The DNA-dependent protein kinase (DNA-PK) requires for activity free ends or other discontinuities in the structure of double strand DNA. In vitro, DNA-PK phosphorylates several transcription factors and other DNA-binding proteins and is thought to function in DNA damage recognition or repair and/or transcription. Here we show that in vitro DNA-PK undergoes autophosphorylation of all three protein subunits (DNA-PKcs, Ku p70 and Ku p80) and that phosphorylation correlates with inactivation of the serine/threonine kinase activity of DNA-PK. Significantly, activity is restored by the addition of purified native DNA-PKcs but not Ku, suggesting that inactivation is due to autophosphorylation of DNA-PKcs. Our data also suggest that autophosphorylation results in dissociation of DNA-PKcs from the Ku-DNA complex. We suggest that autophosphorylation is an important mechanism for the regulation of DNA-PK activity.

In the presence of DNA, the DNA-dependent protein kinase (DNA-PK) exhibits serine/threonine protein kinase activity and phosphorylates many transcription factors and other nuclear proteins, including the tumor suppressor protein p53 (reviewed in Ref. 1). Early studies identified a large polypeptide (previously called p350) that was associated with DNA-PK activity (2, 3). Biochemical studies indicate that p350 is the catalytic subunit of DNA-PK (DNA-PKcs) (2, 3). Interestingly, the cDNA sequence of DNA-PKcs reveals a novel catalytic domain with homology to the phosphatidylinositol 3-kinase family rather than serine/threonine kinases (4). During our preliminary purification of DNA-PK, several other polypeptides appeared to copurify with activity and were phosphorylated in a DNA-dependent manner, including polypeptides of approximately 70 and 80 kDa that were identified as the Ku autoantigen (3). Subsequently, Ku was shown to be the DNA targeting subunit of DNA-PK (5, 6), hence active DNA-PK is a complex of DNA-PKcs, both subunits of Ku, and DNA.

In vitro, DNA-PK requires free ends of DNA for activation (1, 2, 6, 7), suggesting that DNA-PK may be involved in DNA damage recognition and/or DNA repair (8, 9, 10). Consistent with this hypothesis, radiosensitive murine cell lines from x-ray cross-complementing group 5 (XRCC5), which are defective in DNA double strand break repair and V(D)J recombination, lack the Ku p80 subunit (11–16). Also murine Scid (severe combined immunodeficiency) cell line, which are also radiosensitive and defective in DNA double strand break repair and V(D)J recombination, lack DNA-PKcs (17–19). In addition, a radiosensitive human cell line (MOS9) that is defective in DNA double strand break repair lacks DNA-PKcs (20). Moreover, the gene for DNA-PKcs in humans maps to the site of XRCC7, a gene that complements radiosensitivity, defects in DNA double strand break repair and V(D)J recombination in murine Scid cells (21).

However, other data suggest that DNA-PK may perform additional functions. In vitro, DNA-PK can be activated by DNA structures containing bubbles and hairpins (7), suggesting that it may be involved in DNA replication or transcription. DNA-PK phosphorylates many transcription factors including p53, Sp1 (23), fos (1, 24), jun (25), myc (26), serum response factor (27), and the C-terminal domain of RNA polymerase II (1, 5, 28). Also, several studies have suggested that the Ku component of DNA-PK acts as a specific transcription factor (29–32). Although a role for DNA-PK in RNA polymerase II-directed transcription has not been demonstrated, DNA-PK activity is stimulated by transcriptional activator proteins (33). DNA-PK may also play a role in regulating the transcription of ribosomal genes by modulating the activity of RNA polymerase I (34–36). In addition, it plays a role as the DNA targeting subunit of DNA-PK, Ku may also function as a helicase (37) and an ATPase (38).

Despite these advances, relatively little is known about the regulation of DNA-PK activity. Previously, preparations highly enriched for DNA-PKcs (p350) were shown to lose activity following incubation with ATP and DNA (2, 3), suggesting that DNA-PK was inactivated by autophosphorylation. In addition, we showed that Ku copurified with DNA-PK activity and that DNA-PKcs and both subunits of Ku were phosphorylated in a DNA-dependent manner (3). Now that Ku is known to be the DNA targeting component of DNA-PK, we have studied the effects of autophosphorylation on DNA-PK activity using highly purified proteins. Our results suggest that inactivation correlates with autophosphorylation of DNA-PKcs and disruption of the DNA-PK complex.

