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Cleavage and inactivation of DNA-dependent protein kinase catalytic subunit during apoptosis in *Xenopus* egg extracts

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SUMMARY

DNA-dependent protein kinase (DNA-PK) consists of a 460 kDa subunit that contains the catalytic domain (DNA-PKcs) complexed with two polypeptides of 70 kDa and 80 kDa (Ku70 and Ku80) which comprise the Ku autoantigen. DNA-PKcs requires association with DNA via Ku for catalytic activation and is implicated in double strand break repair, V(D)J recombination and transcription. We have utilised a cell-free system of concentrated *Xenopus laevis* egg extracts to investigate the regulation and possible functions of DNA-PK. Recently, we have shown that this system can reproduce events of apoptosis, including activation of an apoptotic protease that cleaves poly(ADP-ribose) polymerase. Here, we report that DNA-PK is rapidly inactivated with the onset of apoptosis in this system. Loss of activity is concomitant with cleavage of the catalytic subunit, whereas the Ku subunits are stable. Cleavage and inactivation of DNA-PKcs is prevented by prior addition of the anti-apoptotic protein Bcl-2 or inhibition of an apoptotic protease that has characteristics of the CPP-32/Ced-3 family of cysteine proteases that cleave poly(ADP-ribose) polymerase. These results suggest that cleavage and inactivation of DNA-PKcs prevents this factor from functioning in DNA repair, recombination or transcriptional regulation during apoptosis.

Key words: DNA-dependent protein kinase, Apoptosis, Programmed cell death, Protease, *Xenopus*

INTRODUCTION

DNA-PK is a nuclear protein-serine/threonine kinase that needs to be associated with DNA to be catalytically active. It is composed of a large subunit of approximately 460 kDa (DNA-PKcs) that contains a catalytic domain related to phosphatidylinositol 3-kinase (Hartley et al., 1995) complexed with a DNA binding component that is the autoantigen Ku (Dvir et al., 1992; Gottlieb and Jackson, 1993). Ku was originally characterized as a nuclear antigen recognised by sera from patients with various autoimmune diseases (Reeves, 1992). It consists of two polypeptides of approximately 70 and 80 kDa (Ku70 and Ku80, respectively) that form a tightly bound heterodimer that binds double-strand breaks and other aberrations in DNA molecules (Blier et al., 1993; Griffith et al., 1992; Mimori and Hardin, 1986; Paillard and Strauss, 1991; Zhang and Yaneva, 1992). Ku recruits DNA-PKcs to the DNA and this binding is required for activation of the kinase (Gottlieb and Jackson, 1993). Genetic mutations leading to the loss of DNA-PKcs or Ku result in hypersensitivity to ionising radiation due to defective double strand break repair; cells lacking DNA-PK are also defective in V(D)J recombination, a process that involves site-specific DNA breaks and rearrangements (Blunt et al., 1995; Boubnov et al., 1995; Finnie et al., 1995; Lees-Miller et al., 1995; Peterson et al., 1995; Rathmell and Chu, 1994; Smider et al., 1994; Taccioli et al., 1994), suggesting that the protein kinase activity of DNA-PK is required during these processes. DNA-PK has also been implicated in trancriptional regulation (Genersch et al., 1995; Giffin et al., 1996; Kuhn et al., 1995).

In addition to DNA-PKcs, several other large proteins homologous in their carboxy-terminal kinase domains have been identified which form a subgroup of the phosphatidylinositol 3-kinase superfamily (Jackson, 1995, 1996; Keith and Schreiber, 1995). One of these proteins, the human *ATM* gene product is mutated in ataxia-telangiectasia (AT) (Savitsky et al., 1995). AT cells have increased genomic instability, are hypersensitive to ionising radiation and are defective in cell cycle checkpoints induced by DNA damage. Other members of this group include the products of *MEC1/ESR1* (Kato and Ogawa, 1994; Paulovich and Hartwell, 1995) and *TEL11* (Greenwell et al., 1995; Morrow et al., 1995) in budding yeast, *Rad3* in fission yeast (Seaton et al., 1992) and *Drosophila Mei-41* (Hari et al., 1995). These gene products have also been implicated in chromosome stability, DNA repair and cell cycle checkpoints, suggesting that members of this subfamily have roles in the detection and/or response to aberations in genomic integrity throughout the eukaryotic kingdom. However,

different members of the subfamily may have distinct functions within the same cell (Jackson, 1996).

DNA damage may also induce apoptosis, or programmed cell death. Other stimuli, including depravation of extracellular survival signals or ligation of Fas, can trigger this process, but most cells undergoing apoptosis exhibit similar changes, including chromatin condensation and nuclear fragmentation, suggesting a common biochemical mechanism. In many cases, genomic DNA is severed into oligonucleosomal fragments that produce a characteristic ladder when separated on agarose gels. Apoptosis in many (perhaps all) cells involves the activation of cysteine proteases related to the Ced-3 gene product of *Caenorhabditis elegans* (Yuan et al., 1993) and human CPP-32 (Fernandes-Alnemri et al., 1994; Nicholson et al., 1995), members of the interleukin-1β-converting enzyme (ICE) family (Kumar, 1995). One of the substrates identified for these proteases is poly(ADP-ribose) polymerase (PARP) (Kaufmann et al., 1993; Lazebnik et al., 1994). PARP has been implicated in sensing DNA damage. It binds to DNA strand breaks and is activated, catalysing the poly(ADP)-ribosylation of itself and possibly other DNA-binding proteins (de Murcia and Menissier de Murcia, 1994), although its precise function is unclear. During apoptosis, PARP is cleaved at a site DEVD↓G releasing the catalytic domain from the DNA-binding domain (Kaufmann et al., 1993; Lazebnik et al., 1994). The isolated catalytic domain is insensitive to DNA damage and the DNA-binding domain can act as a dominant inhibitor by binding to DNA strand breaks (Molinete et al., 1993).

