DNA-dependent Protein Kinase Interacts with Antigen Receptor Response Element Binding Proteins NF90 and NF45*

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The DNA-dependent protein kinase (DNA-PK) is composed of a large catalytic subunit of approximately 470 kDa (DNA-PKcs) and the DNA-binding protein, Ku. Absence of DNA-PK activity confers sensitivity to x-rays and defects in both DNA double-strand break repair and V(D)J recombination. However, the precise function of DNA-PK in DNA double-strand break repair is not known. Here we show, using electrophoretic mobility shift assays, that polypeptides in a fraction purified from human cells interact with DNA-PK and stabilize the formation of a complex containing DNA-PKcs-Ku and DNA. Five polypeptides in this fraction have been identified by amino-terminal sequence analysis and/or immunoblotting. These proteins are NF90 and NF45, which are the 90- and 45-kDa subunits of a protein known to bind specifically to the antigen receptor response element of the interleukin 2 promoter, and the α, β, and γ subunits of eukaryotic translation initiation factor eIF-2. We also show that NF90, NF45, and eIF-2β are substrates for DNA-PK in vitro. In addition, recombinant NF90 promotes formation of a complex between DNA-PKcs, Ku, and DNA, and antibodies to recombinant NF90 or recombinant NF45 immunoprecipitate DNA-PKcs in vitro. Together, our data suggest that NF90, in complex with NF45, interacts with DNA-PKcs and Ku on DNA and that NF90 and NF45 may be important for the function of DNA-PK.

The DNA-dependent protein kinase, DNA-PK, is composed of a large catalytic subunit of approximately 470 kDa (DNA-PKcs) and a DNA binding heterodimer of approximately 70- and 80-kDa subunits called Ku (reviewed in Refs. 1–4). The catalytic subunit, DNA-PKcs, belongs to the phosphatidylinositol 3 kinase family of proteins (5) that includes ATM (the gene product defective in ataxia telangiectasia) and the FKBP-rapamycin-binding protein, FRAP (reviewed in Refs. 6–8). DNA-PK acts as a serine/threonine protein kinase, and several in vitro protein substrates have been identified (reviewed in Refs. 1, 2, and 4). DNA-PK requires ends of double-stranded DNA for activity (1, 9, 10), although hairpins, dumbbells, and DNA constructs containing single-stranded to double-stranded transitions also activate DNA-PK in vitro (11). Absence of any of the DNA-PK protein components confers sensitivity to ionizing radiation and defects in both DNA double-strand break repair and V(D)J recombination (reviewed in Refs. 3, 12, and 13). Although DNA-PK is essential for these processes in vivo, its precise function is at present unknown. One possibility is that upon binding of Ku to a suitable DNA end, DNA-PKcs is recruited to form the active kinase, which may then phosphorylate itself and/or other molecules that are required for DNA end rejoining. Alternatively, the DNA-PK complex may act as a scaffold to which other proteins required for DNA repair may be recruited. In addition to its role in DNA repair, DNA-PK may also play a role in the regulation of transcription by both RNA polymerases I and II (14–17).

To determine the role of DNA-PK in these processes, it is essential to understand how the kinase interacts both with DNA and with other protein partners. The interaction of Ku with ends of DNA has been extensively studied. Ku binds with high affinity to free ends of double-stranded DNA (18–22) as well as to nicked DNA (22, 23), hairpins, and dumbbell structures in vitro (23). Studies of the interaction of Ku with DNA ends have relied in large part on the electrophoretic mobility shift assay (EMSA). Using this assay, Ku forms multiple protein-DNA complexes, the number of which depends on the length of the DNA and the amount of Ku protein (19, 22). In contrast, relatively little is known about the formation of the DNA-PKcs-Ku-DNA complex. Several observations suggest that the interaction between DNA-PKcs and Ku in the absence of DNA is transient or weak. For example, the DNA-PKcs-Ku partner but not DNA-PKcs (29). Other, similar studies have revealed that the product of the avv proto-oncogene interacts with Ku (30); however, the significance of this observation is not known.

Here we used an EMSA to show that the interaction of highly
purified DNA-PKcs with Ku and DNA is weak or transient and that several polypeptides that partially copurify with DNA-PKcs can stabilize complex formation among DNA-PKcs, Ku, and DNA. Five polypeptides have been identified by protein sequence analysis and/or Western blot as NF45 and NF90, which are the 45- and 90-kDa subunits of a protein previously shown to bind specifically to the interleukin 2 promoter in activated T cells (31, 32) and the α, β, and γ subunits of eukaryotic initiation factor eIF-2, a heterotrimeric protein that is required for the initiation of protein synthesis (reviewed in Ref. 33). We provide evidence that NF90 and NF45 interact with DNA-PK in vitro and that NF90, NF45, and the β subunit of eIF-2 are phosphorylated by DNA-PK in vitro. Our data suggest that NF90 and NF45 interact with DNA-PKcs and Ku to form a supercomplex at the ends of DNA and that this interaction may be important for the function of DNA-PK.

