

# Mapping of protein–protein interactions within the DNA-dependent protein kinase complex

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## ABSTRACT

**In mammalian cells, the Ku and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) proteins are required for the correct and efficient repair of DNA double-strand breaks. Ku comprises two tightly-associated subunits of ~69 and ~83 kDa, which are termed Ku70 and Ku80 (or Ku86), respectively. Previously, a number of regions of both Ku subunits have been demonstrated to be involved in their interaction, but the molecular mechanism of this interaction remains unknown. We have identified a region in Ku70 (amino acid residues 449–578) and a region in Ku80 (residues 439–592) that participate in Ku subunit interaction. Sequence analysis reveals that these interaction regions share sequence homology and suggests that the Ku subunits are structurally related. On binding to a DNA double-strand break, Ku is able to interact with DNA-PKcs, but how this interaction is mediated has not been defined. We show that the extreme C-terminus of Ku80, specifically the final 12 amino acid residues, mediates a highly specific interaction with DNA-PKcs. Strikingly, these residues appear to be conserved only in Ku80 sequences from vertebrate organisms. These data suggest that Ku has evolved to become part of the DNA-PK holoenzyme by acquisition of a protein–protein interaction motif at the C-terminus of Ku80.**

## INTRODUCTION

Ku is a heterodimeric protein, comprising two tightly associated subunits of ~70 and ~83 kDa, that has been found in many organisms, ranging from *Saccharomyces cerevisiae* to man (1). Although some information has been obtained regarding the regions of the Ku polypeptides that interact with one another (2–8), very little is known about the molecular mechanism of this interaction. The most highly characterised function of Ku at the biochemical level is its ability to bind avidly to certain disruptions of the DNA double helix in a sequence-independent fashion, including DNA double-strand breaks (DSBs) and single-strand to double-strand transitions (9–12). When complexed with DNA, Ku can interact with an ~460 kDa polypeptide, the

DNA-dependent protein kinase catalytic subunit (DNA-PKcs). DNA-PKcs is a member of the phosphatidylinositol (PI) 3-kinase-like (PIKL) protein kinase family (13) and, together with Ku and DNA, forms a catalytically active DNA-PK complex with serine/threonine protein kinase activity (14–16). Little is currently known about how Ku interacts with DNA-PKcs, although the fact that it is unable to bind DNA-PKcs in the absence of DNA (16) suggests that DNA binding by Ku induces a conformational change that permits the DNA-PKcs interaction. It has been shown that, under certain conditions, DNA-PKcs can bind to double-stranded DNA ends and become activated in the absence of Ku (17,18). This suggests that contacts between DNA-PKcs and DNA play an important role in DNA-PK activation, even in the presence of Ku.

A breakthrough in the understanding of DNA-PKcs/Ku function came with the discovery that defects in these proteins are associated with a subset of mutant mammalian cell lines that are defective in DNA DSB rejoining, and which are profoundly sensitive to ionising radiation and other agents that generate DNA DSBs as their principal lethal lesion (reviewed in 19–21). Furthermore, DNA-PK catalytic activity has been implicated at an early stage of DNA DSB repair in *Xenopus* cell-free extracts (22,23). Cells deficient in DNA-PKcs, Ku80 or Ku70 are also severely impaired in V(D)J recombination, a site-specific genomic rearrangement process that involves the generation of DNA DSBs, and which is required to generate the vast antigen recognition capacity of antibody and T-cell receptor molecules (reviewed in 24,25). Coupled with the observations that Ku displays a very high affinity for double-stranded DNA ends *in vitro*, these data suggest that DNA-PK functions directly in the recognition and resolution of DNA DSBs *in vivo*.

Consistent with the Ku-associated DNA DSB repair pathway being highly conserved throughout eukaryotic evolution, Ku is found in *S.cerevisiae* and is essential for repair of DNA DSBs by the pathway of non-homologous end-joining (reviewed in 21,26). However, there is no clear orthologue of DNA-PKcs encoded by the fully-sequenced *S.cerevisiae* genome. Thus, in yeast, Ku carries out DNA-repair functions independently of DNA-PK. Although it is possible that the functions of mammalian DNA-PKcs are assumed by other members of the PIKL protein kinase family, such as Mec1p and/or Tel1p, there is little evidence to suggest that these interact physically or genetically with Ku. *Saccharomyces cerevisiae* Ku has also been shown to play important roles in telomere length maintenance, and in the

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transcriptional silencing of genes placed close to telomeric DNA (27–31).

Given the importance of DNA-PK in the major pathway of DNA DSB repair in mammalian cells, and our lack of a clear understanding of the protein–protein interactions within the enzyme, we have investigated interactions between Ku70 and Ku80, and between the two Ku subunits and DNA-PKcs. This work reveals that the C-terminal region of Ku80 makes highly specific contacts with DNA-PKcs. Furthermore, functional analysis of the Ku subunits, in conjunction with protein sequence comparisons, suggest that the two Ku subunits are structurally and functionally related to each other. We discuss the importance of these results in regard to the structure, physiological functions and evolution of DNA-PKcs and Ku.

## MATERIALS AND METHODS

### Yeast two-hybrid assay

A large fragment of Ku70 (corresponding to residues 213–590) was PCR amplified and cloned into the yeast two-hybrid ‘bait’ vector pEG202 (E. Golemis) to generate a fusion with the DNA-binding domain Lex202 (Lex-Ku70 $\Delta$ 213–590). This was transformed into a yeast strain, EGY48, containing a genomic *LEU2* gene under the control of three LexA operator sites, as well as the plasmid pSH18-34 (S. Hanes) containing the reporter gene *GAL1-LacZ* downstream of four LexA operators. A HeLa cell expression library in the two-hybrid vector pJG4-5 (generated by J. Gyuris) was then transformed into the yeast. Yeast were then subjected to a two-fold selection procedure, first plating onto medium lacking leucine. Resulting colonies (530) were picked onto fresh plates containing X-gal to screen for activation of the *GAL1-LacZ* reporter. Some of the transformation was plated onto non-selective media to calculate the transformation efficiency at  $>10^7$  total transformants.

