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Double-strand break repair by Ku70 requires heterodimerization with Ku80 and DNA binding functions

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Heterodimers of the 70 and 80 kDa Ku autoantigens (Ku70 and Ku80) activate the DNA-dependent protein kinase (DNA-PK). Mutations in any of the three subunits of this protein kinase (Ku70, Ku80 and DNA-PKcs) lead to sensitivity to ionizing radiation (IR) and to DNA double-strand breaks, and V(D)J recombination product formation defects. Here we show that the IR repair, DNA end binding and DNA-PK defects in Ku70–/– embryonic stem cells can be counteracted by introducing epitope-tagged wild-type Ku70 cDNA. Truncations and chimeras of Ku70 were used to identify the regions necessary for DNA end binding and IR repair. Site-specific mutational analysis revealed a core region of Ku70 responsible for DNA end binding and heterodimerization. The propensity for Ku70 to associate with Ku80 and to bind DNA correlates with the ability to activate DNA-PK, although two mutants showed that the roles of Ku70 in DNA-PK activation and IR repair are separate. Mutation of DNA-PK autophosphorylation sites and other structural motifs in Ku70 showed that these sites are not necessary for IR repair *in vivo*. These studies reveal Ku70 features required for double-strand break repair.

Keywords: double-strand break repair/DNA-dependent protein kinase/heterodimerization/ionizing radiation/Ku

Introduction

The DNA-dependent protein kinase (DNA-PK) is a nuclear DNA-binding complex involved in double-strand break repair events occurring following ionizing radiation (IR) damage or as a result of B and T cell V(D)J recombination in progenitor stages of immune system development (Jin *et al.*, 1997a). Spontaneous damage to chromosomes leading to double-strand breaks is also likely to be repaired by a mechanism involving DNA-PK (Liang *et al.*, 1996). DNA-PK consists of (i) a large serine/threonine protein kinase (DNA-PKcs), encoded by the *scid* gene (Anderson and Lees-Miller, 1992; Blunt *et al.*, 1995; Hartley *et al.*, 1995), and (ii) the Ku autoantigen (Mimori *et al.*, 1981). Ku associates with and dissociates from DNA-PK *in vivo* and *in vitro* (Lees-Miller *et al.*, 1990; Suwa *et al.*, 1994; Finnie *et al.*, 1995), and these processes may be modulated in DNA repair responses (Jin *et al.*, 1997b; Kharbanda *et al.*, 1997).

Ku is a heterodimer of 70 and 80 kDa subunits that binds to DNA ends and other alterations in double-stranded DNA; it binds poorly to nicks and single-stranded DNA [reviewed in Jin *et al.* (1997a)]. Ku can bind to DNA irrespective of whether it is associated with the protein kinase. DNA-PK is DNA-dependent, and the association of Ku with DNA appears to activate the kinase. Thus the properties of Ku and the activity of DNA-PK are both potentially important for DNA repair events. Although the DNA binding and heterodimerization properties of Ku are characteristic of the complex (Wu and Lieber, 1996), the relevance of these properties to IR repair has not been investigated.

By analysing the properties of Ku80-deficient cells, it has been shown that Ku80 is required for double-strand break repair and V(D)J recombination (Getts and Stamato, 1994; Rathmell and Chu, 1994; Taccioli *et al.*, 1994; Boubnov *et al.*, 1995b). Ku80-deficient mice suffer both severe combined immunodeficiency and general growth defects, also indicating a role in V(D)J recombination and DNA repair that may influence cell proliferation (Nussenzweig *et al.*, 1996; Zhu *et al.*, 1996). In the yeast, *Saccharomyces cerevisiae*, Ku70 (HDF1) and Ku80 mutants are vulnerable to DNA repair defects, and both subunits are required to restore DNA repair proficiency and DNA end binding (Milne *et al.*, 1996). Recently, it was shown that murine embryonic stem (ES) cells with gene-targeted deletions of Ku70 were sensitive to IR and had V(D)J recombination defects and deficiencies in DNA end binding (Gu *et al.*, 1997). In Ku70-deficient cell lines, the unmutated Ku80 partner is unstable, as is the Ku70 partner in Ku80-deficient cell lines (Nussenzweig *et al.*, 1996; Gu *et al.*, 1997). Likewise, Ku80-deficient cells have reduced DNA-PK activity (Finnie *et al.*, 1995).

This is the first report of reintroduction of Ku70 into a Ku70-deficient cell line where the features of Ku in IR repair *in vivo* can be studied. In order to understand the molecular properties of Ku, we have undertaken an extensive mutational analysis of the Ku70 gene. We find that DNA binding and Ku heterodimerization functions are generally linked and are found in a core region of the protein. Several epitopes previously suggested to be relevant to Ku properties in IR repair, including DNA-PK phosphorylation sites, a nucleotide binding motif, a leucine repeat and a helix–turn–helix, were all dispensable for Ku functions in complemented cells. Additional mutational analysis shows that the Ku activation of DNA-PKcs correlates well with Ku DNA end binding and heterodimerization, although two mutants separated the restoration of DNA end binding and DNA-PK activity from IR repair. Ku70 participates in DNA repair by virtue of association with Ku80 and DNA binding; the structural integrity of Ku is important for DNA-PK activation.

Results

Complementation of IR-sensitivity, DNA end binding and DNA-PK deficiency of Ku70–/– cells by Ku70 cDNA

Ku70–/– (double knockout, or DKO) cells are sensitive to IR, lack Ku-dependent DNA end binding and fail to allow V(D)J recombination product formation (Gu *et al.*, 1997). They also have significantly diminished levels of Ku80 protein. Therefore, Ku70's role in DNA repair may be linked to the known biochemical features of Ku, namely heterodimerization, DNA binding and DNA-PK activation. To understand better the properties of Ku70 through a mutational analysis, it was important first to demonstrate whether introduction of a wild-type cDNA was capable of restoring the normal IR repair and Ku phenotypes to these cells.

The wild-type human Ku70 cDNA was modified to contain a c-Myc epitope tag at the N-terminus, cloned into a mammalian expression vector (pEFpuro) and introduced into the DKO cell line by electroporation (Materials and methods). Following puromycin selection for 7–10 days, cells were plated for IR cell survival tests with doses ranging from 0 to 300 rad (Boubnov *et al.*, 1995b). The IR resistance of ES cells reconstituted with Ku70 cDNA was comparable to that of ES cells heterozygous for Ku70 (SKO) (Figure 1A). Both cell lines were significantly more radioresistant than the DKO cell line. Introduction of pEFpuro alone led to radiosensitivity that was indistinguishable from that in DKO cells.

Next, a DNA end binding assay was performed to evaluate the ability to form protein–DNA complexes. As for IR repair, DNA end binding was restored in conjunction with reintroduction of the wild-type Ku70 cDNA, but not following stable transfection of pEFpuro alone (Figure 1B).

Crude extracts of Ku80-deficient cells have no biochemically detectable DNA-PK activity (Finnie *et al.*, 1995). Therefore, it might be expected that Ku70-deficient cells would also lack DNA-PK activity. DNA-PK activity was monitored by assessing the wortmannin sensitivity of DNA-dependent p53 peptide phosphorylation (Materials and methods; Lees-Miller *et al.*, 1992; Hartley *et al.*, 1995). We observed that DKO cell extracts have a low level of DNA-stimulated DNA-PK activity whether or not wortmannin is added to the kinase reaction (Figure 1C). In contrast, ES cells reconstituted with Ku70 cDNA yielded about three times more DNA-PK activity in the absence of wortmannin then when it was added to the reaction (Figure 1C). Therefore, wild-type Ku70 cDNA can complement each of these biochemical features of the DNA-PK complex, as well as IR repair.