MATeRIALS AND METHODS

Electrophoretic Mobility Shift Assay—Conditions for the electrophoretic mobility shift assay were similar to those of Blier et al. (22), with the following modifications. The plasmid vector pGEM Zf (Promega) was propagated in JM109 and harvested and purified using Qiagen columns according to the manufacturer’s instructions. DNA probes of 40, 80, and 102 bp were generated by digestion of plasmid DNA with HaeIII. Oligonucleotides were separated using MonoQ 5/5HR chromatography using a linear gradient of 0.61–0.79 M NaCl in 20 mM Tris-HCl (0.15 ml/min, 0.5 mM NaCl/min) for 360 min according to the manufacturer’s recommendations (Pharmacia FPLC Application File: Restriction Enzyme Fragments) and purified using a Mermaid DNA purification kit (Bio 101 Inc., La Jolla, CA). Also, oligonucleotides corresponding to the 40-bp HaeIII fragments were synthesized and gel purified by the DNA sequence facility, University of California. The sequence of the 40-bp oligonucleotide used was 5’-CCCAATTCGC-CCTATATGCTAGCTATTACATCCATCG3’. Synthesized oligonucleotides were combined in equimolar amounts in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0, containing 50 mM NaCl, heated to 95° C for 10 min and annealed overnight at room temperature. Oligonucleotides were end labeled with [γ-32P]ATP (Easy-tide, NEN Life Science Products) using polynucleotide kinase (Life Technologies, Inc.) according to the manufacturer’s instructions. Unincorporated nucleotide was removed using Microspin-G50 columns (Pharmacia) or Centricon 30 microconcentrators (Amicon) and stored in aliquots at –80 °C unless otherwise indicated. All purification steps were performed at 4 °C. FPLC steps were performed at either RT or 4 °C with no detectable difference in results. Phenylmethylsulfonyl fluoride, leupeptin, and apropin were present in 0.1 mM, 20 μg/ml, and 10 μg/ml, respectively, in all buffers and samples up to DEAE. Phenylmethylsulfonyl fluoride (0.2 mM) was present in buffers up to and including the FPLC steps.

Immunoprecipitation—Approximately 20 μg of total protein in the fraction pre-dsDNA cellulose was incubated under the conditions described for EMSA, either with or without 40-bp dsDNA at 5 μg/ml. Antibodies to DNA-PKcs, NF45, NF90, SV40 TAg, or preimmune serum were added as indicated, and immunoprecipitations were carried out under non-denaturing conditions as described (27, 35) except that protein G-Sepharose (Pharmacia) was used. Immunoprecipitated proteins were analyzed using SDS-PAGE as described.

Electrophoresis and Western Blot—SDS gels and Western blot for analysis of DNA-PKcs were as described (25) except that transfer buffer contained 20% methanol. For all other polypeptides, electrophoretic transfer for Western blot was in 25 mM Tris, 200 mM glycine, 20% methanol at 100 V for 35 min. Blots were developed using Enhanced Chemiluminescence (Amersham Corp.) according to the manufacturer’s instructions. Detection of protein in membranes was as described except for Antisera to NF90 and NF45 were as described (32). Antisera to eIF-2γ were a generous gift from Dr J. Hershey (University of California, Davis).

Protein Sequence Analysis—Approximately 10 μg of each of the polypeptides eluting from MonoQ FPLC corresponding to fractions 44–46 (see above and Fig. 3) was run on an SDS 10% polyacrylamide gel that had been pre-aged for 24 h and analyzed by electrophoresis under normal conditions. Polypeptides were then transferred to polyvinylidene difluoride membrane (Bio-Rad; sequencing grade, 0.2 μm) in 10 mM CAPS, 10% methanol (v/v), pH 11; as described (36). The polypeptides were visualized briefly in freshly prepared Coomassie Blue stain and briefly destained in 50% (v/v) methanol 0.05% (v/v) acetic acid. The appropriate bands were excised and sent for protein sequence analysis to either the University of Wisconsin Biotechnology Center (Madison, WI) (p50/52 and p37) or the University of Victoria Sequencing Center (Victoria, British Columbia, Canada) (p75 and p45).

Phosphorylation Reactions—Phosphorylation reactions were as described previously (27). 0.5–1 μg of purified protein was incubated in the presence or absence of purified DNA-PKcs, Ku, and DNA as indicated and analyzed by SDS-PAGE and autoradiography.

