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High Mobility Group Proteins 1 and 2 Can Function as DNA-Binding Regulatory Components for DNA-Dependent Protein Kinase *In Vitro*[1]

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The DNA-dependent protein kinase (DNA-PK) holoenzyme consists of a 470-kDa catalytic subunit (DNA-PKcs), a DNA-binding regulatory component known as Ku protein, and double-stranded DNA (dsDNA) with ends. We previously reported that the activity of DNA-PK *in vitro* is stimulated by non-histone chromosomal high mobility group proteins (HMG) 1 and 2 comprising two similar repeats, termed domains A and B, and an acidic C-terminal. Here we demonstrate that *in vitro* HMG1 and 2 can completely replace Ku protein as the DNA-binding regulatory component of DNA-PK. DNA-PKcs and Ku protein were separately purified from Raji nuclear extracts, and reconstituted into the DNA-PK holoenzyme in the presence of dsDNA. DNA-PKcs alone catalyzed DNA-dependent phosphorylation at a very low but significant level, and HMG1 and 2 markedly stimulated the phosphorylation of α-casein and a specific peptide substrate in a DNA-dependent manner. The HMG2-domains (A+B) polypeptide devoid of the C-terminal acidic region was more effective for DNA-PKcs stimulation than the full-length HMG2, and HMG2-domain A and -domain B polypeptides. Anti(Ku protein) antibodies inhibited the DNA-dependent phosphorylation activity of the DNA-PKcs:Ku protein complex, but not that of DNA-PKcs alone or when it was complexed with HMG1 or 2. These results demonstrate that HMG1 and 2 can function as the DNA-binding regulatory component for DNA-PKcs *in vitro*, and imply that a conformational change of dsDNA, which is elicited by regulatory components, is important for the stimulation of DNA-PK activity of DNA-PKcs.

Key words: DNA-dependent protein kinase, double-stranded DNA, HMG1, HMG2, Ku protein.

The DNA-dependent protein kinase (DNA-PK) holoenzyme is composed of a 470-kDa catalytic subunit (DNA-PKcs) (*1-3*), a heterodimeric DNA-binding component known as Ku protein (p70/p80), and double-stranded DNA (dsDNA) with ends (*4-6*). DNA-PK is a nuclear serine/threonine kinase that catalyzes the phosphorylation *in vitro* of a variety of proteins, including simian virus 40 large T antigen, Sp1, Fos, Jun, c-Myc, p53, pRB, RPA (p34), TBP, TFIIB, and the largest subunit of RNA polymerase II (*4-16*). The minimal essential requirements for the DNA-PK recognition sequence are P-S/T-X and X-S/T-Q (*6, 12*). DNA end-binding protein Ku (p70/p80), which was first discovered as an autoimmune antigen in patients with scleroderma-polymyositis overlap syndrome (*17*), targets DNA-PKcs to DNA under relatively low salt conditions (*4, 5, 18, 19*). DNA-PKcs is a member of the phosphatidylinositol kinase superfamily and although it has been shown to have no lipid kinase activity it is sensitive to wortmannin, a potent inhibitor of phosphatidylinositol 3-kinase (*1, 3*). Interestingly DNA-PKcs has been reported to be a death substrate in most apoptotic processes (*20-24*). Since DNA-PK requires dsDNA with ends or with single- to double-strand transitions for its activity (*25*), DNA-PK has been considered to function in the regulation of DNA replication, transcription, repair, and recombination. It was recently revealed that DNA-PK is involved at least in DNA end-joining in the process of DNA double-strand break repair and V(D)J recombination (*26-32*).

We previously reported that DNA-PK activity in partially purified preparations was stimulated by high mobility group (HMG) proteins 1 and 2 with M_r of about 25,000 (*13*). HMG1 and 2 are abundant, ubiquitously distributed non-histone chromosomal proteins consisting of two similar repeats of DNA binding domains, termed HMG1/2-domains A and B, and an acidic carboxyl-terminal region (*33*). They show highly conserved primary structures in mammals. For example, there is little difference in amino acid sequence between the human and pig HMG2 proteins: the

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Abbreviations: DNA-PK, DNA-dependent protein kinase; DNA-PKcs, catalytic component of DNA-PK; dsDNA, double-stranded DNA; HMG, high mobility group; PMSF, phenylmethylsulfonyl fluoride.

human HMG2 has a serine at residue 167 outside HMG-domain B instead of the glycine in pig HMG2; and it lacks 1 glutamic acid residue in the acidic tail compared with pig HMG2 (*34*). The degree of homology between HMG1 and HMG2 of human and pig origin is about 80% (*34*). HMG1 seems to be involved in transcription (*35*), whereas HMG2 may function in cell proliferation (*36*). It is clear that HMG1 and 2 bind to DNA sequence-non-specifically and bend DNA (*37*). Interestingly HMG1 and 2 *in vitro* enhance not only RAG1 and RAG2-mediated cleavage at a V(D)J recombination signal (*38, 39*), but also both intra-molecular and inter-molecular ligation reactions of DNA double-strand breaks (*40*). Although HMG1 and 2 stimulated DNA-PK activity in preparations containing DNA-PKcs and Ku protein (*13*), it remained to be clarified how DNA-PKcs, Ku, and HMG1 (or HMG2) interact with one another. Therefore, we set out to obtain completely purified preparations of DNA-PKcs, Ku protein, and HMG proteins with no cross-contamination.

Here we show that sequence-non-specific DNA-binding proteins HMG1 and 2 are capable of replacing Ku protein as the regulatory component of DNA-PK *in vitro*. In addition to the inherent enzymological interest, this observation may be useful for clarifying the molecular mechanism of DNA-dependent activation of DNA-PK and the mechanism of DNA end-joining.

