Binding of Inositol Hexakisphosphate (IP$_6$) to Ku but Not to DNA-PKcs*

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The nonhomologous end joining (NHEJ) pathway is responsible for repairing a major fraction of double strand DNA breaks in somatic cells of all multicellular eukaryotes. As an indispensable part of this pathway, Ku has been hypothesized to be the first protein to bind to the DNA ends generated at a double strand break being repaired by this pathway. When bound to a DNA end, Ku improves the affinity of another DNA end-binding protein, DNA-PKcs, to that end. The Ku-DNA-PKcs complex is often termed the DNA-PK holoenzyme. It was recently shown that myo-inositol hexakisphosphate (IP$_6$) stimulates the joining of complementary DNA ends in a cell-free system. Moreover, the binding data suggested that IP$_6$ bound to DNA-PKcs (not to Ku). Here we clearly show that, in fact, IP$_6$ associates with DNA-PKcs but rather with Ku. Furthermore, the binding of DNA ends and IP$_6$ to Ku are independent of each other. The possible relationship between inositol phosphate metabolism and DNA repair is discussed in light of these findings.

Double strand DNA breaks are among the most lethal DNA lesions, and they arise in somatic cells of multicellular eukaryotes spontaneously in the absence of external factors (1, 2). Recently, we demonstrated that oxidative metabolism is the major agent that the IP$_6$ binds to the DNA-PKcs rather than to Ku. This paper is available online at http://www.jbc.org

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† The abbreviations used are: NHEJ, nonhomologous DNA end joining; DNA-PK, DNA-dependent protein kinase; IP$_6$, myo-inositol hexakisphosphate; IP$_3$, myo-inositol 1,4,5-trisphosphate; BSA, bovine serum albumin; ds, double strand; EMSA, electrophoretic mobility shift assay; SPR, surface plasmon resonance.

EXPERIMENTAL PROCEDURES

Inositol Phosphates—IP$_3$ and myo-inositol 1,4,5-trisphosphate (IP$_3$) were purchased from Sigma. Tritiated IP$_3$ ([3H]IP$_3$, 21.0 Ci/mmol) and IP$_6$ ([3H]IP$_6$, 20.5 Ci/mmol) were obtained from PerkinElmer Life Sciences.

Protein Purification—Native DNA-PKcs was purified as described previously (26) except that HeLa cells were used as the source for purification. C-terminal His-tagged Ku70 and nontagged Ku86 were co-expressed in the baculovirus system and purified as described previously (27). Nontagged Ku was expressed and purified as described previously (28) (a gift of Dr. J. Goldberg). The concentration of purified proteins was estimated by comparing bovine serum albumin (BSA) standards on a Coomassie Blue stained SDS-PAGE gel.

In Vitro Immuno Pull-down of Inositol Phosphates—Immuno pull-down of IP$_3$ was performed in 50-µl reactions that contain 10 mM Tris, pH 7.5, 50 mM NaCl, 10 mM MgCl$_2$, 1 mM EDTA, 1 mM dithiothreitol, 10756

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10% glycerol, and 0.1 mg/ml BSA. Anti-Ku70 monoclonal antibody (clone N3H10, NeoMarkers, Fremont, CA), anti-Ku86 monoclonal antibody (clone 111, NeoMarkers), and anti-DNA-PKcs monoclonal antibodies (clone 42-27 and 25-4) were used as indicated to reach a final total antibody amount of 20 μg in each reaction. The rest of the reagents were added to each 50-μl reaction as indicated: 1 pmol of [3H]-IP6 or [3H]-IP3, 25–50 pmol of 35-bp DNA YM-8/YM-9 (blunt ds DNA, YM-8 5’-AGG CTG TGT TAA GTA TCT GCC CTC AGA GG-3’), 6.2–10 pmol of Ku, and 2.5 pmol of DNA-PKcs. Then, 100 μl of 50% slurry of protein G-Sepharose (Amersham Biosciences) and 350 μl of binding buffer were added to make the final volume 500 μl. The pull-down reactions were allowed to proceed for 1 h at 4°C with constant mixing. After washing in the same binding buffer (without BSA) for 4 times (0.8–1 ml of buffer for each time), the beads were resuspended in 200 μl (2 × 100 μl) of binding buffer (without BSA) then mixed into 2 ml of ScintiVerse II scintillation fluid (Fisher Scientific). The amounts of bead-associated [3H] were measured in a liquid scintillation analyzer (model Tri-Carb 2100TR, Packard BioScience, Meriden, CT).