Interaction of DNA-PK with NF90 and NF45

man placebo over three ion exchange resins followed by chromatography on dsDNA-cellulose as described previously (25). Using this methodology, DNA-PKcs and Ku fractionated into two pools, referred to as pool A and pool B, respectively (25). Pool A contained six other predominant polypeptides with molecular masses of approximately 90, 75, 52, 45, 40, and 37 kDa. Polypeptides purified from MonoQ FPLC were further characterized by SDS-PAGE under nondenaturing conditions as described (25), followed by hydrophobic interaction chromatography using a phenyl superose HR5/5 FPLC column (Pharmacia). DNA-PKcs was applied to the phenyl superose column in Buffer A (50 mM Tris-HCl, pH 8.0, containing 50 mM KCl, 1 mM DTT) and eluted with a linear gradient of Buffer A to Buffer B (containing 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM DTT) at 0.7% Buffer B/min. The peak of DNA-PKcs protein eluted at approximately 880 mM ammonium sulfate and was homogeneous when analyzed by silver staining of SDS gels. The 90-, 75-, 52-, 45-, and 37-kDa polypeptides eluted from MonoQ FPLC at approximately 250–300 mM KCl and were combined to form pool C (see Fig. 2). Pool C was dialyzed and applied to MonoQ FPLC under exactly the same buffer conditions as described above for MonoQ chromatography. The 90-, 75-, 52-, and 45-kDa polypeptides eluted from MonoQ FPLC as a broad peak at approximately 200–300 mM KCl (see Fig. 3). Fractions containing these polypeptides were either used alone or combined as indicated. Ku was purified as described previously (25). Purified proteins were dialysed against 25 mM Hepes, 100 mM KCl, 1 mM DTT, pH 7.5, concentrated using Centricron 30 microconcentrators (Amicon) and stored in aliquots at –80 °C unless otherwise indicated. All purification steps were performed at 4 °C. FPLC steps were performed at either RT or 4 °C with no detectable difference in results. Phenylmethylsulfonyl fluoride, leupeptin, and apropin were present in 0.1 mM, 20 μg/ml, and 10 μg/ml, respectively, in all buffers and samples up to DEAE. Phenylmethylsulfonyl fluoride (0.2 mM) was present in buffers up to and including the FPLC steps.

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Purification of RecoRminant His-tagged proteins were purified from E. coli. Conditions for the expression and purification of recombinant His-tagged NF90 (rNF90) and NF45 (rNF45) in bacteria was as described previously (32) with some modification. Briefly, proteins were induced by the addition of isopropyl-1-thio-D-galactopyranoside and harvested using standard methodologies. As found previously (32), both rNF45 and rNF90 were insoluble and were resolubilized from the inclusion bodies in 8 M urea, 50 mM Tris-HCl, pH
Fig. 1. Ku/DNA-PKcs/DNA complex formation on EMSA is stabilized by chemical cross-linkers. Highly purified Ku (5 ng) was incubated with DNA alone (lanes 2, 8, and 14) or with highly purified DNA-PKcs and DNA (lanes 4–7, 10–13, and 16–19). Samples in lanes 3, 9, and 15 contained DNA-PKcs (30 ng) and DNA. Purified DNA-PKcs were present at 10 ng in lanes 4, 10, and 16; 20 ng in lanes 5, 11, and 17; 30 ng in lanes 6, 12, and 18 or 50 ng in lanes 7, 13, and 19. Lane 1 contained probe alone. All samples were incubated with a radiolabeled 40-bp DNA probe for 10 min at RT. Samples in lanes 1–7 were analyzed directly on nondenaturing gels as described under “Materials and Methods.” Samples in lanes 8–13 were treated with glutaraldehyde (final concentration, 0.06% (v/v)) for an additional 5 min at room temperature prior to electrophoresis. Samples in lanes 14–19 were incubated in the presence of the chemical cross-linker BS3 at 1.2 mM for an additional 5 min at room temperature prior to electrophoresis. Lower-case a and b indicate Ku-DNA complexes formed in the absence of chemical cross-linker. Ku-DNA complexes formed in the presence of cross-linker are designated A and B. The putative DNA-PKcs-Ku-DNA complex is designated C.

8.0. Resolubilized proteins in 8 M urea were diluted with an equal volume of 50 mM Tris-HCl, pH 8.0, and loaded onto Ni2+–NTA resin (Qiagen) that had been equilibrated in the same buffer. The elution column was washed with 50 mM Tris-HCl, pH 8.0, containing 10 mM imidazole, and rNF90 and rNF45 were eluted with 50 mM Tris-HCl, pH 8.0, containing 200 mM imidazole. Purity was estimated at >95% by Coomassie Blue staining. Purified proteins were dialyzed into 50 mM Tris-HCl, 50 mM KCl, 5% glycerol, 1 mM DTT and stored in aliquots at −80 °C.

RESULTS

Interaction of Highly Purified DNA-PKcs with Ku and DNA Is Stabilized by Chemical Cross-linkers—The interaction of Ku with DNA has long been studied using the electrophoretic mobility shift assay (18–22); however, relatively little is known regarding the interaction of DNA-PKcs with the Ku-DNA complex. Here we describe an EMSA that can be used to detect complexes between highly purified DNA-PKcs, Ku, and DNA. EMSAs were usually carried out using a 40-bp ds deoxyoligonucleotide probe that was either purified after HaeIII digestion of pGEM plasmid DNA or was synthesized chemically and annealed prior to labeling with [γ32P]ATP as described under “Materials and Methods.” The sequence of this oligonucleotide has no known specific binding sites and therefore is used as a source of free (blunt) dsDNA ends. Under the conditions used in our assay, the purified Ku heterodimer forms two complexes with the 40-bp DNA probe (labeled a and b in Fig. 1, lane 2). Similar protein-DNA complexes have been observed in many previous studies and have been interpreted as being due either to Ku binding to both ends and internal sequences of DNA or to the formation of Ku multimers on the DNA (18–22).