MATERIALS AND METHODS

Materials—[γ-^{32}P]ATP (6,000 Ci/mmol) was purchased from NEN (Boston, MA, USA). Ultrafree-15 centrifugal filter devices were from Millipore (Bedford, MA, USA); DEAE-cellulofine A-800 from Seikagaku Kogyo (Tokyo); dsDNA-cellulose from Sigma (St. Louis, MO, USA); Mono S HR 5/5 and Mono Q HR 5/5 columns from Pharmacia (Uppsala, Sweden); nitrocellulose membrane filters and peroxidase-conjugated antibodies against rabbit IgG and mouse IgG from Bio-Rad (Hercules, CA, USA); peroxidase-conjugated antibodies against human IgG from Sigma (St. Louis, MO, USA); rabbit anti(DNA-PKcs) antibodies from Serotec (Oxford, UK); and enhanced chemiluminescence Western blotting detection reagents from Amersham (Buckinghamshire, UK). The human serum from an autoimmune patient (OM) was used as anti(Ku protein) antibodies (*19*). Sepharose 4B conjugated with purified IgG from anti(Ku protein)-positive patient (KT) serum was used for the immunodepletion study (*19*). A monoclonal antibody raised against pig thymus HMG2, which reacts with domain A in HMG1 and 2 (Shirakawa, H., and Yoshida, M., unpublished results), was employed.

Purification of DNA-PK—All purification procedures were performed at 0-4°C. DNA-PK was partially purified from Raji nuclear extracts as described (*6*), and the partially purified enzyme preparation was concentrated with an Ultrafree-15 centrifugal filter device. The enzyme solution (52 units/0.6 ml) was subjected to centrifugation in a Hitachi RPS-40T rotor for 24 h at 38,000 rpm in a 15-30% glycerol gradient in 11.6 ml of buffer C (50 mM NaP_i, pH 7.5, 0.2 mM EDTA, 0.02% Tween 20, 2 mM dithiothreitol, 0.1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin) containing 0.5 M NaCl. After centrifugation, the contents of the centrifuge tube were slowly pushed out with 1.5 M sucrose, from lower to higher glycerol density. Fractions containing DNA-PKcs (Fraction A with about 21-23% glycerol) were separated from the main Ku protein fractions (Fraction B with about 17-19% glycerol). Fraction A was dialyzed against buffer D (20 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, 5% glycerol, 0.02% Tween 20, 2 mM dithiothreitol, 0.1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin) containing 50 mM KCl. The dialysate was applied to a Mono S HR 5/5 column (see Refs. *11* and *41*), and eluted with 4 ml of a 0.05-1 M KCl linear gradient. The DNA-PKcs fractions at around 0.5 M KCl were pooled and frozen at −80°C. Fraction B was dialyzed against buffer D containing 0.2 M KCl. The dialysate was applied to a column (1 × 3 cm) of dsDNA-cellulose equilibrated with buffer D containing 0.2 M KCl, and proteins were eluted stepwise with 0.2, 0.3, and 0.5 M KCl in buffer D. The Ku protein fractions (10 ml) eluted at 0.5 M KCl were pooled and applied to a column (1 × 3 cm) of DEAE-cellulofine A-800 equilibrated with buffer D containing 0.5 M KCl. The flow-through fraction was dialyzed against buffer D containing 0.05 M KCl, and then applied to a Mono Q HR 5/5 column. Proteins were eluted with 4 ml of a 0.05-1 M KCl linear gradient, and the fractions containing Ku protein at around 0.6 M KCl were frozen at −80°C.

Preparation of HMG Proteins and Truncated Polypeptides—The HMG1 and 2 proteins were purified to homogeneity from pig thymus as described (*42*). Recombinant polypeptides containing HMG2-domain A (1-76 amino acid residues), -domain B (88-164 amino acid residues), and -domains (A+B) (1-164 amino acid residues) were purified from lysates of *Escherichia coli* BL21 cells harboring pGEM plasmids with the corresponding cDNA sequences downstream of the T7 promoter (Yoshioka, K. *et al.*, manuscript submitted; see Ref. *43*).

Protein Determination—Protein was quantitated by the method of Bradford (*44*) with BSA as a standard.

DNA-PK Assay—DNA-PK was assayed essentially as described (*6*). The phosphorylation of synthetic peptide 15 (EPPLSQEAFADLWKK, 10 μg) derived from the human p53 N-terminal sequence (*10*) was carried out in the presence of 0.3 mM [γ-^{32}P]ATP (0.033 μCi/nmol), and the phosphorylation of dephosphorylated α-casein (10 μg) was carried out in the presence of 50 μM [γ-^{32}P]ATP (0.2 μCi/nmol). After the addition of [γ-^{32}P]ATP, the reaction mixture, with a total volume of 20 μl, was incubated at 30°C for 10 min, and then the reaction was terminated by the addition of 10 μl of 30% acetic acid (for peptide 15) or 10 μl of 0.5 M EDTA/10 mM ATP (for α-casein). The solutions containing peptide 15 and α-casein were spotted onto Whatman P81 and Whatman DE81 paper, respectively. After washing extensively with 15% acetic acid (for peptide 15) or 0.25 M K_2HPO_4 (for α-casein), radioactivity was counted with a liquid scintillation counter. One unit of the enzyme was defined as the amount catalyzing the incorporation of 1 nmol phosphate/min into peptide 15 under the assay conditions used.

Immunodepletion—Purified DNA-PKcs was mixed with anti(Ku protein) IgG-Sepharose beads equilibrated with buffer D containing 0.2 M KCl. After standing on ice for 1 h, the mixture was centrifuged at 5,000 rpm for 1 min in a microcentrifuge and the supernatant was removed. In a 16-μl reaction mixture devoid of ATP, DNA-PKcs (0.24 μg) alone or in combination with the purified Ku protein

(0.11 μg), HMG1 (5 μg), or HMG2 (5 μg) was incubated with 0.05 μl of anti(Ku protein) antibodies (OM) (*19*) or 0.05 μl of normal human serum at 0°C for 30 min. The phosphorylation reaction was started by the addition of ATP, in a total volume of 20 μl, as described above.

