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Terminal DNA structure and ATP influence binding parameters of the DNA-dependent protein kinase at an early step prior to DNA synapsis

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ABSTRACT

The DNA-dependent protein kinase catalytic subunit (DNA-PKcs) regulates the non-homologous end-joining pathway of DNA double-strand break repair in mammalian cells. The ability of DNA-PKcs to sense and respond to different terminal DNA structures is postulated to be important for its regulatory function. It is unclear whether discrimination occurs at the time of formation of the initial protein–DNA complex or later, at the time of formation of a paired, or synaptic complex between opposing DNA ends. To gain further insight into the mechanism of regulation, we characterized the binding of DNA-PKcs to immobilized DNA fragments that cannot undergo synapsis. Results showed that DNA-PKcs strongly discriminates between different terminal structures at the time of initial complex formation. Although Ku protein stabilizes DNA-PKcs binding overall, it is not required for discrimination between terminal structures. Base mispairing, temperature and the presence of an interstrand linkage influence the stability of the initial complex in a manner that suggests a requirement for DNA unwinding, reminiscent of the 'open complex' model of RNA polymerase–promoter DNA interaction. ATP and a nonhydrolyzable ATP analog also influence the stability of the DNA-PKcs•DNA complex, apparently by an allosteric mechanism that does not require DNA-PKcs autophosphorylation.

INTRODUCTION

DNA double-strand breaks (DSBs) interrupt chromosome integrity and are potentially lethal to dividing mammalian cells. The chemical structure of the DNA ends varies widely. Ionizing radiation and reactive oxygen species from other sources, which are the predominant natural double-strand break-inducing agents, produce chemically heterogeneous DNA ends that require removal or addition of nucleotides before rejoining (1). The RAG recombination nuclease, another important natural source of DSBs, cuts DNA to produce blunt ends at one side of the incision and distinctive hairpin ends at the other (2,3). Cancer chemotherapy agents, including platinum compounds and topoisomerase II inhibitors, produce DSBs associated with distinctive repair-resistant chemical or protein adducts (4,5).

Despite this structural diversity, one repair pathway, non-homologous end joining (NHEJ), provides the default mechanism for DSB repair in mammalian cells. Moreover, just one polypeptide in this pathway, the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), functions as a key regulator of the repair process [reviewed in (6–9)]. Through its DNA binding and autophosphorylation activities, DNA-PKcs determines whether DSBs will be repaired by NHEJ or shunted to an alternative homologous recombination repair pathway (10–12). DNA-PKcs also controls access to the DNA ends by processing enzymes and, in some cases, regulates their activity through protein–protein interactions and phosphorylation (11–17).

DNA-PKcs cooperates with another repair protein, Ku, to establish a functional complex at DNA ends (18,19). Within this complex, DNA-PKcs is in direct physical contact with terminal nucleotides of the DNA (20–22). DNA-PKcs has a serine-threonine kinase activity that is activated by contact with the DNA ends and that is essential for DSB repair (23). DNA-PKcs catalyzes its own phosphorylation at multiple sites (24,25). This autophosphorylation modulates accessibility of the DNA ends (11,26–28). DNA-PKcs also phosphorylates several other repair proteins, including Artemis, DNA ligase IV and XRCC4 (15–17,29–31), providing another level of regulatory complexity. An intrinsic ability to discriminate between different terminal DNA structures and respond

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through selective phosphorylation of itself and other proteins is postulated to be important for the ability of DNA-PKcs to regulate NHEJ.

In vitro studies using isolated enzyme and defined DNA fragments have provided some insight into the mechanism by which DNA-PK discriminates between terminal structures. Chu and co-workers (32,33) showed that DNA fragments with single-stranded tails activate DNA-PK to particularly high levels, whereas a DNA with a closed hairpin end is a poor activator under the same conditions. Based on these and other data, they proposed an elegant model where the single-stranded tail of one DNA fits into a binding pocket presented by a DNA-PK complex bound to an opposing DNA, signaling the presence of a synaptic complex (33). Although appealing, this model appears to be insufficient to explain other observations in the literature, including the ability of hairpin ends to promote autophosphorylation and inactivation of DNA-PK under other experimental conditions (34). An alternative and somewhat more general model is that activation is an autonomous property of DNA-PK bound to a solitary DNA end. In this model, protein–DNA interactions that are essential for activation take place internally within a single DNA-PKcs-containing complex and do not require one DNA to interact in *trans* with DNA-PK bound to a second DNA.

