Specific interaction of IP₆ with human Ku70/80, the DNA-binding subunit of DNA-PK

Les A. Hanakahi and Stephen C. West¹

Cancer Research UK, London Research Institute,
Clare Hall Laboratories, South Mimms, Hertfordshire EN6 3LD, UK

¹Corresponding author
e-mail: stephen.west@cancer.org.uk

In eukaryotic cells, DNA double-strand breaks can be repaired by non-homologous end-joining, a process dependent upon Ku70/80, XRCC4 and DNA ligase IV. In mammals, this process also requires DNA-PK_{cs}, the catalytic subunit of the DNA-dependent protein kinase DNA-PK. Previously, inositol hexakisphosphate (IP₆) was shown to be bound by DNA-PK and to stimulate DNA-PK-dependent end-joining *in vitro*. Here, we localize IP₆ binding to the Ku70/80 subunits of DNA-PK, and show that DNA-PK_{cs} alone exhibits no detectable affinity for IP₆. Moreover, proteolysis mapping of Ku70/80 in the presence and absence of IP₆ indicates that binding alters the conformation of the Ku70/80 heterodimer. The yeast homologue of Ku70/80, yKu70/80, fails to bind IP₆, indicating that the function of IP₆ in non-homologous end-joining, like that of DNA-PK_{cs}, is unique to the mammalian end-joining process.

Keywords: DNA repair/double-strand breaks/
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Introduction

The repair of double-strand breaks (DSBs) in DNA is essential for the maintenance of genome integrity. The importance of DSB repair is highlighted by the fact that failure to repair a DSB can result in the loss of genetic information, chromosomal translocation and cell death. Repair of DSBs can be effected either by homologous recombination (HR) or by non-homologous end-joining (NHEJ). HR, promoted by proteins of the *RAD52* epistasis group, requires interactions with a sister duplex that restores genetic information lost at the DSB site. In contrast, NHEJ, as its name implies, is a homology-independent process and represents an important DSB repair pathway that is effective during all phases of the cell cycle (Takata *et al.*, 1998; Essers *et al.*, 2000).

In mammals, NHEJ requires the products of the *XRCC5*, *XRCC6* and *XRCC7* genes, which together encode the DNA-dependent protein kinase DNA-PK. *XRCC5* and *XRCC6* encode the 80 and 70 kDa subunits of the Ku70/80 heterodimer (the DNA-binding subunit of DNA-PK), and *XRCC7* encodes the DNA-stimulated protein kinase DNA-PK_{cs} (Weaver, 1996; Chu, 1997; Critchlow and Jackson, 1998). Mammalian cell lines deficient in these proteins exhibit DSB repair defects and are highly sensitive to ionizing radiation (Jackson and Jeggo,

1995). Two other proteins, XRCC4 and DNA ligase IV, which form a stable heterodimer, are also specifically required for NHEJ (Critchlow *et al.*, 1997; Grawunder *et al.*, 1997, 1998). In contrast to mutations in Ku70/80 or DNA-PK_{cs}, mutations in either XRCC4 or DNA ligase IV result in embryonic lethality in the mouse (Frank *et al.*, 1998; Gao *et al.*, 1998).

Although the physiologically relevant targets of DNA-PK remain elusive, many intriguing potential candidates have been identified: (i) phosphorylation of XRCC4 by DNA-PK modulates its DNA-binding activity (Critchlow *et al.*, 1997; Modesti *et al.*, 1999); (ii) autophosphorylation of DNA-PK_{cs}, which results in its inactivation, may play a regulatory role in mammalian NHEJ (Chan and Lees-Miller, 1996); and (iii) multiple phosphorylation of both Ku70 and Ku80 by DNA-PK_{cs} occurs, but the downstream consequences of these modifications are unclear (Yaneva and Busch, 1986; Chan *et al.*, 1999). Yeast homologues for Ku70 (yKu70), Ku80 (yKu80), XRCC4 (LIF1) and DNA ligase IV (DNL4) have been identified and shown to participate in NHEJ (Critchlow and Jackson, 1998). To date, however, no yeast homologue of DNA-PK_{cs} has been identified, making the specific role of DNA-PK_{cs} and the DNA-PK holoenzyme in the mammalian NHEJ reaction of particular interest.