MATERIALS AND METHODS

Protein Purification—The DNA-PKcs and Ku subunits of DNA-PK were purified from human placenta as described (39). DNA-PK Activity Assays—Kinase assays were as described (22) except that the synthetic peptide substrate used was PESQEAFA DLWKK (28). The rate of phosphate incorporation into this peptide is at least twice that of the previously used peptide, EPPLSQEADLWKK K (28). The peptide PESQEAFA DLWKK is not phosphorylated by DNA-PK.
Therefore, we first examined the effect of different molar ratios of DNA-PKcs to Ku on the inactivation of DNA-PK in the presence of ATP and DNA. DNA-PKcs and Ku were purified to homogeneity from human placenta (39) and incubated at molar ratios of 2:1, 1:1, 1:2.5, and 1:5 (DNA-PKcs to Ku) in the presence of ATP, magnesium, and sonicated calf thymus DNA (as described under "Materials and Methods"). Molar ratios were calculated using molecular masses of 450 and 156 kDa for DNA-PKcs and the Ku dimer, respectively. After preincubation for 10 min, aliquots (corresponding to 10% (v/v) of each reaction) were removed and reassayed for kinase activity under standard assay conditions in the presence of the synthetic peptide PESQEAFADLWKK, DNA, and [γ-32P]ATP as described. Regardless of the molar ratio of DNA-PKcs to Ku, preincubation with ATP and DNA resulted in the loss of 60–80% of the original DNA-PK activity (Table I). In all subsequent experiments a molar ratio of 1:1 was employed; however, identical results were obtained using a molar ratio of 1:5 (DNA-PKcs to Ku).

In order to determine whether inactivation of DNA-PK required ATP hydrolysis, purified DNA-PKcs and Ku were preincubated with DNA in the presence of ATP or the nonhydrolyzable ATP analogue AMP-PNP, and at timed intervals aliquots were removed and reassayed for kinase activity as described. DNA-PK activity was rapidly lost after incubation with DNA and ATP but not after preincubation with DNA alone, DNA plus AMP-PNP (Fig. 1A), or ATP alone (data not shown), strongly suggesting that DNA-dependent phosphorylation is required for kinase inactivation. In order to correlate inactivation with phosphorylation of individual DNA-PK proteins, an experiment was performed in parallel using radiolabeled ATP in the preincubation reaction. Aliquots were removed and analyzed by SDS-PAGE using 10% acrylamide gels, followed by autoradiography. DNA-dependent inactivation of DNA-PK was shown to correlate with autophosphorylation of all three subunits of DNA-PK (DNA-PKcs, Ku p70/p80, and Ku p86) (Fig. 1B).

Individually, neither DNA-PKcs nor Ku has significant kinase activity (5, 6, 39), and DNA-PK activity requires assembly of DNA-PKcs onto DNA-bound Ku (42). To determine whether inactivation of DNA-PK was due to autophosphorylation of DNA-PKcs or the Ku component of DNA-PK, purified DNA-PKcs or Ku were added back to autophosphorylation reactions containing inactivated DNA-PK as described under "Materials and Methods." If phosphorylation resulted in inactivation of only one protein component of DNA-PK, then we should be able to

The catalytic subunit and the DNA-binding component of DNA-PK (DNA-PKcs and Ku p70/p80 dimer, respectively) are easily separated during purification under mild chromatographic conditions, hence early extracts of DNA-PK contained predominantly DNA-PKcs (previously called p350) with trace amounts of Ku (2, 3). Preincubation of these extracts with ATP and DNA resulted in loss of DNA-PK activity, suggesting that DNA-dependent phosphorylation of DNA-PK results in inactivation (2, 3). The stoichiometry of DNA-PKcs and Ku in the active DNA-PK complex has not been determined; however, maximum kinase activity toward synthetic peptide substrates requires molar ratios (DNA-PKcs to Ku) of 1:1.3 to 1:1.5 (7, 39).