### Generation of GST-fusion proteins

The Ku70 and Ku80 open reading frames, and fragments of these generated by PCR, were cloned in-frame with the glutathione-binding domain of *Schistosoma japonicum* glutathione *S*-transferase (GST) by sub-cloning into pGEX2TK-P (a derivative of pGEX2TK Pharmacia). Expression from all pGEX2TK-P constructs was carried out in either DH5 $\alpha$  or DS941 strains of *Escherichia coli*. Bacterial cell extracts were produced by sonication in phosphate-buffered saline (PBS; 138 mM NaCl, 3 mM KCl, 10 mM phosphate buffer pH 7.4) containing 1% Triton X-100 (Sigma) and protease inhibitor tablets (Boehringer). GST-fusion proteins were purified by passing bacterial lysates over glutathione–Sepharose beads (Pharmacia) and washing in PBS containing 1% Triton X-100 and then in PBS alone. Proteins were stored at  $-80^\circ\text{C}$  prior to use.

### Preparation of biotinylated bacterial cell extracts

Ku70 and Ku80 were cloned into pET30a (Novagen) to generate an N-terminal hexa-histidine tag, and were expressed in *E. coli* [BL21 (DE3) pLysS]. The soluble protein fraction was prepared by lysing cells in buffer (50 mM phosphate pH 7.5, 150 mM NaCl, 0.1%  $\beta$ -mercaptoethanol, 0.1% Triton X-100, Boehringer Complete Protease Inhibitors). The soluble fraction was labelled with biotin (Sigma) by incubating for 1 h on ice with

0.05 mg biotin per 1 mg of total protein. Unreacted biotin groups were removed by addition of glycine (0.1 M final) after 1 h. Extracts were diluted two-fold in buffer (50 mM Tris pH 8.0, 250 mM NaCl, 0.1%  $\beta$ -mercaptoethanol, 0.1% Triton X-100, Boehringer Complete Protease Inhibitors) before use in ‘pull-down’ assays.

### Peptides

Five peptides derived from the sequence of Ku80 (see Fig. 7) were generated with a Ser-Gly-Ser-Gly linker to a biotin moiety at the N-terminus (Khiron Technologies). The biotin group allows these peptides to be captured from solution by incubation for 15 min with streptavidin-coated para-magnetic Dynabeads (Dyna), which can then be retrieved using a magnet.

### Purification of DNA-PKcs

All steps were performed in buffer D (20 mM HEPES pH 7.6, 2 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 10% glycerol) with a specified concentration of KCl or ammonium sulphate. HeLa cell nuclear extract (Computer Cell Culture Centre, Mons, Belgium) was fractionated over columns of Q-Sepharose (Pharmacia) and heparin–agarose (Sigma). The resulting DNA-PKcs fraction was applied to a column of phenyl-Sepharose (Pharmacia) in 0.5 M ammonium sulphate. Under these conditions, 100% of DNA-PKcs is retained by the column, whilst the majority of contaminating Ku flows through. DNA-PKcs was eluted with a linear gradient of 0.4–0 M ammonium sulphate. Peak DNA-PKcs fractions were applied to a 1 ml Mono-S FPLC column (Pharmacia) as a final purification step. DNA-PKcs fractions were dialysed into buffer D containing 50 mM KCl and stored at  $-80^\circ\text{C}$  prior to use.

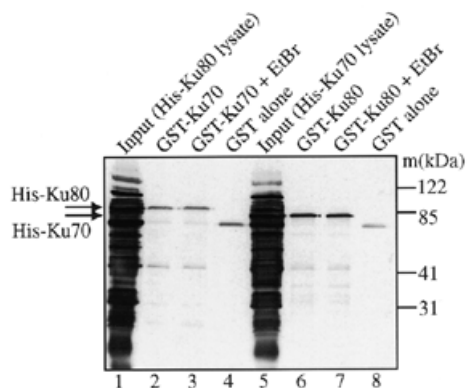
## RESULTS

### A C-terminal region of Ku80 interacts with Ku70 in the yeast two-hybrid assay

To investigate protein–protein interactions between the subunits of Ku, we first employed a yeast two-hybrid assay to screen a human HeLa cell cDNA library for proteins that interact with a large fragment of Ku70 (spanning residues 213–590) containing two putative leucine zipper motifs. Fifty-five positive clones were identified and the library plasmids contained within these were then rescued into *E. coli*. Bacterial colony hybridisation to a radiolabelled Ku80 DNA probe revealed that 40 gave a strong positive signal. These clones were sequenced and all were found to contain a Ku80 partial cDNA. The retrieved plasmids comprised eight distinct Ku80 cDNA clones; all contained the poly(A) tail of the Ku80 cDNA, but differed in their 5' ends. Perhaps surprisingly, the different 5' termini were highly clustered, all falling to within 27 nucleotides of each other. The smallest clone encoded the C-terminal 294 amino acid residues of Ku80, defining this region (amino acid residues 439–732) as sufficient for interaction with Ku70 in this assay.