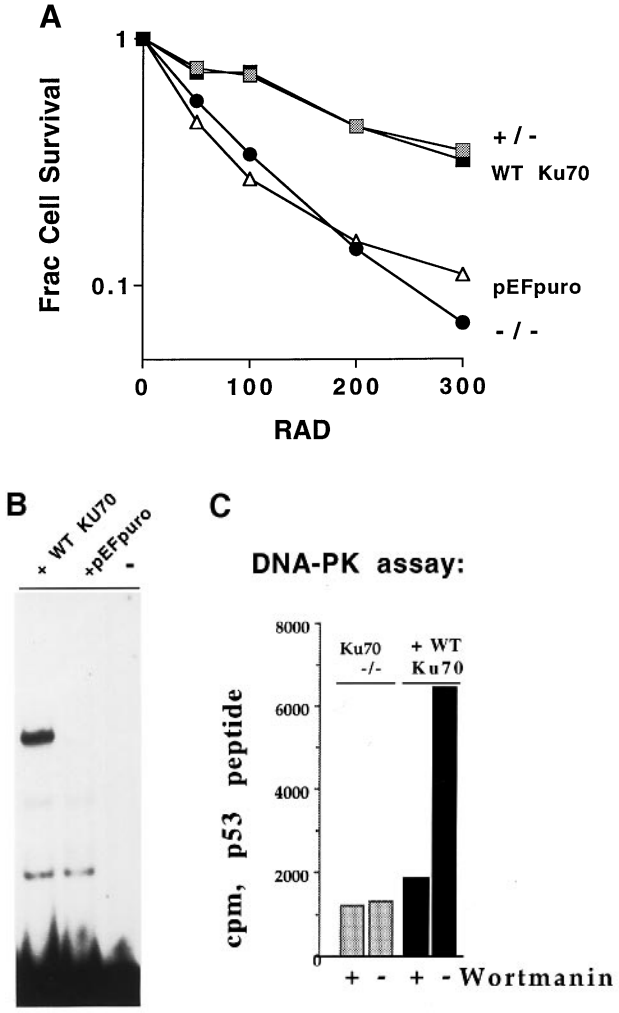

Fig. 1. Complementation of IR-sensitivity, DNA end binding and DNA-PK activity of Ku70–/– (DKO) cells by Ku70 cDNA. (**A**) IR sensitivity of Ku70–/– ES cells complemented by transfection of an epitope-tagged Ku70 cDNA. Ku70+/– (gray boxes), Ku70–/– (filled circles), Ku70–/– complemented with wild-type Ku70 cDNA cloned into pEFpuro (filled boxes), and pEFpuro alone (open triangles) were compared for radiosensitivity in clonotypic cell survival assays. IR doses of 0–300 rad were tested for each cell line. (**B**) Restoration of DNA end binding in Ku70–/– cells complemented for IR repair. Cell extracts from the Ku70–/– cells complemented with wild-type cDNA cloned into pEFpuro or pEFpuro alone, were prepared as described previously (Boubnov *et al.*, 1995). DNA end binding was monitored by a gel shift assay with a radiolabeled DNA probe (Materials and methods). In the lane labeled '–', no protein was added to the DNA end binding reaction. (**C**) DNA-PK activity of DKO and wild-type Ku70 cDNA reconstituted DKO cells. Frozen–thawed cell extracts were prepared and DNA-PK activity assayed by measurement of phosphorylation of a p53 peptide (Materials and methods). Samples were divided into two equal portions prior to assay for comparison of DNA-PK activity in the presence (+) or absence (–) of 100 μM wortmannin.

N- and C-terminal truncations of Ku70 define regions essential for IR repair

The N-terminal regions of Ku70 are not required for DNA end binding and heterodimerization of Ku *in vitro* (Wu and Lieber, 1996), but these authors did not test whether truncated proteins could complement Ku functions *in vivo*. To investigate further the role of the N-terminal portion of Ku70, we prepared four truncation mutants (containing Ku70 amino acid residues 63–609, 122–609, 165–609 or 226–609) by PCR and subcloning into pEFpuro (Materials and methods). As for the wild-type cDNA expression plasmid, pEFpuro-Ku70 truncation mutant derivatives were introduced into DKO cells by electroporation and puromycin selection. None of these four truncation mutants of Ku70 restored IR repair above the level found in parental DKO cells (Figure 2A and Table I). Likewise, nuclear extracts of the mutant cell lines were deficient in DNA end binding activity, like DKO cells (Figure 4A).

We next evaluated whether the Ku70 truncation proteins were produced and able to associate with the other Ku

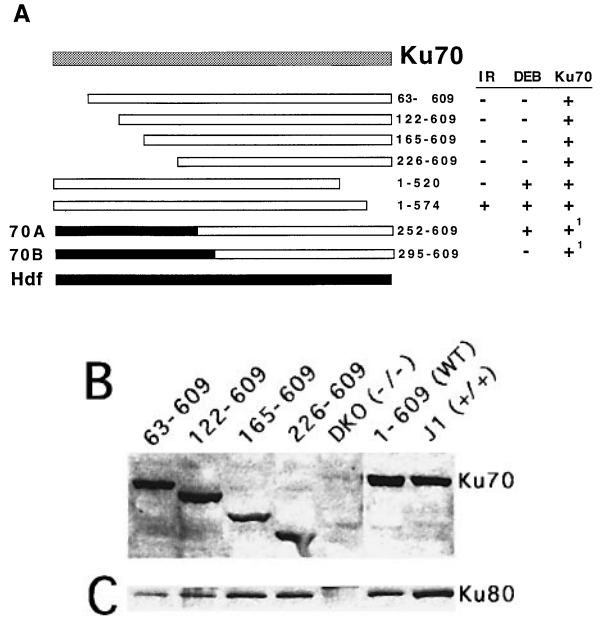

Fig. 2. Properties of deletion mutants of Ku70 and HDF1–Ku70 chimeras. (**A**) N- and C-terminal truncations of Ku70 were constructed in pEFpuro (Materials and methods). Amino acid coordinates of the Ku70 proteins are listed for each truncation. Each truncation mutant contains an N-terminal c-Myc epitope tag (not shown). Chimeric genes encoding the N-terminal fragments of yeast Ku70 (HDF1) and C-terminal portions of human Ku70 were prepared as shown, and introduced into an *S.cerevisiae* expression vector. Ku70–/– DKO cells were then examined for IR repair as in Figure 1, and for DNA end binding and stable production of Ku. Ku70- and Ku80-deficient yeast (DWY315; Δ*hdf1* Δ*ku80*) were examined for DNA end binding. '–' indicates lack of complementation, '+' refers to IR repair, DNA end binding or stable Ku70 derivative protein production, '$+^1$' refers to chimeric proteins produced by *in vitro* translation. (**B**) Immunoblot of lysates from N-terminal truncations and wild-type (WT) cDNA-complemented cells probed with anti-Ku70 antibody, M19. (**C**) Immunoblot of same samples with anti-Ku80 antibody, C20.

subunit, Ku80. Cell lysates were prepared from each of the truncation mutant cell lines and controls. We observed that all four Ku70 truncated proteins were successfully produced, as shown by SDS–PAGE and immunoblotting with an anti-Ku70 antibody (M19) (Figure 2B). The N-terminal deletion proteins were stably formed at nearly the same levels as reintroduced and expressed full-length protein (1-609, WT) whereas DKO cells alone showed no Ku70 protein (Figure 2B). Immunoprecipitation with an anti-Myc antibody recognizing the N-terminal epitope tag of the expressed Ku70 proteins was then used to evaluate the ability of these truncation mutants to associate with Ku80. DKO cells transfected with the full-length Ku70 cDNA were observed to co-precipitate Ku80 (Figure 2C). We found that each of the N-terminal Ku70 truncation proteins stably associated with Ku80 at approximately the same levels as full-length Ku70 protein (Figure 2C). Therefore, Ku heterodimerization epitopes are not principally located in the N-terminal third of Ku70, and full-length Ku70 appears to be required for DNA end binding.

C-terminal truncations of Ku70 were created by introducing stop codons by site-directed mutagenesis. Expression of the C-terminal mutant 36 (aa 1–574) conferred the same IR resistance and DNA end binding as the wild-type cDNA (Figure 2A and Table I). However, mutant 34 (aa 1–520), which was further truncated at the C-terminus, deleting conserved homology domain 9 (Wu and Lieber, 1996; Figure 3), failed to reconstitute IR repair (Figure 2A and Table I). It produced an abnormal DNA end binding complex of higher mobility, consistent with the production of a truncated Ku70 protein. Whereas the C-terminus of Ku70 is not essential for IR repair, DNA end binding or heterodimerization, if C-terminal truncations extend into a region that is conserved among Ku homologs, then these functions are disrupted. Therefore, a substantial fraction of Ku70 (at least residues 63–520, i.e. ~75% of the Ku70 protein) is necessary for its functions in IR repair.

We also constructed chimeras of the genes encoding Ku70 and its *S.cerevisiae* homolog, HDF1 (Feldmann *et al.*, 1993). The N-terminal region of HDF1 was substituted in two ways to make chimeric gene products of roughly similar size. Chimeras 70A (aa 1–261 of HDF1 and 252–609 of Ku70) and 70B (aa 1–295 of HDF1 and 295–609 of Ku70) were formed as described in Materials and methods. These chimeric Ku70 proteins were cloned into the yeast expression vector, pSJ8 (*ADH:HDF1/ku70*, *URA3*), and introduced into DWY315 (Δ*hdf1* Δ*ku80 ura3 leu2*) with the human *ku80* gene (pSJ11, *ADH:ku80*, *LEU2*). We found that 70A + Ku80, but not 70B + Ku80, was competent for DNA end binding in yeast (Figure 4C). In control experiments, wild-type human Ku70 and Ku80 expressed in DWY315 produced a DNA end binding complex (Figure 4B, 'WT'). Both gene constructs were confirmed to produce ~75 kDa gene products by *in vitro* transcription/translation (data not shown). However, the DNA end binding complexes of 70A + Ku80 had higher mobility, and were not as abundant as wild-type Ku in yeast (DWY83). Therefore, addition of N-terminal sequences of the related protein, Hdf1, only partially rescue DNA end binding. Conserved sequences in the N-terminus of Ku70 (Figure 3) are likely to contribute to DNA end binding and heterodimerization properties by forming a proper conformation of the Ku complex.