Recently, we have developed a cell-free system of *Xenop*us egg extracts that reproduces the activation of apoptotic proteases in vitro (Cosulich et al., 1996; Newmeyer et al., 1994). PARP cleavage occurs after 1 hour of incubation of the extracts and about 2 hours before the onset of chromosome condensation and DNA fragmentation. This cleavage is completely blocked by a tetrapeptide derivative (Ac-DEVD-CHO) based upon the cleavage site in PARP that is a potent inhibitor of human CPP-32 and closely related proteases (Nicholson et al., 1995). Interestingly, activation of a *Xenopus* apoptotic PARP protease (XAPP) with similar characteristics to CPP-32/Ced-3 is negatively regulated by the addition of Bcl-2 anti-apoptotic oncoprotein to the extracts (Cosulich et al., 1996). In this report, we have investigated the possible role of DNA-PK in this system. Initially, the egg extracts contain DNA-PK activity, but upon incubation and activation of XAPP, DNA-PK is rapidly inactivated. Inactivation is concomitant with cleavage of the catalytic subunit into two large fragments and is inhibited by prior addition of Ac-DEVD-CHO or Bcl-2. In contrast, the Ku subunits are stable. These results suggest that cleavage and inactivation of DNA-PKcs by a CPP-32/Ced-3-like protease is an early event during the execution of apoptosis in this system.

MATERIALS AND METHODS

Preparation of *Xenopus* egg extracts and HeLa nuclei

Xenopus egg extracts were prepared at 4°C as 10,000 ***g*** supernatants supplemented with an ATP-regenerating system, cytochalasin B, cyclohexamide and 5% (v/v) glycerol (Clarke, 1995; Félix et al., 1993). They were snap frozen in 100 µl aliquots and stored in liquid nitrogen. Extracts prepared in this way contain both nuclear and cytoplasmic proteins present in the eggs. They contain, typically, 30-40 mg protein/ml, as well as lipid vesicles and mitochondria, including a dense fraction required for the initiation of apoptosis (Newmeyer et al., 1994). Incubations were initiated immediately after thawing the extracts at 23°C. Where described, HeLa cell nuclei (Cosulich et al., 1996) were added to the extracts at the start of incubation in 1/10 volume to give a final concentration of 1,000/µl. In most extracts prepared in this way, nuclei undergo condensed chromatin and DNA cleavage typical of apoptosis after 2.5-4 hours of incubation (Cosulich et al., 1996). Where stated, Sf21 cell lysates containing Bcl-2 (Cosulich et al., 1996) were added at 1/100 final dilution. The tetrapeptide aldehyde Ac-DEVD-CHO (SNPE, Strasbourg, France) was dissolved in 100 mM Hepes, pH 7.4, and added to the extracts at the concentrations shown.

DNA-PK assays

DNA-PK activity was assayed using a kit according to the protocol of the manufacturer (Amersham). Briefly, the egg extracts were diluted 1/150 (unless stated otherwise) in the assay to a final concentration of approximately 0.2 mg protein/ml. Assays were carried out at 30°C for 30 minutes (unless stated otherwise) using as an exogenous substrate a peptide derived from a sequence in human p53 (EPPLSQEAFADLLKK) that includes a site phosphorylated by DNA-PK (serine 15) (Lees-Miller et al., 1992). Assays were done in the presence or absence of calf thymus DNA, and DNA-PK activity was calculated as the difference between these values. For the pull-down assay, we used essentially the protocol of Finnie et al. (1995): the extracts were diluted five times in buffer A (25 mM Hepes/KOH, pH 7.9, 50 mM KCl, 10 mM $MgCl_2$, 20% glycerol, 0.1% Nonidet P-40, 1 mM dithiothreitol). The extracts were incubated with 40 µl of preswollen double-stranded DNA-cellulose (Sigma) for 15 minutes at room temperature. After two washes in 1 ml of buffer A, the dsDNA-cellulose was resuspended in 20 µl of buffer A; 5 µl was used for the DNA-PK assay and 15 µl of SDS loading buffer was added to the remaining sample for SDS-PAGE. In all cases, DNA-PK assays were performed at least in duplicate and the means of these values, which agreed within ±10% are shown.

SDS-PAGE and immunoblotting

During incubation of the extracts at 23°C, samples were analysed on polyacrylamide gels and transferred to nitrocellulose membranes for immunoblotting. In the case of nuclear proteins, the nuclei were recovered and washed as described previously (Cosulich et al., 1996). Primary antibodies were a human autoimmune serum recognizing human Ku70 and Ku80 (a gift from W. H. Reeves), polyclonal sera raised against human DNA-PKcs and human Ku70 (Serotec, Oxford, UK) and a monoclonal against Ku80 (Le Romancer et al., 1994). The antigen-antibody complexes were revealed by an enhanced chemiluminescence procedure according to the manufacturer (Amersham).

RESULTS

In an attempt to measure the activity of DNA-PK in *Xenopus* egg extracts we utilised a peptide derived from human p53 containing a site phosphorylated exclusively by DNA-PK (Finnie et al., 1995; Lees-Miller et al., 1992). We found that kinase activity towards this peptide was readily detected in egg extracts and was stimulated about 5-fold by double stranded DNA (Fig. 1A). This DNA-dependent activity was inhibited by wortmannin, an inhibitor of phosphatidylinositol 3-kinase (Fig. 1B). The sensitivity to wortmannin was similar to that reported for mammalian DNA-PK (Hartley et al., 1995), with half-maximal inhibition between 50 and 100 nM. These results indicate that *Xenopus* egg extracts contain an activity closely

