apoptosis (21–24). PARP is one of a few key substrates degraded during the process of apoptosis (23, 25), which additionally include nuclear lamin (26, 27), U1-70 small nuclear ribonucleoprotein (28), fodrin (29), actin (30), topoisomerase 1 (25, 31), and DNA-PKcs (32, 33). In this study, we demonstrate that the catalytic subunit of DNA-dependent kinase is specifically cleaved during CTL-mediated cytolysis. Furthermore, we show that granzyme B or another serine protease activates an ICE-like protease to cleave DNA-PKcs during this process.

Materials and Methods

Reagents. Leupeptin, E-64, pepstatin, tosyl-L-lysine chloromethyl ketone (TLCK), tosyl-L-phenylalanine chloromethyl ketone (TPCK), and 3,4-dichloroisocoumarin (DCI), were obtained from Boehringer Mannheim (Indianapolis, IN). PMSF, iodoacetamide, and *N*-ethylmaleimide were from Sigma Chem. Co. (St. Louis, MO), and Tyr-Val-Ala-Asp-CH₂Cl (YVAD-CMK) from Bachem (Torrance, CA). Protease inhibitor stock solutions were made up as follows: PMSF, 0.2 M in methanol; leupeptin, 1 mg/ml in water; pepstatin, 1 mg/ml in methanol; E-64, 10 mg/ml in a mixture of ethanol/water; TLCK, 20 mM in methanol; TPCK, 40 mM in ethanol; DCI, 50 mM in DMSO; iodoacetamide, 100 mM in DMSO; *N*-ethylmaleimide, 100 mM in DMSO, and YVAD-CMK, 2 mM in DMSO.

The following antibodies were used for immunoblotting of DNA-PKcs. Ab-SLY, a polyclonal antibody raised against amino acid region 1550–1840 of DNA-PKcs; DPK1, a polyclonal antibody raised against amino acid region 2018–2136; Ab9607, a polyclonal antibody against amino acid region 2790–3065; Ab42-47, a monoclonal antibody recognizing the kinase (exact sequence undefined) domain and Ab-FLA an antibody against the whole molecule. A mouse polyclonal antibody that detects both Ku70 and 80 was also used (34).

Induction of Apoptosis During Cytotoxic T Cell Killing. The CTL clone was generated as described previously (35). Clone LC13 is HLA B8-restricted and it recognizes the epitope FLRGRAYGL. In this system CTLs act as both effector cells and target cells (35). For CTL-mediated cytolysis peptide FLRGRAYGL was added to U-shaped microtiter wells (10 mM) containing 10⁵ cells and incubated for 15 min at 37°C before centrifugation at 200 *g* and further incubation at 37°C for the times indicated in individual experiments, before harvesting and extraction of proteins. An irrelevant peptide EENLLDFVRE (10 mM) was used as a control.

An HLA B44-restricted clone, but not clone LC13 recognizes this EBV epitope (35).

Western Blot Analysis. After the various treatments, cells were pelleted at 200 *g*, followed by two washes in ice-cold PBS, pH 7.2, and were then resuspended in extraction buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 25 mM NaF, and 25 mM β-glycerolphosphate, pH 7.5), 0.2% Triton X-100, 0.3% NP-40, 0.1 mM sodium vanadate, 0.1 mM PMSF, leupeptin (5 mg/ml), aprotinin (5 mg/ml), and left on ice for 10 min. After centrifugation at 10,000 *g* for 10 min at 4°C, the supernatant was collected and cell extract was assayed for protein using the Bio-Rad microassay (Bio-Rad Labs., Richmond, CA). One-fifth of a volume of 5 times concentrated sample buffer (0.25 M Tris-HCl, pH 6.8, 0.4 M DTT, 5% SDS, 0.5% Bromophenol Blue, 50% glycerol) was added to each sample and boiled for 5 min before storage at –80°C. Electrophoresis was performed on 20 μg of protein sample on 8% SDS-PAGE. Proteins were then transferred to PVDF membranes and immunoblotting was performed with the appropriate antibody using an ECL kit (Amersham Corp., Arlington Heights, IL). In some cases, blots were reprobbed with different antibodies after stripping in 62.5 mM Tris, 100 mM 2-β-mercaptoethanol/2% SDS, pH 6.7.