Mutational analysis reveals dual requirements for DNA end-binding and heterodimerization in IR repair

To investigate further the subdomains dictating Ku70's properties, we undertook a more comprehensive mutational analysis based on site-specific mutagenesis. Thirty-five single, double or triple amino acid mutants were generated in Myc-tagged Ku70 (Figure 3 and Table I). Each of these mutants was subcloned into pEFpuro and transfected into DKO cells (Materials and methods). As described above, the biochemical and functional properties of Ku were investigated for each cell line.

DNA end binding was tested by preparing extracts from each stably transfected ES cell line (Materials and methods). Of the 35 site-specific mutants, extracts from 12 of these mutants had no or very little detectable DNA end binding activity (Figure 4A and Table I). Site-specific mutants were also cloned into a yeast expression vector and co-transformed with a human Ku80 expression vector into Ku-deficient yeast. Yeast cell extracts were then used in DNA end binding (Figure 4B). The DNA end binding of these mutants in yeast corresponded to that in murine ES cells. Mutations in aa 119–483 had the greatest effect on DNA end binding (Figure 3 and Table I) but, even

Table I. Ku70 site-specific and truncation mutants and complemented functions in Ku70–/– cells

No.	Mutation[a]	IR repair	DNA end binding	Ku70	Ku70–Ku80 heterodimerization	DNA-PK activity
1	EE17KK	++	++			
2	R35G	++	++			
3	S51A	++	++			
4	S51E	++	++			
5	SQS51AQA	++	++			
6	F87G	++	+	++		
7	L119R	–	–	–	–	–
8	C150R	–	–	–	–	–
9	KR164NG	++	++			
10	D192A	++	++			
11	KR253NG	+/–	++			++
12	L263R	–	–	+	–	–
13	VG271ER	–	–	++	–	–
14	S319A	++	+			
15	EE332GN	++	++			
16	L347R	–	–	++	–	–
17	F367A	–	+/–	++	–	–
18	S378R	++	++			
19	L385R	–	–	++	–	–
20	C389S	++	+			
21	A396V	++	++			
22	C398S	–	+/–	+	–	–
23	P408H	+	+			
24	L413R	+/–	–	++	–	–
25	P415R	–	–	+	–	–
26	GF430DA	–	–	+	–	–
27	P438A	++	++			
28	RK445SE	++	++	++		
29	R470D	++	++			
30	L483R	–	–	–	–	
31	L490R	++	++	++		
32	L495R	++	++			
33	D505R	–	+			++
34	L521*	–	++[b]	+		+/–
35	K539E	++	++			
36	K575*	++	++			
37	KSG591AAA	++	++			++
61	63–609	–	–	++	++	–
62	122–609	–	–	++	++	
63	165–609	–	–	++	++	
64	226–609	–	–	++	++	–

The IR sensitivity, Ku70 protein levels, heterodimerization of Ku and DNA-PK assay phenotypes of complemented Ku70–/– DKO cells were assessed as described in the text, and categorized as follows: ++, equivalent to wild-type; +, lower than wild-type; +/–, weak; –, absent.
[a]Mutants are listed in numerical order from the N- to C-terminus of Ku70. Amino acid changes are designated by listing the wild-type residue(s), the amino acid position of the N-terminal residue in the group, and then the introduced mutant amino acid(s). Asterisks denote the introduction of stop codons. Mutants 61–64 are truncations, and contain the indicated amino acid residues.
[b]DNA end binding complex with higher mobility.

within this region, a number of mutants (EE332GN, S378R, C389S, A396V and P408H) retained wild-type complementation ability for DNA end binding.

Mutants unable to complement DNA end binding functions of Ku may arise by several means: an inability to express the Ku70 protein stably, loss of heterodimerization with Ku80, or lack of DNA association. Thus, we next examined these specific DNA end binding negative (DEB^-) mutants for the production of Ku70 protein. We found that the majority (12/15) of the DEB^- mutant ES cells tested positive for Ku70 protein by direct immunoblotting of cell lysates and/or immunoprecipitation with either the anti-Ku70 monoclonal antibody, AG4-7.5, or anti-Myc monoclonal antibody, 9E10, followed by immunoblotting with a goat anti-mouse Ku70 antibody (M19; Figure 5, Table I). Only three mutants, L119R, C150R and L483R (numbers 7, 8 and 30 respectively), did not express Ku70 protein (Figure 5; Table I).

We next evaluated the presence of Ku heterodimers using immunoprecipitation and Western blots. Lysates were immunoprecipitated with AG4-7.5, and scored for an associated Ku80 protein by immunoblotting with a goat anti-mouse Ku80 antibody (M20). Ku70–Ku80 heterodimers, formed by reconstitution with wild-type Ku70 cDNA, are readily detected by this assay (Figure 5). In contrast, none of the DEB^- mutants yielded a stably co-associated Ku80 protein (Figure 5, Table I). Thus, the inability to heterodimerize correlates well with loss of DNA end binding activity. In addition, the levels of Ku70 protein were generally lower for mutants unable to reconstitute heterodimerization (Figure 5), suggesting that heterodimerization is relevant to Ku70 stability. Because

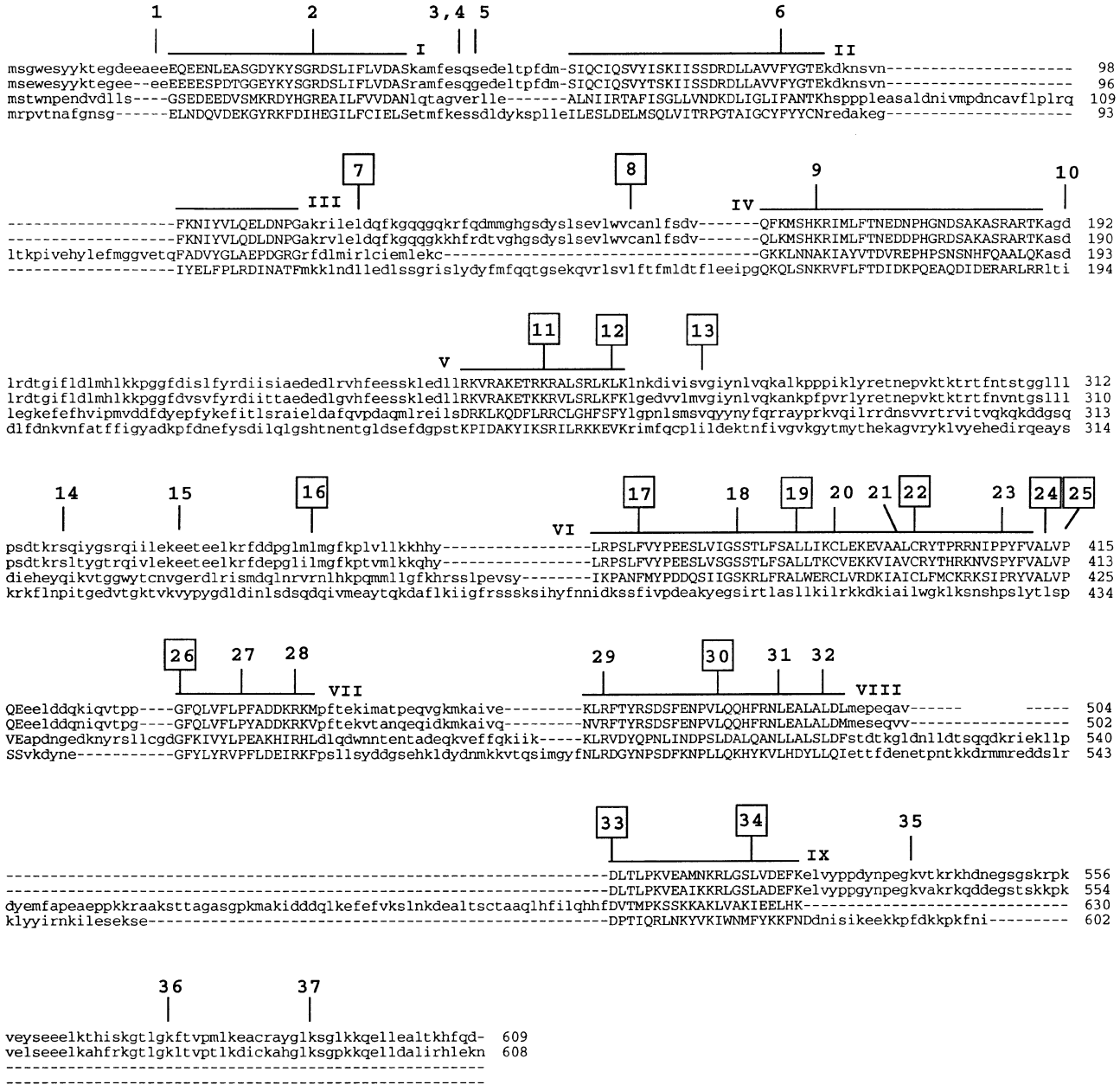

Fig. 3. Ku70 site-specific mutations. Human (top line), mouse (second line), *Drosophila melanogaster* (third line) and *S.cerevisiae* (fourth line) Ku70 homologs were aligned using MACAW as specified previously (Wu and Lieber, 1996). Ku70 domains I–IX of greatest homology are indicated by bold lines. The positions of Ku70 site-specific mutants are indicated by arrows. Mutants displayed in boxes are IR-sensitive; unboxed mutants have wild-type complementation of IR repair, DNA end binding and Ku80 heterodimerization (see text).

the epitopes dictating these properties are spread throughout the Ku70 central region, it is likely that the domain involved in heterodimerization and DNA end binding cannot be subdivided further.