To gain insight into the ability of DNA-PKcs to sense and respond to single-stranded DNA ends within a solitary complex (i.e. without interactions in *trans*), we characterized the assembly of DNA-PKcs•DNA complexes under conditions that preclude synapsis. Our goal is to establish a framework for understanding individual steps leading to enzyme activation, and it was thus important to measure DNA binding and enzyme activity separately from one another. To investigate DNA binding, we immobilized defined DNA fragments at low density on a solid support, then exposed them to Ku and DNA-PKcs to allow complex formation. We measured protein–DNA association by surface plasmon resonance (SPR). Separately, we measured kinase activity in a multiwell plate-based assay that mimicked SPR conditions. Previous studies have validated the use of SPR to study DNA-PKcs binding to DNA (35). Our present work extends this approach to investigate the crucial question of whether DNA-PK is able to sense and discriminate between different terminal structures under conditions that allow formation of an initial complex but preclude progression to a synaptic complex.

Results show that DNA-PKcs strongly discriminates between different terminal structures under these conditions. Binding and activity assays both showed a strong preference for DNA with single-stranded tails over blunt or covalently linked (hairpin) termini. Interestingly, behavior of the enzyme with blunt-end substrate was strongly temperature-dependent, being more like the unpaired substrate at 37°C and more like the hairpin substrate at 25°C. Together, results suggest that DNA-PK promotes transient strand separation to form a stable, active DNA-PK complex. The results are consistent with an 'open complex' model of kinase activation, analogous to that for RNA polymerase–promoter interaction (36). The results also showed that the effect of ATP on DNA-PK is more complex than previously appreciated. ATP affects binding by an allosteric mechanism, in addition to previously described effect of autophosphorylation.

MATERIALS AND METHODS

Purification of DNA-PKcs

DNA-PKcs was purified by a modification of the procedure described by Ding *et al*. (26). A 1 ml Hi-Trap NHS-activated HP column (GE Healthcare, Uppsala, Sweden) was coupled with 20 mg of a Ku80 C-terminal peptide, KGSGEEGGDVD-DLLDMI, where the initial three residues provide an amine coupling group as well as a spacer between the column resin and the start of the Ku sequence. HeLa cell nuclear protein extract (37) (25 ml) was mixed with 25 ml of Buffer A [25 mM HEPES–KOH, pH 7.5, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, protease inhibitors (leupeptin, aprotinin, pepstatin, SBTI at 1 μg/ml) containing 0.2 M KCl] and the mixture was incubated with the Hi-Trap material with tumbling overnight at 4°C. The material was packed in a 1 ml column, which was washed and eluted with a 6 ml linear gradient of 0.15–1 M KCl in buffer A. Fractions (200 μl) were analyzed by SDS–PAGE. DNA-PKcs-containing fractions were pooled, adjusted to 15% trehalose by addition of solid and stored at −80°C.

Expression and purification of Ku protein

Recombinant, non-tagged Ku protein was produced by co-infection of Sf9 cells with VBB2-86Ku and VBB2-70Ku and was purified by sequential Superdex-200, single-stranded DNA-agarose and heparin-agarose chromatography as described previously (38,39).

DNA probes

The following probes were used (Midland Certified Reagent Company Inc., Midland, TX, USA): 5′-GTTACGCGTGCGG-CCAGCCCCCTCGCCT(BiodT)TGGCGAGGGGGCTGGCC-GCACGCGTAAC-3′; 5′-GGCTGGCCGCACGCGTAACGT-TACGCGTGCGGCCAGCCCCCTCGCCT(BiodT)TGGCGA-GGG-3′; 5′-TTTACGCGTGCGGCCAGCCCCCTCGCCT (BiodT)TGGCGAGGGGGCTGGCCGCACGCGTTTT-3′.

