Activation and autoregulation of DNA-PK from structured single-stranded DNA and coding end hairpins

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DNA-dependent protein kinase (DNA-PK) acts through an essential relationship with DNA to participate in the regulation of multiple cellular processes. Yet the role of DNA as a cofactor in kinase activity remains to be completely elucidated. For example, although DNA-PK activity appears to be required for the resolution of hairpin coding ends in variable diversity joining recombination, kinase activity remains to be demonstrated from hairpin ends or other DNA structures. In the present study we report that DNA-PK is strongly activated from hairpin ends and structured single-stranded DNA, but that the phosphorylation of many heterologous substrates is blocked efficiently by inactivation of the kinase through autophosphorylation. However, substrates that bound efficiently to single-stranded DNA such as p53 and replication protein A were efficiently phosphorylated by DNA-PK from structured DNA. DNA-PK also was found to be active toward heterologous substrates from hairpin ends on double-stranded DNA under conditions where autophosphorylation was minimized. These results suggest that the role of DNA-PK in resolving coding end hairpins is likely to be enzymatic rather than structural, expand understanding of how DNA-PK binding to structured DNA relates to enzyme activity, and suggest a mechanism for autoregulatory control of its kinase activity in the cell.

DNA-dependent protein kinase (DNA-PK) is a Ser/Thr kinase required for the resolution of the hairpin coding ends in variable diversity joining [V(D)J] recombination and for correct DNA end joining in nonhomologous DNA (1). Roles for DNA-PK also have been proposed in DNA replication and the regulation of specific gene transcription by RNA polymerases I and II (2–4). Although physiological substrates for DNA-PK remain to be demonstrated, kinase activity appears to be essential to DNA-PK function in recombination, DNA repair, and transcriptional regulation (5).

DNA-PK is comprised of two components: a large catalytic subunit (DNA-PK_{cs}), which binds DNA with low affinity (6), and the Ku antigen (Ku70/Ku80), which binds specifically to DNA ends, sequences, and structural transitions in B-form DNA with high affinity (3, 7). DNA-PK_{cs} is a member of the large phosphatidylinositol 3-kinase-related kinase family with several other kinases, including the ataxia telangiectasia gene product and ataxia telangectasia and RAD-3-related kinase (8). Ku appears to be essential for DNA-PK_{cs} function *in vivo* and likely acts by promoting the recruitment of DNA-PK_{cs} to DNA ends and sequences from which the kinase is activated (3, 9, 10). Ku also contains limited DNA helicase activity and can induce structural transitions in DNA flanking sequence-specific DNA-PK binding sites (11, 12). Whether Ku helicase activity contributes to the activation of DNA-PK_{cs} from DNA ends is not known.

DNA-PK_{cs} is activated at DNA ends in the presence and absence of Ku and from specific Ku DNA binding sites when recruited by Ku (6, 13). Activation of DNA-PK_{cs} from DNA ends is further stimulated by the presence of unpaired bases at the ends of double-stranded DNA (14), whereas a Ku-induced structural transition in DNA may be required for the activation of DNA-PK_{cs} from specific DNA sequences (12).

Although Ku and DNA-PK_{cs} associate specifically with coding end hairpins and kinase activity appears to be required for hairpin resolution and joining (5, 15), the activation of DNA-PK from coding end hairpins or alternative DNA structures remains to be established. Although one early report (9) suggested that DNA-PK could be activated through association with closed DNA ends containing four nucleotide loops and from structural transitions from B-form DNA, most studies have concluded that DNA-PK and purified DNA-PK_{cs} are inactive from structured DNA, single-stranded DNA, and hairpin coding ends (15–18).

DNA-PK activity is induced on DNA damage (2). It also is regulated through the cell cycle (19). Regulation in both cases appears to be mediated postranslationally, as the levels of DNA-PK_{cs} and Ku in the cell do not fluctuate appreciably. The regulation of DNA-PK *in vitro* at DNA ends through the autophosphorylation of DNA-PK_{cs} suggests one possible means of DNA-PK regulation *in vivo* (20).

In the present work we demonstrate the activation of DNA-PK from structured single-stranded DNA and closed hairpin structures of the type found in V(D)J coding end intermediates, in the absence of free DNA ends. Phosphorylation of heterologous substrates occurred in competition with the autoinactivation of DNA-PK through autophosphorylation of DNA-PK_{cs} and was strongly favored by colocalization of the substrate with DNA-PK to the DNA. These results indicate a mechanism for the involvement of DNA-PK activity in the resolution and joining of hairpin coding ends.

Materials and Methods

Reagents and Substrates. DNA-PK and the p53 peptide substrate were obtained from Promega, and heat shock protein 90 (hsp90) was from StressGen Biotechnologies, Victoria, Canada. Recombinant full-length p53 and $p53_{\Delta 30}$ were expressed as fusion proteins from pGEX-6P1 and purified on Glutathione Sepharose 4B (Amersham Pharmacia) essentially as described (21), then cleaved from the glutathione *S*-transferase with PreScission protease. Purity of the preparations exceeded 85% as determined by SDS/PAGE.