We next examined the IR sensitivity of each of the 35 site-specific mutants. Dose–response curves for wild-type Ku70 cDNA-complemented DKO cells and DKO cells were used as positive and negative controls. We found that the radiosensitivity of complemented DKO cells corresponded to the biochemical features of Ku described above. Mutants in the core region involved in DNA end binding and heterodimerization restored IR resistance and their ability to do so corresponded exactly with their ability to reconstitute DNA end binding (Table I). Likewise, mutants from this region that had lost DNA end binding capacity, also lost the ability to repair IR damage. Thus, a central region of Ku70 appears most affected by mutations able to reduce significantly or eliminate IR repair (Figure 3 and Table I). An estimate of the minimum Ku70 core region required for IR repair, DNA end binding and heterodimerization is 168 residues, aa 263–430.

Ku heterodimerization/DNA end binding mutants lack DNA-PK activity

From the above analysis of 41 site-specific and truncation mutants, we found 19 mutants with little or no ability to

reconstitute IR repair in ES DKO cells (Table I). Yet, most of these mutants detectably produced a Ku70 protein. Therefore, we tested whether the Ku70 mutant proteins were sufficient to activate DNA-PK independently of DNA end binding and Ku heterodimerization. Cell extracts from each of the IR-sensitive mutants were examined for DNA-PK activity in an *in vitro* DNA-dependent protein kinase assay (Materials and methods). As for introduced wild-type Ku70 cDNA, we used the relative inhibition by wortmannin to characterize the DNA-stimulated DNA-PK kinase activity. Ku70 proteins with mutations in the core region (aa 253–430) were unable to restore DNA-PK activity if they were also unable to reconstitute DNA end binding and heterodimerization (Table I). Therefore, in this region, Ku-specific properties (DNA end binding and heterodimerization) correspond exactly with the restoration of DNA-PK activity and IR repair.

Two exceptions to the general correspondence between IR repair, DNA end binding and heterodimerization were found. Mutants KR253NG and D505R restored DNA end binding, but failed to reconstitute IR repair adequately (Table I). If Ku is not functionally independent of DNA-PK in IR repair, then these mutants should also be negative for DNA-PK activity. Cell extracts from KR253NG and D505R ES cells were compared with complemented wild-type Ku70 ES cells for DNA-PK function. We observed that KR253NG and D505R reconstituted the DNA-PK p53 peptide kinase activity (Table I). Therefore, KR253NG and D505R are not likely to disrupt the ability of the DNA-PK complex to form and be activated by DNA. Instead, the mutations may identify epitopes or structural regions of Ku70 that either interact directly with other proteins or cause a structural change that affects DNA-PK interactions with this/these factor(s) in IR repair.

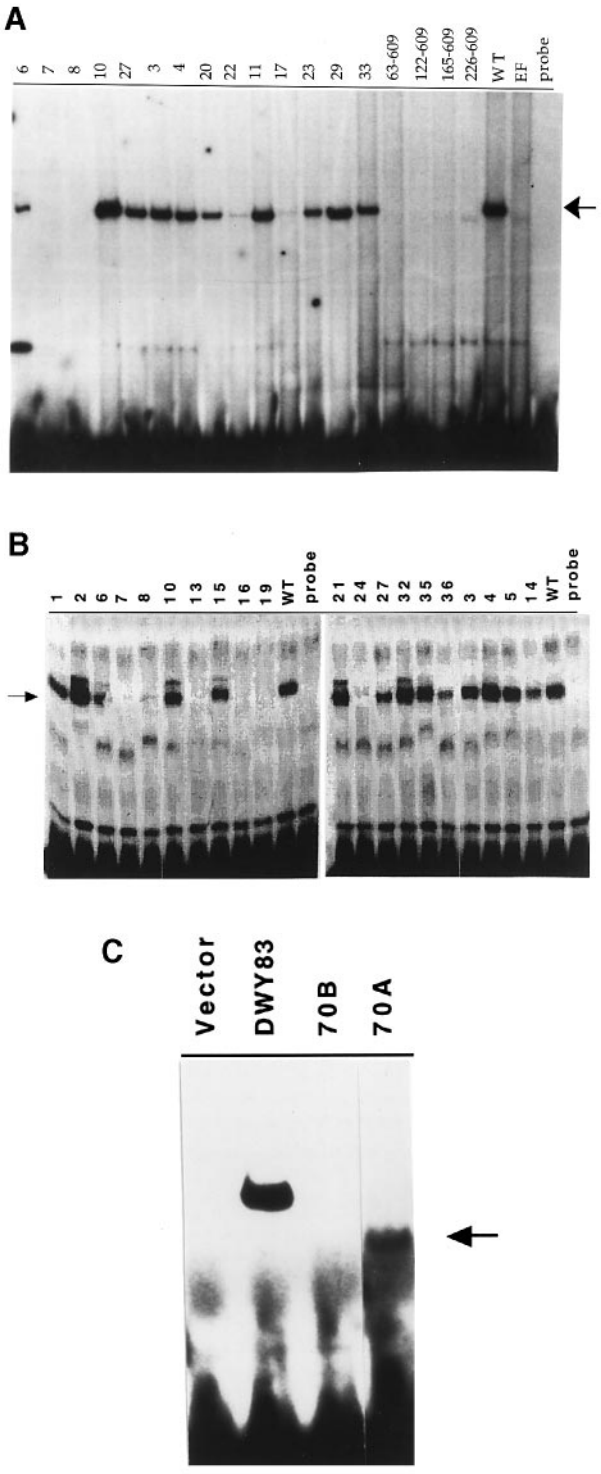

Fig. 4. Ku DNA end binding assays for Ku site-specific and deletion mutants. (**A**) Cell lysates from stably transfected ES cells with Ku70 mutants were prepared as described in Materials and methods. DNA end binding was measured as in Figure 1. Arrow shows the Ku DNA end binding complex. Ku70 truncation mutants are listed according to Figure 2A, and site-specific mutants described in Table I. WT, wild-type Ku70 cDNA-transfected DKO, EF, pEFpuro-transfected DKO; 'probe', no extract control. (**B**) DNA end binding extracts were prepared from yeast DWY315 (Δ*hdf1* Δ*ku80*) cells transformed with Ku70 mutants and controls in a yeast expression vector. Human Ku80 cDNA in a yeast vector was always co-transformed with Ku70 derivatives. WT, wild-type human Ku70 cDNA; –, vectors only. See Table I and Figure 3 for descriptions of mutants. (**C**) DWY315 transformed with either Hdf1–Ku70 chimeras 70A or 70B with human Ku80. DWY83 is a wild-type yeast strain. Vector, pDB20 (*URA3*) + pDBL (*LEU2*) control plasmids (Milne *et al.*, 1996).

Ku70 DNA-PK phosphorylation consensus sites are unnecessary for IR repair

Ku is phosphorylated by DNA-PK when the kinase is activated by the DNA–Ku–DNA-PKcs association or in anti-Ku immunoprecipitation complexes (Lees-Miller *et al.*, 1990; Boubnov *et al.*, 1995a; Chan and Lees-Miller, 1996). Ku70 is phosphorylated five times more than Ku80, arguing that Ku70 phosphorylation could be significant for Ku and/or DNA-PK function. Inspection of human Ku70 reveals two potential DNA-PK phosphorylation sites based on the consensus SQ (Anderson and Lees-Miller, 1992). Only S51Q52 is conserved between the human and mouse proteins (Figure 6).