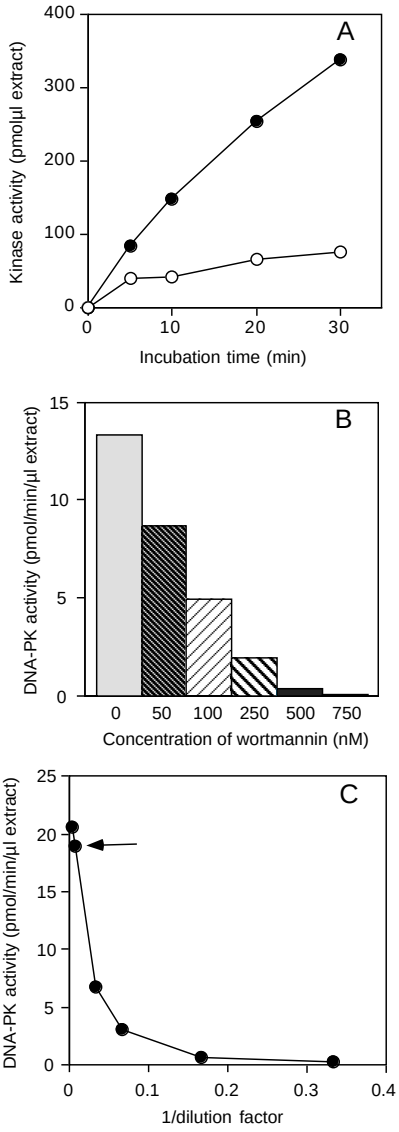

Fig. 1. DNA-PK activity in *Xenopus* egg extracts. (A) Time course of DNA-PK activity assayed in the *Xenopus* system. Egg extract (at a final dilution of 1/150) was incubated at 30°C with the p53 peptide in the presence (●) or absence (○) of dsDNA for the times shown. (B) Inhibiton of DNA-PK activity by wortmannin. Incubations were carried out as in A in the presence of dsDNA and the concentration of wortmannin shown. (C) DNA-PK activity was assayed using different concentrations of egg extract. The activity at each final dilution of the extract in the assay is shown. The arrow indicates the concentration of extract used in other experiments (1/150 final dilution).

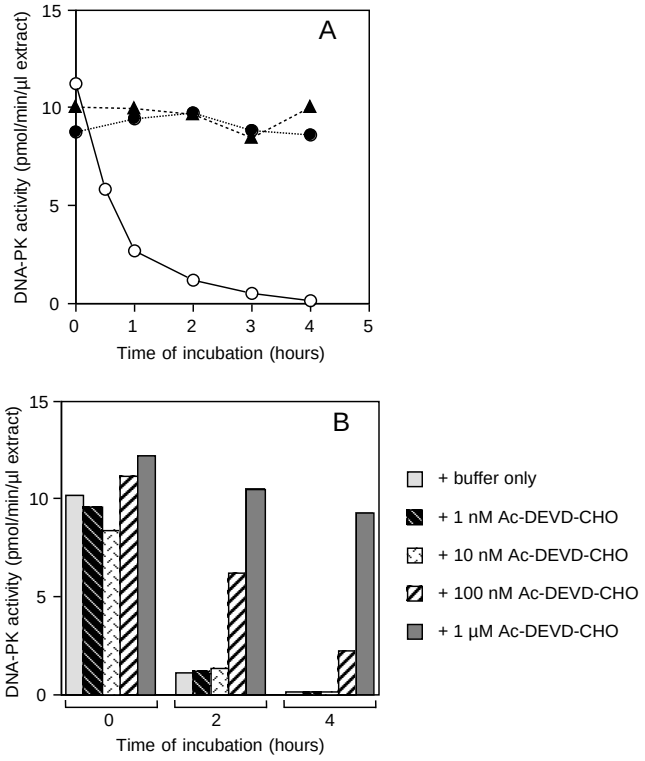

Fig. 2. DNA-PK is inactivated upon incubation of apoptotic *Xenopus* egg extracts. (A) Egg extract was incubated at 23°C with the addition of lysate from Sf21 cells expressing Bcl-2 (1/100 final dilution, ●), 1 µM Ac-DEVD-CHO (▲) or buffer as a control (○). Samples were removed at the times shown and assayed for total DNA-PK activity. (B) Inhibition of DNA-PK inactivation by Ac-DEVD-CHO. Incubations were carried out with the addition of Ac-DEVD-CHO at the concentrations shown and samples removed for assay of DNA-PK at the start of the incubation and after 2 and 4 hours.

related to mammalian DNA-PK. However, DNA-PK activity was strongly inhibited when high concentrations of extracts were used in the assay (Fig. 1C). Inhibition was not relieved by adding more DNA or the protein phosphatase inhibitor microcystin-LR (1 µM) to the assays (data not shown), suggesting that it was not due to competition for DNA binding sites or dephosphorylation of the substrate during the assay. It remains possible that inhibition was due to the presence of competing endogenous substrates for DNA-PK or inhibitors of the kinase present in the extracts. In the experiments described below, DNA-PK assays were carried out for 30 minutes using extracts diluted 150-fold. Under these conditions, kinase activity was not greatly dependent on the concentration of the extracts and was approximately linear with respect to time (Fig. 1A).

Previously, we and others have shown that incubation of *Xenopus* egg extracts may result in apoptotic chromosome condensation and DNA fragmentation in exogenous nuclei added to the extracts (Cosulich et al., 1996; Newmeyer et al., 1994). We examined DNA-PK activity during the incubation of *Xenopus* egg extracts that undergo apoptotic DNA fragmentation after 2.5 hours (not shown; Cosulich et al., 1996). We found that DNA-dependent activity was dramatically decreased by approximately 50% within 30 minutes and decreased to less than 10% after 3 hours (Fig. 2A). In contrast, DNA-PK activity was stable in egg extracts that did not exhibit apoptosis (not shown). In extracts that undergo apoptosis, chromosome condensation and DNA fragmentation is prevented by addition of the anti-apoptotic oncoprotein Bcl-2 at the start of the incubation (Cosulich et al., 1996; Newmeyer et al., 1994). Strikingly, Bcl-2 added in Sf21 cell lysates completely stabilised DNA-PK activity in the extracts (Fig. 2A), strongly suggesting that the inactivation of DNA-PK is related to the onset of apoptosis.