DNA-Binding Assay with dsDNA-Cellulose—Ku protein (0.4 μg) or HMG1 (5 μg) was treated with 30 μl of a dsDNA-cellulose suspension (50%, v/v) in buffer A (50 mM Hepes-KOH, pH 7.7, 0.6 mM EDTA, 0.6 mM EGTA, 7.5 mM $MgCl_2$, 1 mM dithiothreitol, 10% glycerol, and 0.01% Tween 20) for 10 min at room temperature. DNA-PKcs (1.3 μg) was added to the mixture, with a total volume of 50 μl, with subsequent incubation for 5 min at room temperature. The mixture was centrifuged at 15,000 rpm for 1 min in a microcentrifuge, and the resulting pellet was washed three times with buffer A containing 0.15 M KCl. After standing for 10 min in 30 μl of SDS-sample buffer (62.5 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, and 2% 2-mercaptoethanol), the mixture was centrifuged at 15,000 rpm for 1 min in a microcentrifuge, and then the supernatant was subjected to electrophoresis on a 0.1% SDS/7.5% and 12.5% discontinuous polyacrylamide gel.

After SDS-PAGE, Western blot/enzyme immunoassaying was performed essentially as described (*22*). After blocking of the nitrocellulose membrane, it was incubated with an appropriate dilution of rabbit anti(DNA-PKcs) antibodies (1:1,000), anti(Ku protein) antibodies (OM, 1:1,000) or monoclonal antibodies against HMG1 and 2 (1:10,000) for 1 h, and then with peroxidase-conjugated antibodies against rabbit IgG (1:50,000), human IgG (1:10,000), or mouse IgG (1:10,000) for 1 h. The antigen-antibody complex was detected with enhanced chemiluminescence reagents. Following enhanced chemiluminescence detection of DNA-PKcs with the antibody, the membrane was sequentially reprobed with anti(Ku protein) and anti(HMG1 and 2) antibodies. Bound primary and secondary antibodies were removed from the membrane with 2 M glycine-HCl (pH 2.8).

RESULTS

Reconstitution of DNA-PK with DNA-PKcs and Ku Protein—We have separately purified DNA-PKcs and Ku protein from a nuclear extract of Raji Burkitt's lymphoma cells. From Fraction A of a 15-30% glycerol gradient (see "MATERIALS AND METHODS"), DNA-PKcs was further purified by use of a Mono S HR 5/5 column. A single protein peak corresponding to DNA-PKcs was eluted from the column at about 0.5 M KCl. From Fraction B of the 15-30% glycerol gradient, Ku protein was purified by sequential use of dsDNA-cellulose, DEAE-cellulofine, and Mono Q HR 5/5 columns. A single protein peak corresponding to Ku protein was eluted from the columns at about 0.6 M KCl. The purified preparations of DNA-PKcs and Ku protein were subjected to electrophoresis on a 0.1% SDS/7.5% polyacrylamide gel. The DNA-PKcs preparation gave a single polypeptide of 470 kDa, and the Ku protein preparation gave two polypeptides corresponding to heterodimers, p70 and p80 (Fig. 1A). For the purified DNA-PKcs preparation, no bands reactive with anti(Ku protein) antibodies were detected on Western blot/enzyme immunoassaying (data not shown), suggesting the absence of contaminating Ku protein.

Using the purified DNA-PKcs and Ku protein, the phosphorylation of peptide 15 and α-casein as substrates was determined in the presence and absence of sonicated calf thymus DNA. Purified DNA-PKcs alone showed a very low, but significant level of DNA-dependent kinase activity (Fig. 1, B and C). The addition of an equimolar amount of Ku protein to DNA-PKcs markedly stimulated DNA-PK in

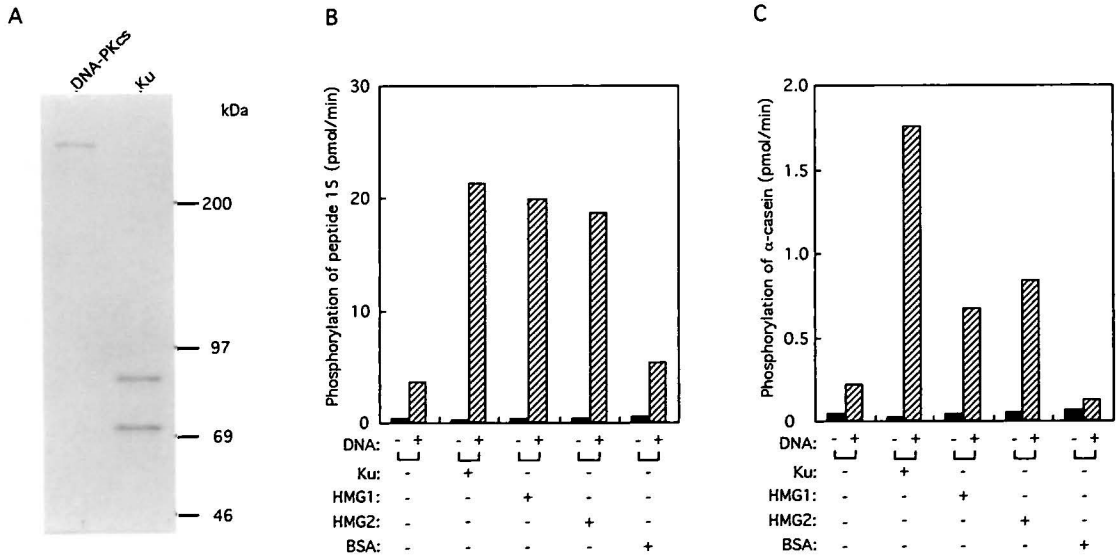

Fig. 1. **Reconstitution of DNA-PK with DNA-PKcs and Ku protein, and replacement of Ku protein with HMG1/2.** (A) Purified DNA-PKcs and Ku protein (1 μg each) were subjected to electrophoresis on a 0.1% SDS/7.5% polyacrylamide gel and then stained with Coomassie Brilliant Blue R-250. (B, C) The reaction mixture (20 μl) containing DNA-PKcs (26 nM) alone or in combination with Ku protein (26 nM), HMG1 (10 μM), HMG2 (10 μM), or BSA (10 μM) was incubated in the absence (filled bars) or presence of 0.4 μg of sonicated calf thymus DNA (hatched bars) at 30°C for 10 min. DNA-PK activity was determined with peptide 15 (B) or α-casein (C) as a substrate. Experimental results essentially identical to (B) and (C) were obtained twice more.