We determined whether DNA-PK autophosphorylation of Ku70 was consistent with an SQ consensus by phosphoamino acid analysis and phosphopeptide mapping. LAZ388 human B cells were lysed and immunoprecipitated with an anti-Ku80 antibody, GE2-9.5, followed by an *in vitro* kinase reaction with [γ-^{32}P]ATP (Materials and methods). After fractionation on a 10% SDS–polyacrylamide gel, a ^{32}P-labeled Ku70 gel slice was excised and complete trypsin protease digestion conducted. ^{32}P-labeled phosphopeptides were next fractionated by two-dimensional electrophoresis. Two Ku70 peptides of very similar

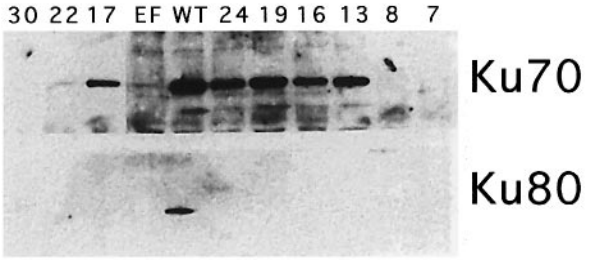

Fig. 5. Analysis of Ku70 production and Ku70–Ku80 heterodimerization. Ku70 mutants stably expressed in ES DKO cells were examined for the production of Ku gene products and the formation of Ku heterodimers. Mutants that were IR-sensitive and/or DNA end binding negative were tested for Ku protein levels (Table I). Cell lysates were prepared in NP40 lysis buffer, immunoprecipitated with anti-Ku70 monoclonal antibody AG4-7.5, fractionated by 10% SDS–PAGE, electrophoretically transferred and immunoblotted with anti-Ku70 antibody, M19 (**A**), or anti-Ku80 antibody, M20 (**B**), as described in the text.

mobility were discriminated at pH 8.9, indicating a high negative charge (Figure 6A). Phosphoamino acid analysis and chromatography showed that these peptides were phosphorylated exclusively on serine residues (Figure 6B). Peptidesort computer projection of the Ku70 tryptic peptide (aa 47–74) containing SQS at residues 51–53 is predicted to have a significant negative charge (–4) at pH 8.9, and this charge is increased to –6 upon phosphorylation at serine. These peptides are expected to have charges of +2 (unphosphorylated) and +1 (phosphorylated) at pH 1.9, as was seen (data not shown). The tryptic fragment containing the non-conserved S319,Q320 would not be consistent with this electrophoretic pattern. Therefore, it is likely that the observed phosphorylation of Ku70 by DNA-PK is restricted and at residues 51–53 (SQS).

To determine whether Ku70's functions in IR repair depend on its ability to be phosphorylated at DNA-PK consensus serines, site-specific mutations were generated as specified in Materials and methods. Single and double point mutants of serine residues were created at residues 51–53 (SQS) or 319 and 320 (SQ), giving AQS 51–53, EQS 51–53, AQA 51–53 and AQ 319–320. Each mutant was then stably transfected into ES DKO cells and its IR sensitivity was evaluated as above. If phosphorylation of Ku70 is required for IR repair, it would be expected that serine to alanine Ku70 mutants would be unable to complement IR sensitivity of DKO cells. In contrast, the substitution of glutamate may mimic serine phosphorylation, and produce an IR-competent protein. Each of the phosphorylation-inactivating mutants AQS 51–53, AQA 51–53 and AQ 319–20 was found to reconstitute IR repair capacity and DNA end binding to DKO cells to the same extent as wild-type Ku70 cDNA (Figures 6C and 4A; Table I). Likewise, mutant EQS 51–53 did not alter IR repair or DNA end binding (Table I). Therefore, Ku70 phosphorylation at DNA-PK consensus sites is required for IR repair. Phosphorylation could be significant for other Ku functions in the cell.

Examination of additional motifs of Ku70 for a role in IR repair

Inspection of the Ku70 open reading frame reveals several potentially relevant structural motifs that have been pointed out previously (Reeves and Sthoeger, 1989; Chou *et al.*, 1992; Wu and Lieber, 1996). Using mutagenesis, we tested whether some of these residues could be involved in Ku's functions.

Previously it was shown that *in vitro* DNA binding of purified Ku requires reduced sulfhydryl residues that may be in direct contact with DNA (Zhang and Yaneva, 1993). Two cysteines are well conserved between human, mouse and *Drosophila* Ku70 in domain VI within the core region implicated in DNA end binding and heterodimerization (Figure 3; Wu and Lieber, 1996). Cysteine mutations (C389S and C398S) were generated to determine whether an -SH group might be necessary for Ku70 stability and functions. We observed that C389S was able to complement DKO cells for IR repair, whereas C398S was not (Table I). Therefore, only C398 may be critical for Ku70 function.

A leucine zipper-like region has been identified between residues 483 and 497 of human Ku70 (Reeves and Sthoeger, 1989). This motif is within conserved region VIII between human, mouse, *Drosophila* and yeast Ku70 proteins (Figure 3; Wu and Lieber, 1996). To assess whether there is any significance for repeated leucines in this region, we constructed individual leucine to arginine changes at positions 483, 490 and 495 (mutant nos 30–32; Figure 3). L490R and L495R were equivalent to wild-type in terms of IR repair and DNA end binding (Table I). In contrast, L483R was unable to complement either DKO IR repair or DNA end binding but, because this mutant did not stably produce Ku70, the importance of the residue in a repeat structure cannot be assessed. Yet, it is likely that a leucine repeat is not important for Ku functions since L490R gave wild-type reconstitution of IR repair and DNA end binding.

It has been argued that a C-terminal helix–turn–helix motif was responsible for the DNA binding properties of Ku (Chou *et al.*, 1992). In fact, both Ku70 and a C-terminal peptide of Ku70 protein bind to DNA in Southwestern blots. A triple mutation, KSG591AAA (see footnote to Table I for nomenclature of mutations), within the helix–turn–helix eliminates Ku70 DNA binding in these *in vitro* experiments. To assess the significance of this motif in DNA repair, we synthesized the same mutation (KSG591AAA, mutant 37), and observed that KSG591-AAA-complemented DKO cells were completely reconstituted for IR repair and DNA end binding (Table I). Therefore, the putative C-terminal helix–turn–helix is not essential for double-strand break repair.

The C-terminus of human and mouse Ku70 also contains a motif thought to be a nucleotide binding site based on the Koonin-type ATPase consensus (Koonin, 1993). Within this consensus, residues GKV (538–540) might bind nucleotides via the lysine residue. Ku also has DNA helicase and ATPase properties that may be dependent on such a nucleotide binding consensus sequence (Cao *et al.*, 1994; Tuteja *et al.*, 1994). Therefore, we created the mutation K539E, to eliminate the putative nucleotide-binding site. DKO cells reconstituted with K539E Ku70 had IR repair and DNA end binding functions equivalent to those reconstituted with the wild-type Ku70 protein (Table I). Thus, the putative nucleotide binding motif of Ku70 is not essential for DNA repair.

Discussion

DNA end binding and heterodimerization of Ku70

Our study is indicative of the complex relationship between Ku subunit associations, DNA end binding and the ability to functionally complement DSB repair defects of Ku70–/– cells. The N-terminal truncation mutants are not capable of either IR repair or DEB, whereas each of these mutant proteins are not disrupted in Ku80 heterodimerization (Figures 2 and 4). On the other hand, with the Ku70 site-specific mutants there is a general correspondence between Ku80 heterodimerization, DEB and the complementation of IR repair of ES Ku70–/– DKO cells (Table I). Given the large number of mutations of Ku70 studied here to come to these findings, the correlations between heterodimerization, DNA end binding and IR repair are likely to be significant. With regard to its role in DNA repair, we argue that Ku must function as a heterodimer.

Previous *in vitro* evidence indicates that Ku70 in complex with Ku80 binds DNA more efficiently than Ku70 alone (Griffith *et al.*, 1992; Wu and Lieber, 1996). UV-crosslinking and other studies suggest that it is Ku70 rather than Ku80 that contacts the DNA more closely (Chou *et al.*, 1992; Zhang and Yaneva, 1992). Our mutagenesis study suggests that the association between DNA and Ku70 depends primarily on (i) the formation of direct DNA contacts by residues in Ku70 and (ii) the conformation of the Ku70–Ku80 heterodimer. A potentially significant residue for Ku70 DNA contact may be C398 in domain VI (Figure 3), because one or more reduced cysteine residues are necessary for DNA binding *in vitro* (Zhang and Yaneva, 1993). The conservative C398S mutation eliminates the ability to form a reduced sulfhydryl group, possibly used in direct association with DNA. The C398S mutation blocked DNA end binding, but did not destabilize Ku70 protein (Table I).

Mutations that disrupted DNA end binding and heterodimerization were found over a sizeable part of the Ku70 protein (aa 263–520) (Figure 3). This may mean either that DNA contacts are not restricted to a narrow region of Ku70, or that a large portion of the protein is necessary for heterodimerization. Similarly, DNA contacts may not be limited to one subunit of Ku. Considering the role of Ku in DNA end binding, principally relevant to DNA repair and V(D)J recombination, perhaps Ku associates with DNA ends and other structures by wrapping DNA around the heterodimer.