DNAs. *Hin*dIII-linearized pBluescript (linpBlue) DNA was obtained by extraction of *Hin*dIII-digested plasmid (Qiagen, Chatsworth, CA) from 0.8% agarose gels. Single-stranded M13 DNA

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Abbreviations: DNA-PK, DNA-dependent protein kinase; DNA-PK_{cs}, DNA-PK catalytic subunit; V(D)J, variable diversity joining; linpBlue, *Hin*dIII-linearized pBluescript; ssM13, single-stranded M13 DNA; hsp90, heat shock protein 90; RPA, replication protein A.

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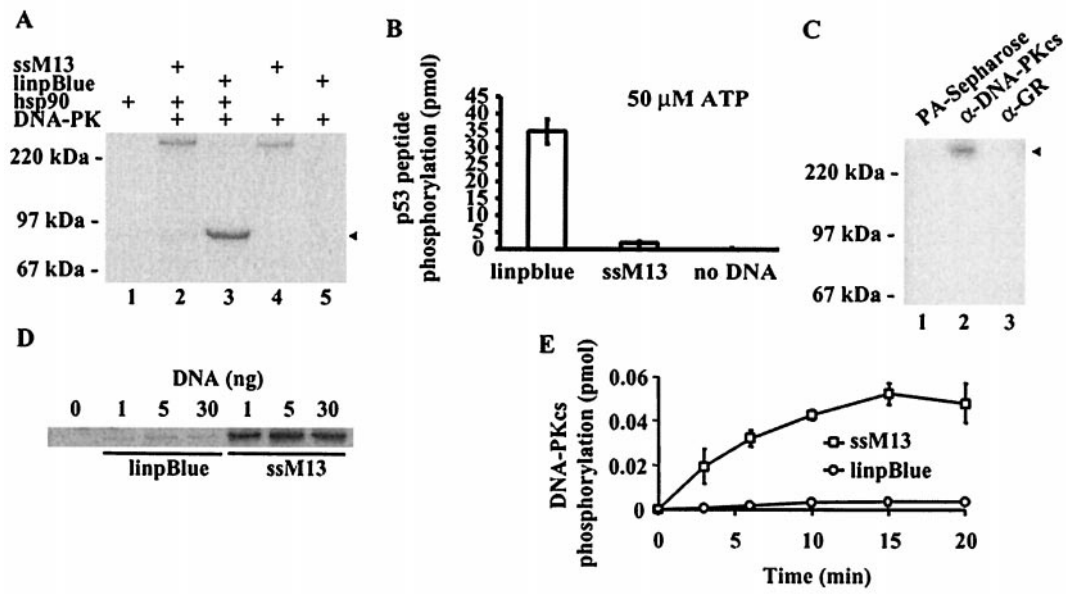

Fig. 1. Preferential autophosphorylation of DNA-PK_{cs} on ssM13 at 50 μM ATP. (*A*) SDS/PAGE (8%) analysis of proteins phosphorylated by DNA-PK in the presence of 10 ng linpBlue or ssM13 and hsp90 (arrowhead) as indicated above the autoradiograph. (*B*) DNA-PK-dependent phosphate incorporation into a p53 peptide substrate in the absence of DNA or in the presence of 10 ng linpBlue or ssM13 determined by PhosphorImage analysis of 40% alkaline PAGE gels. Data points correspond to the mean of two determinations ± SD. (*C*) Autophosphorylation of DNA-PK_{cs} in the presence of 10 ng of ssM13 and γ^{32}P ATP was assessed by SDS/PAGE analysis of material precipitated by Protein A-Sepharose (lane 1) or after incubation with DNA-PK_{cs} antibody AB-4 (α-DNA-PKcs, lane 2) or glucocorticoid receptor antibody $BuGR_2$ (lane 3). The arrowhead indicates the position of DNA-PK_{cs}. (*D*) Analysis of the autophosphorylation of DNA-PK_{cs} in the presence of 0–30 ng linpBlue or ssM13 as indicated is compared with autophosphorylation in the absence of DNA. (*E*) Twenty-minute time-course analysis of phosphate incorporation into DNA-PK_{cs} in the presence of 10 ng of ssM13 (□) or linpblue (○). Phosphate incorporation was quantified by PhosphorImage analysis of SDS/PAGE gels as in *B*. Data points correspond to the mean of two determinations ± SD.