In Vitro Pull-down of Inositol Phosphates by DNA Beads—In each 50-μl reaction (contains the same buffer components as the immune pull-down reactions), 50 pmol of 5’-biotinylated 35-bp DNA YM-8/YM-9, 6.2 pmol of Ku, and 1 pmol of inositol phosphate were used. 100 μl of 50% streptavidin agarose (Sigma) were then added into each reaction. The binding, washing, and scintillation counting steps were carried out as described above.

Electrophoretic Mobility Shift Assay (EMSA)—The gel shift assay was performed as described previously (20), and 0.5 nm labeled YM-6/ YM-7 (20) and 1 nm Ku were used for all the reactions. IP6 and IP3 were diluted in H2O first and then added to the reaction mixtures to the indicated final concentrations.

Surface Plasmon Resonance (SPR)—The SPR experiment was done as described previously (7, 20). A 5’-biotinylated 35-bp DNA (sequence is the same as YM-8/YM-9) was immobilized on the streptavidin-coated surface of the sensor chip (Sensor chip SA, Biacore, San Diego, CA). Ku was diluted in the running buffer and injected at a flow rate of 5 μl/min for 4 min. The injection of Ku was repeated to ensure that the surface-immobilized DNA on the sensor chip was saturated with Ku. Then DNA-PKcs (to a final concentration of 6.1 nM) and additional reagents (1 mM ATP, 1 mM MgCl2, and 0.1 nM IP6 or IP3, final concentration indicated) were mixed in the running buffer and injected at 5 μl/min for 4 min. Proteins bound to the sensor chip were allowed to dissociate for 6 min before the surface was regenerated with 0.05% SDS. The resulting sensograms were edited using the BlAevalua software (version 3.0).

RESULTS

Ku, but Not DNA-PKcs, Binds to IP6—A previous study on the association of DNA-PK and IP6 used a DNA-PK preparation that contained Ku and, possibly, contaminating DNA fragments (25). We were interested in testing whether binding of IP6 to DNA-PK was dependent on Ku and/or DNA ends. Native DNA-PKcs and recombinant Ku were purified as described previously. DNA-PKcs was immobilized on protein G-Sepharose beads via monoclonal antibodies against it. Tritiated-IP6 was incubated with these Immobeads in the absence or presence of Ku and a 35-bp DNA (under the buffer conditions specified under “Experimental Procedures”). The DNA length of 35 bp was chosen, because this permits the binding of Ku and DNA-PKcs simultaneously to the same DNA molecule (7). The radioactivity associated with the extensively washed beads was then measured using a liquid scintillation counter. Surprisingly, IP6 showed no association with DNA-PKcs alone, above the low level of background binding to the protein G-Sepharose beads (Fig. 1A, histogram bar 2 versus bar 1). This lack of association between IP6 and DNA-PKcs was obtained regardless of the presence of the 35-bp linear DNA (Fig. 1A, bar 3 versus bar 2). When IP6 was added to DNA-PKcs-immobilized beads along with Ku, there was only a near background level of binding detected (Fig. 1A, bar 4), in agreement with our observation that Ku does not associate with DNA-PKcs in the absence of DNA (see below).

Interestingly, when the binding of IP6 to DNA-PKcs was tested in the presence of Ku and 35-bp DNA, a substantial amount of binding was observed (Fig. 1A, bar 5). This could be because Ku induces a conformational change in DNA-PKcs to permit its binding of IP6. Alternatively, IP6 might bind at the interface between Ku and DNA-PKcs when both are present on DNA, because 35 bp is long enough for Ku and DNA-PKcs to co-localize (7). Alternatively, IP6 might simply bind to Ku. To test this, we omitted DNA-PKcs entirely and examined the binding of IP6 to bead-immobilized Ku via monoclonal anti-Ku antibodies. In this case, we observed a very high level of IP6 binding (Fig. 1A, bar 6). The specificity of binding of IP6 to Ku was tested with another inositol polyphosphate, IP3, and only a background level of binding was detected (Fig. 1A, bar 7).

![IP6 Binds to Ku](image-url)
Hence, it appeared that IP₆ was in fact binding to Ku rather than to DNA-PKcs.