Deletion of as little as 63 amino acids from the Ku70 N-terminus eliminates IR repair and DEB (Figures 2A and 4A). In contrast, the same N-terminal region is not necessary for heterodimerization since these truncated proteins continue to associate with Ku80 (Figure 2C). There are several explanations for the formation of Ku heterodimers without restoring DNA end binding properties. First, the N-terminal region may encode DEB motifs directly. It was previously reported that in an *in vitro* translated 48 kDa Ku70 N-terminal truncation mutant (amino acids 254–609) was active for DEB, even though

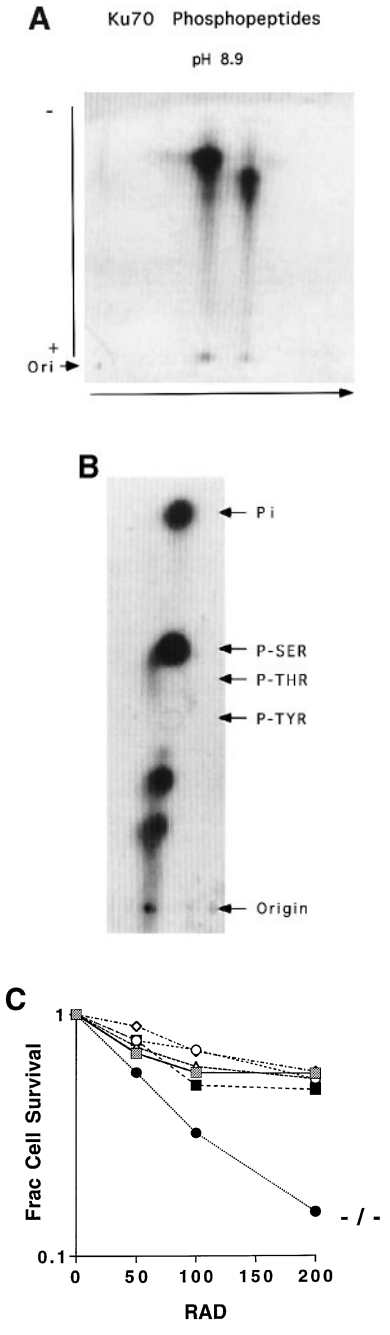

Fig. 6. Ku70 phosphorylation by DNA-PK and functional requirements for phosphorylation sites in IR repair. (**A**) Phosphopeptides of Ku70 formed by DNA-PK autophosphorylation *in vitro*. DNA-PK in LAZ388 human B cell lysates was immunoprecipitated with anti-Ku monoclonal antibody, GE2-9.5, and incubated in an *in vitro* kinase assay with [γ-^{32}P]ATP. Following fractionation of the reaction products by 8% SDS–PAGE, trypsin proteolysis of Ku70-containing gel slices were conducted. Extracted phosphopeptides were washed, dried and loaded at the origin (Ori) for electrophoresis (vertical) and organic pyridine chromatography (horizontal). (**B**) Phosphoaminoacid analysis of Ku70 phosphopeptides from Part A treated by exhaustive acid hydrolysis, electrophoresis and chromatography. Inorganic phosphate (Pi), phosphoserine (P-SER), phosphothreonine (P-THR) and phosphotyrosine (P-TYR) controls run with the sample were detected with ninhydrin staining. (**C**) IR cell survival assays of DKO cells complemented with DNA-PK phosphorylation site mutants. Mutations of candidate Ku70 DNA-PK consensus phosphorylation sites were constructed in pEFpuro as above. Stably transfected ES DKO cells with these derivatives were then compared in IR clonotypic cell survival assays as in Figure 1 over a dose ranging from 0 to 200 rad. –/– (DKO, ●); SKO (▨); wild-type Ku70 cDNA-complemented DKO (SQS 51–53 and SQ 319–320; ■); AQS 51–53 (◇); AQA 51–53 (○); AQ 319–320 (△).

at a reduced level and an altered DEB complex mobility from wild-type Ku70/Ku80 (Wu *et al*., 1996). Also, we found no site-specific mutants in the amino acid 1–263 region that affected DEB levels alone (Table I). Instead, point mutations in this region generally produced Ku70 derivatives that were wild-type or all complementation and biochemical tests (Table I). Thus it is unlikely that the N-terminal region of Ku70 determines DEB directly.

An alternative explanation for the loss of DEB with N-terminal truncation mutants is that they fail to form Ku heterodimers with a 'correct' conformation *in vivo*. In support of this notion, the 70A Hdf/Ku70 chimera formed a DEB complex (Figure 4C). The presence of a chimeric protein with roughly similar size and a related N-terminal amino acid composition to the normal protein may restore a Ku70 conformation which is now sufficient for DEB. The altered mobility of the DEB complex is likely to be caused by the conformational changes of the chimera. Thus, consistent with the finding of a core region in the C-terminal half of Ku70 for heterodimerization and DEB (Wu *et al*., 1996), the gross integrity of the Ku70 molecule in the N-terminus does contribute to an appropriate conformation for DEB that relates to its ability to function in IR repair. Also, the absence of N-terminal epitopes may lead to conformational changes that impede DNA-PK assembly. We found that DNA-PK activity could not be reconstituted with either Ku70 (63–609) or Ku70 (226–609) (Table I).

The C-terminal 20 kDa of Ku70 (aa 439–609) was sufficient for Ku80 association by two-hybrid analysis (Wu and Lieber, 1996). In contrast, we found nine mutated residues in the region 263–430 that reduced Ku's ability to heterodimerize (Figure 3, Table I). The 263–430 region corresponds well with the region that is most highly conserved between Ku70 and the *S.cerevisiae* HDF1 protein (Feldmann and Winnacker, 1993), suggesting that it might be significant for this basic property of Ku70. It is possible that the C-terminal 20 kDa does contain a portion of the heterodimerization epitopes, but that it is not sufficient for functional associations *in vivo*. Unfortunately, the only mutant that we identified in this region that influenced DNA end binding (L483R) did not stably produce Ku70 (Table I), and thus C-terminal epitopes governing heterodimerization were not identified.

A limited analysis of Ku80 mutants has similarly suggested that a core region of the Ku80 protein is necessary for IR repair (Errami *et al.*, 1996). Ku80 mutants with in-frame deletions of 46 and 84 amino acid residues were unable to heterodimerize or undergo DNA end binding. Also, N-terminally truncated Ku80, comprising residues 334–732, can complex with Ku70 and facilitate DNA end binding *in vitro* (Wu and Lieber, 1996). Further truncation of Ku80 (aa 449–732) associates with Ku70 as judged by a yeast two-hybrid analysis, but no longer binds DNA ends *in vitro* (Wu and Lieber, 1996). Unfortunately, these truncations have not yet been tested in Ku80-deficient cells to determine the efficacy of IR repair *in vivo*. In any case it appears that the bulk of Ku80 is also required for DNA end binding and heterodimerization.

Several significant amino acid changes were tested in our analysis that would certainly be expected to perturb secondary structures involving the mutated residues by charge or polarity changes. In particular, in three of the domains with the greatest degree of similarity between eukaryotic Ku70 (domains VI–VIII), highly conserved residues could be mutated with no apparent effects on Ku function (Figure 3). S378R, C389S, A396V, P408H (domain VI), P438A, RK445SE (domain VII) and R470D, L490R, L495R (domain VIII) were all wild-type for IR repair and DNA end binding (Table I). Considering that Ku70 and Ku80 appear functionally intertwined, the interpretation of mutational analyses such as these will probably have to await X-ray diffraction studies of Ku.

DNA-PK functions of Ku70

Our data support the notion that DNA-PK activity requires the proper assembly of a Ku70–Ku80 heterodimer. Ku70–/– cells were found to have inactive DNA-PK that could be restored by addition of the wild-type Ku70 cDNA (Figure 1). Mutant Ku70 proteins that disrupt heterodimerization and DNA end binding were unable to rescue DNA-PK activity, even though many of these mutants produced Ku70 protein (Table I, Figure 5). Thus, Ku70 cannot act alone in stimulating DNA-PK, consistent with previous observations from Ku80-deficient cells, where DNA-PK is inactive (Smider *et al.*, 1994; Finnie *et al.*, 1995; Errami *et al.*, 1996; Nussenzweig *et al.*, 1996). Furthermore, lack of DNA-PK activity correlates well with an inability to restore IR repair. The C-terminal truncation mutant L521* (containing residues 1–520) could be an exception to this general conclusion as DKO cells complemented with this mutant were IR-sensitive, yet retained DNA end binding ability (Table I). However, complexes of this mutant with Ku80 have an abnormal DNA end binding mobility and only partially activate DNA-PK (Table I). These phenotypes may suggest that the truncated mutant 1–520 produces a Ku70 that is poorly active in all respects, rather than grossly interfering with any one property in particular.