We next used this assay to examine the interaction of highly purified DNA-PKcs with Ku and DNA. The stoichiometry of the interaction between Ku and DNA-PKcs is not known; however, roughly equal amounts of DNA-PKcs monomer (470 kDa) and Ku heterodimer (156 kDa) are required for maximum catalytic activity (11, 25). For our studies, we therefore used amounts of purified DNA-PKcs and Ku that gave molar ratios of approximately 1. In the presence of a 1.3-fold molar excess of DNA-PKcs to Ku, the predominant bands seen on EMSA migrated in the same position as the bands formed with Ku alone (Fig. 1, lanes 4–7); however, a faint band of slower mobility was often observed (labeled C in Fig. 1, lanes 6 and 7). Addition of a 2.5-fold molar excess of DNA-PKcs did not result in increased formation of band C under these conditions (data not shown). Variation of the salt concentration between 50 and 150 mM and inclusion of up to 10 mM DTT in the binding reaction had little or no effect on the formation of complex C under these conditions. Addition of magnesium chloride at 1–5 mM had no significant effect on complex formation (data not shown). At least five different preparations of highly purified DNA-PKcs and Ku were used for EMSA reactions, and in no case was a strong band observed at position C.

We therefore explored the possibility that protein cross-linking agents might enhance the formation of a DNA-PKcs-Ku-DNA complex. Recent studies using EMSA to analyze complexes formed between p53 and its cognate DNA binding sequence have used low concentrations of glutaraldehyde to stabilize the protein-DNA complex (37). Under the conditions used by Tegtmeier and colleagues (37), protein cross-linking was specific for p53 and DNA. We therefore investigated the use of glutaraldehyde on the formation of the DNA-PKcs-Ku-DNA complex. Purified DNA-PKcs and Ku were incubated with DNA as above, and after 10 min, glutaraldehyde was added to a final concentration of 0.06% (v/v). After a further 5 min of incubation, the sample was analyzed by electrophoresis as described above. Addition of glutaraldehyde to Ku resulted in a slight increase in the mobility of the Ku-DNA complexes (Fig. 1, complexes A and B; compare lane 8 to lane 2), consistent with formation of a more compact structure (37). Significantly, addition of glutaraldehyde to reactions containing DNA-PKcs and Ku resulted in increased amounts of a complex with slower mobility that migrated in the approximate position of complex C (Fig. 1, lanes 10–13). These data suggest that glutaraldehyde stabilizes or promotes the formation of a complex between DNA-PKcs-Ku and DNA. Because glutaraldehyde may promote formation of nonspecific protein-protein and protein-DNA interactions, we used the chemical cross-linker BS3 in place of glutaraldehyde. BS3 interacts with free amino groups over a distance of approximately 11 Å. Like glutaraldehyde, addition of BS3 to reactions containing DNA-PKcs, Ku, and DNA resulted in a significant increase in the amount of the slower migrating complex C (Fig. 1A, lanes 16–19). Similar results were obtained using a 80-bp dsDNA oligonucleotide but not with a 20-bp DNA molecule (data not shown), suggesting that about 40 bp is required for stable complex formation in this assay. Chemical cross-linkers did not promote indiscriminate cross-linking between DNA-PKcs and DNA (Fig. 1) between BSA and either Ku or DNA-PKcs, or among heat-denatured DNA-PKcs, Ku, and DNA (data not shown), suggesting that under these conditions, the observed interactions are specific. Formation of protein-DNA complexes after cross-linking was greatly reduced by excess linear dsDNA but not by closed circular plasmid DNA (data not shown). These properties are consistent with the known properties of Ku-DNA complexes.
Identification of Polypeptides That Interact with DNA-PK—One of the proposed functions of DNA-PK is to recruit other proteins to the site of DNA damage. The inability of highly purified DNA-PKcs to form a stable complex with Ku and DNA in the absence of chemical cross-linkers suggested to us that this EMSA might be used to detect proteins that interact with and stabilize the DNA-PKcs-Ku-DNA complex. We previously described the purification of DNA-PK from HeLa cells (24) and human placenta (25). In each case, several polypeptides co-fractionated with DNA-PKcs on double-stranded DNA-cellulose chromatography, and several of these polypeptides were phosphorylated by DNA-PK \textit{in vitro} (24). Currently, our laboratory purifies DNA-PK from human placenta (25), and because polypeptides of a similar size were found to co-purify with DNA-PKcs during this purification procedure also, we were curious as to whether these polypeptides might interact with the DNA-PKcs-Ku-DNA complex.