### Analysis of interactions between bacterially expressed Ku70 and Ku80 deletion derivatives

In a complementary approach to the yeast two-hybrid technique, we devised an *in vitro* ‘pull-down’ assay. Ku70 and Ku80 were expressed as GST-fusion proteins in *E. coli* (GST–Ku70 and GST–Ku80) and purified on glutathione–Sepharose beads. Unfractionated bacterial cell extracts containing low amounts



**Figure 1.** Both Ku70 and Ku80 interact specifically with their heterodimerisation partner in GST 'pull-down' assays. The figure shows a nitrocellulose membrane, probed with streptavidin conjugated to horseradish peroxidase (HRP) to detect biotinylated proteins. Unfractionated, biotinylated bacterial cell extracts (50  $\mu$ g) expressing histidine-tagged Ku70 and Ku80 (His-Ku70 and His-Ku80) are shown in lanes 1 and 5 respectively. Full-length Ku70, expressed as a fusion protein with GST (GST-Ku70; 1  $\mu$ g), and bound to glutathione-Sephareose beads, was incubated for 3 h at 4°C with extract containing His-Ku80 (500  $\mu$ g total protein). The beads were washed in seven changes of NETN buffer (180 mM NaCl, 25 mM EDTA, 20 mM Tris pH 8.0, 0.5% NP-40). Proteins retained by the GST-Ku70 beads in the absence or presence of 0.2 mg/ml ethidium bromide (EtBr) are shown in lanes 2 and 3. Similar binding reactions were performed using GST-Ku80 and extracts containing His-Ku70 (lanes 6 and 7). Control binding reactions were carried out using beads bound to GST (5  $\mu$ g) and bacterial lysates containing either His-tagged Ku80 (lane 4) or His-tagged Ku70 (lane 8).

of histidine-tagged His-Ku70 or His-Ku80 were generated and labelled with biotin (Materials and Methods; expression conditions were chosen to ensure that His-Ku70 or His-Ku80 represented only a small percentage of the total protein in these extracts). Interaction assays were then conducted by incubating either GST-Ku80 beads with bacterial lysate containing His-Ku70, or GST-Ku70 beads with His-Ku80 lysate.

As shown in Figure 1, of the large number of biotinylated proteins present in crude extracts (lanes 1 and 5), only a single major species was retained on the beads in 'pull-downs' with either GST-Ku70 (lane 2) or GST-Ku80 (lane 6). Smaller amounts of a few lower molecular weight proteins were, sometimes, also obtained. Identification of the major interacting proteins in lanes 2 and 6 as Ku80 and Ku70, respectively, was confirmed by quantitative western blotting with anti-Ku subunit antisera (data not shown). In control binding reactions using GST alone, neither Ku subunit was retained from the bacterial lysate (Fig. 1, lanes 4 and 8). The binding of GST-Ku70 or GST-Ku80 to its partner subunit in the extract was not inhibited by ethidium bromide, which has been shown to disrupt some protein-DNA complexes (32), suggesting that this interaction is direct.

#### Sequence analysis of Ku80 and Ku70 suggests that they are structurally related

To develop a logical approach for constructing Ku deletion mutants, we analysed the human Ku70 and Ku80 protein sequences for candidate interaction motifs. This analysis revealed a number of sequence motifs that are conserved between all Ku70 and Ku80 proteins. These conserved motifs,

which we have called the primary homology regions (PHRs), occur in the same order and with highly conserved spacing in all of the Ku70 and Ku80 homologues (Fig. 2). A subset of these homologies has been recently identified by Dynan and Yoo (1). The PHRs contain a number of highly conserved proline and glycine residues (particularly PHR3, PHR4 and PHR5; Fig. 2), consistent with these being conserved structural regions of the Ku subunits.

The sequence conservation and co-linearity of the PHR motifs, suggests that Ku70 and Ku80 may have the same overall structure, and might be derived from a common ancestor. Notably, the separation of PHR2 and PHR3 is between 250 and 284 amino acid residues in all known Ku70 and Ku80 sequences, so perhaps this region is required to achieve a critical alignment of the PHR1-2 region with the PHR3-4-5 region in the tertiary structure of the protein. The region separating PHR4 and PHR5 contains some weak similarity between the Ku70 and Ku80 sequences and is conserved in size. The regions between the PHRs may therefore exhibit structural similarities that are not easily detectable in the primary amino acid sequence. These regions have therefore tentatively been designated secondary homology regions (SHRs) as illustrated in Figure 2. The Ku70 and Ku80 sequences show greatest divergence at their C-termini. Specifically, there is a region of similarity exclusive to the Ku80 sequences that we have designated the Ku80-specific C-terminal region (Ku80-CTR; Fig. 2), and a region of similarity only between the Ku70 sequences which we have called the Ku70-specific C-terminal region (Ku70-CTR).

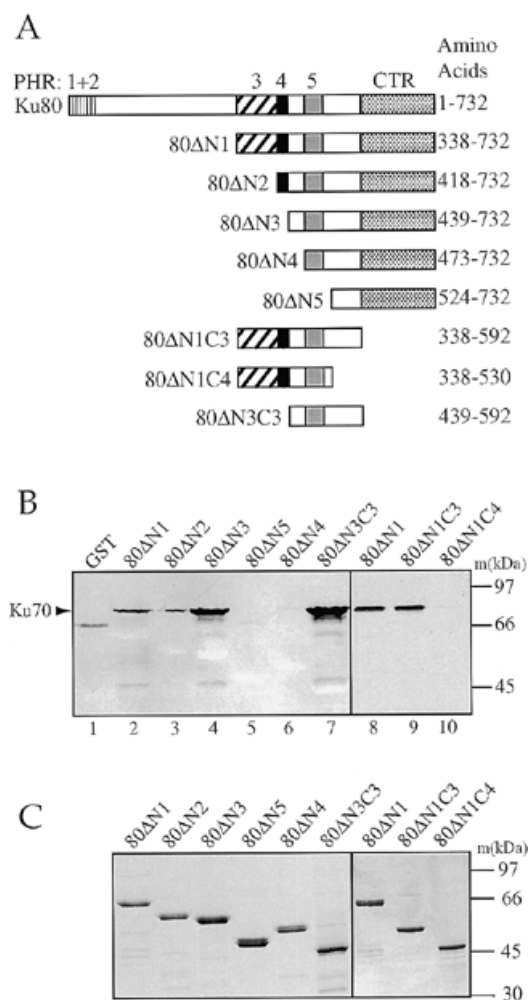
The region we have identified as interacting with Ku70 in the two-hybrid assay includes SHR2, PHR5, SHR3 and the Ku80-CTR, making these attractive candidates for Ku subunit-interaction motifs. As outlined below, we have used the information from the alignment in the construction of a range of Ku70 and Ku80 deletion mutants that contain one or more of the PHRs/SHRs to study their potential roles in mediating interactions between the Ku subunits, and between Ku and DNA-PKcs.