Surface plasmon resonance

The probes were dissolved in Buffer B, which was prepared by adding 150 mg/ml solid trehalose to a solution of 50 mM HEPES–KOH, pH 7.5, 1 mM EDTA, 10 mM $MgCl_2$, 100 mM KCl and 1 mM DTT (Addition of trehalose results in approximate final concentrations of 44 mM HEPES–KOH, 0.87 mM EDTA, 8.7 mM $MgCl_2$, 87 mM KCl, 0.87 mM DTT and 13% trehalose). A total of 250 RU of each biotinylated oligonucleotide [1 RU = 0.8 pg/mm^2 of chip surface (40)] was coupled to Flow Cell 2 of a streptavidin-coated sensor chip. Flow Cell 1 served as an unliganded control. The data were collected on a Biacore X instrument (Biacore, Uppsala, Sweden). To regenerate the sensor chips between experiments, the flow cells were washed with acidic (10 mM glycine, pH 3) and basic (1 M NaCl, 50 mM NaOH) solutions. In addition, each experiment was performed with at least two different chips. Except where indicated otherwise in the figure legends, binding was performed at 37°C. BIAEvaluation software was used for quantification of binding parameters.

Microwell kinase assays

Steptavidin-Coated Clear Strip Plates (Pierce, Rockford, IL) were washed with PBS-T (0.1% Tween-20) buffer for 5 min at 37°C and rinsed with Buffer B. An aliquot of 100 pmol of oligonucleotide was added per well in 100 μl of kinase buffer and incubated for 10 min at 37°C. The wells were blocked with 100 μl of 3% BSA for 5 min at 37°C and washed with 100 μl of kinase buffer for 5 min at 37°C. Reaction mixture (32 μl) containing 30 ng DNA-PKcs in 1.25× Buffer B was distributed to each well and pre-incubated for 5 min at 37°C. The ATP concentration was adjusted to 12.5 μM by addition of [γ-^{32}P]ATP (20 Ci/mmol) in a volume of 8 μl. The reactions were terminated with 10 μl of sample buffer [NuPage LDS sample buffer (4×), Invitrogen] and analyzed by 6% SDS–PAGE. Phosphoprotein products were detected by PhosphorImager analysis. Note that a lower ATP concentration was used than in the SPR experiments because the resulting higher specific activity facilitated detection of the radiolabeled product. Prior work shows that 12.5 μM ATP is at or above the k_m (37), and the use of the lower ATP concentration was therefore not expected to affect overall conclusions from the experiment.

RESULTS

Experimental design

We previously demonstrated the ability to assemble oriented DSB repair complexes using oligonucleotides blocked at one end with a streptavidin–biotin complex. This constrains protein interaction in such a way that a single repair complex can assemble at the opposite end of the fragment (39). In addition to assuring a unique orientation with respect to the underlying DNA sequence, this design eliminates concerns over interference between two repair complexes formed at opposite ends of the same oligonucleotide. There is evidence that such interference can significantly affect interpretation of results of activity assays (34).

Our original studies were performed using duplex oligonucleotides prepared by annealing two separately synthesized strands, one of which incorporated a biotin group at the 5′ end. Here, we updated the probe design to take advantage of a new phosphoramidite reagent that allows incorporation of biotin at an internal site in an oligonucleotide probe. The new probes were designed as single molecules that fold to form a duplex DNA with a biotin in a hairpin structure at one end (Figure 1A). The new design provides a singly biotinylated duplex DNA probe without the need for gel purification of an annealed product. These DNAs were tethered to a streptavidin-coated support, with the other end free for DNA-PK complex assembly. In this new generation of probes, the 12 nt sequence adjacent to the free end was identical to the highly active 'f12' substrate used by Hammarsten and co-workers (41). Additional internal sequence, proximal to the biotin, was the same as used previously (20).