The Ku70/80 heterodimer binds avidly to DNA termini in a structure-specific manner (Dyran and Yoo, 1998). X-ray crystallographic studies of the Ku70/80 heterodimer bound to DNA indicate that substantial subunit–subunit contacts lead to the formation of a highly charged channel through which the DNA passes (Walker *et al.*, 2001). Primary sequence alignments of Ku70 and Ku80 show that while Ku70 is well conserved (Weaver, 1996), Ku80 appears to be more divergent across distantly related species. The C-terminal region of Ku80, however, is well conserved between mammals (Gell and Jackson, 1999). One interpretation of these observations is that Ku80 mediates interactions that are unique to the mammalian NHEJ apparatus, possibly to fulfil roles that relate to mammalian physiology, such as V(D)J joining and immunoglobulin isotype switch recombination. Interestingly, it is the extreme C-terminal region of Ku80 that associates with DNA-PK_{cs} (Gell and Jackson, 1999; Singleton *et al.*, 1999). However, despite the fact that our understanding of Ku70/80 ranges from the genetic to the atomic level, the precise role of Ku70/80 in the repair of DSBs remains enigmatic.

Previously, we reported the participation of an inositol polyphosphate, inositol hexakisphosphate (IP₆), in an *in vitro* DNA-PK-dependent NHEJ reaction that recapitulates NHEJ in mammalian cells, and demonstrated that purified DNA-PK binds IP₆ (Hanakahi *et al.*, 2000). We show here that IP₆ is bound specifically by the Ku70/80 DNA-binding subunit of DNA-PK. Furthermore, it is

Fig. 1. Specific binding of IP₆ by purified DNA-PK. Binding reactions contained 5000 U of purified DNA-PK (Promega) and 100 nM [³H]IP₆, in the presence or absence of unlabelled competitor as indicated. Complexes were separated by gel filtration through Superdex 200. [³H]IP₆ was detected by scintillation counting. (A) DNA-PK with [³H]IP₆ only. (B) As (A), but in the presence of a 100-fold excess of IS₆. (C) As (A), but in the presence of a 10-fold excess of IP₆. (D) Control indicating the mobility of [³H]IP₆ in the presence of non-specific marker proteins.

shown that the binding of IP₆ results in a change to the proteolytic cleavage pattern of the Ku70/80 heterodimer, suggestive of a conformational change. Such an alteration is likely to be important for the regulation and/or the mechanism of action of the mammalian NHEJ apparatus.

Results

Specific recognition of IP₆ by DNA-PK

It has been shown previously that purified DNA-PK binds IP₆, an inositol phosphate that stimulates DNA-PK-dependent NHEJ *in vitro* (Hanakahi *et al.*, 2000). This interaction was demonstrated by the altered mobility of [³H]IP₆ in the presence and absence of DNA-PK during gel filtration chromatography (Figure 1, compare A with D). However, because IP₆ is a small, highly phosphorylated (and therefore highly charged) compound, it is possible that interactions mediated by high charge density could be a source of non-specific IP₆ binding. To rule out this possibility, competition experiments were carried out using either an excess of unlabelled IP₆ or IS₆, a compound that presents the same 6-carbon inositol ring, with a charge to mass ratio similar to that of IP₆, but displaying sulfate rather than phosphate groups. Previously, it was shown that IS₆ fails to stimulate NHEJ *in vitro* (Hanakahi *et al.*, 2000). We found that a 10-fold excess of IP₆ was an

Fig. 2. Interaction of IP₆ with components of the DNA-PK holoenzyme. Ku70/80 (300 nM), DNA-PK_{cs} (300 nM) and [³H]IP₆ (100 nM) were mixed and incubated as described in Materials and methods. The products were then resolved by gel filtration through Superdex 200. [³H]IP₆ was detected by scintillation counting; Ku70/80 and DNA-PK_{cs} were detected by western blotting. (A) Binding of IP₆ by DNA-PK. (B) Reactions were carried out as described for (A), but in the presence of dsDNA (300 nM).

effective competitor to the interaction between DNA-PK and [³H]IP₆ (Figure 1C), whereas a 100-fold excess of IS₆ was not (Figure 1B). These observations confirm the specificity of the DNA-PK–IP₆ interaction.