#### Analysis of the regions of Ku80 and Ku70 required for heterodimerisation

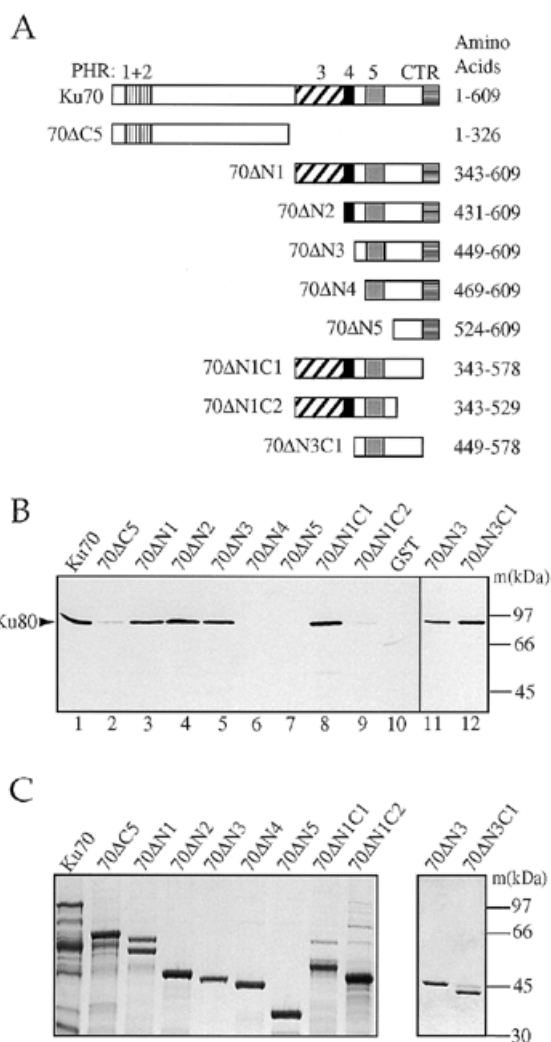
We tested a range of Ku80 deletion derivatives (Fig. 3A) and a range of Ku70 deletion derivatives (Fig. 4A) expressed as GST-fusion proteins, for their abilities to affinity purify the full-length Ku subunit partner from a crude bacterial extract. As shown in Figure 3B, the GST-Ku80 derivatives 80 $\Delta$ N1, 80 $\Delta$ N2 and 80 $\Delta$ N3 all interacted with Ku70, demonstrating that PHRs 1-4 of Ku80 are not required for this interaction (lanes 2-4). Furthermore, the Ku80-CTR is also not required, as demonstrated with the derivatives 80 $\Delta$ N1C3 and 80 $\Delta$ N3C3 (Fig. 3B; compare lane 4 with 7, and lane 8 with 9). Thus, a region of Ku80 comprising SHR2, PHR5 and SHR3 is sufficient to mediate interactions with full-length Ku70. Deletion of this region from either the N- or C-terminus, however, rendered the Ku80 deletion derivative unable to interact with Ku70 (exemplified by 80 $\Delta$ N4, 80 $\Delta$ N5 and 80 $\Delta$ N1C4 in Fig. 3B; lanes 5, 6 and 10).

Interaction assays performed using GST-Ku70 deletion derivatives and full-length Ku80 demonstrate that the N-terminal half of Ku70, comprising PHR1, PHR2 and SHR1 (70 $\Delta$ C5) interacts very poorly with Ku80 (Fig. 4B, lane 2). By contrast, the C-terminal region (70 $\Delta$ N1) binds Ku80 efficiently (lane 3). Further subdivision of the C-terminal region of GST-Ku70





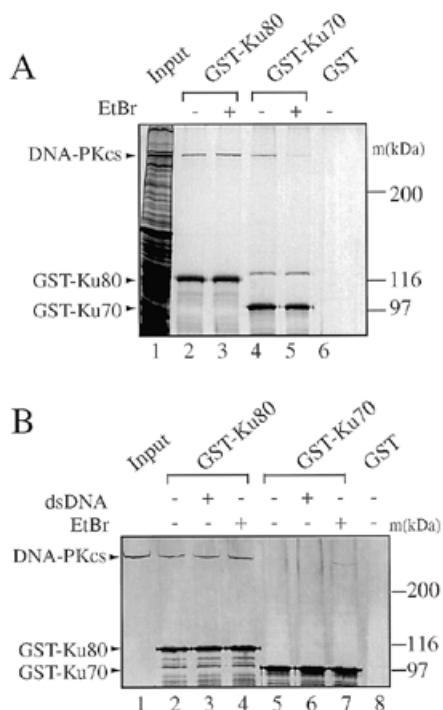
**Figure 3.** Defining a region of Ku80 that interacts with full-length Ku70. A range of Ku80 deletion derivatives (~2 μg of each), expressed as GST-fusion proteins and bound to glutathione–Sepharose beads, were incubated for 3 h at 4°C with biotinylated bacterial cell extracts (500 μg protein) expressing Ku70 (histidine-tagged). The beads were washed in NETN buffer (Fig. 1) and proteins retained by the beads were resolved by SDS–PAGE and transferred to nitrocellulose. (A) Diagram of GST–Ku80 deletion derivatives used for these interaction assays. The positions of the PHRs within each derivative are indicated by shading (see Fig. 2). Full-length Ku80 is represented by the uppermost bar. (B) Nitrocellulose membrane, probed with HRP-conjugated streptavidin, showing the amount of Ku70 protein retained by each GST–Ku80 deletion derivative. Ku70 (arrow) is, essentially, the only protein retained by Ku80 derivatives. Note that ‘negative staining’ bands can be seen, due to the GST-fusion proteins on the membrane. To control for protein loading, the membrane was stained with Coomassie blue (not shown). (C) Coomassie-stained gel of purified GST–Ku80 deletion derivatives used in these interaction assays.