On the other hand, two mutants were found that separated DNA-PK activity from IR repair (KR253NG and D505R), arguing that these processes are not always together. It is interesting that KR253NG and D505R fall within the conserved domains V and IX of Ku70. Within these domains, residues R254 and D505 are conserved in human, mouse, *Drosophila* and yeast Ku70. Since mutations of residues 253, 254 and 505 do not seem to disturb DNA-PK activity, these sites may be involved in interaction with unknown proteins that may associate functionally with Ku.

DNA-PK autophosphorylation

Autophosphorylation of DNA-PK inactivates DNA-PK *in vitro* (Chan and Lees-Miller, 1996). Each DNA-PK component (DNA-PKcs, Ku70 and Ku80) is phosphorylated, causing disassociation of the kinase subunit from Ku (Chan and Lees-Miller, 1996). Likewise, phosphorylation of DNA-PKcs by the tyrosine kinase c-Abl, causes disassociation and inactivation of DNA-PK (Jin *et al.*, 1997b). Reactivation of DNA-PK requires the addition of unphosphorylated DNA-PKcs, but not unphosphorylated Ku. Also, Ku that cannot be phosphorylated by DNA-PK, as occurs in *scid* and MO59J human cells that are DNA-PK deficient (Boubnov *et al.*, 1995a; Lees-Miller *et al.*, 1995), is competent for DNA end binding, indicating that this property does not require DNA-PK phosphorylation.

Our findings regarding the role of Ku70 DNA-PK consensus phosphorylation sites strongly argue that Ku phosphorylation by DNA-PK is not required for IR repair. Further, mutations in these sites do not disturb Ku DNA end binding. A tentative conclusion from these data is that Ku autophosphorylated products are not sufficiently limiting in mammalian cells, and thus may have no physiological negative effects on IR repair. Considering that activation of DNA-PK by a DNA break is probably a 'local' event in DNA repair, only a fraction of all the Ku in the cell may be expected to be phosphorylated at any time.

Other functions of Ku70

We found that several C-terminal motifs are not essential for DNA repair functions, since the nucleotide binding motif mutant K539E, the helix–turn–helix disrupting mutant KSG591AAA, and the 1–575 C-terminal truncation mutant had normal functions (Table I and Figure 2). If Ku's function in eukaryotes is primarily in DNA repair, then these data would appear to be consistent with the observed divergence between mammalian, yeast and *Drosophila* Ku70 proteins at the C-terminus. Similarly, the N-terminus of Ku70 is an acidic region that may be necessary for DNA end binding and IR repair. We changed the net charge in this region by introduction of lysine residues in place of glutamic acid. Mutant 1 (EE17KK), which significantly alters the charge of this domain, had normal IR repair and DNA end binding properties (Table I). Thus, it is likely that a strongly acidic domain at the N-terminus is not necessary for DNA repair. Instead, the C- and N-termini of Ku70 may contribute to other properties of Ku. Ku has been implicated in transcriptional control in a variety of circumstances (Jin *et al.*, 1997). Also, Ku-deficient yeast are temperature-sensitive for growth and arrest in S phase (Feldmann *et al.*, 1993). Likewise, Ku80-deficient embryonic fibroblasts have decreased proliferation potential (Nussenzweig *et al.*, 1996). It has also been shown that telomeres are shorter in Ku70- or Ku80-deficient yeast (Bolton and Jackson, 1996; Porter *et al.*, 1996). These phenotypes are not all likely to be attributable to double-strand break repair deficiencies. One possibility would be that some other DNA binding function may be required for these other roles. For example, Ku has sequence-specific DNA binding properties that may negatively regulate the transcriptional activity of the MMTV promoter (Giffin *et al.*, 1996) or activate transcription at other promoters (McConnell and Dynan, 1996).

Materials and methods

Ku70 truncations, chimeras and point mutants in expression vectors

An N-terminal epitope-tagged Ku70 gene product was formed using wild-type human Ku70 cDNA. Double-stranded oligonucleotides encoding the c-Myc (9E10) epitope were cloned at the *Afl*III site of human Ku70, overlapping the initiator methionine in the SRαKu70 expression vector (Boubnov *et al.*, 1995b), creating pSJ4. We used pEFpuro, a derivative of pEF-BOS (Mizushima and Nagata, 1990), where the puromycin-resistance gene was subcloned into a unique *Sal*I site, as a mammalian expression vector. Ku70 cDNAs, excised with *Bam*HI and made blunt-ended with Klenow polymerase, were subcloned into pEFpuro at a unique *Not*I site flanking the elongation factor II promoter.

Ku70 N-terminal truncation mutants were created by PCR using the primers SJ61 (CCACATGTTGACACCTTTTGACATG), SJ62 (CCACATGTTTCTAGAGCTTGACCAG), SJ63 (CCACATGTTCAAGATGAGTCATAAG) and SJ64 (CCACATGTTCATCAGCATAGCAGAG) each paired with the 3′ primer SJ65 (CCATCGATGTCAGTCCTGGAAGTGC), using pSJ4 as a template. Primers SJ61–64 contain a 5′ *Afl*III site and SJ65 contains a *Cla*I site for subcloning into expression vectors. The four N-terminal truncations of Ku70, Myc-Ku70(63–609), Myc-Ku70(122–609), Myc-Ku70(165–609) and Myc-Ku70(226–609), were created using SJ61–64 respectively.

Chimeric proteins between *S.cerevisiae* HDF1 (Ku70) and human Ku70 were formed by PCR and subcloning into yeast expression vectors as follows. The N-terminal portions of yeast HDF1 were created by PCR to yield a product containing a 5′ *Bam*HI site, the entire yeast *ADH* promoter, and the first 250–280 residues of HDF1 and 3′ *Not*I sites added in-frame. A primer 5′ to the *Bam*HI site in the template pSJ17 (Milne *et al.*, 1996) (SJ81, GTGGCGGCCGCGTCTTCTCCTGTTATAGG) and a 3′ primer containing an internal *Not*I site (SJ82, GTGGCGGCCGCGCTCGATGAACGGAACCC) were used. Likewise, C-terminal portions of human Ku70 (aa 252–609 and aa 295–609) were synthesized by PCR with 5′ oligonucleotides [SJ83 (GTGGCGGCCGCCAGGAAGCGAGCACTC) or SJ84 (GTGGCGGCCGCACCAGTGAAAACCAAGACC) containing 5′ *Not*I sites] and a 3′ oligonucleotide (4559, TGAGAAGATGCGGCCAGCAA) located in the *URA3* gene of pSJ10, a yeast expression vector for the human Ku70 gene. Following PCR, HDF1 N-terminal fragments were digested with *Bam*HI and *Not*I, and human Ku70 C-terminal fragments were digested with *Not*I and *Xho*I, and gel purified. Combinations of HDF1 *Bam*HI–*Not*I and human Ku70 *Not*I–*Xho*I fragments were then subcloned into a *Bam*HI- and *Xho*I-digested *S.cerevisiae* expression vector, pSJ8, in a three-part ligation. The two resulting chimeric Ku70 proteins were 70A (aa 1–261 of HDF1 and aa 252–609 of human Ku70) and 70B (aa 1–307 of HDF1 and aa 295–609 of human Ku70). Chimeric Ku70 proteins were expressed from the *ADH1* promoter.

Ku70 site-specific mutants were formed by incorporating mutant oligonucleotides by strand extension reactions using the pAlter plasmid mutagenesis kit (Promega), as described by the supplier. pSJ5 was derived by subcloning a Myc-Ku70 *Bam*HI fragment of pSJ4 into the pAlter *Bam*HI site. pSJ5 was then the template for all mutagenesis. Oligonucleotides (25- to 36-mers) were synthesized containing one, two or three base changes from Ku70 in the center. Following the application of the mutagenesis strategy, each mutant was identified by DNA sequencing. Mutant Ku70 genes were subcloned into pEFpuro for evaluation of the reconstitution of murine DKO ES cell defects, or into pSJ8 (*ADH* promoter:*ku70*, *URA3*) for examination in Ku-deficient *S.cerevisiae* (Milne *et al.*, 1996).

Cell culture, transfection and IR tests of ES cells

DKO (ES Ku70–/–) and single knockout (ES Ku70+/–) cells and derivatives were propagated as described by Gu *et al.* (1997). Electroporation was used to introduce Ku70 plasmids stably into the genome of DKO cells. Approximately 50 μg of pEFpuro-mutant Ku70 DNA was linearized with *Sca*I, ethanol precipitated and resuspended in 25 μl of sterile H_2O. DKO cells (1×10^7) were collected by trypsinization, washing and resuspension in 0.8 ml DME medium without serum. Electroporation was conducted with a Bio-Rad Gene Pulser (23 kV, 500 μF), and the surviving cells were plated for 24 h in ES cell growth medium. Puromycin (2 μg/ml) was added for 2 days, followed by feeding the cells with medium containing 1 μg/ml puromycin until colonies formed. Each transfection yielded, on average, 200–500 colonies. Colonies were pooled from the transfections for functional and biochemical analysis. Clonogenic cell survival was used to assess IR sensitivity of DKO cells and derivatives. Each cell line was plated at 500 cells/well in duplicate and IR doses of 0, 50, 100, 200 and 300 rad applied the following day. The number of surviving cells was scored by crystal violet staining and quantified after 7 days.