Binding of IP₆ by DNA-PK is mediated by Ku70/80

Because DNA-PK_{cs} is a member of the phosphoinositol-3-kinase (PI3K)-related family of protein kinases, we speculated previously that DNA-PK_{cs} might function as the IP₆-binding subunit of the heterotrimeric DNA-PK holoenzyme (Hanakahi *et al.*, 2000). To determine the validity of this hypothesis, ³H-labelled IP₆ was incubated with DNA-PK (i.e. a mixture of DNA-PK_{cs} and Ku70/80, both purified from HeLa cells) in the absence or presence of a 65 bp duplex. The products of the binding reactions were again analysed by gel filtration. In the absence of DNA, conditions in which DNA-PK fails to assemble, the Ku70/80 and DNA-PK_{cs} subunits exhibited different mobilities, as detected by western blot analysis. We found that [³H]IP₆ co-migrated with the peak of Ku70/80, and not with that of DNA-PK_{cs} (Figure 2A). These results indicate that Ku70/80, rather than DNA-PK_{cs}, is the primary IP₆-binding component of DNA-PK.

In contrast, in the presence of DNA, on which the DNA-PK complex assembles, a larger [³H]IP₆-containing complex was observed (Figure 2B). Western blot analyses of the [³H]IP₆-containing fractions confirmed the presence of Ku70/80 and DNA-PK_{cs} in this large complex. As expected, the absorbance profile at 260 nm also showed the presence of double-stranded (ds) DNA in the same fractions (data not shown), indicating that IP₆ is incorporated into the DNA-PK heterotrimer assembled on DNA.

Fig. 3. Specific interaction of IP₆ with Ku70/80. Spin-column analysis of the binding of [^3H]IP₆ by Ku70/80. Following centrifugation, the [^3H]IP₆ present in the void volume indicates the amount bound by Ku70/80. (A) Binding reactions were carried out as described in Materials and Methods using [^3H]IP₆ (100 nM) and Ku70/80 (filled squares) or DNA-PK_{cs} (open diamonds). (B) Binding reactions were carried out with Ku70/80 (100 nM) and [^3H]IP₆ (100 nM), and supplemented with unlabelled IP₆ (1, 3 and 10 times molar excess) or unlabelled IS₆ (10 times molar excess).

Additional binding experiments using surface plasmon resonance assays showed that the interaction of IP₆ with Ku70/80 or DNA-PK had no significant effect on the ability of these proteins to interact with DNA (data not shown). These results demonstrate that DNA-PK is capable of coordinated interaction with both DNA and IP₆.

Although IP₆ interacts strongly with DNA-PK, an analysis of the DNA-stimulated protein kinase activity of DNA-PK_{cs} showed that addition of IP₆ to DNA-PK had no effect on the reaction kinetics, Mg²⁺ dependence or DNA dependence of its kinase activity (data not shown). Additionally, IP₆ had no effect on the inactivation of DNA-PK by autophosphorylation.

Binding specificity

Using Ku70/80 purified from HeLa nuclear extracts, we next carried out a direct assessment of its ability to bind specifically to IP₆ using spin-column assays. As shown in Figure 3A, the amount of IP₆ bound was directly proportional to the amount of Ku70/80. As expected, the catalytic subunit of DNA-PK, DNA-PK_{cs} alone, failed to bind [^3H]IP₆ under the same conditions. We also found that unlabelled IP₆ effectively competed with [^3H]IP₆ to block complex formation with Ku70/80, whereas IS₆ did not (Figure 3B). Further control experiments indicated that DNA binding by Ku70/80 had no effect on the formation

Fig. 4. Trypsin proteolysis mapping of Ku70/80 and DNA-PK_{cs} in the presence and absence of IP₆. (A) Trypsin digestion of Ku70/80. Protein (1.3 μg) was digested in the presence or absence of IP₆ (10 μM) using the following amounts of trypsin: 0, 1.6, 4, 16, 44 and 132 ng (lanes a–f). The arrow shows a proteolysis product that is formed only in the absence of IP₆. (B) Trypsin digestion of DNA-PK_{cs}. Protein (1.3 μg) was digested with trypsin as described in (A). Proteins were detected by western blotting.

of the Ku70/80–IP₆ complex, and that the efficiency of binding of IP₆ to Ku70/80 was the same when Ku was alone or incorporated into DNA-PK assembled on DNA termini (data not shown). These results show that the Ku70/80 heterodimer binds specifically to IP₆, and that the heterodimer provides the primary inositol phosphate binding site in DNA-PK.