DNA-PK Activity Assay. Whole cell extracts were prepared from CTL cells treated with FLRGRAYGL epitope according to procedures described previously (36). DNA-PK activity was assayed with a synthetic peptide corresponding to the NH₂-terminal region of human p53 gene product (PESQAEFADLWKK) at 0.25 mM as described previously (36). Each sample was also assayed in the presence of non-substrate peptide EPPLSEQFADLWKK. Incorporation of phosphate into this peptide was less than 5% of that for the substrate peptide, and values were subtracted from the appropriate samples.

In vitro, DNA-PK Cleavage by Granzyme B and CPP32 Proteases. DNA-PKcs was purified as described previously (34). Ku proteins were also prepared as part of this protocol. Purified granzyme B was used in the cleavage studies (37, 38). This enzyme was shown to be free of other granzyme activities such as tryptase (grA) and Met-ase. The recombinant form of the ICE-like protease CPP32 was prepared as a bacterial lysate as described previously (39). For cleavage of DNA-PKcs 10 ng of purified granzyme B was incubated with 0.2 μg of purified protein in a 20-μl reaction volume containing 10 mM Hepes, pH 7.4, 0.1 mM EDTA, and 5 mM DTT for 2 h at 37°C. Incubations also contained sonicated calf thymus DNA (0.2 μg). The reactions were terminated by addition of 5 ml of loading buffer (5 times concentrated) followed by separation on 8% SDS-PAGE and immunoblotting was carried out with DPK1 antibody. Ku (0.2 μg) was incubated with 10 ng of purified granzyme B as described for DNA-PKcs. Incubations also contained DNA-PKcs (0.2 μg) and sonicated calf thymus DNA (0.2 μg). Immunoblotting was carried out with polyclonal antibody against Ku (34). For cleavage with CPP32, 2 μl of bacterial lysate containing the recombinant protein was incubated with DNA-PKcs (0.2 μg), Ku (0.2 μg), and sonicated calf thymus DNA (0.2 μg), in a 20-μl reaction volume containing 25 mM Hepes, pH 7.5, 5 mM EDTA, 5 mM DTT, and 0.1% 3-[(3-cholamidopropyl) dimethylamino]-1-propanesulfonate (CHAPS) for 20 min at 37°C.

Preparation of Extracts for DNA-PK Cleavage In Vitro. Cell extracts were prepared from CTL clone LC13 cells treated with specific epitope FLRGRAYGL 4 h post-treatment. Cells were pelleted at 200 *g*, followed by two washes in ice-cold PBS, pH 7.2, and then lysed by sonication in lysis buffer containing 25

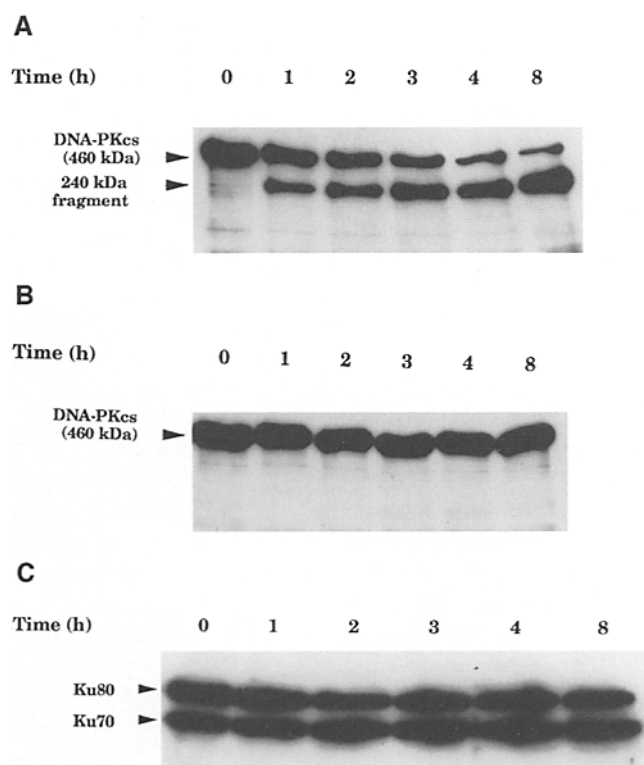
mM Hepes, pH 7.5, 5 mM EDTA, 2 mM DTT, 0.1% CHAPS, and 1 mM PMSF, 2 mg/ml each of aprotinin, leupeptin, and pepstatin. The lysates were centrifuged at 16,000 *g* for 10 min, and the supernatants collected and stored at -20°C until required. For cleavage of DNA-PKcs, extracts (4 μg of total protein) were preincubated with either leupeptin (210 mM), PMSF (1 mM), YVAD-CMK (10 mM), *N*-ethylmaleimide (5 mM), or iodoacetamide (5 mM) for 30 min at 37°C before addition of DNA-PKcs (0.2 μg) and Ku (0.2 μg) and sonicated calf thymus DNA (0.2 μg) and then incubated for a further 1 h at 37°C in a 20-ml reaction volume before separation on 8% SDS-PAGE and immunoblotting was carried out with DPK1 antibody.