Briefly, the purification scheme involves homogenization of whole placenta in high salt buffer and ammonium sulfate precipitation followed by anion and cation exchange chromatography. These steps are followed by a second round of chromatography on a weak anion exchange resin (DEAE-CL6B) in the presence of magnesium. Under these conditions, DNA-PKcs, Ku, and several other polypeptides no longer bind to the resin, whereas the majority of proteins are retained on the DEAE resin as in the initial anion exchange step (24, 25). In the next purification step, DNA-PK and the associated polypeptides from the flow through of the magnesium-DEAE column were fractionated by gradient elution on double-stranded DNA cellulose. Several polypeptides in addition to DNA-PKcs eluted from dsDNA cellulose chromatography between 0.2 and 0.4 mM KCl, including polypeptides of approximately 90, 75, 52, 50, 45, and 37 kDa. These fractions have previously been designated as pool A (25). Polypeptides in pool A were further chromatographed on MonoQ FPLC as described under “Materials and Methods.” DNA-PKcs eluted from MonoQ FPLC at approximately 150–200 mM KCl, and the fraction containing the 90-, 75-, 52-, 50-, 45-, and 37-kDa polypeptides eluted at approximately 250–300 mM KCl (Fig. 2, pool C). The SDS-PAGE conditions used often failed to resolve the 52- and 50-kDa polypeptides, in which case they appeared as a single band, which is labeled p50/52 (see, for example, Fig. 2).

Polypeptides in pool C were then further purified over MonoS FPLC as described under “Materials and Methods.” Silver staining of fractions 42–64 from MonoS chromatography again revealed six major polypeptides migrating at approximately 90, 75, 50, 52, 45, and 37 kDa (Fig. 3). No DNA-PKcs or Ku was present in these MonoS fractions as judged by lack of DNA-PK activity and by the absence of DNA-PKcs and Ku proteins by Western blot (data not shown). Fractions 44–64 in Fig. 3 were pooled and assayed for their ability to be phosphorylated by DNA-PK. Fig. 4A shows a Coomassie-stained SDS-10% acrylamide gel of the phosphorylation reactions, and Fig. 4B shows the corresponding autoradiogram. In the absence of added DNA-PKcs and Ku, no endogenous phosphorylation was observed, but addition of DNA-PK resulted in DNA-dependent phosphorylation of the 90-, 50/52-, and 45-kDa polypeptides (Fig. 4, A and B, lanes 5 and 6). Under the conditions used, p90 and p75 were separated from phosphorylated Ku80; however, p50 and p52 co-migrated. A similar sample was therefore run for a longer period of time to separate the 50- and 52-kDa polypeptides (Fig. 4, C and D). These data show that p50 is phosphorylated by DNA-PK, whereas p52 and p37 are not.

It is perhaps interesting to note that the extent of autophosphorylation of DNA-PKcs and both Ku subunits is decreased in the presence of the added proteins from MonoS FPLC (Fig. 4B). We have previously shown that DNA-PK loses activity when autophosphorylated and that loss of kinase activity is reduced in the presence of a suitable peptide substrate (27). Our data suggest that the presence of the 90-, 50/52-, and 45-kDa polypeptides may reduce autophosphorylation and hence inactivation of DNA-PK. This would also suggest that these polypeptides may be better substrates for DNA-PK than either DNA-PKcs or Ku.

To determine whether the polypeptides present in fractions 44–64 from MonoS FPLC were able to interact with DNA-PK, these fractions were added to purified DNA-PKcs and Ku and analyzed using the EMSA. In this case, however, chemical cross-linkers were not included at any point. Aliquots of individual fractions from MonoS FPLC were added to the DNA probe (in the absence of chemical cross-linker) either alone (Fig. 5A, lanes 2–9) or in the presence of Ku plus DNA-PKcs.
after the dye had reached the end of the gel to separate p50 and p52. Samples in lanes 1 and 2 contained purified DNA-PKcs and Ku; lanes 3 and 4 contained a pool of fractions 44–64 from MonoS FPLC; lanes 5 and 6 contained DNA-PKcs, Ku, and the MonoS pool. Samples in odd-numbered lanes were incubated in the presence of DNA, and even-numbered lanes were incubated without DNA. Samples were run on 10% polyacrylamide SDS-PAGE and stained with Coomassie Blue. p75 is shown migrating below Ku80. B, autoradiogram corresponding to the gel shown in panel A; C, a sample similar to that shown in A and B was incubated under phosphorylating conditions as described for A and then run on an SDS-10% polyacrylamide gel for 10 min after the dye had reached the end of the gel to separate p50 and p52. Samples in lanes 1 and 2 contained the MonoS pool alone, and samples in lanes 3 and 4 contained the MonoS pool plus DNA-PK. Samples in odd-numbered lanes were incubated in the presence of DNA, and samples in even-numbered lanes were incubated without DNA. Shown is a portion of a silver-stained gel. The sample shown in C contained a higher proportion of p50, p52, and p37 than Ku70 and p45; hence, phosphorylation of Ku70 and p45 was diminished relative to A and B. D, autoradiogram corresponding to the gel shown in panel C.

(Fig. 5A, lanes 12–19). In the absence of DNA-PKcs and Ku, none of the MonoS fractions supported formation of a complex, although some retardation of the DNA probe was observed (Fig. 5A, lanes 3–5). Addition of aliquots from individual fractions 46–62 to reactions containing DNA-PKcs, Ku, and DNA resulted in dramatic formation of a slower migrating protein-DNA complex at the expense of the Ku-DNA complexes a and b (labeled D in Fig. 5A, lanes 13–17). Addition of the MonoS fractions to DNA-PKcs alone did not induce formation of additional complexes (Fig. 5B). However, addition of the MonoS fractions to Ku and DNA did result in enhanced formation of the Ku-DNA complexes (Fig. 5B), suggesting that these polypeptides may induce a modest change in the interaction of Ku with DNA.