IP₆ binding alters the proteolytic cleavage pattern of Ku70/80

To determine whether the binding of IP₆ might induce conformational changes in Ku70/80 and thus stimulate mammalian NHEJ, partial proteolysis mapping of Ku70/80 was carried out in the presence or absence of IP₆. When the Ku70/80 heterodimer was digested with trypsin (which cleaves at basic residues), we observed that both subunits exhibited increased resistance to digestion in the presence of IP₆ (Figure 4A). In contrast, no differences in the trypsin proteolysis profile of DNA-PK_{cs} in the absence or presence of IP₆ were observed (Figure 4B), demonstrating that the increased resistance of Ku70/80 to trypsin was not due to blocking of tryptic cleavage sites by the non-specific binding of IP₆ to exposed basic residues. A similar increase in resistance to proteolytic digestion in the presence of IP₆ was observed using V8 protease, which cleaves at acidic residues (Figure 5).

When Ku70/80 was bound to DNA, we found that the complex was more resistant to digestion with trypsin compared with unbound Ku70/80. The data for the Ku80 subunit are shown in Figure 6, and similar results were

Fig. 5. V8 protease mapping of Ku70/80 in the presence and absence of IP₆. Reactions were carried out as described in Figure 4, using the following amounts of V8 protease: 0, 4, 16, 48, 148 and 444 ng (lanes a–f). In the lower panels, a slight cross-reactivity of the anti-Ku70 polyclonal antiserum to Ku80 can be seen.

Fig. 6. Trypsin proteolysis mapping of Ku70/80–DNA complexes. Reactions were carried out as described in the legend to Figure 4, using Ku70/80 in the presence or absence of sheared calf thymus DNA. Proteins were detected with western blotting using anti-Ku80 polyclonal antibodies.

obtained for the Ku70 subunit. The proteolysis profile for the Ku70/80 heterodimer bound to DNA was unaffected by the presence or absence of IP₆ (Figure 6). When DNA-PK holoenzyme was assembled on DNA and probed with trypsin, we again saw that IP₆ had no effect on Ku70/80 (data not shown).

The increased resistance of Ku70/80 to protease digestion in the presence of IP₆ may indicate that the heterodimer adopts an overall more compact structure in the presence of the cofactor. The change, however, appeared particularly marked in the Ku80 subunit because a trypsin proteolytic cleavage product observed in the absence of IP₆ was not observed in its presence (Figure 4A, αKu80, arrow). Taken together, these data indicate that Ku70/80 undergoes structural changes upon association with IP₆, leading us to suggest that the stimulation of NHEJ by IP₆ may be due, at least in part, to these alterations.

Binding of IP₆ by Ku70/80 requires the intact mammalian heterodimer

To investigate further whether Ku70/80 may hold the key to the participation of IP₆ in mammalian NHEJ, we next determined whether the yeast homologue of Ku70/80,

Fig. 7. Specificity of IP₆ for mammalian Ku70/80. Spin-column analysis of the binding of [³H]IP₆ by Ku70/80. Following centrifugation, the [³H]IP₆ present in the void volume indicates the amount bound by Ku70/80. Error bars represent the standard deviation of three independent measurements. (A) Comparison of the binding of human Ku70/80 (100 nM) or its yeast homologue yKu70/80 (1 μM) with [³H]IP₆ (100 nM). (B) Comparison of the [³H]IP₆ (100 nM) binding ability of purified recombinant Ku70 (500 nM), recombinant Ku80 (500 nM), recombinant Ku70/80 containing a C-terminal truncated Ku80 subunit [rKu70/80(T); 100 nM], recombinant Ku70/80 containing a full-length Ku80 subunit [rKu70/80(FL); 100 nM], and Ku70/80 purified from HeLa cells [Ku70/80 (HeLa); 100 nM].

yKu70/80, could interact with IP₆. As previously noted, the primary sequence of Ku70 is relatively well conserved between yeast and mammals, whereas Ku80 is more divergent. As shown in Figure 7A, using the same binding conditions for Ku70/80 and yKu70/80, we were unable to detect the interaction of [³H]IP₆ with yKu70/80. These *in vitro* findings are supported by observations showing that *Saccharomyces cerevisiae* mutants with defects in the biosynthesis pathways of IP₆ exhibit normal NHEJ (B.Llorente and L.Symington, personal communication). The observation that yKu70/80 fails to bind IP₆ demonstrates that IP₆ binding by Ku70/80 is unique to the mammalian NHEJ reaction, further reinforcing the relationships between IP₆ binding by Ku70/80 and the specificity of IP₆ for mammalian NHEJ.