One of the first events apparent during apoptosis in egg

extracts is the rapid activation of an apoptotic protease that cleaves PARP (Cosulich et al., 1996). This protease is inhibited by Ac-DEVD-CHO, a tetrapeptide aldehyde based on the site of cleavage in PARP that is a potent inhibitor of cysteine proteases related to human CPP-32 and *C. elegans* Ced-3 (Cosulich et al., 1996; Nicholson et al., 1995), indicating that the *Xenopus* protease is a similar enzyme. Activation of this *Xenopus* apoptotic PARP protease (XAPP) and PARP cleavage is greatly delayed by Bcl-2 (Cosulich et al., 1996), indicating that at least one of the functions of Bcl-2 is to negatively regulate the activation of apoptotic proteases. We therefore examined the effect of Ac-DEVD-CHO on the inactivation of DNA-PK. When 1 μM Ac-DEVD-CHO was added at the start of the incubation, DNA-PK activity was maintained at initial levels for at least 4 hours (Fig. 2A). At 100 nM, Ac-DEVD-CHO partially inhibited inactivation, whereas 10 nM was ineffective (Fig. 2B). Both the time course of DNA-PK inactivation and its sensitivity to Ac-DEVD-CHO is similar to the characteristics of PARP cleavage in these extracts (Cosulich et al., 1996; S. C. Cosulich et al., unpublished). Taken together, these results strongly suggest that the inactivation of DNA-PK in apoptotic egg extracts is due to the activation of a protease that also cleaves PARP, is negatively regulated by Bcl-2 and has very similar characteristics to CPP-32/Ced-3-like proteases.

We investigated the cause of inactivation by examining the stability of the components of DNA-PK during incubation of the extracts. For this experiment, we incubated HeLa nuclei in the *Xenopus* egg extracts under the same conditions used to examine the cleavage of human PARP in the nuclei (Cosulich et al., 1996). At certain times during the incubation, samples were removed, and the nuclei were recovered by centrifugation. Nuclear proteins were analysed by SDS-PAGE and immunoblotted using polyclonal antibodies that recognise components of human DNA-PK. At the start of the incubation, a single high molecular mass polypeptide was detected by an antibody to the 460 kDa catalytic subunit of DNA-PK (DNA-PKcs). However, following incubation of the nuclei in the extracts, two reactive polypeptides of approximately 170 kDa and 300 kDa appeared with a concomitant decrease in the amount of intact DNA-PKcs (Fig. 3A). This suggested that human DNA-PKcs was being cleaved to produce two major products during incubation of the extracts, similar to the cleavage of DNA-PKcs in cultured human cells undergoing apoptosis (Casciola-Rosen et al., 1995; Song et al., 1996). When Ac-DEVD-CHO or Bcl-2 was added at the start of the incubation and the nuclear proteins analysed after 3 hours, the cleavage of DNA-PKcs and the appearance of the lower molecular mass fragments was significantly inhibited (Fig. 3B). By contrast, Ku70 and Ku80 present in the nuclei were stable during the course of the incubation (Fig. 3C). These results show that human DNA-PKcs in nuclei added to the extracts is cleaved with very similar kinetics to the loss of DNA-PK activity and is also sensitive to inhibitors and regulators of XAPP.

Since we had measured the total activity of *Xenopus* DNA-PK in the extracts in the absence of intact nuclei, we wished to confirm that inactivation was due to cleavage of the *Xenopus* catalytic subunit. Using the antibody to human DNA-PKcs, we could detect a weak signal from an endogenous polypeptide present in the extracts that comigrated on SDS-PAGE with