Microsequencing. Purified DNA-PKcs was digested with either CPP32 or extracts from cells undergoing apoptosis as described above. Fragments were separated by 8% SDS-PAGE followed by transfer to PVDF membranes. The 150- and 120-kD fragments generated by DNA-PKcs cleavage after digestion with CPP32 and the 150-kD fragment generated by digestion of DNA-PKcs with cell extracts from CTL clone CL13 cells were sequenced using an 473A Gas Phase sequencer (Applied Biosystems, Inc., Foster City, CA).

Results and Discussion

We have recently shown that the catalytic subunit (DNA-PKcs) of DNA-dependent protein kinase (DNA-

PK) is preferentially degraded in Burkitt's lymphoma and other cell types undergoing apoptosis (32). The DNA-binding component of this enzyme, Ku, remained intact and inhibitor and *in vitro* studies implicated the ICE-like protease CPP32 in the degradation of DNA-PKcs. Members of the ICE-like family of proteases require the presence of an Asp residue at the P1 position for cleavage (22), a property shared with the cytotoxic T cell-specific protease granzyme B making it unique among serine proteases (1). Since granzyme B is necessary for the induction of DNA fragmentation and apoptosis in CTL-mediated killing it was possible that DNA-PKcs might be a direct target for this enzyme or it could involve the activation of an ICE-like protease such as CPP32 to cleave DNA-PKcs. To address this question we employed Epstein-Barr Virus-specific CTL (clone LC13) which we have demonstrated previously to be effective targets for their own lysis when incubated in the presence of a specific epitope but not in the presence of irrelevant epitopes (35). The results in Fig. 1 *A* demonstrate that when CTL clone LC13 is incubated with cognate peptide FLRGRAYGL a time-dependent degradation of DNA-PKcs to a 240-kD fragment is observed when immunoblotting is carried out with antibody DPK1 prepared against amino acid region 2018-2136 of the pro-



PKcs using an ECL kit (Amersham). (B) Time course in the presence of irrelevant peptide. Clone LC13 was also incubated in the presence of an irrelevant peptide (EENLLDFVRE, 10 mM). An HLA B44-restricted clone but not clone LC13 recognizes this EBV epitope (35). (C) Lack of degradation of Ku70 and Ku80, the DNA-binding component of DNA-PK, during apoptosis in CTL clone LC13 in the presence of specific peptide with time after exposure. (D) Time course of DNA-PK activity in CTL clone LC13 incubated with specific epitope (FLRGRAYGL). DNA-PK activity was assayed with a synthetic peptide (PESQAEFADLWKK) substrate as described previously (47). Each sample was also assayed in the presence of non-substrate peptide EPP-LSEQFADLWKK.