To determine whether either of the Ku70/80 subunits alone could interact with IP₆, recombinant Ku70 and Ku80 were prepared and analysed for their ability to bind

[³H]IP₆. As shown in Figure 7B, neither the Ku70 nor the Ku80 subunit formed a stable complex with IP₆. As indicated by the Ku70/80 crystal structure (Walker *et al.*, 2001), substantial contacts exist between the Ku70 and Ku80 subunits within the heterodimer and it is likely that both subunits undergo substantial structural rearrangement upon heterodimer formation. In view of this, we cannot rule out the possibility that the soluble proteins that we have purified after overexpression may be inappropriately folded and do not reflect the polypeptides in the intact heterodimer. Consistent with this possibility, reconstitution of an active Ku70/80 heterodimer is known to require the co-expression of both subunits *in vivo*.

It has been demonstrated that the C-terminal domain of Ku80 is required for DNA-PK_{cs} association (Gell and Jackson, 1999; Singleton *et al.*, 1999), but not for heterodimer formation or for DNA-binding activity (Walker *et al.*, 2001). To assess the contribution of the C-terminal domain of Ku80 in IP₆ binding, we analysed the IP₆-binding ability of purified recombinant Ku70/80 containing a 19 kDa C-terminal truncation in the Ku80 subunit. As shown in Figure 7B, the Ku70/80 heterodimer with the Ku80 truncation [rKu70/80(T)] bound IP₆ in a manner that was comparable to Ku70/80 purified from HeLa cells [Ku70/80 (HeLa)] and recombinant Ku70/80 incorporating a full-length Ku80 subunit [Ku70/80(FL)]. Indeed, the rKu70/80(T) bound approximately two times more IP₆ than a comparable amount of Ku70/80 from HeLa, although this difference may reflect the amount of active Ku70/80 in the two preparations, rather than any real differences in their ability to bind inositol phosphate. These data demonstrate that, like heterodimer assembly and DNA binding, the binding of IP₆ by Ku70/80 is not dependent on the presence of the C-terminal domain of Ku80.

Discussion

In previous studies, it was shown that IP₆ stimulates DNA-PK-dependent NHEJ *in vitro*, and that purified DNA-PK specifically recognizes and forms a stable complex with IP₆ (Hanakahi *et al.*, 2000). Although DNA-PK_{cs} is a protein kinase, it shares sequence homologies with members of the phosphatidylinositol 3-kinase (PI-3K)-related kinase family. This homology is particularly strong within the catalytic core domain of the PI-3K family, which is known to bind and phosphorylate the phosphoinositol headgroup of phosphatidylinositol (Hunter, 1995). Given the obvious potential for recognition of inositol phosphate by DNA-PK_{cs}, it was interesting to find that Ku70/80, and not DNA-PK_{cs}, acts as the IP₆-binding subunit of DNA-PK.

The participation of the DNA-PK holoenzyme in NHEJ is unique to mammals. While yeast homologues for Ku70/80 (yKu70/80), XRCC4 (LIF1) and DNA ligase IV (DNL4) have been identified and shown to participate in NHEJ (Critchlow and Jackson, 1998), no yeast homologue of DNA-PK_{cs} has been identified. Our demonstration that yKu70/80 fails to interact with IP₆ indicates that the IP₆-binding motif in Ku70/80, like the participation of DNA-PK_{cs}, is restricted to NHEJ in mammals.

Interactions between Ku70/80 and DNA-PK_{cs} have been shown to involve the C-terminal domain of Ku80

(Gell and Jackson, 1999; Singleton *et al.*, 1999). This region of Ku80 is particularly interesting as it is a part of Ku70/80 that is highly divergent between yeast and mammals, and yet is very highly conserved within mammals (Gell and Jackson, 1999). As the binding of IP₆, the presence of the C-terminal domain of Ku80 and DNA-PK_{cs} itself are all unique to the mammalian NHEJ system, it seemed plausible that the C-terminus of Ku80 might be responsible for IP₆ binding and for interactions with DNA-PK_{cs}. We found, however, that deletion of the 19 kDa C-terminal domain of Ku80 had little effect on the ability of the Ku70/80 heterodimer to bind IP₆. Future studies to localize the IP₆-binding site within Ku70/80 will be an important first step in understanding the consequences of IP₆ binding, and provide a key to help elucidate its role in mammalian NHEJ.