(ssM13) was purchased from New England Biolabs. A 43-bp oligonucleotide containing coding end-like hairpins with no unpaired nucleotides was generated by ligation of an internal nick resulting from the annealing-mediated folding over of complementary strands from each end of the 86-mer (5′-ACTGTGGATCGACGTAG*CTACGTCGATCCACAGTGG-CATGCGTCTTGGTTCGTATCGTAG*CTACGATACGA-ACCAAGACGCATGCC-3′). Closed circular molecules were purified by two rounds of electroelution from a denaturing polyacryamide gel (6 M urea, 40% formamide, 8% acrylamide). The 43-bp double-stranded linear oligonucleotide used was of the same sequence (corresponding to the italicized portion of the hairpin sequence above and its antisense complement) and was prepared by electroelution of the double-stranded oligonucleotide from a native 8% polyacryamide gel. To obtain a DNA template with a single hairpin end and a blocked linear end, an 86-mer of the same sequence labeled both 5′ and 3′ with biotin (Gene Link, Hawthorne, NY) was synthesized beginning from the CTACG that forms one end of the double-strand linear oligonucleotide, annealed, and purified as for the linear oligonucleotide. Thus all three oligonucleotides used have the same sequence, but differ specifically in the structure of the DNA ends. All DNAs were quantified by spectrophotometry before use in kinase assays. The free end of the biotin-labeled single hairpin oligonucleotide was fixed by preincubation of the oligonucleotide with streptavidin-acrylamide beads (Sigma).

Kinase Assays. Assays were performed at 30°C for 10 min in the presence of 0.042 μM of γ-^{32}P ATP (3,000 Ci/mmol) and 10 units of DNA-PK in 20 μl of reaction buffer (50 mM Hepes/100 mM KCl/10 mM $MgCl_2$/0.2 mM EGTA, pH 7.5) unless otherwise indicated. Substrate concentration was 3 μM for p53 peptide and 0.2 μM for p53, hsp90, and replication protein A (RPA) unless otherwise indicated. The concentration and composition of the DNA substrates were as indicated. In many experiments, the final ATP concentration was adjusted to 50 μM with unlabeled ATP. For the p53 peptide, phosphorylation was analyzed by alkaline gel electrophoresis (40%) using a minigel adaptation of a published protocol (22). For protein substrates, the kinase reactions were resolved by SDS/PAGE (8–12%) and visualized by autoradiography. Quantification was performed by PhosphorImager analysis in the presence of a series of γ-^{32}P ATP standards.

Immunoprecipitation. DNA-PKcs immunoprecipitation was performed with Ab-4 (NeoMarkers, Fremont, CA) or an antibody against the glucocorticoid receptor ($BuGR_2$, Affinity BioReagents, Neshanic Station, NJ) according to standard protocols.

Results

Activation of DNA-PK from Single-Stranded DNA. ssM13 is covalently closed circular single-stranded DNA that adopts a highly complex structure that maximizes available base-pairing within the single DNA strand (23, 24). To begin an examination of how non-B-form DNA might lead to the activation of DNA-PK we compared the ability of purified DNA-PK holoenzyme (containing both Ku and DNA-PK_{cs}) to phosphorylate two known DNA-PK substrates from DNA ends and ssM13 (Fig. 1). Phosphorylation of hsp90 was strongly stimulated in the presence of linpBlue plasmid DNA (Fig. 1*A*, lane 3). By contrast, the addition of ssM13 did not promote hsp90 phosphorylation (Fig. 1*A*, lane 2). This result is similar to what has been described for other single-stranded DNAs (9, 12, 14, 17, 18). We also obtained the same result with the p53 peptide substrate that is used most frequently as a substrate for DNA-PK (Fig. 1*B*). Unexpectedly, however, ssM13 stimulated the incorporation of ^{32}P into a large factor migrating near the top of the SDS/PAGE gel that did not

appear upon addition of linpBlue and was independent of hsp90 (Fig. 1*A*, lanes 2 and 4).

The large size of the labeled factor suggested that ssM13 may have specifically promoted the autophosphorylation of DNA-PK_{cs}. This was confirmed by the selective immunoprecipitation of the high molecular mass factor with an antibody to DNA-PK_{cs} (Fig. 1*C*).

Previously it has been suggested that rapid autophosphorylation of DNA-PK_{cs} at double-stranded DNA ends leads to the inactivation of DNA-PK activity (20). However, this autocatalytic DNA-PK activity was characterized on sheared calf thymus DNA whose composition and structure was not detailed. Direct comparison of DNA-PK_{cs} autophosphorylation on ssM13 DNA and linpBlue confirmed that DNA-PK_{cs} autophosphorylation demonstrated a strong preference for ssM13 as a cofactor under our reaction conditions (Fig. 1 *D* and *E*). One nanogram of M13 DNA was sufficient to promote strong autophosphorylation of DNA-PK_{cs}, whereas little autophosphorylation was observed in the presence of 30 ng of linpBlue (Fig. 1*D*). Autophosphorylation of DNA-PK_{cs} was rapid on ssM13, being 40% complete within 3 min at 30°C, and plateauing between 6 and 10 min (Fig. 1*E*). By contrast autophosphorylation of DNA-PK_{cs} on linpBlue had not risen above 5% of the level obtained with ssM13 DNA by 20 min. Further, autophosphorylation of DNA-PK_{cs} on the two DNAs occurred within the same tryptic peptides, indicating that phosphorylation occurred at similar sites on the two DNAs (S.S. and R.J.G.H, unpublished observation).