We wanted to rule out the possibility that the six-amino acid histidine affinity tag on Ku70 might be responsible for the association between IP₆ and recombinant Ku. To test this, we compared the binding of IP₆ to purified Ku with and without the tag, and the association of IP₆ to these different preparations of Ku was indistinguishable (Fig. 1B). Therefore, the binding of IP₆ to Ku is completely unaffected by the affinity tag.

DNA Does Not Enhance or Inhibit the Binding of IP₆ to Ku—To investigate the role of DNA in the interaction between IP₆ and Ku, we used a monoclonal antibody against Ku70 to immobilize the Ku heterodimer. The level of binding was quite high, regardless of whether the 35-bp ds DNA was present (Fig. 2A, bars 1 and 2). Immobilization with an anti-Ku86 monoclonal antibody gave indistinguishable results (Fig. 2A, bars 3 and 4). As shown above, the binding of IP₆ to Ku was specific because IP₇ failed to bind (Fig. 2A, bar 5). As was the case for anti-DNA-PKcs monoclonal antibodies (Fig. 1, bars 1–4 and 7), none of the anti-Ku antibodies bound to IP₆ (data not shown) nor to IP₇ (Fig. 2A, bar 5). These data clearly indicate that IP₆ binds to Ku rather than to DNA-PKcs, and this binding is independent of the presence of DNA.

Further evidence for the binding of IP₆ to Ku can be observed when the experiments are configured in a manner where a linear DNA fragment was immobilized on streptavidin-agarose beads via a 5'-biotin linkage, and Ku was added to these DNA beads. IP₆ associated with the Ku-DNA beads, while IP₇ did not (Fig. 2B, bars 1 and 2). The streptavidin-agarose beads coated with biotinylated DNA could pull down the Ku-IP₆ complex as efficiently as protein G-Sepharose coated with anti-Ku antibodies (Fig. 2B, bar 1 versus bars 3 and 5). This is additional conclusive evidence that IP₆ binds to Ku, and this interaction is unaffected by DNA.

IP₆ Does Not Alter the Binding Properties of Ku to DNA Ends—We and others have previously done detailed studies of the binding of Ku to DNA ends. Recently, we showed that on linear DNA long enough to bind two Ku molecules (two-site linear DNA), the second Ku molecule loads with a 14-fold higher equilibrium constant (20). Namely, the two Ku molecules bind cooperatively to a two-site DNA molecule. We were interested in testing whether the binding properties of the Ku-IP₆ complex loading onto this two-site DNA would be altered compared with the loading of Ku alone onto the same DNA. To test this, concentrations of Ku and two-site DNA were chosen such that about 50% of Ku would bind to DNA; under these conditions, each 45-bp DNA molecule contains either one or two Ku molecules. The concentration of IP₆ was then varied over a 100,000-fold range that surrounded the physiologic concentration range of IP₆ in eukaryotic cells. Neither the noncooperative first Ku binding nor the cooperative second Ku binding was altered by IP₆ (Fig. 3A). Hence, it does not appear that IP₆ alters the association of Ku with DNA ends.

Ku has been designated “the DNA-binding subunit” of DNA-PK holoenzyme due to the fact that it enhances the binding of DNA-PKcs to a Ku-bound DNA end and stimulates the kinase activity of DNA-PKcs (9). Therefore, the binding of IP₆ to Ku might affect the fraction of DNA-PKcs that is in a complex with Ku and DNA. The kinase activity of DNA-PK on a synthetic substrate is not altered by IP₆ (25). To investigate the possibility that IP₆ might interfere or enhance the binding of DNA-PKcs to a Ku-bound DNA end, an SPR experiment was designed. The SPR technology allows the real-time monitoring of the changes in the mass of macromolecules associated with a surface. In our experiment, a biotinylated 35-bp ds DNA was immobilized on a streptavidin-coated surface. Ku was then allowed to bind to the free end of the DNA molecules on the surface to saturation or near saturation (achieved by two consecutive injections of the same Ku solution). The binding of tritiated IP₆ to Ku was measured as the residual radioactivity of 3H retained on the extensively washed beads. A control (bar 5) with anti-Ku70 and anti-Ku86 immunobeads and IP₆ is shown. B, His-tagged Ku was immobilized onto streptavidin-agarose beads with biotinylated 35-bp DNA (bars 1 and 2) or protein G-Sepharose beads with anti-Ku70 (bars 3 and 4) or anti-Ku86 antibody (bars 5 and 6). Then the association of tritiated IP₆ or IP₇ with Ku was examined. The scheme of the reactions is shown on the bottom of the figure. "b" indicates biotinylation. The hexagons represent inositol phosphate, and ovals represent Ku.