When the structural consequences of IP₆ binding by Ku70/80 were analysed, we found that both subunits of the heterodimer were more resistant to proteolytic cleavage. As shown in Figure 4A, the most notable changes in Ku70/80 induced by IP₆ binding were observed in the trypsin proteolysis profile of Ku80. While it is possible that the binding of negatively charged IP₆ to specific basic residues in the Ku80 subunit might prevent trypsin accessibility, it is equally likely that the overall increased resistance to protease digestion is due to the establishment of a more compact Ku70/80 structure. In contrast, changes to the proteolytic cleavage pattern of DNA-PK_{cs} were not observed. However, we do not exclude the possibility that the structure of DNA-PK_{cs} might alter upon interaction with the Ku70/80–IP₆ complex. Indeed, changes to Ku70/80, or to the DNA-PK holoenzyme in general, are likely to be important factors in the stimulation of NHEJ reactions by IP₆.

What could changes to the structure of Ku70/80 achieve? First, conformational changes in Ku70/80 could result in functional changes to the DNA-binding activity of DNA-PK or its mode of interaction with the break site. Once Ku70/80 has bound to duplex DNA, it is thought to translocate in from the break site, thereby facilitating the binding of DNA-PK_{cs} to the extreme terminal region (Hammarsten and Chu, 1998; West *et al.*, 1998). Interestingly, the crystal structure of the Ku70/80–DNA complex shows that Ku can cradle two turns of DNA while leaving much of the surface area of the DNA exposed to the environment (Walker *et al.*, 2001). The mechanism by which Ku then promotes end-to-end interactions is presently unknown, but its ability to confine DNA movement to a single path is likely to be a key determinant in the alignment of DNA termini. The effect of IP₆ on the localization of Ku and whether it facilitates interactions between Ku heterodimers bound to break sites is an issue that requires further study.

Secondly, conformational changes in Ku70/80 in response to IP₆ binding could affect the protein–protein interaction potential of Ku70/80. In addition to its interaction with DNA-PK_{cs}, Ku70/80 interacts with a host of other factors that might, directly or indirectly, affect the efficiency of NHEJ. For example, an interaction between Ku70/80 and the Werner syndrome protein (WRN) has been described that alters the specificity of the WRN exonuclease (Li and Comai, 2000). It has also been shown that terminal deoxynucleotidyl transferase

(TdT) is recruited to sites of V(D)J joining by Ku70/80 (Purugganan *et al.*, 2001), where TdT is thought to add random nucleotides to recombination junctions. Ku70/80 also interacts with the telomeric factor Trf1 and plays an important role in telomere maintenance (Bailey *et al.*, 1999; Hsu *et al.*, 1999, 2000; Gasser, 2000). Conformational changes in Ku70/80 could affect this interaction and impinge upon whether Ku70/80 binds Trf1 and acts at telomeres, or alternatively participates in NHEJ. Finally, *in vitro* studies have shown that Ku70/80 stimulates DNA ligation (Ramsden and Gellert, 1998) and participates in the recruitment of the XRCC4–DNA ligase IV complex to DNA termini (McElhinny *et al.*, 2000). It is easy to see how a change in Ku70/80 conformation might affect these interactions, resulting in the observed stimulation of NHEJ. Detailed analysis of the conformational changes that result from IP₆ binding by Ku70/80 and how these changes affect the ability of Ku70/80, or that of the DNA-PK holoenzyme in general, to promote NHEJ will be necessary before this critically important repair process can be understood at the molecular level.

Materials and methods

Proteins

Ku70/80 and DNA-PK_{cs} were purified essentially as described for DNA-PK (Dvir *et al.*, 1993). All purification steps were performed at 4°C in L buffer [25 mM HEPES pH 7.6, 2 mM MgCl₂, 0.5 mM EDTA, 20% glycerol, 1 mM dithiothreitol (DTT), 1 mM sodium metabisulfite and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)]. Briefly, nuclear extracts were prepared from 50 l of HeLa cells and fractionated using DEAE–Sephacrose, SP–Sephacrose, heparin–agarose, dsDNA–agarose and phosphocellulose chromatography. At each step, fractions were assayed for DNA-PK activity using the Promega Signa-Tect assay system. To separate Ku70/80 from DNA-PK_{cs}, the active fractions from phosphocellulose were applied to a phenyl–Sephacrose column in L buffer containing 1.0 M NH₄(SO₄)₂ and eluted with a 1.0–0 M NH₄(SO₄)₂ gradient. DNA-PK_{cs} and Ku70/80 were then further purified by Mono Q and Mono S chromatography using linear KCl gradients. Peak fractions were pooled and dialysed against L buffer lacking MgCl₂. These preparations are referred to as Ku70/80 and DNA-PK_{cs}.