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**Table I**

Effect of preincubation of DNA-PKcs with different molar equivalents Ku on DNA-PK activity

| Molar ratio of DNA-PKcs to Ku | DNA-PK activity | 0 min | 10 min |
|-------------------------------|-----------------|-------|--------|
| 2:1                           |                 | 1242  | 524    |
| 1:1                           |                 | 1826  | 462    |
| 1:2.5                         |                 | 1880  | 396    |
| 1:5.0                         |                 | 2107  | 511    |

**RESULTS**

The catalytic subunit and the DNA-binding component of DNA-PK are easily separated during purification under mild chromatographic conditions, hence early extracts of DNA-PK contained predominantly DNA-PKcs (previously called p350) with trace amounts of Ku (2, 3). Preincubation of these extracts with ATP and DNA resulted in loss of DNA-PK activity, suggesting that DNA-dependent phosphorylation of DNA-PK results in inactivation (2, 3). The stoichiometry of DNA-PKcs and Ku in the active DNA-PK complex has not been determined; however, maximum kinase activity toward synthetic peptide substrates requires molar ratios (DNA-PKcs to Ku) of 1:1.3 to 1:1.5 (7, 39).

2 D. W. Chan and S. P. Lees-Miller, unpublished data.
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**Fig. 1.** Inactivation of DNA-PK requires ATP hydrolysis and correlates with autophosphorylation. DNA-PKcs (0.04 μg/μl) and Ku (0.013 μg/μl) were preincubated (molar ratio, 1:1) in 25 mM Hepes, 70 mM KCl, 10 mM magnesium chloride, 1 mM dithiothreitol, 0.2 mM EGTA, 0.1 mM EDTA, 10 μg/ml calf thymus DNA, and 0.25 mM ATP as described in Table I. A, after 0, 1, 2, 3, 5, or 10 min, aliquots of 2 μl (equivalent to 0.1 μg of DNA-PK total protein) were removed and assayed under standard kinase assay conditions in the presence of synthetic peptide (PESEQEAFDLWKK), calf thymus DNA, and radiolabeled ATP (●). In an identical experiment, activity was determined after preincubation of DNA-PK with DNA, magnesium, and the nonhydrolyzable ATP analogue AMP-PNP (○) or with DNA and magnesium alone (△). DNA-PK activity was calculated as a percentage of original activity. The specific activity at time 0 was 1300 units/mg. In these experiments the concentrations of reagents in the final assay were increased by 10% due to carry over from the preincubation reaction. B, in an identical experiment carried out in parallel, DNA-PK was incubated as above, but in the presence of [γ-32P]ATP. At the timed intervals, aliquots (equivalent to 0.16 μg of DNA-PK proteins) were removed and analyzed by SDS-PAGE on 10% acrylamide gels followed by autoradiography. Shown are separate exposures for DNA-PKcs (room temperature, 5 h) and Ku (overnight at –80 °C with intensifying screens).

The mechanism by which Ku binds to DNA is not known; however, Southwestern analysis suggests that the 70-kDa subunit of Ku (Ku p70) can interact directly with DNA (41). We therefore used this assay to determine whether autophosphorylated Ku retains its ability to interact with DNA. DNA-PK complex (molar ratio of DNA-PKcs/Ku, 1:1) was incubated under phosphorylation-inactivated conditions (ATP plus DNA), transferred to nitrocellulose, and probed with a 40-base pair radiolabeled DNA probe. In the experiment shown, incubation under phosphorylation conditions for 5 min resulted in loss of >80% of original DNA-PK activity. Southwestern analysis shows that Ku p70 interacts with DNA under conditions that promote DNA-dependent inactivation of DNA-PK (Fig. 3A). After autoradiography, the blot was stripped and reprobed with antibodies to Ku. During the phosphorylation reaction, we estimate that at least 70% of the Ku p80 polypeptide was shifted into a slower migrating form (Fig. 3B). This form of Ku p80 migrates coincidentally with 32P-labeled Ku p80 and is sensitive to treatment with alkaline phosphatase (data not shown), strongly suggesting that it represents the phosphorylated form of Ku p80. These data suggest that autophosphorylation of Ku does not affect its ability to interact with DNA.