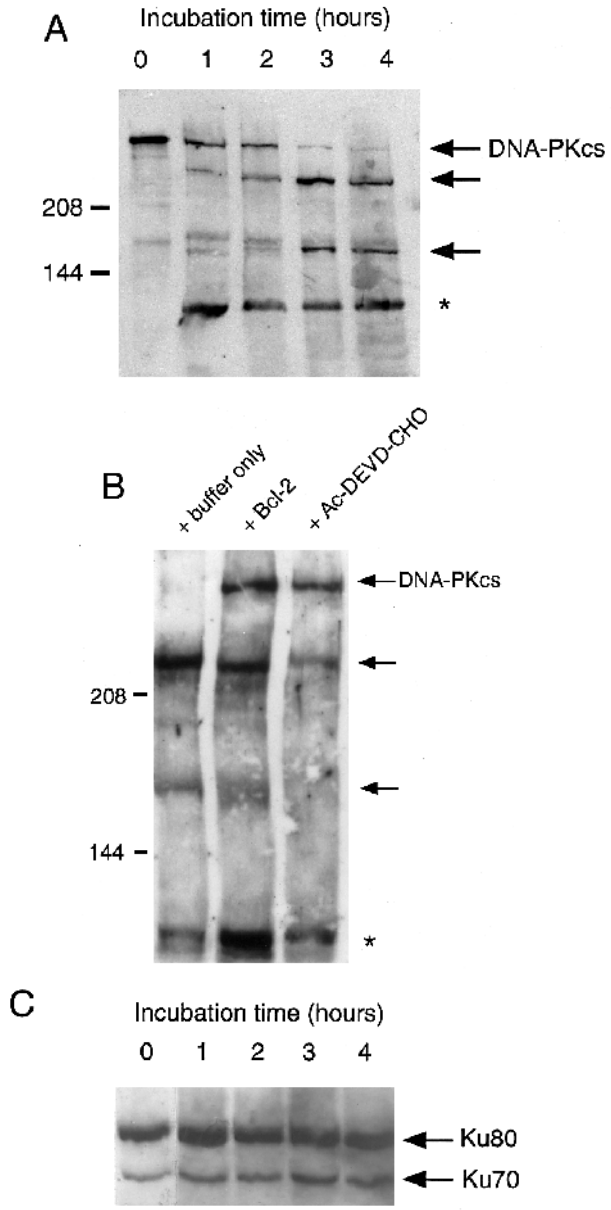

Fig. 3. Human DNA-PKcs is cleaved during apoptosis in *Xenopus* egg extracts. *Xenopus* egg extract was supplemented with isolated HeLa cell nuclei (1,000/μl) and incubated at 23°C. At the times indicated, nuclei were recovered by centrifugation and proteins were analysed by SDS-PAGE and immunoblotting. (A) Samples were run on a 6% polyacrylamide gel, transferred to nitrocellulose and detected with a polyclonal antibody recognizing DNA-PKcs. The positions of the molecular mass marker proteins are indicated on the left (in kDa). The positions of intact DNA-PKcs and of two fragments generated during the incubation are indicated on the right by arrows. The asterix indicates a unidentified reactive *Xenopus* protein that becomes associated with the nuclei by 1 hour and is stable during the incubation. (B) Addition of Bcl-2 protein in Sf21 cell lysate or Ac-DEVD-CHO at the start of the incubation inhibits the subsequent cleavage of DNA-PKcs. Incubations were carried out for 3 hours as above (+buffer only) or with the addition of Bcl-2 (1/100 dilution) or 1 μM Ac-DEVD-CHO and analysed as in A. (C) Ku subunits are stable during the incubation. Samples from the same experiment as A were separated on a 10% polyacrylamide gel, transferred to nitrocellulose and detected with a human autoimmune serum recognizing both the 70 and 80 kDa subunits of Ku.

human DNA-PKcs (not shown). To ensure that this polypeptide was DNA-PKcs, we partially purified and concentrated *Xenopus* DNA-PK using a modification of the pull-down technique used to assay DNA-PK in extracts of cells with low levels of activity (Finnie et al., 1995). Using this technique, we recovered DNA-PK activity from non-incubated extracts specifically bound to DNA-cellulose beads (Fig. 4A). As found for total activity present in the extracts, DNA-PK activity associated with DNA-cellulose dramatically decreased when the extracts were incubated prior to the addition of the beads. This loss of activity in the precipitates was partially prevented by the addition of Ac-DEVD-CHO or Bcl-2 to the extracts at the start of the incubation (Fig. 4A). When the precipitates were immunoblotted with the anti-DNA-PKcs antibody, we found that a high molecular mass polypeptide that bound specifically to DNA-cellulose and probably corresponds to *Xenopus* DNA-PKcs was initially present in the precipitates, but was apparently cleaved with similar kinetics to human DNA-PKcs during incubation of the extracts (Fig. 4B). A large fragment, similar to that resulting from the cleavage of human DNA-PKcs, was generated during the incubation and bound to DNA-cellulose. Another polypeptide that bound specifically to DNA-cellulose was detected on the blots, but it was detected in the precipitates even without incubation of the extracts and prior to DNA-PKcs cleavage. It may therefore be due to another reactive *Xenopus* protein present in the precipitates. *Xenopus* homologues of Ku70 and Ku80 were present in the DNA-cellulose precipitates throughout the course of the same incubation (Fig. 4C).

DISCUSSION

In this report, we have investigated the activity of DNA-dependent protein kinase in a cell-free system prepared from *Xenopus* egg extracts that faithfully reproduces many of the events of apoptosis (Cosulich et al., 1996; Newmeyer et al., 1994). We find that these extracts initially contain an activity with very similar characteristics to mammalian DNA-PK, and antibodies to the catalytic and Ku subunits of the kinase recognise polypeptides of very similar size to those in mammalian cells. During the onset of apoptosis, we observed a rapid inactivation of DNA-PK activity. Our results strongly indicate that inactivation is due to proteolytic cleavage of the catalytic subunit during apoptosis. In contrast to a previous report of the loss of Ku in apoptotic cells (Ajamani et al., 1995), we find that the Ku subunits are stable during apoptosis in this system. Cleavage of DNA-PKcs requires the activity of a protease with very similar characteristics to a CPP-32/Ced-3-like protease that cleaves PARP. Importantly, cleavage of DNA-PKcs is prevented by prior addition of Bcl-2 protein, reinforcing our previous conclusions (Cosulich et al., 1996) that Bcl-2 protects against apoptosis, at least in part, by inhibiting the activation of apoptotic proteases.

The similarities between the cleavage and inactivation of DNA-PKcs and PARP during apoptosis are striking. PARP is cleaved at a site DEVD↓G that lies in a bipartite nuclear localisation signal (NLS) (Fig. 5), releasing the catalytic domain from the DNA-binding domain (Kaufmann et al., 1993; Lazebnik et al., 1994). Studies using truncated versions of PARP suggest that the fragment containing the DNA-binding domain continues to bind DNA and acts as a dominant inactive inhibitor of the binding of PARP and possibly other molecules to DNA ends (Molinete et al., 1993). A similar sequence (DEVD↓N) is present in human DNA-PKcs which is also in the context of basic residues which form a putative NLS (Fig. 5). Cleavage at this site would generate fragments corresponding approximately to the sizes observed in our experiments. The smaller carboxyl-terminal fragment predicted to contain the catalytic domain may be released from the DNA, although in our experiments examining *Xenopus* proteins bound to DNA cellulose (Fig. 4B) it remains possible that this