Recombinant Ku70 was produced using a baculovirus overexpression system. Ku70-encoding virus was used to infect Sf9 cells for 4 days (McElhinny *et al.*, 2000). At this time, the cells were lysed by Dounce homogenization in 50 mM Tris–HCl pH 8.0, 0.3 M NaCl, 1 mM EDTA, 10 mM imidazole and 10 mM β-mercaptoethanol, with 0.1 mM PMSF. Insoluble material was removed by ultracentrifugation and the soluble His-tagged Ku70 was purified by affinity chromatography on a Talon (Clontech) metal chelating column using a 10 mM–1.0 M imidazole gradient. Ku70 was detected by SDS–PAGE gels followed by Coomassie Blue staining. The sample was dialysed into L buffer (without Mg²⁺) containing 50 mM NaCl. Further purification and concentration were achieved by fractionation over a 1 ml Hi-Trap Q column (Pharmacia). Samples were dialysed into the same buffer containing 0.1 M NaCl for storage.

Recombinant Ku80 was produced from the Ku80 cDNA cloned into pET11a (Wu and Lieber, 1996). *Escherichia coli* BL21 DE3 pLysS cells carrying the Ku80 plasmid were grown for 3 h at 37°C, and then chilled for 15 min on ice to reduce the culture temperature. IPTG (1 mM) was then added and the culture incubated for 19 h at 25°C. Under these conditions, however, very little Ku80 was found to be soluble and considerable losses were accepted. The cells were lysed by freezing–thawing followed by sonication, and the remaining soluble Ku80 was purified essentially as described above for Ku70. Protein concentrations were determined by the Bradford assay.

Ling Chen and Alan Tomkinson generously provided purified yKu70/80, Jonathan Goldberg provided Ku70/80 containing the C-terminal truncated form of Ku80, DNA-PK holoenzyme was purchased from Promega, and the recombinant Ku70/80 containing the full-length Ku80 subunit was purchased from Trevigen.

IP₆ binding assays

Binding reactions (100 μl) between components of DNA-PK and IP₆ were carried out in the presence or absence of a 65 bp duplex (McElhinny *et al.*, 2000) in H buffer (50 mM HEPES pH 8.0, 40 mM KOAc, 10% glycerol, 0.1 M KCl, 1 mM DTT) for 20 min at 4°C. Aliquots (50 μl) were applied to a Superdex 200 gel filtration column run at 4°C on a SMART system (Pharmacia) in H buffer at a flow rate of 40 μl/min. Gel filtration standards were purchased from Bio-Rad.

Spin-column binding assays were performed as described (Kavran *et al.*, 1998) but with some modifications. Binding reactions (60 μl) were carried out in H buffer for 30 min at 4°C, and 50 μl aliquots were applied to Bio-Spin 30 Tris columns (Bio-Rad) that had been equilibrated in H buffer. Proteins with bound [³H]IP₆ were spun through the column, and the bound counts in the flow through were measured by scintillation counting. [³H]IP₆ was purchased from NEN. IP₆ and IS₆ were purchased from Calbiochem and Sigma, respectively.

Antibodies and western blotting

Anti-DNA-PK_{cs} antibodies were purchased from Serotec and used at a dilution of 1:2000. Anti-Ku70 and anti-Ku80 antibodies were purchased from Serotec and used at a dilution of 1:4000. Anti-Ku70 monoclonal antibodies were purchased from NeoMarkers and used at a dilution of 1:500. Proteins were detected by western blotting followed by enhanced chemiluminescence (NEN).

Proteolysis mapping

Trypsin (Sigma) and V8 protease (Promega; sequencing grade Endoproteinase Glu-C) were used at the concentrations stated. Digests were carried out in 40 μl reactions at 37°C for 10 min (trypsin) or 12.5 min (V8) in H buffer. IP₆ was present at 10 μM where indicated. Calf thymus DNA (0.4 μg) was present where indicated. Reactions were stopped by addition of an equal volume of SDS–PAGE gel loading buffer, and the products were resolved by 10% SDS–PAGE followed by western blotting.

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