To confirm that DNA-PKcs was present in complex D, fractions 44–64 from MonoS FPLC were pooled and added to DNA binding reactions containing purified DNA-PKcs and Ku. Purified IgG from either immune serum (antibody DPK1 raised to amino acids 2018–2135 of DNA-PKcs) or preimmune serum was added to samples prior to electrophoresis as described under “Materials and Methods.” Addition of IgG from DPK1 serum but not from preimmune serum resulted in abrogation of complex D, either by preventing complex formation or by supershifting complex D into the well (Fig. 6, lanes 7 and 8). The presence of Ku in complexes a, b, and D was confirmed by Western blot (data not shown).

These data strongly suggest that components of fractions 44–64 from MonoS chromatography can interact with DNA-PKcs and Ku in vitro and stabilize the formation of a larger protein-DNA complex that contains DNA-PKcs, Ku, and DNA. The polypeptide in these fractions that most closely tracks with the ability to form the slower migrating protein-DNA complex, complex D, is p90. However, because this activity was spread over at least 17 fractions, multiple polypeptides could be involved. We therefore proceeded to identify each of the major polypeptides present in the MonoS fraction.

Identification of the Polypeptides in the MonoS Fraction—Approximately 10 μg of each of the six predominant polypeptides found in fractions 44–64 from MonoS FPLC was transferred to polyvinylidene difluoride membrane and analyzed by amino-terminal sequence analysis as described under “Materials and Methods.” Amino-terminal sequence was obtained for four of the polypeptides, and in each case, the sequence obtained corresponded precisely to known gene products. In the protein preparation used to obtain the sequence, considerably more p75 than p90 was observed; therefore, only p75 was sequenced. The amino-terminal 10 amino acids from polypeptide p75 were identical to the amino terminus of a 90-kDa polypeptide known as NF90, and polypeptide p45 was identified as NF45 (Table I). NF90 and NF45 are 90- and 45-kDa proteins, respectively, that were first identified as nuclear factors that bind to a cis-acting response element of the interleukin 2 promoter, known as the antigen receptor response element, in T cells that have been stimulated with ionomycin and phorbol myristate acetate (31, 32). Moreover, in vitro transcription assays indicated that NF90 and NF45 modulate expression from the interleukin 2 promoter (31). However, NF90 and NF45 are also found in the nucleus of nonstimulated cells (31), suggesting that they may be widely expressed.

p37 was identified as the α subunit of eukaryotic initiation factor eIF-2. The 50- and 52-kDa polypeptides were not separated in the sample sent for sequencing; therefore, both polypeptides were present. Only one sequence was read from these polypeptides, and this was identified as the γ subunit of eukaryotic translation initiation factor, eIF-2. eIF-2 forms a ternary complex with GTP, initiator methionine-tRNA, and the 40S ribosomal subunit, which then recruits the 60S ribosomal subunit and mRNA to initiate protein translation (reviewed in Ref. 38). The α subunit of eIF-2 is phosphorylated by the dsDNA-dependent protein kinase and possibly other protein kinases (reviewed in Refs. 38 and 39). We have shown by direct amino acid sequencing that p37 corresponds to the α subunit of eIF-2 and that the γ subunit of eIF-2 is present in the polypeptide doublet composed of p50 and p52 (Table I). The predicted molecular mass of the β subunit of eIF-2 is 38 kDa; however, this polypeptide is known to migrate aberrantly on SDS-PAGE, and it often co-migrates with the 52-kDa γ subunit (40). Also, the amino terminus of eIF-2β is amino-terminally blocked (39,
40). These observations are therefore consistent with our results and provide an explanation for why only one sequence was obtained from the p52/p50 polypeptides. Aberrant migration of p50 on one-dimensional gels is consistent with the behavior of eIF-2β (39). Also, phosphorylated p50 migrated on two-dimensional gel electrophoresis close to actin, which has a pI of 5.5 (data not shown), consistent with the known behavior of eIF-2β, which has a pI of 5.9 (39). We therefore conclude that p50 corresponds to the β subunit of eIF-2 and is a substrate for DNA-PK in vitro (Fig. 4).

Antibodies to recombinant NF90 and NF45 were provided by one of us (P. N. K.), and antibodies to purified human eIF-2β and γ subunits were obtained from Dr. John Hershey (University of California, Davis). Antibodies to NF90 cross-reacted with both p75 and p90 across fractions 44–60, suggesting that p75 is related to NF90, perhaps being a proteolytic breakdown product (Fig. 7A). The presence of NF45 was confirmed by Western blot in fractions 44–60 of the MonoS FPLC fractions.
DNA induced the formation of a slower migrating complex (Fig. 8, lane 17, labeled D) that co-migrated with complex D previously observed using DNA-PKcs-Ku-DNA and the MonoS fractions. Antibodies to eIF-2γ cross-reacted with the 50-kDa polypeptide (p50) that is phosphorylated by DNA-PK and antibodies to eIF-2γ reacted with p52 in fractions 48–58 (Fig. 7C and data not shown). From these experiments, we conclude that the protein fraction purified from MonoS FPLC that contains polypeptides capable of interacting with DNA-PKcs and Ku contains NF90, NF45, and the α, β, and γ subunits of eIF-2.