The experimental design called for comparison of three different probes, which were similar internally but differed in the structure of the free end. One probe had a fully base-paired region at the free end. Two others were chosen to represent especially good and poor activators, as identified in prior solution studies: one had a 3 nt unpaired region at the free end (predicted to be a good activator), and the other had a closed hairpin structure at the free end (predicted to be a poor activator). The sequence of the probes is shown in Figure 1A, and the naming convention is explained in the figure legend.

Ku and DNA-PKcs binding measured by SPR

We measured protein–DNA binding in an SPR assay. One of two channels of a streptavidin-coated sensor chip was loaded with probe DNA, and the other was left blank as a control. After washing, analyte containing the protein of interest was flowed over the chip, and time-dependent association with the DNA-coated surface was measured over a 2–3.5 min interval (the association phase). The flow of analyte was then stopped and replaced by the same buffer as was used for initial washing, and time-dependent dissociation was measured (the dissociation phase). The buffer used throughout the experiment was designed to be compatible with both binding and activity assays; it contained divalent cation (required for kinase activity) as well as a polyol, trehalose (as a protein stabilizer).

We characterized the binding of Ku and DNA-PKcs separately. Our prior studies showed that probes of the size used here (up to 28 nt) allow binding of either a single Ku dimer or a single Ku–DNA-PKcs complex. A 28mer duplex is slightly too small to allow formation of a complex containing two Ku dimers (20,39). Figure 1B shows binding of Ku to each of the three DNA probes. After an ∼3 min association phase, binding to all three chips reached about the same level (±20%). Dissociation was negligible under the conditions tested. The association curve for the blunt-end probe had a slightly different shape than the other two, perhaps reflecting ability of this probe (which has the longest duplex section) to accommodate slow binding of a second Ku, but this was not investigated further. The nearly equivalent binding to the three probes was expected, as prior work using other assays has shown that Ku–DNA binding is not affected by the structure of the DNA ends (42,43).

We next pre-saturated the surface of the chips with Ku and measured recruitment of DNA-PKcs to form the initial DNA-PK complex (Figure 1C). The curves were quite different with the different probes. With the hairpin probe (h3B-27cp), DNA-PKcs bound rapidly, established a plateau and dissociated rapidly. With the other two probes, DNA-PKcs binding reached higher values during the association phase and dissociated much more slowly. Results demonstrate that introduction of DNA-PKcs into the system provides an ability to discriminate between different DNA ends that exceeds discrimination with Ku alone.

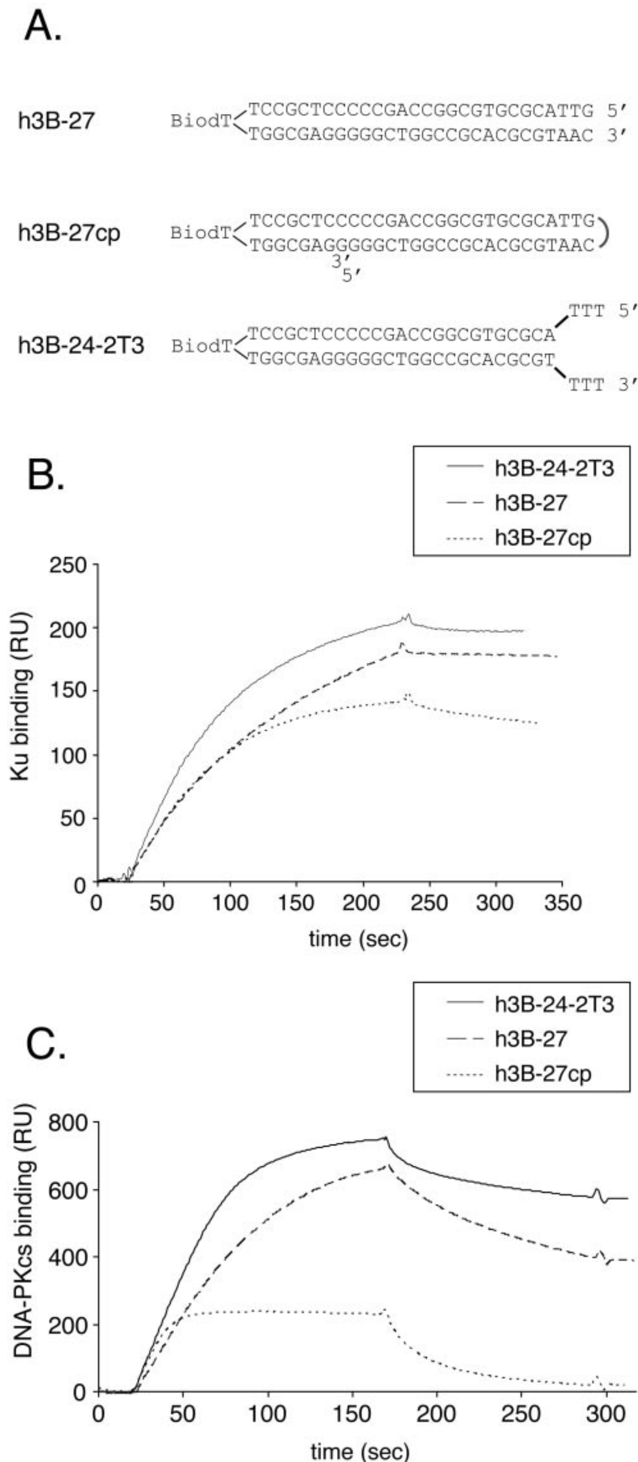
DNA-PKcs discriminates between end structures in the absence of Ku