FIG. 2. DNA does not interfere with the binding of IP₆ to Ku. A, His-tagged Ku was immobilized onto immunobeads via anti-Ku70 (bars 1 and 2) or anti-Ku86 (bars 3 and 4) monoclonal antibody in the presence or absence of 35-bp DNA. The binding of tritiated IP₆ to Ku was measured as the residual radioactivity of 3H retained on the extensively washed beads. A control (bar 5) with anti-Ku70 and anti-Ku86 immunobeads and IP₆ is shown. B, His-tagged Ku was immobilized onto streptavidin-agarose beads with biotinylated 35-bp DNA (bars 1 and 2) or protein G-Sepharose beads with anti-Ku70 (bars 3 and 4) or anti-Ku86 antibody (bars 5 and 6). Then the association of tritiated IP₆ or IP₇ with Ku was examined. The scheme of the reactions is shown on the bottom of the figure. "b" indicates biotinylation. The hexagons represent inositol phosphate, and ovals represent Ku.

allowed to bind to the free end of the DNA molecules on the surface to saturation or near saturation (achieved by two consecutive injections of the same Ku solution). The binding of DNA-PKcs was tested in the presence of IP₆ or IP₇. Neither the association phase (the ascending phase starting with the injection of DNA-PKcs) nor the dissociation phase (the descending phase starting with the termination of injection) was altered by IP₆. This suggests that IP₆ does not affect DNA-PKcs in the association with the Ku-DNA complex.
the same fragments, given that Ku-DNA complexes bind DNA-PKcs 100-fold more efficiently than DNA alone can bind to DNA-PKcs.

The Potential Significance of Ku-IP6 Interaction—IP6 was reported to enhance the efficiency of joining of compatible DNA ends by crude cell extracts (25), and our study suggests that this might occur through association with Ku. One possible significance of this interaction could be that IP6 alters some unidentified function(s) of Ku. A second possibility is that the Ku-IP6 complex might interact with other factors in a way such that the overall NHEJ efficiency could be stimulated. Ku has been reported to physically and functionally interact with DNA-PKcs (only in presence of DNA ends) (9), DNA ligase IV-XRCC4 complex (29, 30), and the Werners helicase, WRN (31, 32). The alteration of the activities of any of these enzymes might result in a profound effect on the outcome of NHEJ. The elucidation of the function of Ku-IP6 interaction awaits further studies.

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FIG. 3. IP6 does not interfere with the binding of Ku to DNA or DNA-PKcs, to the Ku-DNA complex. A. EMSA of His-tagged Ku and a 45-bp duplex DNA in presence of various concentrations of IP6 or IP3 is shown. The positions of the free probe, one Ku-bound DNA and two Ku-bound DNA, are indicated in the figure. B. Sensorgrams of three consecutive protein injections are shown. In both cases, the injection of Ku was repeated to ensure saturation of the DNA surface. The start of each injection and the termination of the last injection are marked by arrows.

DISCUSSION
Ku Binds to IP6—These results demonstrate that IP6, but not IP3, binds to Ku, and neither IP6 nor IP3 associate with DNA-PKcs. Previous work indicated that IP6 enhanced the efficiency of DNA end joining in a cell extract system that was sensitive to inhibitors of DNA-PKcs (25). In that study when the mixture of IP6 and DNA-PK was fractionated by a gel filtration column, one peak of IP6 (detected by scintillation counting of each fraction) and the peak of DNA-PK (determined by assaying DNA-PK activity of each fraction) co-migrated. In light of our data, which indicate that Ku actually associates with IP6, one plausible explanation for the earlier observation could be that there was some contaminating DNA in the commercial DNA-PK preparation that allowed the association of Ku and DNA-PKcs and, therefore, the association of IP6 and DNA-PK.

We have previously shown that DNA-PKcs and Ku do not associate in the absence of DNA ends (27), and this observation has been confirmed by our laboratory (7). In the current study, the failure to co-immunoprecipitate the Ku-IP6 complex by DNA-PKcs immunobeads (Fig. 1, bar 4) further supported this point. Trace levels of Ku-DNA-PKcs associations seen by some laboratories may actually be due to low levels of contaminating DNA that can co-purify with DNA-PKcs if specific purification steps are not included to remove the DNA. The contaminating DNA can then serve to bind both Ku and DNA-PKcs on