**Figure 4.** Defining a region of Ku70 that interacts with full-length Ku80. GST–Ku70 derivatives (~2 μg) were incubated, as described in Figure 3, with biotinylated extracts containing Ku80. (A) Diagram of GST–Ku70 deletion derivatives used for these interaction assays. The position of the PHRs within each derivative is indicated by shading (see Fig. 2). Full-length Ku70 is represented by the uppermost bar. (B) Nitrocellulose membrane, probed with HRP-conjugated streptavidin, indicating the amount of Ku80 protein retained by each GST–Ku70 deletion derivative. Ku80 (arrow) is, essentially, the only protein retained by Ku70 derivatives. To control for protein loading the membrane was stained with Coomassie blue (not shown). (C) Coomassie-stained gel of purified GST–Ku70 deletion derivatives used in these interaction assays.

DNA (15,16). The basis for the Ku–DNA–PKCs interaction, however, has not been studied in detail. To investigate possible interactions between the individual Ku subunits and DNA–PKCs, we performed ‘pull-down’ assays from HeLa cell nuclear extract using GST–Ku70 or GST–Ku80 bound to glutathione–Sepharose beads. As shown in the silver-stained gel in Figure 5A, of the many proteins in the crude extract

(lane 1), DNA–PKCs was the only protein retrieved by affinity purification with GST–Ku80 beads (lanes 2 and 3). Identification of the retrieved species as DNA–PKCs was verified by quantitative western blot analyses (not shown). GST–Ku70 beads also affinity purified DNA–PKCs, although less efficiently than GST–Ku80, as well as a second polypeptide of ~116 kDa, the identity of which is the subject of current investigation (lanes 4



**Figure 5.** (A) Interactions between the Ku subunits and proteins in a HeLa nuclear extract (NE). GST-Ku80 (0.5 µg) or GST-Ku70 (0.5 µg) bound to glutathione-Sepharose beads was incubated for 3–4 h at 4°C with 100 µg of HeLa NE in Z' buffer (25 mM HEPES pH 6.7, 120 mM KCl, 12.5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 0.1% Nonidet P-40, 20% glycerol). The beads were subsequently washed extensively in buffer W (25 mM HEPES pH 6.7, 150 mM KCl, 2 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 0.4% Nonidet P-40). Proteins remaining bound to the beads are shown in lanes 2–5 of this silver-stained SDS-polyacrylamide gel (the GST-Ku70 and GST-Ku80 fusion proteins can also be seen). Lane 1 shows 10 µg of NE. Ethidium bromide (0.2 mg/ml final) was added to the binding reactions shown in lanes 3 and 5. (B) Silver-stained SDS-polyacrylamide gel showing protein retained on GST-Ku80 beads (0.5 µg) or GST-Ku70 beads (0.5 µg) after incubation for 3 h at 4°C with 0.4 µg of purified DNA-PKcs in Z' buffer. The beads were washed in buffer W. Binding reactions for lanes 3 and 6 contained 20 ng/µl sheared genomic DNA (dsDNA), those for lanes 4 and 7 contained 0.2 mg/ml ethidium bromide. Lane 1 shows 0.12 µg of DNA-PKcs. Notably, in binding reactions carried out at lower KCl concentrations some binding of DNA-PKcs to GST-Ku70 was observed (data not shown). The significance of these interactions has not, however, been investigated further.

and 5). By contrast, no proteins were recovered using beads containing GST alone (Fig. 5A, lane 6).

It has been reported previously that Ku70 is capable of interacting with DNA in the absence of a heterodimerisation partner (8,33) and that Ku heterodimers bound to DNA are able to specifically associate with one another (34). The possibility therefore existed that the interactions we had observed between DNA-PKcs and the individual Ku subunits were indirect, and mediated by DNA and/or the Ku heterodimer present in the HeLa nuclear extract. To address the potential involvement of DNA, we used ethidium bromide. Notably, the interaction between Ku80 and DNA-PKcs was unaffected by ethidium bromide (Fig. 5A, lane 3). By contrast, the interaction between DNA-PKcs and Ku70 was substantially reduced by ethidium bromide