The dramatic increase of DNA-PK_{cs} autophosphorylation in the presence of ssM13 suggested that rapid inactivation of the kinase may have precluded phosphorylation of the heterologous substrates. Therefore, we hypothesized that limiting reaction conditions that would decrease the autophosphorylation of DNA-PK_{cs} might allow us to detect the phosphorylation of other substrates in the presence of ssM13.

One simple way to titrate DNA-PK activity while maintaining our ability to detect phosphorylation was to decrease the concentration of ATP in the reaction while simultaneously increasing the specific activity of the ^{32}P γATP used (Fig. 2*A*). Decreasing the ATP concentration in our reactions 1,200-fold (from 50 μM to 0.042 μM) led to a proportional decrease in the molar incorporation of phosphate in DNA-PK_{cs}, while still allowing for the detection of phosphorylation (Fig. 2*A*). Further, at 0.042 μM ATP DNA-PK_{cs} autophosphorylation proceeded in an essentially linear manner for at least 30 min, a significant extension in the catalytic lifetime of the kinase. Under these conditions of limiting ATP, ssM13 was revealed to induce the phosphorylation of the p53 peptide substrate to a level equal to that obtained with linpBlue (Fig. 2*B*).

DNA-PK is a relatively inefficient kinase with a very high k_m for heterologous substrates (25). Previously, we and others have shown that colocalization to DNA and protein–protein interactions can dramatically enhance substrate phosphorylation by DNA-PK (9, 21, 26). Full-length recombinant p53 binds strongly to single-stranded DNA, but high affinity binding to double-stranded DNA depends on additional postranslational modification (27). By contrast to the p53 peptide, phosphorylation of full-length p53 showed a dramatic preference for ssM13 at 0.042 μM ATP (Fig. 3*A*, lanes 1–3). Indeed, even at 50 μM ATP, p53 phosphorylation was ≈5-fold higher in the presence of ssM13 than linpblue (Fig. 3*A*, lanes 4–6).

$p53_{\Delta 30}$ contains a C-terminal truncation of 30 aa that abrogates single-stranded DNA binding without affecting the DNA-PK phosphorylation sites in the N terminus of p53 (27). To directly assess the contribution of the binding of p53 to ssM13 toward its phosphorylation by DNA-PK, we compared the phosphorylation of equimolar amounts of p53, $p53_{\Delta 30}$, and the p53 peptide substrate (Fig. 3*B*). At a substrate concentration of 0.2 μM, only full-length p53 was appreciably phosphorylated in the presence of ssM13 at 50 μM ATP.

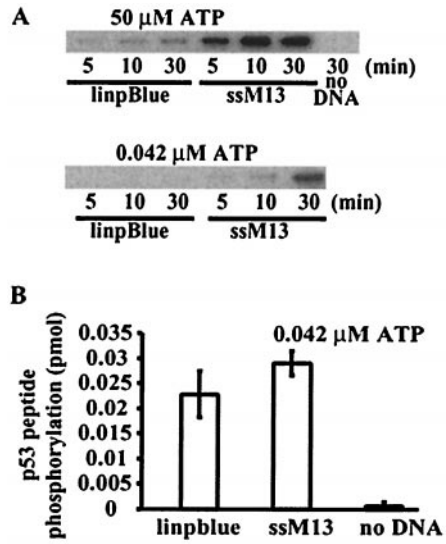

Fig. 2. DNA-PK activity at reduced ATP concentration. (*A*) Autoradiographs of 8% SDS/PAGE gels showing DNA-PK_{cs} autophosphorylation at 50 μM γ[^{32}P]ATP (*Upper*, specific activity 5 Ci/mmol) and 0.042 μM γ[^{32}P]ATP (*Lower*, specific activity 6,000 Ci/mmol) in the presence of 10 ng of linpBlue or ssM13 over a 30-min time course. (*B*) DNA-PK-dependent phosphorylation of p53 peptide substrate at 0.042 μM ATP in the presence of 10 ng linpBlue or ssM13, quantified by PhosphorImage analysis of 40% alkaline PAGE gels. Data points correspond to the mean of two determinations ± SD.

A second single-stranded DNA binding substrate of DNA-PK is RPA, which is phosphorylated in the 34-kDa subunit (28, 29). RPA is not phosphorylated by DNA-PK in the presence of linear double-stranded DNA alone, but p34 has been shown to be efficiently phosphorylated upon coincubation of RPA with single- and double-stranded DNA (30). However, whether double-stranded DNA, added as a cofactor for DNA-PK, is required for RPA phosphorylation was not determined. We observed that ssM13 alone was sufficient for p34 phosphorylation by DNA-PK (Fig. 3*C*).

These results establish that DNA-PK is active from structured single-stranded DNA toward heterologous substrates. They also indicate that targeting of the substrates to DNA is one key factor for efficient phosphorylation.