We next determined whether the ability to discriminate between DNA ends is intrinsic to DNA-PKcs, or whether Ku is also required. Results showed that DNA-PKcs had an even greater ability to discriminate between DNA ends than when tested in the presence of Ku. Binding to the hairpin probe was weak; binding to blunt probe was intermediate; and binding to the unpaired probe was the highest (Figure 2). The finding that the ability to discriminate between

DNA ends is intrinsic to DNA-PKcs is consistent with results of prior solution-phase assays (33,41,44) and extends these findings by demonstrating that discrimination is manifested at an initial protein–DNA binding step.

The rank order of DNA-PKcs binding avidity (unpaired > blunt > hairpin) correlated with the potential for strand separation at the termini, suggesting that localized DNA melting may be required for formation of a stable initial complex. In this case, binding to the blunt probe might be strongly temperature dependent, by analogy to *Escherichia coli* RNA polymerase, which exhibits strongly temperature-dependent binding when it forms an 'open complex' with promoter DNA (36). Less temperature dependence should be seen with the unpaired and hairpin probes, which are structurally constrained in an open and closed conformation, respectively.

We tested DNA-PKcs binding to the three DNAs at 25 and 37°C. At the lower temperature, binding to the blunt-end probe (h3B-27) was reduced almost to the level seen with hairpin (compare Figure 2A and B). Binding to the unpaired probe (h3B-24-2T3) was affected by temperature, but to a lesser extent (note different scales in Figure 2A and B). Binding to the hairpin probe (h3B-27cp) was only slightly affected by temperature. Results are therefore consistent with the model that formation of a stable initial complex involves localized DNA melting.



Destabilization of DNA-PKcs binding by ATP and a nonhydrolyzable ATP analog

We next tested the effect of ATP on formation and stability of the initial DNA-PKcs•DNA complex. In solution-phase assays, incubation of DNA-PK, DNA and ATP leads to kinase inactivation by an autophosphorylation mechanism (45). The autophosphorylation is believed to occur in *trans*, i.e. where one DNA-PKcs molecule phosphorylates a second molecule bound to an opposing DNA end (22,25). We would not expect *trans* phosphorylation to be possible in the initial DNA-PK complex where pairing, or synapsis, of DNAs is constrained.