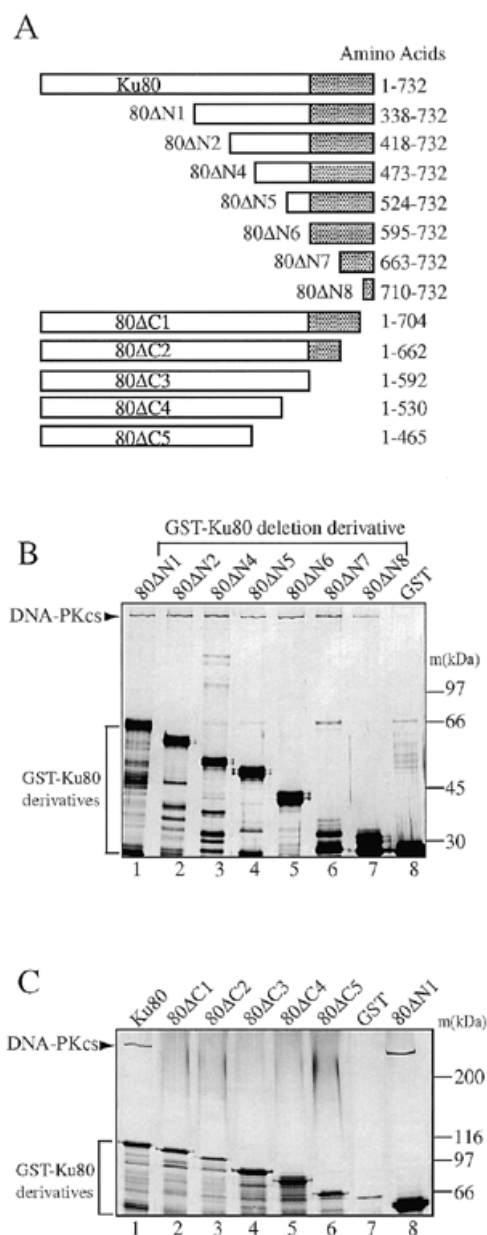
treatment (lane 5), indicating that this interaction is dependent upon protein–DNA interactions. To investigate the possible involvement of Ku heterodimer in the nuclear extract, we probed western blots of proteins bound to the GST-Ku70 or GST-Ku80 beads with Ku-specific antibodies. These studies revealed ethidium bromide-sensitive binding of endogenous Ku to GST-Ku70, but only trace amounts of Ku binding to GST-Ku80 in either the presence or absence of ethidium bromide. Furthermore, we have found that GST-Ku70, but not GST-Ku80, can interact with purified Ku heterodimer in the presence of DNA and that this binding is inhibited by ethidium bromide. Taken together, the above data suggest that the interaction between GST-Ku70 and DNA-PKcs in these assays is dependent on DNA and Ku heterodimer.

To assess the potential involvement of other polypeptides in mediating interactions between GST-Ku80 and DNA-PKcs, we carried out binding reactions using an essentially homogeneous preparation of DNA-PKcs in place of nuclear extract (NE; this preparation was found, by quantitative western blotting, to contain 1 Ku molecule per ~840 DNA-PKcs molecules; data not shown). Significantly, these interaction studies revealed that purified DNA-PKcs interacts efficiently with GST-Ku80 (Fig. 5B, lanes 2–4), but not with GST-Ku70 (Fig. 5B, lanes 5–7) under the conditions employed. Furthermore, we were unable to detect any endogenous Ku contamination in these interaction complexes using either polyclonal rabbit sera or monoclonal antibodies raised against the Ku subunits (data not shown). Taken together, the data suggest that there is a direct interaction between Ku80 and DNA-PKcs.

### The Ku80 C-terminal region interacts with DNA-PKcs

To define which region(s) of the Ku80 polypeptide is responsible for the interaction with DNA-PKcs, we performed interaction assays with purified DNA-PKcs and a battery of GST-Ku80 deletion derivatives bound to glutathione-Sepharose beads. A diagram of the Ku80 deletion derivatives used is shown in Figure 6A. We found that sequential deletions from the N-terminus of Ku80 (80ΔN1–N6) had little or no effect on DNA-PKcs binding (Fig. 6B, lanes 1–5). Significantly, 80ΔN6 comprises amino acid residues 595–732 of Ku80 and corresponds to the C-terminal region of human Ku80, the Ku80-CTR, that has no homology to Ku70 sequences (Fig. 2). Analysis of C-terminal Ku80 protein fragments smaller than 80ΔN6 as GST-fusions was complicated by difficulties in expressing these small fragments. Nevertheless, Figure 6B shows interaction of two of these derivatives, 80ΔN7 and 80ΔN8, with DNA-PKcs (lanes 6 and 7). The expression construct encoding 80ΔN8 was designed to express only 23 amino acid residues corresponding to the extreme C-terminus of Ku80, yet it was still able to interact specifically with DNA-PKcs (compare lane 7 with the control 'pull-down' in lane 8).

To analyse further the role of the Ku80 C-terminal region in the DNA-PKcs interaction, we generated Ku80 derivatives representing a series of deletions from the C-terminus. Figure 6C shows the result of sequential deletion of 0, 28, 70, 140, 202 or 267 amino acid residues from the C-terminus of Ku80 (lanes 1–6, respectively). Strikingly, removal of only 28 amino acid residues rendered the resulting protein unable to bind DNA-PKcs (compare lanes 1 and 2), and no binding was recovered using any of the larger deletions (lanes 3–6). Taken together, these data reveal that DNA-PKcs interacts with the



**Figure 6.** The extreme C-terminus of Ku80 contains a DNA-PKcs interaction domain. Homogeneous DNA-PKcs protein (0.4  $\mu$ g) was incubated with a range of GST-Ku80 deletion derivatives (0.5–2.0  $\mu$ g) in Z' buffer (Fig. 5). The beads were washed extensively in buffer W (Fig. 5). (A) A diagram of Ku80 deletion derivatives used. (B) Silver-stained SDS-polyacrylamide gel showing DNA-PKcs protein bound by Ku80 derivatives deleted at the N-terminus. The GST-Ku80 deletion protein used in each 'pull-down' is visible in the bottom half of the gel as indicated. (C) Silver-stained gel showing that DNA-PKcs protein is not bound by Ku80 derivatives deleted from the C-terminus (lanes 2–6). Lane 1 shows DNA-PKcs retained by full-length Ku80. GST alone (5  $\mu$ g) is unable to bind DNA-PKcs (lane 7); however, it does bind to a bacterial protein of ~66 kDa during the expression/purification procedure and this band is visible clearly in lane 7 (see also Fig. 1, lanes 4 and 8; Fig. 3C, lane 1). The binding reaction between GST-Ku80ΔN1 and DNA-PKcs is duplicated [(B) lane 1; (C), lane 8] for comparison between gels.