To examine the extent to which DNA-PK_{cs} autophosphorylation benefited from the colocalization of multiple kinase molecules on ssM13, we examined the effect of DNA concentration on autophosphorylation (Fig. 3*D*). Although a 10-fold increase in ssM13 reduced DNA-PK_{cs} autophosphorylation approximately 3-fold, suggesting a strong DNA colocalization component to DNA-PK_{cs} autophosphorylation on ssM13, a similar increase in linpblue had little effect on the more modest autophosphorylation detected from DNA ends. Interestingly, further increases in ssM13 concentration had little additional effect on DNA-PK_{cs} autophosphorylation (data not shown).

Autophosphorylation of DNA-PK_{cs} on ssM13 Inactivates Kinase Activity. The identification of DNA-PK substrates that were efficiently phosphorylated in the presence of ssM13 allowed us to test directly whether the autophosphorylation of DNA-PK_{cs} on single-stranded DNA resulted in kinase inactivation (Fig. 4). p53 was added to reactions together with ^{32}P γATP tracer after preincubation of DNA-PK with ssM13 and 50 μM unlabeled ATP. Incorporation of ^{32}P into both p53 and DNA-PK_{cs} decreased linearly with increasing preincubation, such that a

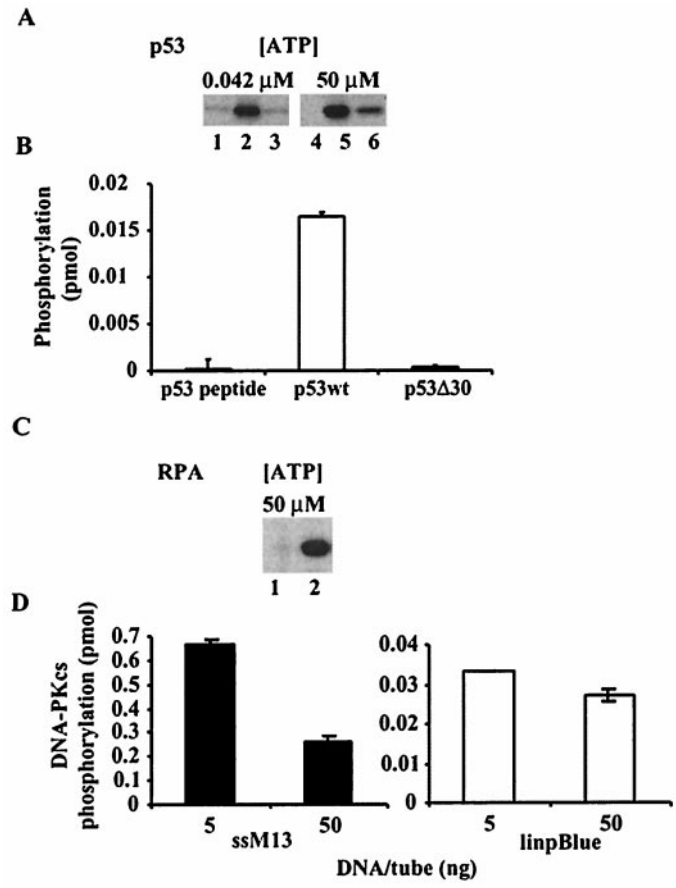

Fig. 3. DNA binding facilitates substrate phosphorylation by DNA-PK. (*A*) DNA-PK-dependent phosphorylation of recombinant p53 in the absence of added DNA (lanes 1 and 4) and in the presence of 10 ng of ssM13 (lanes 2 and 5) or 10 ng linpblue (lanes 3 and 6) at 0.042 μM ATP (lanes 1–3) and 50 μM ATP (lanes 4–6). (*B*) Quantification of DNA-PK-mediated phosphorylation of full-length p53, $p53_{\Delta 30}$, and p53 peptide at a concentration of 0.2 μM incubated with DNA-PK and 10 ng ssM13. (*C*) DNA-PK-dependent phosphorylation of the p34 subunit of recombinant RPA at 50 μM ATP in the absence (lane 1) and presence of 10 ng of ssM13 (lane 2). (*D*) Quantification of autophosphorylation of DNA-PK_{cs} at 50 μM ATP in the presence of 5 and 50 ng of ssM13 (*Left*) or linpBlue (*Right*).