a DNA-dependent manner (Fig. 1, B and C). These results are consistent with previous observations (*5, 11, 41, 45*). We conclude that the DNA-PK holoenzyme can be reconstituted from its constituents: DNA-PKcs, Ku protein, and dsDNA.

Replacement of Ku Protein with HMG1 and 2 in DNA-PK—Like Ku protein, HMG1 and 2 stimulated the activity of DNA-PKcs in a DNA-dependent manner (Fig. 1, B and C). Although HMG1 and 2 are substrates for DNA-PK *in vitro* (*13*), phosphorylated HMG1 and 2 bound to neither P81 paper nor DE81 paper under the assay conditions used. Therefore, the DNA-PK activity detected with DNA-PKcs and HMG1/2 only resulted from the phosphorylation of exogenous peptide 15 or α-casein. BSA (Fig. 1, B and C) and histone H1 (data not shown) at the same concentration as that of HMG1 or 2 had no effect on the activity of DNA-PKcs. These results suggest that HMG1 and 2 can replace Ku protein as the regulatory component of DNA-PK *in vitro*.

Anti(Ku Protein) Antibodies Inhibit Ku-Dependent Phosphorylation but Not HMG1/2-Dependent Phosphorylation—To exclude the possibility that the purified DNA-PKcs preparation contained a very low level of Ku protein, the purified preparation of DNA-PKcs was treated with Sepharose beads conjugated with anti(Ku protein) IgG, even though Ku protein was not detectable on Western blot/enzyme immunoassaying (see above). DNA-PKcs immunodepleted with anti(Ku protein) IgG-Sepharose retained the DNA-PK activity, and this activity was not affected by the addition of anti(Ku protein) antibodies (Fig. 2A). The Ku-dependent phosphorylation was, of course, inhibited by anti(Ku protein) antibodies, but not by normal human serum (Fig. 2B). In contrast, the HMG1 (or HMG2)-dependent phosphorylation in a mixture of DNA-PKcs, HMG1 (or HMG2), and dsDNA did not significantly change on incubation with anti(Ku protein) antibodies (Fig. 2, C and D). Since the antibody specifically inhibited DNA-PK activity on the reconstitution of DNA-PKcs and Ku protein, but not DNA-PK activity in a mixture of DNA-PKcs and HMG1 (or HMG2), we conclude that HMG1 and 2 can function as the DNA-binding regulatory component instead of Ku protein in DNA-PK *in vitro*.

Ku Protein and HMG1/2 Dose-Dependence of the Activation of DNA-PKcs—The addition of increasing amounts of Ku protein to DNA-PKcs resulted in a marked increase in DNA-PK activity (Fig. 3A). The phosphorylation of peptide 15 was saturated at a molar ratio of about 1.3 mol Ku protein/mol DNA-PKcs, which was calculated based on molecular masses of 160 and 470 kDa for Ku protein and DNA-PKcs, respectively. The addition of 10 μM HMG1 and 2 increased DNA-PK activity 6- to 10-fold (Fig. 3, B and C). Even in the presence of 10 μM HMG1 and