Ku80-CTR, and that the final 28 amino acid residues of this region are necessary and sufficient for this interaction.

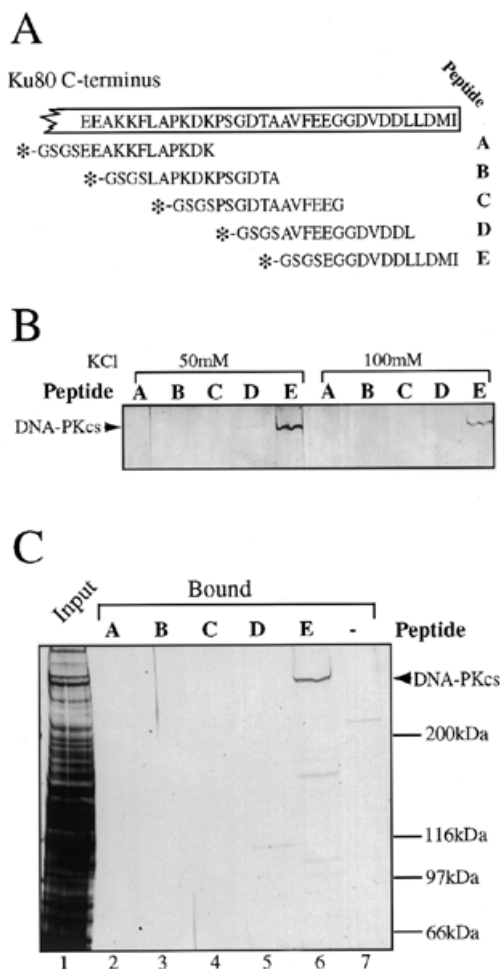
### A peptide from the extreme C-terminus of Ku80 is sufficient to mediate highly specific and efficient interactions with DNA-PKcs

To define more precisely the region of Ku80 that interacts with DNA-PKcs, we synthesised a series of peptides (peptides A–E) whose sequence was derived from the extreme C-terminus of Ku80, and which were modified at the N-terminus by the addition of a biotin moiety (Fig. 7A; Materials and Methods). To assess interactions between these peptides and DNA-PKcs, we incubated each peptide with a homogenous preparation of DNA-PKcs then added paramagnetic beads conjugated with streptavidin to capture the peptide via the biotin-streptavidin interaction. As shown in the silver-stained gel provided in Figure 7B, peptide E, comprising the final 12 amino acid residues at the C-terminus of Ku80, interacted directly with DNA-PKcs at both 50 and 100 mM KCl. In contrast, none of the other peptides (peptides A–D) exhibited significant DNA-PKcs binding.

To investigate the specificity of the DNA-PKcs-peptide interaction, we assessed the ability of each of the five peptides to retrieve proteins from a crude unfractionated HeLa cell nuclear extract (Fig. 7C). Strikingly, peptide E was able to affinity purify predominantly a single protein species of high molecular weight (Fig. 7C, compare input nuclear extract in lane 1 with peptide E eluate in lane 6). Western blotting confirmed the retrieved protein to be DNA-PKcs, and revealed that the faint lower band, seen at ~150 kDa, corresponds to a DNA-PKcs breakdown product (data not shown). Additional studies have shown that peptide E is able to deplete 60–70% of DNA-PKcs from HeLa nuclear extract, but that some DNA-PKcs always remains unbound. Importantly, DNA-PKcs affinity purified using peptide E was active only with addition of purified Ku and double-strand DNA ends, demonstrating that DNA was not present in the 'pull-down' binding reactions (data not shown). Peptides A–D and a variety of control peptides all failed to retrieve specifically any proteins from nuclear extract (Fig. 7C, lanes 2–5; data not shown). Together, these results demonstrate that the extreme C-terminal 12 amino acid residues of Ku80 are sufficient to mediate a direct and highly specific interaction with DNA-PKcs.

### DISCUSSION

We have used sequence alignments between Ku70 and Ku80 to design Ku subunit deletions in order to investigate functional domains of the two polypeptides. Thus, we have identified a region in Ku70 (amino acid residues 449–578) and one in Ku80 (residues 439–592) that mediates contact within the Ku heterodimer. Strikingly, these regions map to analogous regions of the two proteins and share sequence homologies (SHR2, PHR5 and SHR3; Fig. 2). These data suggest that the Ku subunits may have similar structures and interact with one another through a pseudo-homodimerisation mechanism. The existence of distinct Ku70 and Ku80 genes in mammals, flies, nematode worms and yeast suggests that the two proteins may have arisen through a gene duplication that occurred before the last common ancestor of *S.cerevisiae* and metazoa. The absence of clear Ku homologues in the fully sequenced genomes of various bacteria and archaea suggests that Ku