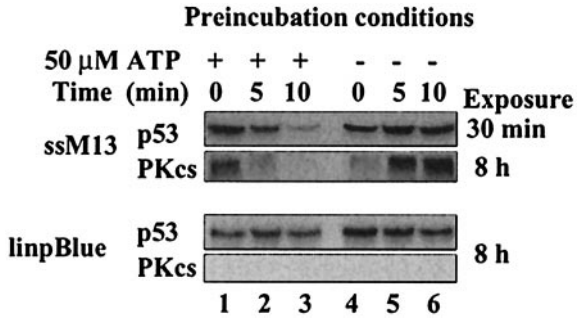

Fig. 4. DNA-PK_{cs} is preferentially autoinactivated upon incubation with ssM13. Phosphorylation of recombinant p53 (p53) and DNA-PK_{cs} (PKcs) in the presence of 10 ng ssM13 or 10 ng linpBlue. The complete reaction mixtures were preincubated with 50 μM ATP for 0 (lane 1), 5 (lane 2), or 10 (lane 3) min at 30°C before the addition of $\gamma[^{32}P]$ATP (specific activity 6,000 Ci/mmol) to a final specific activity of 5 Ci/mmol, followed by incubation for an additional 10 min at 30°C. Complete reaction mixtures lacking ATP were preincubated for 0 (lane 4), 5 (lane 5), or 10 (lane 6) min at 30°C. After addition of 50 μM ATP (specific activity 5 Ci/mmol) the incubations were continued for an additional 10 min at 30°C. Exposure times of the SDS/PAGE gels were as indicated to the right.

10-min preincubation prevented additional autophosphorylation of DNA-PK_{cs} and decreased the phosphorylation of p53 by over 90% (Fig. 4, lanes 1–3). This result depended on ATP, as preincubation of DNA-PK in the absence of ATP had no effect on subsequent phosphorylation of p53 and DNA-PK_{cs} (Fig. 4, lanes 4–6). Interestingly, when p53, DNA-PK, and ssM13 were mixed simultaneously, p53 phosphorylation occurred in preference to DNA-PK autophosphorylation (Fig. 4, lane 4). By contrast to ssM13, preincubation of DNA-PK with linpBlue and 50 μM ATP had no effect on the subsequent phosphorylation of p53 nor did it induce appreciable DNA-PK_{cs} autophosphorylation (Fig. 4 *Lower*).

Activation of DNA-PK from Hairpin Ends. An apparent paradox of DNA-PK function is that although kinase activity appears to be required for the resolution of coding end hairpins, kinase activity has not been observed from these structures (15). As the sharp DNA loop that occurs at the closed hairpin end is reminiscent of snapback structures that occur in ssM13, we tested whether the inability to detect phosphorylation of substrates from DNA hairpin ends might also be caused by the inactivation of DNA-PK_{cs} through autophosphorylation. A covalently closed circular 86-nt single-stranded microcircle with a perfectly complementary sequence that anneals to form a 43-bp double-stranded oligonucleotide with perfect hairpin ends was prepared (Fig. 5*A*). At 50 μM ATP, this oligonucleotide was unable to promote the phosphorylation of the p53 peptide substrate (data not shown), as reported for other hairpin structures (15). However, DNA-PK_{cs} autophosphorylation was promoted at least as well as with ssM13 (Fig. 5*B Upper*, lanes 2 and 4). By contrast, DNA-PK_{cs} was minimally autophosphorylated on a linear double-stranded oligonucleotide of the same size and sequence (Fig. 5*B*, lane 3), whereas the p53 peptide was efficiently phosphorylated (data not shown). At 0.042 μM ATP, however, the hairpin oligonucleotide promoted phosphorylation of the p53 peptide with the same efficiency as the linearized 43-bp oligonucleotide (Fig. 5*B Lower*) and ssM13 (data not shown).

One limitation of this experiment was that the presence of two closely spaced hairpins on the oligonucleotide differed from the natural state of the hairpins in V(D)J recombination and may have encouraged the autophosphorylation/autoinactivation of DNA-PK_{cs} by allowing the accumulation of two molecules of DNA-PK. Therefore we prepared an additional oligonucleotide of the same sequence, but with a single hairpin. The other end was linear, but secured through the presence of streptavidin-acrylamide beads, which previously has been shown to block the access of both Ku and DNA-PK_{cs} to DNA ends (14, 31). Autophosphorylation of DNA-PK_{cs} was reduced 4-fold in the presence of the streptavidin-bound oligonucleotide containing the single hairpin, showing that most of the autophosphorylation was between two DNA-PK_{cs} molecules. However, the residual autophosphorylation was still stronger than from the linear double-stranded 43-mer (Fig. 5*C Upper*).

To test the effect of this reduction in DNA-PK_{cs} autophosphorylation on the phosphorylation of heterologous substrates, we compared phosphorylation of the p53 peptide in the presence of these oligonucleotides at 50 μM ATP (Fig. 5*C Lower*). Addition of biotin/streptavidin to one end of the double-stranded linear 43-bp oligo had no significant effect on the strong phosphorylation of the p53 peptide. By contrast, lower, but significant, phosphorylation was detected from oligonucleotides containing two hairpins. Interestingly, whereas a further increase in p53 peptide phosphorylation was observed with a streptavidin-blocked single hairpin, p53 peptide phosphorylation still did not reach the level predicted by the decease in DNA-PK_{cs}