We next used co-immunoprecipitation experiments to determine whether autophosphorylation affects interactions between the DNA-PK proteins. To ensure that the presence of DNA did not affect the ability of our antibodies to immunoprecipitate the appropriate antigen, we first incubated DNA-PKcs or Ku alone under standard assay conditions in either the absence or the presence of DNA. Following immunoprecipitation with the respective antibodies, complexes were analyzed by Western immunoblot. Rabbit polyclonal antibodies to DNA-PKcs (DPK1) immunoprecipitated DNA-PKcs in the presence of the absence of DNA (Fig. 4A). Similarly mouse polyclonal antibodies to Ku also recognized Ku in the presence or the absence of DNA (Fig. 4B). Next, DNA-PKcs and Ku were combined and incubated in the presence or the absence of DNA (with no added ATP), immunoprecipitated, and analyzed by

![Figure 2](image-url)
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Fig. 3. Southwestern/Western blot of autophosphorylated Ku. DNA-PKcs (0.0375 μg/μl) and Ku (0.0125 μg/μl) were preincubated in the presence of ATP and DNA as described. After 0, 2, and 5 min, aliquots corresponding to 0.75 μg of DNA-PKcs and 0.25 μg of Ku were removed and separated on SDS-PAGE. Proteins were transferred to nitrocellulose, renatured, and incubated with a labeled 40-base pair DNA probe as described under “Materials and Methods.” A, autoradiogram showing binding of DNA to Ku p70. B, the same nitrocellulose membrane was stripped and probed with antibodies to Ku (Ab24). Immunodetection was using Enhanced Chemiluminescence (Amersham Corp.). The arrow indicates the presence of the phosphorylated form of Ku p80.

Fig. 4. DNA-PKcs and Ku co-immunoprecipitate only in the presence of DNA. DNA-PKcs (1.5 μg) and Ku (0.5 μg) were incubated under standard assay conditions in the absence or the presence of DNA as indicated. Reactions contained DNA-PKcs alone (A), Ku alone (B), or DNA-PKcs plus Ku (C and D). Proteins were immunoprecipitated with antibodies to DNA-PKcs (DPK1) (A and D) or Ku (Ab24) (B and C). Immunocomplexes were analyzed by Western blot using antibodies to DNA-PKcs (A and C) or Ku (B and D).

Western blot as above. Antibodies to Ku co-immunoprecipitated DNA-PKcs only in the presence of DNA. Similarly, antibodies to DNA-PKcs co-immunoprecipitated Ku only in the presence of added DNA (Fig. 4D). These data are consistent with previous findings (42) and strongly suggest that the DNA-PK holoenzyme only forms or is stabilized in the presence of DNA.