**Figure 7.** The final 12 amino acid residues of Ku80 are sufficient to bind DNA-PKcs. **(A)** A diagram of the C-terminus of human Ku80, amino acid residues 699–732, and the sequence of five peptides (peptides A–E) generated with an N-terminal biotin group (asterisk) **(B)** Interactions between peptides A–E and purified DNA-PKcs. Each peptide ( $2 \times 10^{-11}$  mol) was incubated for 2 h 45 min at 4°C with 400 ng ( $\sim 10^{-12}$  mol) of purified DNA-PKcs in Z' buffer (Fig. 5) containing either 50 mM KCl (first five lanes) or 100 mM KCl (next five lanes). Peptides were then captured on streptavidin-conjugated beads and washed in buffer W (Fig. 5) containing the same concentration of KCl as the binding reaction. Any protein remaining bound to the peptide/beads was eluted in SDS and resolved on a 6% SDS–polyacrylamide gel as shown. **(C)** Silver-stained SDS–polyacrylamide gel showing protein bound by peptides A–E following incubation with HeLa NE. Each peptide was incubated for 2 h 45 min at 4°C with 100  $\mu$ g of HeLa cell nuclear extract in Z' buffer containing 50 mM KCl, followed by capture of the peptide onto streptavidin-conjugated beads and washing in Z' buffer (50 mM KCl). Proteins retained by peptides A–E are shown in lanes 2–6. An aliquot of 10  $\mu$ g of NE is loaded in lane 1. In a control binding reaction, magnetic beads alone interacted weakly with a band migrating just above the 200 kDa marker (lane 7) and this interaction was suppressed by binding of biotinylated peptide to the beads suggesting it may represent an interaction with a nuclear protein mediated by streptavidin.

evolved subsequent to the divergence of the eukaryotic and prokaryotic lineages.

Previously, little was known about the region(s) of Ku responsible for its interaction with DNA-PKcs. We have found that Ku80 makes direct and specific contacts with DNA-PKcs.

Furthermore, we have shown that the major DNA-PKcs binding domain of Ku80 is located at the extreme C-terminus of the molecule, and that the final C-terminal 12 amino acid residues of Ku80 are sufficient to mediate this highly specific interaction. Indeed, the interaction between DNA-PKcs and the C-terminal region of Ku80, either expressed as a GST fusion or presented as a biotinylated peptide, can be used to affinity purify DNA-PKcs to virtual homogeneity in one step from crude unfractionated human nuclear extract (Figs 5A and 7C). This novel affinity purification step will be of use for future analyses of DNA-PK, particularly where source material is limited.

Consistent with the DNA-PKcs interaction motif we have identified being of physiological importance, recent mutational and functional analyses have demonstrated that a region comprising 178 residues at the C-terminus of Ku80 is extremely important for the interaction of the Ku heterodimer with DNA-PKcs *in vivo* (35). Perhaps surprisingly, we find that C-terminal fragments of Ku80 in isolation bind to DNA-PKcs independently of DNA. In contrast, the interaction between the native Ku heterodimer and DNA-PKcs occurs only in the presence of DNA (15,16). An attractive model to explain this difference is that the DNA-PKcs interaction region of Ku80 is masked in the native Ku heterodimer and only becomes exposed when a conformational change takes place upon DNA binding. Interestingly, we have observed interactions between Ku70 and DNA-PKcs in low salt buffers (<100 mM KCl; data not shown), but the possible importance of these has not been investigated. It is possible that DNA-PKcs contacts formed by Ku70 in cooperation with the Ku80 subunit are of functional significance.

The Ku80-CTR neither participates in homodimerisation (Figs 3 and 4), nor contributes to the DNA-binding domain, which comprises the C-terminal approximately two-thirds of both Ku subunits (2,7,8,35). In addition, contact between this region and DNA-PKcs suggests that the Ku80-CTR may constitute a protein domain exposed on the surface of the Ku heterodimer. It is therefore tempting to speculate that the Ku80-CTR, and possibly the Ku70-CTR, contact other proteins involved in DNA DSB repair, or play a role in additional functions that have been ascribed to Ku, such as regulating telomeric functions and controlling chromatin structure (25,36). Indeed, the *Caenorhabditis elegans* and *S.cerevisiae* Ku80-CTRs, although lacking the DNA-PKcs interaction motif, share some sequence similarities that may correspond to such protein interaction sites.

Significantly, genes encoding orthologues of DNA-PKcs have been found in human, mouse, hamster, horse and *Xenopus laevis* (1), but are not evident in *S.cerevisiae*, or in the virtually complete genome sequence of *C.elegans*. This difference is mirrored by the state of the C-terminus of Ku80 in these organisms. Thus, the final 12 amino acid residues of human Ku80 that we have identified as interacting with DNA-PKcs are strikingly conserved in mouse and hamster Ku80, and in the recently identified *Xenopus* Ku80 homologue (37) but are absent from the *C.elegans* and *S.cerevisiae* Ku80 homologues (Fig. 8). DNA-PKcs and the Ku80 DNA-PKcs interaction motif have, therefore, been found only in vertebrates. These observations suggest that Ku may have evolved to function as a subunit of a relatively recently evolved activity, DNA-PK, by acquiring a protein–protein interaction domain at the C-terminus of the Ku80 subunit. What cellular changes might have been linked to this event? Perhaps the evolution of DNA-PK was part of a





**Figure 8.** Multiple sequence alignment of the C-terminal regions of Ku80 homologues showing high sequence conservation between mammalian (*Cricetus griseus*, *Mus musculus* and *Homo sapiens*) and *X.laevis* Ku80 homologues in the final 13 amino acid residues. In contrast, the *C.elegans* and *S.cerevisiae* Ku80 homologues lack these sequences. The location of the DNA-PKcs interaction motif defined within human Ku80 (the final 12 amino acid residues) is indicated.

development of the DNA DSB repair machinery to carry out specialised end-joining functions in V(D)J recombination, a process which appeared early in vertebrate evolution (38). Alternatively, the evolution of DNA-PK may be connected to a more fundamental change to DNA-repair processes that occur in all cells, not just the subset of cells that undergo V(D)J recombination. This change is a switch in the predominant DNA DSB repair pathway, from homologous recombination, as observed in yeast, to non-homologous end-joining, as occurs in mammalian cells.

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