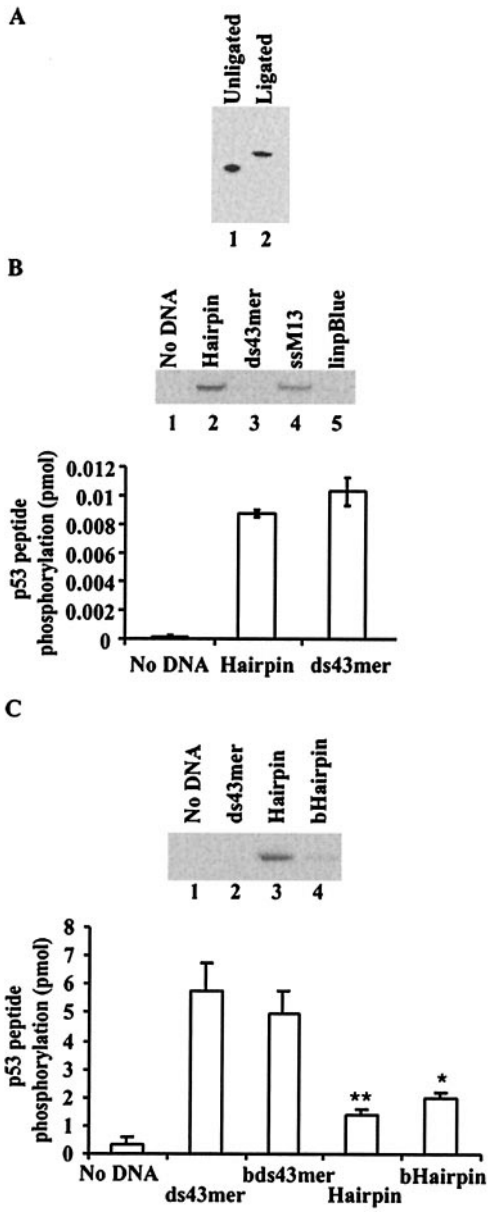

Fig. 5. Activation of DNA-PK from hairpin ends. (*A*) Autoradiograph of a 6 M urea, 40% formamide PAGE gel (8%) showing the purified 86-nt covalently closed circular single-stranded DNA after ligation that anneals into a 43-bp double-stranded DNA molecule with perfect hairpin ends (ligated) and the linear oligonucleotide starting material (unligated). (*B*) (*Upper*) Autophosphorylation of DNA-PK_{cs} at 50 μM ATP in the absence of DNA (lane 1) or in the presence of 10 ng of the 43-bp hairpin oligonucleotide (lane 2), a 43-bp linear double-stranded oligonucleotide of the same sequence (lane 3), ssM13 (lane 4), or linpBlue (lane 5). (*Lower*) Quantification of p53 peptide phosphorylation by DNA-PK at 0.042 μM ATP in the absence of added DNA (No DNA) and in the presence of 10 ng of the 43 bp hairpin and double-stranded oligonucleotide (ds43mer). Data points correspond to the mean of two determinations ± SD. (*C*) (*Upper*) Autophosphorylation of DNA-PK_{cs} at 50 μM ATP in the absence of DNA (lane 1) or the presence of 10 ng of a 43-bp double-stranded oligonucleotide that was linear at both ends (lane 2), contained two hairpin ends (lane 3), or contained a single hairpin and single linear end that was labeled 3′ and 5′ with biotin and bound to streptavidin-acrylamide (lane 4). (*Lower*) Quantification of p53 peptide phosphorylation at 50 μM ATP in the absence (No DNA) and presence of 10 ng of the linear double-stranded 43-bp oligonucleotide oligonucleotide (ds43mer), the same oligonucleotide biotinylated at one end and bound to streptavidin-acrylamide (bds43mer), the 43-bp oligonucleotide containing two hairpin ends (Hairpin), and the 43-bp oligonucleotide containing a single hairpin and linear end biotinylated and bound to streptavidin-acrylamide (bHairpin). *, $P < 0.0.05$ vs. hairpin; **, $P < 0.05$ vs. no DNA.

autophosphorylation. Thus although the phosphorylation of the p53 peptide confirmed the activation of DNA-PK toward heterologous substrates from hairpin ends, the activity of the kinase from hairpins may differ from its activity from DNA ends.

Discussion

Our results indicate that DNA-PK is strongly activated from DNA structures that resemble coding end hairpins and from structured single-stranded DNA. The phosphorylation of heterologous substrates from these DNA structures *in vitro* occurred in competition with rapid autophosphorylation of DNA-PK_{cs} that inactivated kinase activity and was strongly favored by colocalizing the substrate with the kinase on DNA. These results provide an indication of how DNA-PK activity may function in mediating the resolution of coding ends in V(D)J recombination.