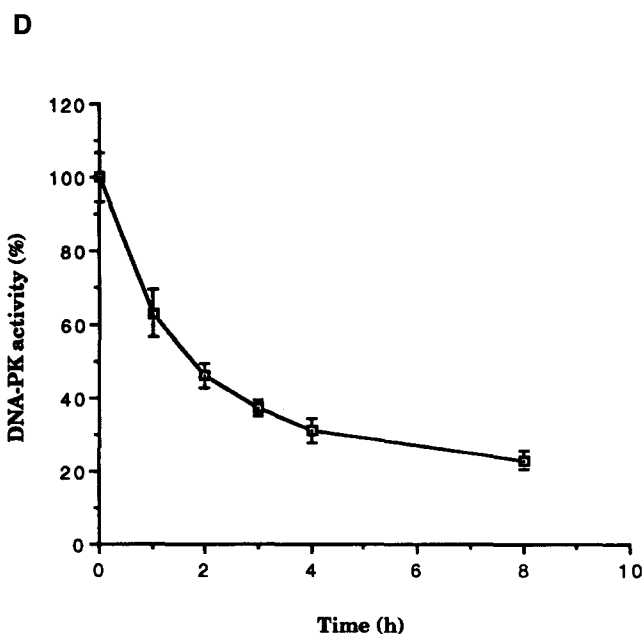


Figure 1. (A) Time course of cleavage of DNA-PKcs after exposure of Epstein Barr Virus-specific CTL clone LC13 to specific peptide. The CTL clone was generated as described previously (35). Extracts were prepared as described in Materials and Methods, electrophoresed on 8% SDS-PAGE and immunoblotting was performed with DPK1 antibody which recognizes a peptide containing amino acids 2018-2136 in DNA-

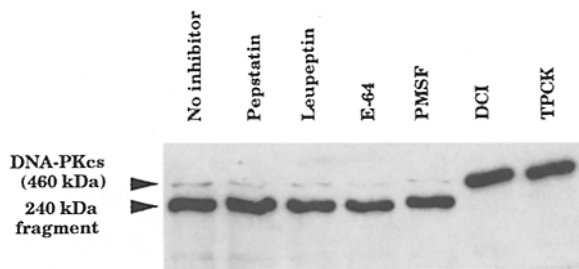


Figure 2. Effect of protease inhibitors on DNA-PKcs cleavage in CTL clone LC13 undergoing apoptosis. Cells were exposed to the following inhibitors pepstatin (3.6 mM), leupeptin (210 mM), E-64 (50 mM), PMSF (1 mM), DCI (200 mM), and TPCK (200 mM) for 30 min before exposure to specific peptide for 15 min followed by harvesting 4 h later. DNA-PKcs was detected with DPK1 antibody.

tein (40). After 4 h incubation with FLRGRAYGL ~60% degradation of DNA-PKcs is observed which agrees well with the extent of specific lysis at this time point as measured by chromium release (35). The size of this fragment is comparable to that occurring in other cell types exposed to a variety of agents that cause apoptosis (32). Exposure of CTL clone LC13 to a non-specific epitope (EENLLD-FVRE) which fails to elicit lysis in this clone did not cause any degradation of DNA-PKcs up to 8 h incubation (Fig. 1 B). Under these conditions there was no evidence for degradation of either Ku70 or Ku80, the DNA-binding component of DNA-PK (Fig. 1 C).

As expected, the activity of DNA-PK in CTL clone LC13 was markedly reduced with time after the addition of cognate epitope FLRGRAYGL, being reduced to 31% of that in untreated cells after 4 h (Fig. 1 D). This reduction in activity paralleled the degradation of DNA-PKcs and is also in agreement with data observed in etoposide treated Burkitt's lymphoma cells (32). In an attempt to identify the nature of the protease involved in DNA-PKcs degradation, we employed a number of protease inhibitors. TPCK (cysteine and serine protease inhibitor) and DCI (serine protease inhibitor) prevented apoptosis during CTL killing (results not shown), and were also capable of inhibiting the degradation of DNA-PKcs, while other inhibitors such as leupeptin, pepstatin, PMSF, and E64 failed to do so (Fig. 2). It was not possible to use iodoacetamide and *N*-ethylmaleimide, two cysteine protease inhibitors, with whole cells because of their toxicity. These results suggested that a serine type protease, such as granzyme B, might be responsible for the degradation of DNA-PKcs (41). To substantiate this further, purified DNA-PKcs was exposed to granzyme B. In the presence of Ku, DNA-PKcs was degraded to a fragment of similar size (240 kD) to that observed in CTL undergoing apoptosis, as detected with DPK1 antibody (Fig. 3 A, lane 3). When Ku was excluded from the incubation mix cleavage was less effective with an intermediate-sized fragment being evident (Fig. 3 A, lane 2). Use of an antibody specific for Ku (34) demonstrated that there was some cleavage of both Ku70 and Ku80 by granzyme B under these conditions (Fig. 3 B, lane 2). No

degradation of Ku was observed in CTL (Fig. 3 B, lane 3) or Burkitt's cells (Fig. 3 B, lane 4) undergoing apoptosis or when the purified complex was exposed to CPP32 (lane 5).