DNA end binding assay

In mammalian cells, DNA end binding was conducted essentially as previously described (Boubnov *et al.*, 1995b). Cell lysates were prepared from approximately 10^7 cells by lysis in Nonidet P40 (NP40) lysis buffer [50 mM Tris, pH 8.0, 0.5% NP40, 150 mM NaCl, 10 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin] on ice for 15 min. The NaCl concentration was then adjusted to 500 mM by adding NaCl. Lysates were next pre-cleared by adjusting to 6% polyethylene glycol 8000 for 10 min on ice, and microcentrifugation for 15 min at 4°C, yielding extracts of ~5 mg/ml.

A 70 bp radiolabeled DNA substrate was prepared by digesting pSKII with *Not*I and *Xho*I, filling in with [α-^{32}P]dCTP and dGTP, dATP and dTTP, gel purification and elution. For DNA end binding, 1–2 μg of protein extract was mixed with 0.175 ng radiolabeled probe, 1 μg covalently closed circular plasmid DNA, in 1× binding buffer (20 mM Tris–Cl, pH 7.5, 10 mM EDTA, 10% glycerol), 150 mM NaCl, in a volume of 10 μl at room temperature for 10 min. DNA end binding reactions were separated on 5% polyacrylamide gels in 1× TGE (50 mM Tris, 380 mM glycine, pH 8.5, 10 mM EDTA).

For DNA end binding reactions using yeast extracts, the wild-type *S.cerevisae* strain DWY83, a Ku-deficient strain, DWY315 (Δ*ku70* Δ*ku80*) (Milne *et al*., 1996), and human Ku70- plus Ku80-complemented derivatives were examined. DWY315 was co-transformed with human Ku70 (pSJ10, *ADH*:*ku70*, *URA3*) and Ku80 (pSJ11, *ADH*:*ku80*, *LEU2*) to allow expression of both human Ku subunits. Yeast strains were propagated, and yeast DNA end binding extracts were prepared as described (Milne *et al.*, 1996).

In vitro kinase assay and phosphopeptide analysis

LAZ388 human B cell lysates were formed in NP40 lysis buffer as above, immunoprecipitated with an anti-Ku80 monoclonal antibody, GE2-9.5, and washed in NP40 lysis buffer four times. Ku–DNA-PKcs complexes precipitate under these conditions. Samples were washed once in 1× kinase buffer [25 mM Tris–Cl, pH 7.5, 75 mM NaCl, 10 mM $MgCl_2$, 0.2 mM EGTA, 0.2 mM EDTA, 1 mM dithiothreitol (DTT)], and resuspended in 1× kinase buffer with 100 μM ATP and [γ-^{32}P]ATP (75 μCi, 6000 Ci/mmol) to a final volume of 50 μl, and incubated at 30°C for 10 min. Radiolabeled proteins were separated by 10% SDS–PAGE and visualized by autoradiography. Phosphorylated Ku70 was excised as a gel slice and prepared for phosphopeptide and phosphoamino acid analysis as described by Coligan *et al.* (1995).

DNA-dependent protein kinase assays were performed from ES cell extracts prepared by a freeze–thaw lysis procedure originally described by Finnie *et al.* (1995) with the following modifications. Approximately 1–2×10^7 cells were washed in PBS and pelleted, and either stored at −80°C or lysed immediately. Lysis was conducted by resuspension in 100 μl of FT buffer (20 mM HEPES, pH 7.5, 200 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 1 μg/ml leupeptin, 4 μg/ml pepstatin, 1 μg/ml aprotinin, 0.5 mM PMSF and 25% glycerol), freezing on dry ice, and thawing at 30°C. Freezing–thawing was repeated twice more. Lysates were cleared by centrifugation in a Beckman J2-21 centrifuge with a JA-18.1 rotor for 10–15 min at 13 000 r.p.m. Supernatants were fractionated on 0.6 ml Sephacryl 300 columns in FT-150 buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 1 μg/ml leupeptin, 4 μg/ml pepstatin, 1 μg/ml aprotinin, 0.5 mM PMSF and 5% glycerol) and the void volume collected in the first 400 μl. Samples were diluted with FT-0 (20 mM HEPES, pH 7.5, 0.2 mM EDTA, 1 mM DTT, 1 μg/ml leupeptin, 4 μg/ml pepstatin, 1 μg/ml aprotinin, 0.5 mM PMSF and 5% glycerol) to 0.8 ml and mixed with 200 μl of double-stranded DNA beads [1:1 (v/v) slurry in FT-0] by rocking at 4°C for 60 min. The DNA bead-associated fraction was washed twice in 1 ml of 1× kinase buffer in a microcentrifuge. The DNA bead pellet was divided into two fractions and was used immediately for kinase assays. DNA-dependent protein kinase activity associated with the DNA beads was measured according to Lees-Miller *et al.* (1990) as follows. DNA bead pellets (50 μl) were mixed with 25 μl of 2× kinase buffer, 20 μl of 250 μg/ml p53 peptide (EPPLSQEAFADLWKK), ATP to 50 μM, [γ-^{32}P]ATP (75 μCi, 6000 Ci/mmol), with or without 100 μM wortmannin (Biomolecules) to a final volume of 50 μl. The reactions were incubated at 30°C for 10 min and two 20 μl aliquots spotted on P81 phosphocellulose filter circles (Gibco-BRL), washed four times with 75 mM orthophosphoric acid and twice with H_2O, and radioactivity was quantified by liquid scintillation counting.

Detection of Ku subunits by immunoblotting and immunoprecipitation

Approximately 2×10^6 cells were trypsinized, washed in medium, washed in PBS, pelleted, and either stored at –80°C or lysed immediately. For immunoprecipitation analysis, cell pellets were lysed in 600 μl NP40 lysis buffer on ice for 30 min. Nuclei were removed by centrifugation in a Beckman J2-21 centrifuge with a JA-18.1 rotor for 15 min at 13 000 r.p.m. Aliquots of supernatants were removed for immunoblot analysis without immunoprecipitation. Immunoprecipitations were conducted with an anti-Ku70 monoclonal antibody, AG4-7.5, or an anti-Myc monoclonal antibody, 9E10, for 60 min on ice, followed by binding of rabbit anti-mouse antibody, and absorption on to protein A–Sepharose. Protein A beads were washed twice in 1 ml of NP40 lysis buffer and resuspended in 25 μl of 2× SB (120 mM Tris–Cl, pH 6.8, 4% SDS, 0.002% bromophenol blue, 20% glycerol, 718 mM 2-mercaptoethanol), boiled, and fractionated by 10% SDS–PAGE. Also, aliquots of cell lysates were diluted 1:1 with 2× SB, boiled and fractionated similarly. Gels were transferred to PVDF membranes (NEN) using a Bio-Rad semi-dry electrophoretic transfer apparatus at 20 V for 45 min in 48 mM Tris–Cl, 39 mM glycine, 1.3 mM SDS, 20% methanol, pH 9.2. Blots were blocked in PBS + 3% bovine serum albumin (BSA) overnight. Primary antibodies, anti-Ku70 (M19; Santa Cruz Biotech) and/or anti-Ku80 (C20 or M20, Santa Cruz Biotech) were used at 5–10 μg/ml in PBS for 60 min at room temperature. Blots were then washed five times in PBS + 0.2% Tween-20. Blots were then incubated with horseradish peroxidase conjugated protein A/G (Pierce) for 30–40 min in PBS + 1% BSA, and extensively washed in PBS + 0.2% Tween-20. Next, blots were developed using a Chemiluminescence Renaissance kit (NEN) according to the supplier's instructions. Signals were detected on Kodak XR1 film.

In vitro transcription/translation

HDF1/Ku70 chimeric protein constructions were tested for their ability to produce hybrid proteins *in vitro* prior to introduction into yeast with Ku80. PCR was conducted using a 5′ HDF primer containing T7 RNA polymerase promoter, ribosome binding site, and ATG in-frame with the N-terminal amino acid residues of HDF1: GGATCCTAATACGACTCACTATAGGGAGACCACCATGCGGCCAGTCACTAATGCATTTGGC. A 3′ primer from the C-terminus of human Ku70 was used (TCACCTGGCGTAGTCGGGCACGTCGTAGGGGTAGTCCTGGAAGTGCTTGGT). PCR products were gel purified and used in coupled T7 polymerase *in vitro* transcription/translation with [^{35}S]methionine according to the supplier's instructions (Promega).

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