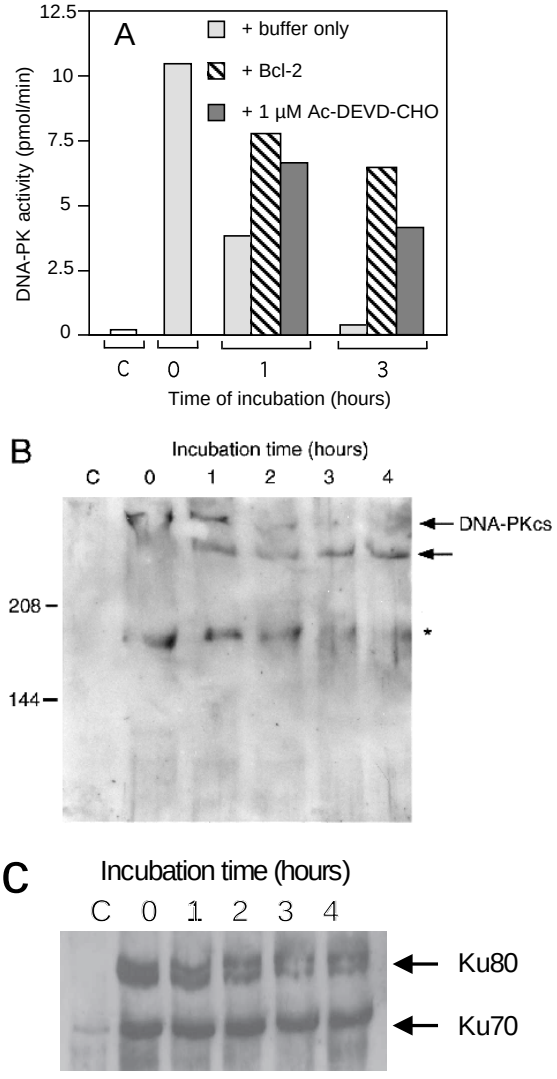

Fig. 4. Inactivation and cleavage of *Xenopus* DNA-PKcs. Egg extract was incubated as in Fig. 3A but without added nuclei. DNA-PK was recovered on dsDNA-cellulose beads at the times shown. Control precipitations used cellulose beads (C). (A) DNA-PK activity recovered from incubations following the addition of Bcl-2, Ac-DEVD-CHO or buffer at the start of the incubation. (B) Immunoblot of precipitated proteins analysed as in Fig. 3A with an antibody to DNA-PKcs. The asterix indicates an unidentified reactive *Xenopus* protein that specifically associates with the DNA-cellulose. (C) Immunoblot of precipitated proteins analysed with a polyclonal serum recognizing *Xenopus* Ku70 and a monoclonal antibody recognizing *Xenopus* Ku80.

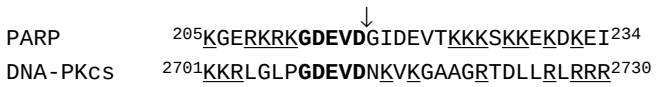

Fig. 5. Alignment of amino acid sequences from a region of human DNA-PKcs (residues 2,701-2,730) with the region containing the site of cleavage in human PARP (residues 205-234). Residues identical in the two sequences are shown in bold and basic residues are underlined. The site of cleavage in PARP between aspartic acid-217 and glycine-218 is indicated by an arrow.

fragment was obscured by another reactive protein or was simply not detected by the antibody. However, the larger, amino-terminal fragment appears to remain associated with DNA, probably via the Ku subunits, and this complex might have a dominant inhibitory effect, or could perhaps carry out other roles either through functional domains (as yet unidentified) within this fragment or through other activities of Ku. Recently, Song et al. (1996) have found a very similar cleavage and inactivation of DNA-PKcs in cultured human cells undergoing apoptosis. These authors have shown that CPP-32 protease cleaves purified human DNA-PKcs and the primary site of cleavage is DEVD↓N. These authors also found that Ku subunits were stable in apoptotic cells.

The recognition of cleavage sites in substrates by ICE-related proteases absolutely requires an aspartic acid residue immediately amino-terminal to the cleavage site, although the residue carboxy-terminal to the cleavage site is variable. The amino acid residue at position P_4 is critical in determining the recognition of a site by particular enzyme (Nicholson et al., 1995). In both DNA-PKcs and PARP the residues P_1-P_4 at the cleavage sites are conserved, with an aspartic acid residue at P_4. Our experiments have also shown that the timing of cleavage and the sensitivity to specific protease inhibitors is very similar for DNA-PK and PARP. Together, these results strongly suggest that a very similar or identical enzyme acts on both substrates in *Xenopus* egg extracts, and this protease is likely to be closely related to human CPP-32. However, the precise identity of the enzymes responsible for cleavage of PARP and DNA-PKcs in either *Xenopus* or human cells remains to be determined. A number of other proteins are specifically cleaved during apoptosis, but the relationship between the proteases that carry out these reactions is also unclear: there may be the sequential activation of a number of different proteases (Cosulich et al., 1996; Martin and Green, 1995).

Although *Xenopus* DNA-PKcs and Ku subunits have not yet been cloned and sequenced, our studies and previous reports of DNA-PK activity (Finnie et al., 1995; Walker et al., 1985) and Ku proteins (Higashiura et al., 1993) in *Xenopus* cell extracts suggest that the structure and function of DNA-PK is in all probability highly conserved. DNA-PK activity and Ku are present in other eukaryotes indicating that their functions may be universal (Jackson, 1996). DNA-PKcs is cleaved and inactivated in *Xenopus* egg extracts (this study) and in human cells undergoing apoptosis (Casciola-Rosen et al., 1995; Song et al., 1996), suggesting the controlled proteolysis of DNA-PK may be a common feature of apoptosis in eukaryotic cells.

The cleavage and inactivation of DNA-PK during apoptosis would be expected to switch off the functions of the kinase in DNA repair, recombination and transcriptional control. When DNA-PK is lost in mammalian cells due to genetic mutations it leads to an increased sensitivity to cell killing by treatments that induce DNA double strand breaks. Loss of DNA-PK during the early stages of protease activation may potentiate the apoptotic response to DNA damage. It also remains possible that DNA-PK plays a direct role in the suppression of apoptosis. Alternatively, cleavage of DNA-PKcs and other proteins such as PARP that are involved in sensing DNA damage may be required to prevent their massive activation when DNA fragmentation occurs during the terminal stages of apoptosis. Further experiments will be required to determine the precise role of the cleavage and inactivation of DNA-PKcs in apoptosis.

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REFERENCES

Ajmani, A. K., Satoh, M., Reap, E., Cohen, P. L. and Reeves, W. H. (1995). Absence of autoantigen Ku in mature human neutrophils and human promyelocytic leukemia line (HL-60) cells and lymphocytes undergoing apoptosis. *J. Exp. Med.* **181**, 2049-2058.

Blier, P. R., Griffith, A. J., Craft, J. and Hardin, J. A. (1993). Binding of Ku protein to DNA. Measurement of affinity for ends and demonstration of binding to nicks. *J. Biol. Chem.* **268**, 7594-7601.