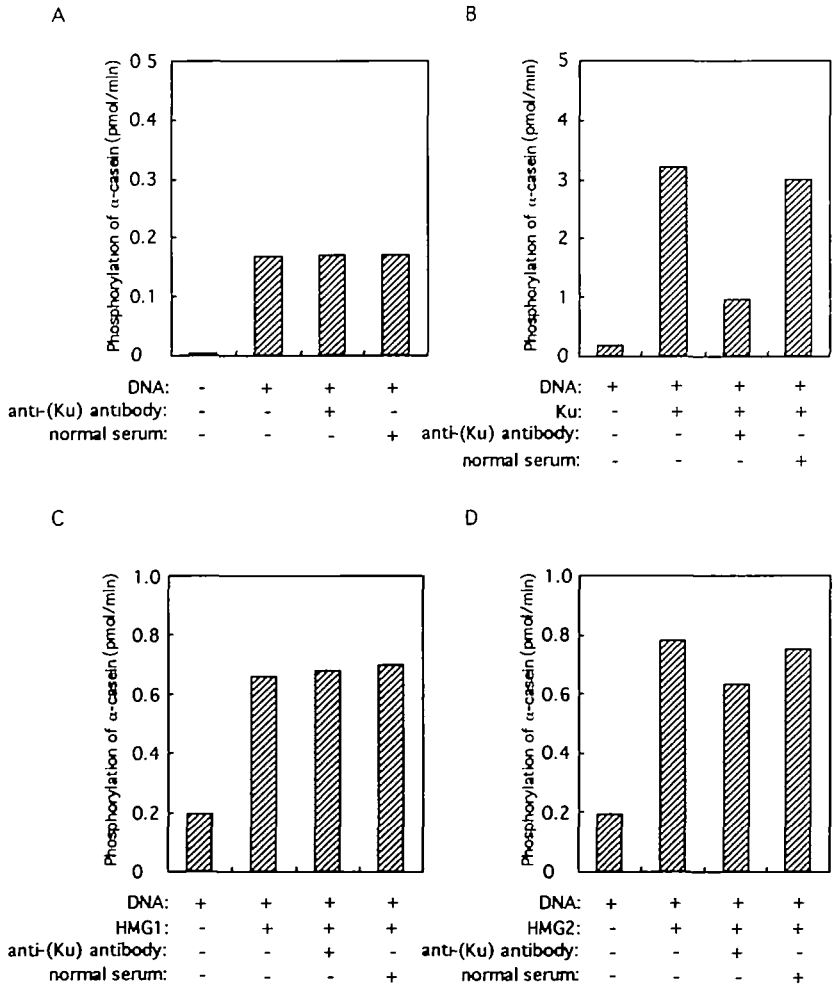

Fig. 2. **Effect of anti(Ku protein) antibodies on Ku-dependent and HMG-dependent phosphorylation.** DNA-PKcs (26 nM) alone (A) or in combination with Ku protein (37 nM) (B), HMG1 (10 μM) (C), or HMG2 (10 μM) (D) was preincubated with 0.05 μl of anti(Ku protein) antibodies (OM) or 0.05 μl of normal human serum in a 16-μl reaction mixture without ATP. The phosphorylation reaction was started by the addition of ATP, and DNA-PK activity was determined with α-casein as described under "MATERIALS AND METHODS." DNA (0.4 μg) was added when indicated (+). These experiments were repeated twice with virtually identical results.

2, corresponding to about 380 mol HMG1 or 2 per mol DNA-PKcs, the DNA-PK activity determined with peptide 15 and α-casein was not saturable. The DNA-PK activity of the DNA-PKcs:HMG1 or 2 complex with peptide 15 as a substrate was comparable to that in the presence of Ku protein, but when α-casein was used the activation by HMG1 or 2 was lower than that with Ku protein (Fig. 3, B and C).

Stimulation of DNA-PK Activity by HMG1/2 in the Presence of Ku Protein—Since HMG1 and 2 stimulate DNA-PKcs in place of Ku protein as a DNA-binding component, we examined whether the HMG proteins and Ku protein influence the kinase activity synergistically or complementarily. In the presence of sub-optimal concentrations of Ku protein, the kinase activity was enhanced by additional HMG1 and 2 (Fig. 4A). In the presence of an excess amount of Ku protein, however, HMG1 and 2 only slightly stimulated the DNA-PK activity determined with peptide 15 and α-casein (Fig. 4, B and C). These results imply that the effects of Ku protein and HMG1 (or HMG2) on DNA-PKcs are complementary to each other.

Interaction of DNA-PKcs with dsDNA in the Presence of HMG1 and 2—Ku protein is known to target DNA-PKcs to dsDNA (*4, 5, 19, 45*). The Ku:dsDNA:DNA-PKcs complex is stable in 0.15 M KCl but not in 0.5 M KCl (*19*). We examined whether or not HMG1 and 2 target DNA-PKcs to dsDNA as in the case of the Ku:dsDNA:DNA-PKcs complex. Ku protein and HMG1 bound to dsDNA in the presence of 0.15 M KCl, while DNA-PKcs alone was not retained on dsDNA under these conditions (Fig. 5). In the presence of Ku protein, DNA-PKcs was capable of binding to dsDNA strongly, whereas it bound slightly, albeit significantly, to dsDNA in the presence of large amounts of HMG1 (Fig. 5). These results suggest that the effect of HMG1 or 2 as to the targeting of DNA-PKcs to dsDNA is weaker than that of Ku protein.

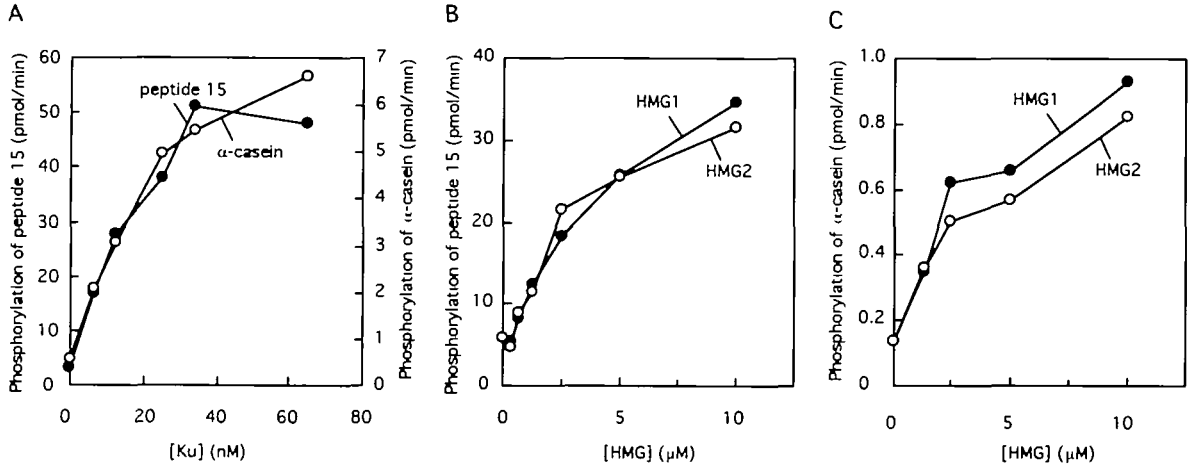

Fig. 3. **Ku protein and HMG1/2 dose-dependence of the activation of DNA-PKcs.** DNA-PK activity was determined with DNA-PKcs (26 nM) and the indicated concentrations of Ku protein (A), HMG1, or HMG2 (B, C) in a reaction mixture containing peptide 15 (A, B) or α-casein (A, C) as a substrate.