Use of an antibody that detected all regions of DNA-PKcs (Ab-FLA) also revealed only a single degradation fragment of 240 kD after incubation of DNA-PKcs with granzyme B (Fig. 3 C, lane 2). Furthermore, the same cleavage pattern was observed when purified DNA-PKcs was incubated with granzyme B at 10 ng over the time range 0–3 h (Fig. 3 D; lanes 1–6) or when purified DNA-PKcs was incubated with granzyme B over the dose range 0–20 ng for a period of 2 h (Fig. 3 E; lanes 1–6). Use of other antibodies specific for different regions of DNA-PKcs also failed to detect fragments other than the 240-kD fragment (results not shown). When Ab-FLA was used to detect DNA-PKcs in extracts from CTL undergoing apoptosis two fragments of ~240 and 150 kD were observed (Fig. 3 C, lane 3). This pattern was similar to that observed with extracts from Burkitt's lymphoma cells undergoing apoptosis induced by etoposide (Fig. 3 C, lane 4). The ICE-like protease CPP32, when incubated with purified DNA-PKcs, also produced fragments of 240 and 150 kD and an additional strong band was observed at 120 kD (Fig. 3 C, lane 5). It was not possible to detect the 120-kD band in either the CTL or Burkitt's cell lanes under these conditions. However, we have previously shown that a weaker 120-kD band is produced during apoptosis in Burkitt's cells (32). To further distinguish between a role for granzyme B or an ICE-like protease in this process, extracts were prepared from CTL exposed to specific peptide for 4 h. As observed in cells undergoing apoptosis, these extracts also degraded DNA-PKcs to a 240-kD fragment as detected with DPK1 antibody (Fig. 4, lane 2). YVAD-chloromethylketone (CMK), a specific inhibitor of ICE-like proteases, iodoacetamide and *N*-ethylmaleimide, cysteine protease inhibitors, prevented the degradation of DNA-PKcs by CTL extracts (Fig. 4, lanes 5–7, respectively). Neither DCI nor TPCK were able to prevent the degradation of DNA-PKcs by CTL extracts (Fig. 4, lanes 8 and 9, respectively), in contrast to the results obtained in whole cells where both inhibitors were effective in preventing DNA-PKcs degradation and onset of apoptosis (Fig. 2). These data suggest that DNA-PKcs is a direct target for an ICE-like protease in CTL-mediated cytotoxicity and that granzyme B participates in the process further upstream, possibly activating the ICE-like protease.

We have previously shown that both extracts from Burkitt's lymphoma cells undergoing etoposide-induced apoptosis and CPP32 incubated with DNA-PKcs gave rise to 150- and 120-kD fragments (32). Furthermore, NH₂-terminal sequencing revealed that the site of cleavage that generated the 150-kD fragment was DEVD↓N (amino acids 2709–2713) in both cases. The 120-kD fragment is produced from within the 150-kD fragment by cleavage at DWVD↓G (amino acids 2979–2983). Both sequence motifs would be expected to be recognized by ICE-like proteases (22, 42). Microsequencing of the 150-kD fragment generated from incubation of DNA-PKcs with CTL ex-