Blunt, T., Finnie, N. J., Taccioli, G. E., Smith, G. C., Demengeot, J., Gottlieb, T. M., Mizuta, R., Varghese, A. J., Alt, F. W., Jeggo, P. A., et al. (1995). Defective DNA-dependent protein kinase activity is linked to V(D)J recombination and DNA repair defects associated with the murine scid mutation. *Cell* **80**, 813-823.

Boubnov, N. V., Hall, K. T., Wills, Z., Lee, S. E., He, D. M., Benjamin, D. M., Pulaski, C. R., Band, H., Reeves, W., Hendrickson, E. A. and Weaver, D. T. (1995). Complementation of the ionizing radiation sensitivity, DNA end binding, and V(D)J recombination defects of double-stranded break repair mutants by p86 Ku autoantigen. *Proc. Nat. Acad. Sci. USA* **92**, 890-894.

Casciola-Rosen, L. A., Anhalt, G. J. and Rosen, A. (1995). DNA-dependent protein kinase is one of a subset of autoantigens specifically cleaved early during apoptosis. *J. Exp. Med.* **182**, 1625-1634.

Clarke, P. R. (1995). Preparation of Xenopus egg extracts and their utilization in cell cycle studies. In *Cell Cycle: Materials and Methods* (ed. M. Pagano), pp. 103-116. Springer-Verlag, Heidelberg.

Cosulich, S. C., Green, S. and Clarke, P. R. (1996). Bcl-2 regulates activation of apoptotic proteases in a cell-free system. *Curr. Biol.* **6**, 997-1005.

de Murcia, G. and Menissier de Murcia, J. (1994). Poly(ADP-ribose) polymerase: a molecular nick-sensor. *Trends Biochem. Sci.* **19**, 172-176.

Dvir, A., Peterson, S. R., Knuth, M. W., Lu, H. and Dynan, W. S. (1992). Ku autoantigen is the regulatory component of a template-associated protein kinase that phosphorylates RNA polymerase II. *Proc. Nat. Acad. Sci. USA* **89**, 11920-11924.

Félix, M.-A., Clarke, P. R., Coleman, J., Verde, F. and Karsenti, E. (1993). *Xenopus* egg extracts as a system to study mitosis. In *The Cell Cycle: A Practical Approach* (ed. P. Fantes and R. Brooks), pp. 253-283. IRL Press, Oxford.

Fernandes-Alnemri, T., Litwack, G. and Alnemri, E. S. (1994). CPP32, a novel human apoptotic protein with homology to Caenorhabditis elegans cell death protein Ced-3 and mammalian interleukin-1β-converting enzyme. *J. Biol. Chem.* **269**, 30761-30764.

Finnie, N. J., Gottlieb, T. M., Blunt, T., Jeggo, P. A. and Jackson, S. P. (1995). DNA-dependent protein kinase activity is absent in xrs-6 cells: implications for site-specific recombination and DNA double-strand break repair. *Proc. Nat. Acad. Sci. USA* **92**, 320-324.

Genersch, E., Eckerskorn, C., Lottspeich, F., Herzog, C., Kuhn, K. and Poschl, E. (1995). Purification of the sequence-specific transcription factor CTCBF, involved in the control of human collagen IV genes: subunits with homology to Ku antigen. *EMBO J.* **14**, 791-800.

Giffin, W., Torrance, H., Rodda, D. J., Préfontaine, G. G., Pope, L. and Haché, R. J. G. (1996). Sequence-specific DNA binding by Ku autoantigen and its effects on transcription. *Nature* **380**, 265-268.

Gottlieb, T. M. and Jackson, S. P. (1993). The DNA-dependent protein kinase: requirement for DNA ends and association with Ku antigen. *Cell* **72**, 131-42.

Greenwell, P. W., Kronmal, S. L., Porter, S. E., Gassenhuber, J., Obermaier, B. and Petes, T. D. (1995). TEL1, a gene involved in controlling telomere length in S. cerevisiae, is homologous to the human ataxia telangiectasia gene. *Cell* **82**, 823-829.

Griffith, A. J., Blier, P. R., Mimori, T. and Hardin, J. A. (1992). Ku polypeptides synthesised in vitro assemble into complexes which recognise ends of double-stranded DNA. *J. Biol. Chem.* **267**, 331-338.

Hari, K. L., Santerre, A., Sekelsky, J. J., McKim, K. S., Boyd, J. B. and Hawley, R. S. (1995). The mei-41 gene of D. melanogaster is a structural and functional homolog of the human ataxia telangiectasia gene. *Cell* **82**, 815-821.

Hartley, K. O., Gell, D., Smith, G. C. M., Zhang, H., Divecha, N., Connely, M. A., Admon, A., Lees-Miller, S. P., Anderson, C. W. and Jackson, S. P. (1995). DNA-dependent protein kinase catalytic subunit: a relative of phosphatidylinositol 3-kinase and the ataxia telangiectasia gene product. *Cell* **82**, 849-856.

Higashiura, M., Takasuga, Y., Yamashita, J. and Yagura, T. (1993). A protein homologous to human p70-protein is required for reconstitution of Xenopus sperm pronuclei. *Chromosome Res.* **1**, 27-36.

Jackson, S. P. (1995). Ataxia-telangiectasia at the crossroads. *Curr. Biol.* **5**, 1210-1212.

Jackson, S. P. (1996). The recognition of DNA damage. *Curr. Opin. Genet. Dev.* **6**, 19-25.

Kato, R. and Ogawa, H. (1994). An essential gene, ESR1, is required for mitotic cell growth, DNA repair and meiotic recombination in Saccharomyces cerevisiae. *Nucl. Acids Res.* **22**, 3104-3112.

Kaufmann, S. H., Desnoyers, S., Ottaviano, Y., Davidson, N. E. and Poirier, G. G. (1993). Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. *Cancer Res.* **53**, 3976-3985.

Keith, C. T. and Schreiber, S. L. (1995). PIK-related kinases: DNA repair, recombination and cell cycle checkpoints. *Science* **270**, 50-51.