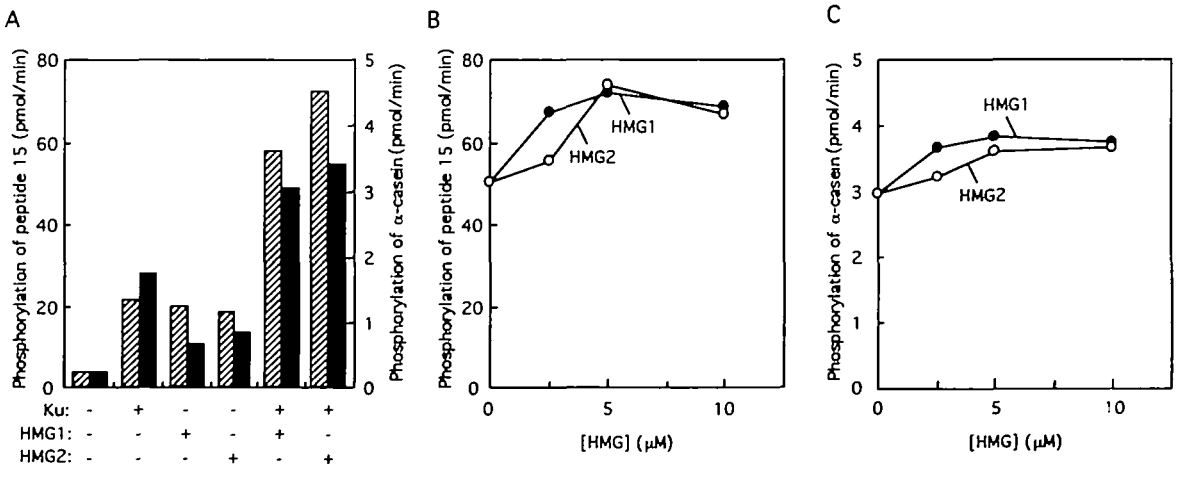

Fig. 4. **Influence of HMG1 and 2 on DNA-PK activity in the presence of Ku protein.** (A) In the presence of a sub-optimal concentration of Ku protein (20 nM), HMG1 (10 μM) and HMG2 (10 μM) were added to DNA-PKcs (26 nM). DNA-PK activity was determined with peptide 15 (hatched bars) or α-casein (filled bars) as a substrate. (B, C) DNA-PK activity was determined with peptide 15 (B) or α-casein (C) as a substrate in the presence of an excess amount of Ku protein (66 nM) in combination with varying concentrations of HMG1 and 2.

A Single HMG-Domain Stimulates DNA-PKcs—In order to determine which regions of HMG1 and 2 are necessary for the stimulation of DNA-PKcs, we employed bacterially produced HMG2-domain A, -domain B, and -domains (A+B) polypeptides. DNA-PKcs was similarly stimulated by these recombinant HMG2 polypeptides (Fig. 6A). Among these polypeptides (Fig. 6B), and full-length HMG1 and 2 (Fig. 3), HMG2-domains (A+B) composed of a tandem array of two DNA binding domains, A and B, and a basic linker (l) region (77-87 amino acid residues) was predominant for the stimulation of DNA-PKcs, and its optimal concentration was determined to be about 3 μM (Fig. 6B), which corresponds to about 100 mol HMG2-domains (A+B) per mol DNA-PKcs. This predominant effect of HMG2-domains (A+B) lacking the acidic carboxyl terminus may be due to its higher affinity for DNA. Using a BIAcore™ instrument, the K_d values (M) for 30-bp DNA of HMG2-domain A, -domain B, and -domains (A+B), and full-length HMG2 were recently estimated to be $(1.5\pm0.3)\times10^{-4}$, $(1.7\pm0.4)\times10^{-4}$, $(4.8\pm0.6)\times10^{-7}$, and $(6.1\pm1.5)\times10^{-7}$, respectively (Yoshioka, K. *et al.*, manuscript submitted; see Ref. *43*). At higher concentrations, however, the stimulatory effect of HMG2-domains (A+B) was markedly decreased (Fig. 6B), suggesting that the acidic terminal is functionally important for the HMG1 and 2 proteins (see Ref. *46*). These results indicate that the HMG-domain A or -domain B is capable of stimulating DNA-PKcs and that the HMG2-domains (A + B) polypeptide is the most active of the HMG2 polypeptides examined as to stimulation of DNA-PKcs *via* DNA, which is comparable to the binding affinity to DNA (*43*; Yoshioka, K. *et al.*, manuscript submitted). The serine residue at position 99 (Pro-Ser-Ala) in domain B of HMG1 and 2 (*33*) is likely to only be a target for DNA-PK (*13*), suggesting no relationship between the stimulatory effect on DNA-PKcs and the susceptibility to phosphorylation by DNA-PK.

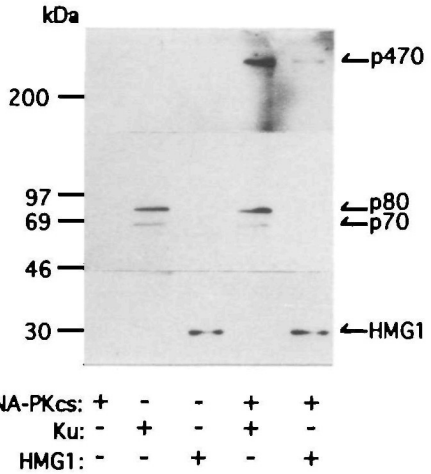

Fig. 5. **DNA binding of DNA-PKcs in the presence of Ku protein or HMG1.** Ku protein (0.4 μg) and HMG1 (5 μg) were treated with dsDNA-cellulose, and then DNA-PKcs (1.3 μg) was added to the mixture. After washing with buffer A containing 0.15 M KCl, the bound proteins were subjected to electrophoresis on 0.1% SDS/7.5% and 12.5% discontinuous polyacrylamide gels. Western blot/enzyme immunoassaying with anti(DNA-PKcs) antibodies, anti(Ku protein) antibodies, and monoclonal antibodies reacting with HMG1 and 2 was performed as described under "MATERIALS AND METHODS." DNA-PKcs was run in a 7.5% polyacrylamide gel, and Ku protein and HMG1 in a 12.5% polyacrylamide gel.