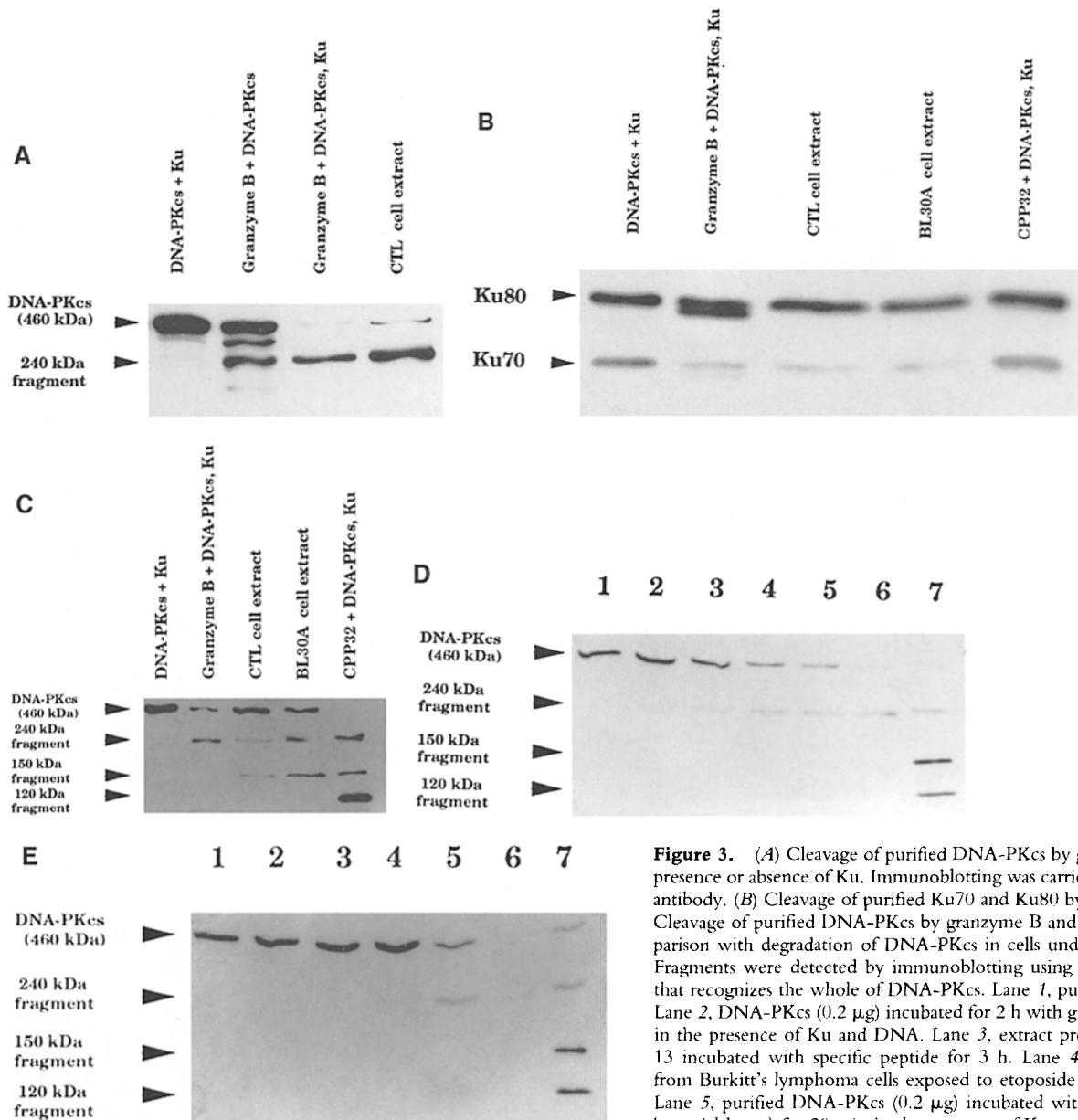


Figure 3. (A) Cleavage of purified DNA-PKcs by granzyme B in the presence or absence of Ku. Immunoblotting was carried out with DPK1 antibody. (B) Cleavage of purified Ku70 and Ku80 by granzyme B. (C) Cleavage of purified DNA-PKcs by granzyme B and CPP32 and comparison with degradation of DNA-PKcs in cells undergoing apoptosis. Fragments were detected by immunoblotting using Ab-FLA antibody that recognizes the whole of DNA-PKcs. Lane 1, purified DNA-PKcs. Lane 2, DNA-PKcs (0.2 μ g) incubated for 2 h with granzyme B (10 ng) in the presence of Ku and DNA. Lane 3, extract prepared from clone 13 incubated with specific peptide for 3 h. Lane 4, extract prepared from Burkitt's lymphoma cells exposed to etoposide (68 mM) for 3 h. Lane 5, purified DNA-PKcs (0.2 μ g) incubated with CPP32 (2 μ l of bacterial lysate) for 20 min in the presence of Ku and DNA. (D) Time course of cleavage of purified DNA-PKcs by granzyme B in the presence of Ku and comparison with degradation of purified DNA-PKcs by CPP32 in the presence of Ku. Immunoblotting was carried out with Ab-FLA antibody. Lanes 1–6, purified DNA-PKcs (0.2 μ g) incubated with granzyme B (10 ng) in the presence of Ku and DNA for 0, 0.5, 1, 1.5, 2, 3 h. Lane 7, DNA-PKcs (0.2 μ g) incubated with CPP32 (2 μ l of bacterial lysate) for 20 min in the presence of Ku and DNA. (E) Effect of different doses of granzyme B on cleavage of purified DNA-PKcs in the presence of Ku after 2 h incubation and comparison with degradation of purified DNA-PKcs by CPP32 in the presence of Ku. Immunoblotting was carried out with Ab-FLA antibody. Lanes 1–6, purified DNA-PKcs (0.2 μ g) incubated with granzyme B at 0, 1.25, 2.5, 5, 10, and 20 ng for 2 h in the presence of Ku and DNA. Lane 7, DNA-PKcs (0.2 μ g) incubated with CPP32 (2 μ l of bacterial lysate) for 20 min in the presence of Ku and DNA.