NF90 and NF45 Interact with DNA-PK—Recombinant His-tagged NF90 and NF45 proteins were purified from bacteria as described and assayed for their ability to interact with DNA-PKcs and Ku, and DNA in the EMSA in the absence of chemical cross-linkers. Incubation of recombinant NF90, recombinant NF45, or recombinant NF90/NF45 (combined) with DNA in the absence of DNA-PK, did not result in formation of a significant protein-DNA complex (Fig. 8, lanes 4–7). Similarly, no protein-DNA complexes were observed when recombinant NF90/NF45 proteins were added to DNA-PKcs and DNA (Fig. 8, lanes 12–15) or to Ku and DNA (Fig. 8, lanes 8–11), although in some experiments, a modest increase in the amount of Ku band b relative to Ku band a was observed (data not shown). Significantly, addition of recombinant NF90 to DNA-PKcs, Ku, and DNA induced the formation of a slower migrating complex (Fig. 8, lane 17, labeled D′) that co-migrated with complex D previously observed using DNA-PKcs-Ku-DNA and the MonoS fractions. Results are expressed using the amino acid single-letter codes. Uppercase letters represent assigned amino acids, and lowercase letters represent ambiguous sequence assignments. X refers to unidentified amino acids. In each case, 10 or 15 amino acids were read, and in each case, all positively identified amino acids were an exact match to the sequences of the following known gene products: NF90 (GenBank accession no., U10323); eIF-2β-γ (GenBank accession no., L19161); the reported cDNA sequence is MPGLSCRFYQHKFPE, suggesting that the amino-terminal methionine had been lost and that amino acids 1, 4, 5, and 6 in the sequence obtained correspond to P, S, C, and R, respectively; and eIF-2-α (GenBank accession no., J02645; the reported cDNA sequence is MAGGEAGVTLGQPHLSR, suggesting that the amino-terminal methionine has been lost and that the assignments of T and G at positions 8 and 10, respectively, in the read sequence are correct).
PKcs, NF90, or NF45 (data not shown). Effect on the ability of preimmune sera or the monoclonal DNA-PKcs appeared to be preferentially immunoprecipitated NF90 and NF45 being a heterodimer. In the experiment shown, absence of Ku and DNA. Antibodies to NF90 or NF45 immunoprecipitating that NF90 and NF45 may interact with DNA-PKcs in the absence of Ku and DNA. Antibodies to NF90, NF45, and eIF-2. Immunoprecipitates were analyzed by Western blot using a rabbit polyclonal antibody to DNA-PKcs (DPK1) or NF90 (Fig. 9, lane 8). Interestingly, DNA-PKcs immunoprecipitated with NF90 and NF45 in the presence and absence of DNA, suggesting that NF90 and NF45 may interact with DNA-PKcs in the absence of Ku and DNA. Antibodies to NF90 or NF45 immunoprecipitated both p90 and p75 (Fig. 9B), consistent with NF90 and NF45 being a heterodimer. In the experiment shown, DNA-PKcs appeared to be preferentially immunoprecipitated with NF90 in the presence of DNA; however, this was not a consistent finding. The presence or absence of DNA had no effect on the ability of preimmune sera or the monoclonal antibody to SV40 T antigen to immunoprecipitate either DNA-PKcs, NF90, or NF45 (data not shown).

**DISCUSSION**

DNA-PK is required for DNA double-strand break repair; however, its precise function is as yet unknown. The large size of the DNA-PK complex suggests that DNA-PK may act as a scaffold to which other proteins are recruited. We have used highly purified proteins in an EMSA to show that under the conditions of this assay, very highly purified DNA-PKcs and Ku only form a stable complex on DNA in the presence of chemical cross-linkers. Furthermore, this protein-DNA complex has properties similar to those previously reported for Ku and DNA-PK. Our data also show that DNA-PKcs does not interact with DNA in the absence of Ku, even in the presence of cross-linkers (Fig. 1, lanes 3-15), consistent with previous reports using UV cross-linking (10). Double-stranded DNA of 40 and 80 bp readily supported DNA-PK complex formation, whereas complex formation was less efficient on dsDNA of 20 bp (data not shown), suggesting a minimum size requirement of about 30 bp. In our hands, DNA-PKcs-Ku complexes that formed on DNA of 100 bp or greater did not enter the gel (data not shown).