DISCUSSION

We have separately purified DNA-PKcs and Ku protein from Raji nuclear extracts, and found that HMG1 and 2 activate DNA-PKcs in the absence of Ku protein. This means that HMG1 and 2 can function as the DNA-binding regulatory component in place of Ku protein in the DNA-PK holoenzyme *in vitro*. Previously we reported that DNA-PK in preparations containing DNA-PKcs and Ku protein was stimulated several-fold on the addition of HMG1 or HMG2, or recombinant HMG-domain A, -domain B, or -domains (A+B) (*13*). In our subsequent studies, however, the stimulation ratio was found to vary widely from one preparation to another of the partially purified DNA-PK. At present we understand that the influence of HMG1 and 2 on the DNA-PK activity of DNA-PKcs:Ku fluctuates depending on the level of Ku protein in the DNA-PK preparations (see Fig. 4). The following observations exclude the possibility that the stimulation of DNA-PKcs by HMG1 and 2 was dependent on the presence of a trace

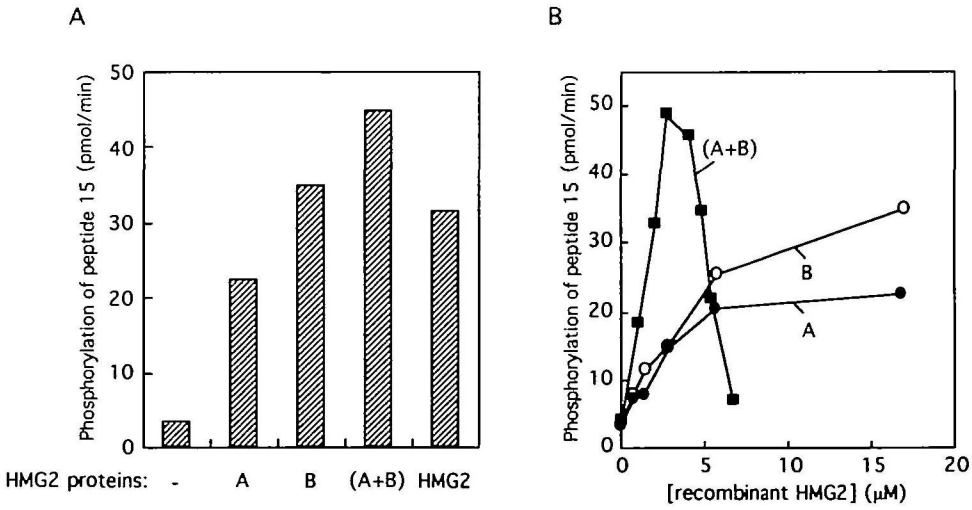

Fig. 6. **Stimulation of DNA-PK activity by recombinant HMG2 polypeptides.** (A) The reaction mixture (20 μl) containing DNA-PKcs (26 nM) alone or in combination with HMG2-domain A (17 μM), HMG2-domain B (17 μM), HMG2-domains (A+B) (4 μM), or HMG2 (5 μM) was incubated in the presence of 0.4 μg of sonicated calf thymus DNA. DNA-PK activity was determined with peptide 15 as a substrate. (B) To the reaction mixture containing DNA and DNA-PKcs as in (A), HMG2-domain A, HMG2-domain B, or HMG2-domains (A+B) was added at the indicated concentrations.

amount of Ku protein contaminating the preparation of DNA-PKcs, pig thymus HMG1 or HMG2: (i) Ku protein could not be detected on immunoblot analysis after SDS-PAGE of the DNA-PKcs and HMG proteins; (ii) DNA-PKcs treated with anti(Ku protein) IgG-Sepharose was similarly stimulated by HMG1 and 2 (Fig. 2); (iii) anti(Ku protein) antibodies capable of inhibiting the activity of the DNA-PKcs:Ku protein had no effect on DNA-PKcs alone or in combination with HMG1 or 2 (Fig. 2); and (iv) DNA-PKcs was stimulated by recombinant HMG2-domain A, -domain B, and -domains (A+B), which had been produced in *E. coli* cells containing no Ku protein (Fig. 6).

In the reconstituted DNA-PK, the molar ratio of Ku protein to DNA-PKcs was about 1.3 (Fig. 3), which is similar to the stoichiometry observed by others (*41*). In contrast, two orders of magnitude more HMG1 and 2 per mol of DNA-PKcs is required for the activation of DNA-PKcs (Fig. 3, B and C). Mutant cells devoid of Ku p80 and devoid of DNA-PKcs are deficient in DNA end-joining for DNA double-strand break repair and V(D)J recombination (*26-32*). Since these mutant cells show similar phenotypes and exhibit no significant DNA-PK activity in nuclear extracts (*28*), HMG1 and 2 might not function *in vivo*, at least in the DNA end-joining process as the regulatory protein in DNA-PK in place of Ku protein. Since a higher amount of HMG1 or 2 is required for the activation of DNA-PKcs, high expression of the HMG1 or 2 gene in Ku-deficient cells might rescue these mutations. Our results suggest that some of the myriad of functions ascribed to DNA-PK *in vivo* may result from the interaction of DNA-PKcs with other DNA-binding proteins, such as HMG1 and 2. KARP-1, which comprises a Ku 86-kDa polypeptide with a 9 kDa long amino-terminus, seems to function as a regulatory component of DNA-PK *in vivo* (*47*).