A previous study using sheared calf thymus as a source of DNA cofactor was the first to demonstrate that autophosphophosphorylation of DNA-PK_{cs} could inactivate DNA-PK (20). By contrast, we obtained minimal autophosphorylation of DNA-PK in the presence of a carefully prepared linear double-stranded DNA plasmid. Rather, our results indicated that autoinactivation of DNA-PK occurs preferentially in the presence of structured single-stranded DNA and DNA containing closed hairpin ends. We cannot directly comment on the state of the calf thymus DNA used in the previous study. However, we also have noted minimal autophosphorylation of DNA-PK_{cs} using calf thymus DNA prepared by restriction with the enzyme *Hae*III (data not shown). Lastly, in the previous study, Ku also was found to be phosphorylated, albeit to a much lesser extent. Although not evident in the exposures provided here, we noted a similar low level of Ku phosphorylation that was proportional to the DNA-PK_{cs} phosphorylation on both DNA forms (data not shown).

At least two possibilities are suggested by these results. The first is that the interaction of DNA-PK_{cs} with structured DNA is distinct from its interaction with DNA ends and leads to a protein conformation that specifically encourages autophosphorylation. A second is that the highly structured nature of ssM13 and the presence of two hairpin ends in our initial oligonucleotide constructs promoted a concentration of kinase within single DNA molecules that encouraged intermolecular kinasing. The DNA competition experiments and the use of DNA templates containing only a single free hairpin end showed that the majority of the autophosphorylation observed was intermolecular and likely resulted from concentration of the kinase on the structured DNA. In both instances significant residual autophosphorylation remained under the modified conditions, suggesting that at least some intramolecular DNA-PK_{cs} autophosphorylation also may occur. However, it is also possible that remaining autophosphorylation in the DNA competition experiments were facilitated by Ku-Ku interactions that have been reported (32), although the fixing of the single hairpin oligonucleotide to streptavidin-acrylamide beads decreases the likelihood of a significant contribution from these interactions.

Although autophosphophosphorylation of DNA-PK_{cs} occurred rapidly *in vitro*, it remains to be determined to what extent DNA-PK_{cs} autophosphorylation occurs in the cell. We note, however, that single-stranded DNA structures likely occur at their highest concentration during S phase, which is coincident with a reduction in DNA-PK activity that occurs in the absence of a changes in the levels of Ku or DNA-PK_{cs} (19, 33). Although DNA-PK_{cs} is a phosphoprotein in cellular extracts, it is not yet clear to what extent the kinase is maintained in a catalytically inactive state. A recent report suggests that DNA-PK may be activated by phosphatase treatment (34). DNA-PK activity also has been found to be induced subsequent to DNA damage (2), suggesting that autophosphorylation of the kinase may be reversed by an inducible phosphatase activity or may depend on its specific targeting to DNA ends.

Our results with p53 and RPA indicate that the phosphorylation of heterologous substrates by DNA-PK from structured single-stranded DNA is likely to be significantly enhanced through mechanisms that assist in the recruitment of the substrate to the kinase. We have previously shown a similar result for the phosphorylation of glucocorticoid receptor by DNA-PK in cis from specific DNA sequences (21). Such interactions may prove to be significant for determining specific substrate phosphorylation by DNA-PK *in vivo* and could compensate for the high k_m of DNA-PK for substrates (25) and its very relaxed specificity (S/TQ) (35).

Specific substrate phosphorylation by DNA-PK also may be promoted through specific protein–protein interactions. Recently, we have shown that a specific protein–protein interaction between the POU homeodomain of octamer transcription factor 1 and Ku70 strongly promoted the phosphorylation of Oct-1 within the POU-specific domain by DNA-PK from DNA ends (26). Interestingly, we failed to observe significant phosphorylation of the Oct-1 POU domain from a hairpin coding end (data not shown). This result is consistent with our previous observation that the Oct-1-Ku interaction was specific for DNA end-bound Ku and was not detected when Ku was bound to a DNA sequence (26). Thus these results suggest that substrate accessibility to DNA-PK may be controlled both through specific interaction of the substrate with DNA and specific protein–protein interactions with the kinase complex that may be differentially influenced by the form of DNA to which the kinase is associated. Several additional proteins, including c-abl (36), have been shown to interact with Ku or DNA-PK_{cs}. How binding affects the phosphorylation of these proteins remains to be determined.

Lastly, although p53 peptide phosphorylation was readily detected from a hairpin end, the level of phosphorylation observed when autophosphorylation was titrated through fixing the hairpin oligonucleotide to a solid support suggests the possibility that substrate recognition by DNA-PK also may be directly influenced by the DNA form to which it is associated. Alternatively, we cannot yet completely exclude the possibility that the addition of streptavidin to one end of the hairpin oligonucleotide inhibited DNA-PK activity in a manner that was not observed with the linear oligonucleotide.

Together our results demonstrate how DNA-PK becomes activated from hairpin structures resembling coding end hairpin intermediates in V(D)J recombination and suggest several potential mechanisms that may be used to restrict substrate phosphorylation. The exact targets of DNA-PK in recombination remain to be determined, but seem likely to include one or more of the factors that are directly involved in cleavage and end joining, including potentially the newly identified factor artemis (37).

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