Next, DNA-PKcs and Ku were combined and preincubated under either phosphorylating conditions (plus DNA and ATP) (Fig. 5, A–D, lanes 2) or nonphosphorylating conditions (plus DNA and the nonhydrolyzable analogue, AMP-PNP) (Fig. 5, A–D, lanes 3), and following immunoprecipitation, immunocomplexes were analyzed by Western blot as above. In the first experiment, DNA-PK complex was immunoprecipitated using antibodies to Ku (Fig. 5, A and B), and immunocomplexes were analyzed by Western blot with antibodies to DNA-PKcs (Fig. 5A) or Ku (Fig. 5B). Under nonphosphorylating conditions (in the presence of AMP-PNP and DNA), both DNA-PKcs and Ku were immunoprecipitated (Fig. 5, A and B, lane 3); however, preincubation of DNA-PKcs and Ku under phosphorylating conditions resulted in a drastic reduction in DNA-PKcs that co-precipitated with antibodies to Ku (Fig. 5A, lane 2). Under phosphorylating conditions Ku p80 was present as a doublet (Fig. 5B, lane 2, arrow), consistent with our previous observation (Fig. 3) that phosphorylated Ku p80 has a slower migration on SDS-PAGE. Similar results were obtained using three different polyclonal antibodies to Ku. In reciprocal experiments, reactions containing DNA-PK complex, DNA, and ATP or AMP-PNP were immunoprecipitated with an antibody to DNA-PKcs. Western blots with DNA-PKcs showed that DNA-PKcs immunoprecipitated equally under phosphorylating or nonphosphorylating conditions (Fig. 5C, lanes 2 and 3), whereas the majority of the Ku was only immunoprecipitated by antibodies to DNA-PKcs in reactions carried out in the absence of phosphorylation (Fig. 5D, lanes 2 and 3). Therefore to summarize, under conditions that promoted DNA-dependent phosphorylation, DNA-PKcs did not co-immunoprecipitate with antibodies to Ku (Fig. 5A, lane 2), and Ku did not immunoprecipitate with antibodies to DNA-PKcs (Fig. 5D, lane 2), whereas under nonphosphorylating conditions (but in the presence of DNA) both antisera immunoprecipitated both proteins (Fig. 4, C and D, and Fig. 5, A–D, lane 3). These data suggest that autophosphorylation of DNA-PKcs leads to disruption of the DNA-PKcs-Ku complex.
DNA-PKcs is responsible for phosphorylation-induced inactivation. Together, these data suggest that phosphorylation of conditions in which approximately 70% of the Ku is phosphorylated shows that Ku p70 retains its ability to interact with DNA under our assay conditions.2 In many substrate proteins, DNA-PK phosphorylates serine or threonine residues that precede a glutamine (1, 22). The cDNA sequence of DNA-PKcs predicts 26 potential DNA-PK phosphorylation sites (4), and the Ku subunits contain six potential phosphorylation sites (45, 46). Attempts to map these sites are in progress. Our preliminary data show that autophosphorylation of DNA-PKcs occurs at multiple sites, any of which might be critical to kinase inactivation.

What then might be the biological significance of inactivation of DNA-PK? Indeed, why should DNA-PK inactivate itself? It has been proposed that one of the functions of the DNA-PK complex is in the rejoining of DNA ends during V(D)J recombination (44) and DNA double strand break repair. According to this model, active DNA-PK assembles at or close to the recombination site where it interacts with and presumably phosphorylates other proteins involved in DNA repair and/or recombination. The large size of DNA-PKcs suggests that it might act as a scaffold for interaction with other proteins (4). Possible candidate proteins might include RAG 1, RAG 2, or nucleases or other proteins involved in V(D)J recombination or DNA double strand break repair. In addition, we and others have shown that DNA-PKcs preferentially phosphorylates DNA-bound substrates (22, 23, 28). We speculate that under certain conditions, perhaps in the absence of a suitable substrate, DNA-PK undergoes autophosphorylation of DNA-PKcs and Ku proteins, leading to inactivation of DNA-PKcs and disruption of the DNA-PK complex. This could allow other proteins involved in DNA repair or V(D)J recombination to interact with the DNA breaks site or with other phosphorylated proteins, including perhaps DNA-bound phosphorylated Ku. Perhaps, therefore, one function of autophosphorylation is to make phosphorylated Ku more accessible to new protein partners. Alternatively, phosphorylation could affect some other property of Ku, for example helicase activity (37). Also phosphorylated DNA-PKcs, once liberated, might be free to pursue some other function in the cell. Interestingly, the cDNA sequence of DNA-PKcs reveals homology to the phosphatidylinositol 3-kinase family. To date, phosphatidylinositol 3-kinase activity has not been demonstrated for either DNA-PKcs or DNA-PK holoenzyme (4); however, it is possible that autophosphorylation could affect phosphatidylinositol 3-kinase activity of DNA-PKcs alone or in combination with other proteins. Other evidence suggests that DNA-PK is involved in transcription (1, 5, 6, 18, 19, 23–26) and apoptosis,3 and a similar model could be proposed for the role of autophosphorylation in the interaction

3 Q. Song, S. P. Lees-Miller, S. Kumar, N. Zhang, D. W. Chan, G. C. M. Smith, S. P. Jackson, E. S. Alnemri, G. Litwack, and M. F. Lavin, submitted for publication.
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