The abundant, chromosomal Ku protein widely distributed in eukaryotic cells is one of two DNA-end binding proteins, the other of which is poly(ADP-ribose) polymerase. HMG1 and 2 are also nuclear proteins distributed ubiquitously in higher eukaryotic cells. Obviously, heterodimeric Ku protein, and monomeric HMG1 and 2 can bind to DNA in a sequence non-specific manner. The ability of HMG1 or 2 to target DNA-PKcs to dsDNA with ends seems to be weaker than that of Ku protein, even with a higher concentration of HMG1 or 2 (Fig. 5), although the ability of HMG1 or 2 as to the stimulation of DNA-PKcs was comparable to that of Ku protein. Since HMG1 and 2 preferentially bind to supercoiled, cruciform or cisplatin-damaged DNA (*48-50*), the ability of HMG1 and 2 to stimulate DNA-dependent phosphorylation may be increased by the structures of DNA. However, the supercoiled DNA examined was ineffective for the activation of DNA-PKcs:HMG1 or 2, as in the case of DNA-PKcs:Ku protein (data not shown). The K_d value of Ku protein for linear dsDNA was estimated to be around 10^{-9} M (*51*), which is much lower than those of HMG1 and 2 (about 10^{-6} M) (*43*). These differences in biochemical and biophysical properties between Ku protein and the HMG proteins seem to reflect their ability to target DNA-PKcs to dsDNA.

The ability of HMG2-domains (A+B) to stimulate DNA-PKcs was predominant among those of full-length HMG2 and the truncated polypeptides examined (Figs. 3 and 6). This superiority of HMG2-domains (A+B) composed of two HMG domains and a basic l-region (77-87 amino acid residues) parallels its DNA-binding activity. Compared with those of HMG2-domain A ($K_d = 1.5 \times 10^{-4}$ M) and -domain B ($K_d = 1.7 \times 10^{-4}$ M), the DNA-binding affinity of HMG2-domains (A+B) ($K_d = 4.8 \times 10^{-7}$ M) was about 300-fold higher and that of HMG2-domain A plus l-region (Al) ($K_d = 3.2 \times 10^{-5}$ M) was 5-fold higher (Yoshioka, K. *et al.*, manuscript submitted). These findings imply that the tandem array of HMG-domains A and B connected with the l-region is required for not only effective binding with DNA but also efficient stimulation of DNA-PKcs. In addition, the acidic carboxyl terminus of HMG1 and 2 seems to reduce the DNA-binding ability, and to be important for regulation of the conformational change of DNA (*43*). The acidic carboxyl tail of HMG1 has been reported to be necessary for stimulation of the expression of reporter genes in COS-1 cells (*46*). As can be seen in Fig. 6, the stimulatory effect of HMG2-domains (A+B) was markedly decreased at higher concentrations, suggesting that the acidic carboxyl terminus is important for regulation of the appropriate conformation of DNA in DNA-PKcs stimulation. Since HMG1 and 2 are capable of inducing DNA-bending (*37*) and unwinding (Yoshioka, K. *et al.*, manuscript submitted), such a change in the DNA conformation may be required for the tight binding of DNA-PKcs to DNA. It is of interest that the physiological, regulatory component, Ku protein, exhibits DNA helicase activity (*52*).

Peterson *et al.* (*53*) have reported that DNA-PKcs is stimulated by the GAL4 amino-terminal 147 amino acids [GAL4 (1-147)] and heat shock transcription factor 1 (HSF1) even in the absence of Ku protein, for which specific sequences are required outside the internal DNA binding domains. In the case of HSF1, a tandem repeat of the specific DNA sequence, the heat shock element, is effective for stimulation. The mechanism of activation of DNA-PKcs by HMG1 and 2 seems to be different from that in the case of GAL4 and HSF1: (i) Ku protein and GAL4/HSF1 synergistically stimulate DNA-PKcs, but the stimulation by Ku protein and HMG1 (or HMG2) is complementary (Fig. 4), suggesting that Ku protein and HMG1 (or HMG2) function through a similar activation mechanism; (ii) GAL4 and HSF1 contain transcriptional activation domains essential for the activation of DNA-PKcs, whereas HMG1/2 binds to DNA sequence-non-specifically; (iii) in the case of GAL4 and HSF1, of course, the dsDNA required for DNA-PK activation contains the corresponding specific DNA sequences; and (iv) in contrast to HMG1 and 2, GAL4 and HSF1 have been reported to be unable to enhance the binding of DNA-PKcs to DNA (*53*). Our preliminary experiments indicated that the sequence non-specific DNA-binding proteins examined, such as proliferating cell nuclear antigen, histone H1 and XPA protein, are ineffective as the regulatory component of DNA-PKcs.

Although the DNA binding regulatory components of DNA-PK, such as Ku protein and HMG1 (or HMG2), target DNA-PKcs to DNA, the molecular anatomy of the activation of DNA-PKcs by Ku protein, HMG1 or HMG2 remains obscure. Recently, we found that poly(ADP-ribose) polymerase, another DNA end-binding protein, acts as the regulatory component of DNA-PK instead of Ku protein (Teraoka, H., Yumoto, Y., and Yoshida, M., unpublished results). Since these sequence non-specific DNA-binding proteins, *i.e.* Ku protein, HMG1 (or HMG2), and poly-

(ADP-ribose) polymerase, are independently able to activate DNA-PKcs in the presence of dsDNA with ends, we imagine that DNA-PKcs might not directly bind to one of these regulatory proteins, even in the presence of dsDNA, but rather it is likely to bind preferentially to a putative, common conformation of dsDNA, which is induced by one of these regulatory components. This idea seems to be supported by recent atomic-force microscopy studies showing that DNA-PKcs and Ku protein bind to dsDNA at adjacent positions along with the DNA chain (*45*) without the tight protein-protein interaction typically observed in the subunit structures of allosteric enzymes. In addition, gel-shift analysis with 18-bp DNA indicated that DNA-PKcs and Ku protein were not able to bind with each other, and the DNA-PK activity of DNA-PKcs alone was not increased by the addition of Ku protein in the presence of 18-bp DNA (*45*), implying no physical interaction between DNA-PKcs:18-bp DNA and Ku protein:18-bp DNA. Taken together, a conformational change of DNA, which is induced by Ku protein or HMG1 (or HMG2), seems to be important for the activation of DNA-PKcs. We are interested in what kind of DNA conformation elicited by Ku or other DNA-binding proteins is required for the activation of DNA-PKcs.

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