This assay was then used to identify proteins that interact with DNA-PK and stabilize complex formation. We have identified a protein fraction from human cells that stabilizes the formation of a slowly migrating complex on EMSA in the absence of added chemical cross-linkers. This protein fraction has been purified extensively through several chromatographic stages and two FPLC columns in the presence of the nonionic detergent Tween 20. The major polypeptides in this fraction have been identified as NF90, NF45, and the α, β, and γ subunits of eIF-2. Fractions containing NF90 and NF45 tracked most closely with the activity capable of interacting with DNA-PK, and recombinant NF90 induced formation of a DNA-PKcs-Ku-DNA complex on EMSA. Because neither recombinant NF90, NF45, nor the purified MonoS polypeptides alone had significant affinity for the Ku-DNA complex, our data suggest that the interaction with the DNA-PK complex occurs via DNA-PKcs. Immunoprecipitation data showing that NF90 and NF45 can interact with the DNA-PKcs in the presence or absence of DNA further support the notion that the interaction is likely mediated through DNA-PKcs rather than Ku.

NF90 and NF45 were initially purified as a dimer (31, 32), and antibodies to NF90 coimmunoprecipitate NF45 and vice versa (Fig. 9, lanes 4-9, and data not shown). However, recombinant NF45 did not support formation of complex D’ on EMSA (Fig. 8), suggesting that the interaction with DNA-PKcs is mediated through the NF90 subunit. We therefore propose a model in which NF90 interacts with DNA-PKcs to form a complex between NF90-NF45 and DNA-PKcs and that this complex can interact with Ku in the presence of DNA. The Ku heterodimer forms multiple protein-DNA complexes on EMSA, the number of which depends both on the length of DNA and on the protein concentration (19-22). These protein-DNA complexes may represent Ku bound to either ends of DNA or bound at internal

**FIG. 9.** Antibodies to recombinant NF90 and recombinant NF45 immunoprecipitate DNA-PKcs. Approximately 20 μg of protein from the pre-dsDNA cellulose fraction was incubated as described for EMSA DNA binding in a volume of 20 μl, plus or minus 5 μg/ml 40-bp DNA probe as indicated. The following antibodies were added for immunoprecipitation: lanes 1 and 2, a mouse polyclonal antibody to recombinant NF90; lanes 3 and 4, a mouse polyclonal antibody to recombinant NF45; lanes 5 and 6, monoclonal antibody 42-27 to DNA-PKcs; lane 7, preimmune mouse serum; and lane 8, monoclonal antibody to SV40 T antigen. Lane 9 contained 5 μg of pre-DNA cellulose sample alone. Immunoprecipitations were as described (29) except that protein G-Sepharose (Pharmacia) was used. Immunoprecipitates were analyzed by Western blot using a rabbit polyclonal antibody DPK1 to DNA-PKcs (A). The blot was stripped and probed with a rabbit polyclonal antibody to recombinant NF90 (B).
sites after translocation after end binding. Alternatively, the complexes may represent different oligomeric forms of Ku. Our data suggest that the DNA-PKcs-Ku-DNA-containing complexes form preferentially from Ku complex b (Figs. 1 and 5 and data not shown). Attempts are currently under way to determine the precise composition of the different Ku-DNA and DNA-PKcs-Ku-DNA complexes.

Both NF45 and the 90 kDa polypeptide that cross-reacts with antibodies to NF90 are phosphorylated by DNA-PK in vitro (Fig. 4). We also show that the amino-terminal 10 amino acids of the 75 kDa polypeptide (called p75 in Figs. 2–4, 7, and 9) corresponds exactly to the predicted amino-terminal sequence of NF90 (Table I). Moreover, p75 is recognized by antibodies to recombinant NF90 both in Western blot and immunoprecipitation and is immunoprecipitated by antibodies to NF45 (Figs. 7 and 9). However, p75 was not phosphorylated by DNA-PK in vitro, suggesting either that p90 and p75 are variants of NF90 or that p75 has suffered proteolysis during the purification that results in loss of a carboxyl-terminal polypeptide of about 15 kDa that contains the DNA-PK phosphorylation sites. Interestingly, addition of phosphatase to extracts from stimulated T cells that contain NF90 and NF45 resulted in reduced binding to the antigen receptor response element sequence (31), suggesting that NF90 and NF45 may be phosphoproteins and that their function may be regulated by phosphorylation in vivo. DNA-PK is the first protein kinase known to phosphorylate NF90 and NF45 in vitro. Although many in vitro substrates have been identified for DNA-PK (reviewed in Refs. 1, 2, and 4), identification of in vivo substrates for DNA-PK has proved difficult. Potential substrates such as replication protein A and p53 may be phosphorylated less efficiently in cell lines that lack significant DNA-PK activity (41), keeping with a role for eIF-2 in the nucleus in addition to its role in translation. Also, it has recently been shown that the yeast TOR1 and TOR 2 proteins, which are related to DNA-PKcs by virtue of the carboxyl-terminal phosphatidylinositol-3 kinase domain, play a role in translation initiation (48), and TOR2 signals to the GTP exchange/GTPase proteins RHO and ROM to control cytoskeletal reorganization in response to nutrient availability (49). These observations set a precedent for a functional relationship between phosphatidylinositol 3-kine homologues, guanine nucleotide exchange proteins, and translational initiation.

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