Kuhn, A., Gottlieb, T. M., Jackson, S. P. and Grummt, I. (1995). DNA-dependent protein kinase: a potent inhibitor of transcription by RNA polymerase I. *Genes Dev.* **9**, 193-203.

Kumar, S. (1995). ICE-like proteases in apoptosis. *Trends Biochem. Sci.* **20**, 198-202.

Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G. and Earnshaw, W. C. (1994). Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* **371**, 346-347.

Le Romancer, M., Reyl, D. F., Cherifi, Y., Pigeon, C., Bottari, S., Meyer, O. and Lewin, M. J. (1994). The 86-kDa subunit of autoantigen Ku is a somatostatin receptor regulating protein phosphatase-2A activity. *J. Biol. Chem.* **269**, 17464-17468.

Lees-Miller, S. P., Sakaguchi, K., Ullrich, S. J., Appella, E. and Anderson, C. W. (1992). Human DNA-activated protein kinase phosphorylates serines 15 and 37 in the amino-terminal transactivation domain of human p53. *Mol. Cell. Biol.* **12**, 5041-5049.

Lees-Miller, S. P., Godbout, R., Chan, D. W., Weinfeld, M., Day, R. R., Barron, G. M. and Allalunis-Turner, J. (1995). Absence of p350 subunit of DNA-activated protein kinase from a radiosensitive human cell line. *Science* **267**, 1183-1185.

Martin, S. J. and Green, D. R. (1995). Protease activation during apoptosis: death by a thousand cuts? *Cell* **82**, 349-352.

Mimori, T. and Hardin, J. A. (1986). Mechanism of interaction between Ku protein and DNA. *J. Biol. Chem.* **261**, 10375-10379.

Molinete, M., Vermeulen, W., Burkle, A., Menissier-de Murcia, J., Kupper, J. H., Hoeijmakers, J. H. and de Murcia, G. (1993). Overproduction of the poly(ADP-ribose) polymerase DNA-binding domain blocks alkylation-induced DNA repair synthesis in mammalian cells. *EMBO J.* **12**, 2109-2117.

Morrow, D. M., Tagle, D. A., Shiloh, Y., Collins, F. S. and Heiter, P. (1995). HAT1/TEL1, an S. cerevisiae homologue of the human gene mutated in ataxia-telangiectasia, is functionally related to the yeast checkpoint gene MEC1. *Cell* **82**, 831-840.

Newmeyer, D. D., Farschon, D. M. and Reed, J. C. (1994). Cell-free apoptosis in Xenopus egg extracts: inhibition by Bcl-2 and requirement for an organelle fraction enriched in mitochondria. *Cell* **79**, 353-364.

Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A. et al. (1995). Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* **376**, 37-43.

Paillard, S. and Strauss, F. (1991). Analysis of the mechanism of interaction of simian Ku protein with DNA. *Nucl. Acids. Res.* **19**, 5619-5624.

Paulovich, A. G. and Hartwell, L. H. (1995). A checkpoint regulates the rate of progression through S phase in S. cerevisiae in response to DNA damage. *Cell* **82**, 841-847.

Peterson, S. R., Kurimasa, A., Oshimura, M., Dynan, W. S., Bradbury, E. M. and Chen, D. J. (1995). Loss of the catalytic subunit of the DNA-dependent protein kinase in DNA double-strand-break-repair mutant mammalian cells. *Proc. Nat. Acad. Sci. USA* **92**, 3171-3174.

Rathmell, W. K. and Chu, G. (1994). Involvement of the Ku autoantigen in the cellular response to DNA double-strand breaks. *Proc. Nat. Acad. Sci. USA* **91**, 7623-7627.

Reeves, W. H. (1992). Antibodies to the p70/p80 (Ku) antigens in systemic lupus erythematosus. *Rheumatic. Dis. Clin. North. Am.* **18**, 391-414.

Savitsky, K., Bar-Shira, A., Gilad, S., Rotman, G., Ziv, Y., Vanagaite, L., Tagle, D. A., Smith, S., Uziel, T., Sfez, S. et al. (1995). A single ataxia telangiectasia gene with a product similar to PI 3-kinase. *Science* **268**, 1749-1753.

Seaton, B. L., Yucel, J., Sunnerhagen, P. and Subramani, S. (1992). Isolation and charaterization of the Schizosaccharomyces pombe rad3 gene involved in the DNA damage and DNA synthesis checkpoints. *Gene* **119**, 83-89.

Smider, V., Rathmell, W. K., Lieber, M. R. and Chu, G. (1994). Restoration of X-ray resistance and V(D)J recombination in mutant cells by Ku cDNA. *Science* **266**, 288-291.

Song, Q., Lees-Miller, S. P., Kumar, S., Zhang, N., Chan, D. W., Smith, G. C. M., Jackson, S. P., Alnemri, E. S., Litwack, G. and Lavin, M. F. (1996). DNA-dependent protein kinase catalytic subunit: a target for an ICE-like protease in apoptosis. *EMBO J.* **15**, 3238-3246.

Taccioli, G. E., Gottlieb, T. M., Blunt, T., Priestley, A., Demengeot, J., Mizuta, R., Lehmann, A. R., Alt, F. W., Jackson, S. P. and Jeggo, P. A. (1994). Ku80: product of the XRCC5 gene and its role in DNA repair and V(D)J recombination. *Science* **265**, 1442-1445.

Walker, A. I., Hunt, T., Jackson, R. J. and Anderson, C. W. (1985). Double-stranded DNA induces the phosphorylation of several proteins including the 90 000 mol. wt. heat-shock protein in animal cell extracts. *EMBO J.* **4**, 139-145.

Yuan, J., Shaham, S., Ledoux, S., Ellis, H. M. and Horvitz, H. R. (1993). The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. *Cell* **75**, 641-652.

Zhang, W.-W. and Yaneva, M. (1992). On the mechanisms of Ku protein protein binding to DNA. *Biochem. Biophys. Res. Commun.* **186**, 574-579.

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