tracts demonstrated that cleavage occurred at the same DEVD↓N site, providing further support for the direct involvement of an ICE-like protease in DNA-PKcs degradation during T cell killing.

In the present study we have demonstrated for the first time that DNA-PKcs is an important substrate in CTL-mediated cytolysis and its cleavage in this intact cell system is exclusively due to an ICE-like protease. Although both the catalytic and regulatory subunits of DNA-PK are cleaved

by granzyme B *in vitro*, this enzyme is not a physiological substrate for granzyme B. Direct cleavage of DNA-PKcs is mediated by an ICE-like protease, and granzyme B, which plays a major role in CTL killing, appears to be involved upstream from this protease. Recent studies suggest that this is the case. Darmon et al. (12) demonstrated that granzyme B cleaved *in vitro* translated CPP32, and the cleaved product was active and capable of cleaving PARP an important substrate in apoptosis. Proteolytic activation of CPP32 by

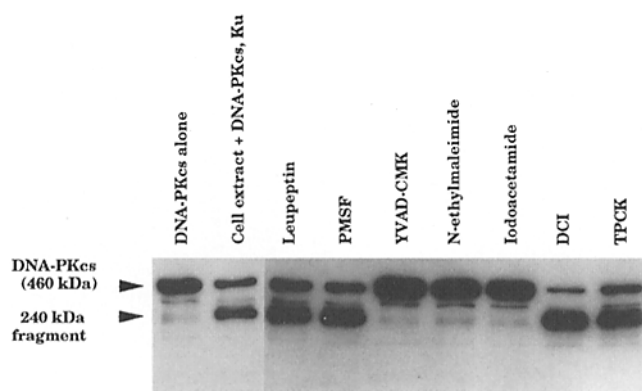


Figure 4. Effect of inhibitors on degradation of purified DNA-PKcs by extracts prepared from CTL clone LC13 undergoing apoptosis induced with specific peptide epitope. Cell extracts were prepared from CTL clone LC13 cells treated with specific peptide, 4 h post-treatment. Immunoblotting was carried out with DPK1 antibody.

granzyme B was also demonstrated by Quan et al. (13). In addition, the Nedd2 precursor can be processed by purified granzyme B (Harvey, N.L., J.A. Trapani, and S. Kumar, unpublished observation). CPP32 has been shown to

cleave PARP (21–24), and DNA-PKcs represents only the second substrate for this enzyme described here in CTL-killing and during apoptosis in Burkitt's lymphoma cells treated with etoposide (32). Since both of these enzymes are implicated in DNA repair (43–50) their loss would facilitate the onset of DNA fragmentation and apoptosis. However, since PARP knockout mice (51) and SCID mice (lacking active DNA-PKcs) are able to develop essentially normally it seems likely that these proteins are not the only targets during apoptosis. Indeed, other substrates such as nuclear lamin, fodrin, actin, and the U1-70-kD protein are cleaved during apoptosis (26–30). As suggested by Martin and Green (52) "too many cuts in too many places result in a critical change in the cell and it suddenly collapses into apoptotic death." All of these data point to the importance of several critical targets, not all of which are required to be degraded in response to a single apoptotic stimulus. We have shown that, in CTL killing, activation of an ICE-like protease by granzyme B or another serine protease leads to the degradation of one such critical substrate (DNA-PKcs). Clearly, it is important to identify other substrates involved in this form of T cell killing in order to understand the nature of the pathway(s) involved.

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