We tested the effect of ATP and a nonhydrolyzable analog, AMP-PNP, in the SPR binding assay. We used the unpaired probe because of the high avidity of DNA-PKcs for this DNA. ATP strongly influenced DNA-PKcs binding (Figure 3A). Plateau binding values were lower, and dissociation was much more rapid. The nonhydrolyzable AMP-PNP analog also had an effect, although less dramatic. The observation

Figure 1. (**A**) DNA molecules used in the study. Each probe was synthesized as a single self-complementary molecule with an internal 5-biotinyl-deoxythymidine (BiodT) residue. Sequences were identical internally but differed at the free end of the folded molecule. The convention for naming probes was as follows: each formed a hairpin with a 3 nt loop at the apex of the self-annealed region, and the loop had a single biotinylated nucleotide (thus, h3B). The loop was adjacent to a 24 or 27 nt duplex region (thus, h3B-24 or h3B-27). Probes had one of three different structures at the free terminus. The probe with a plain blunt end was designated as h3B-27, a circularly permutated probe with a closed hairpin at both ends and a nick in the central duplex was designated h3B-27cp, and the unpaired probe with the same total length and three unpaired T residues at the 5′ and 3′ termini was designated h3B-24-2T3. (B and C) Assembly of initial DNA-PK complex monitored by SPR. Equal amounts of DNA (250 RU) were immobilized via biotin-streptavidin linkage to Channel 2 of an SPR chip. Channel 1 served as an unliganded control. During the indicated association phase, the indicated proteins were flowed over the surface of the chip at 37°C in kinase reaction buffer without ATP, as described in Materials and Methods. Prior to and following the association phase, buffer alone was flowed over the chip. SPR signal (Channel 2 minus Channel 1) was plotted as a function of time. Except as noted, all curves are averages of duplicates. (**B**) Binding of Ku. Ku alone (9 nM) was present in the association phase. (**C**) Binding of DNA-PKcs to surface pre-saturated with Ku. Ku (36 nM) was flowed over the surface of the chip for 120 s, sufficient for binding to approach a plateau value (data not shown). DNA-PKcs (1 nM) was injected immediately (within ∼10 s of the end of the Ku association phase), and SPR signal was measured as a function of time. The trace for h3B-27 represents only one measurement, instead of the usual average of duplicates.

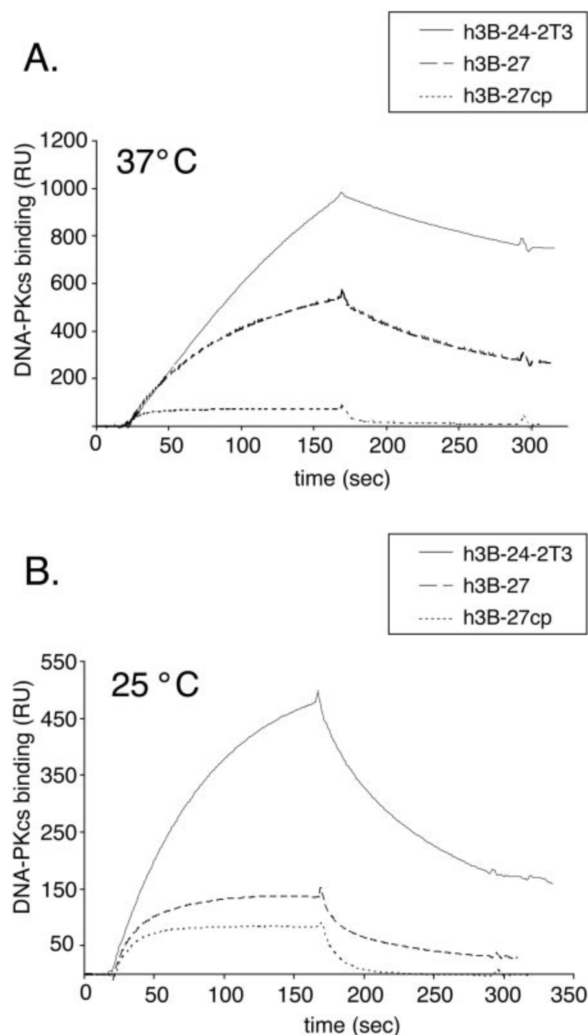


Figure 2. Comparison of DNA-PKcs binding to different DNAs. (**A**) Binding at 37°C. DNA-PKcs (1 nM) was present in the association phase. (**B**) Same as panel A except experiment was performed at 25°C.

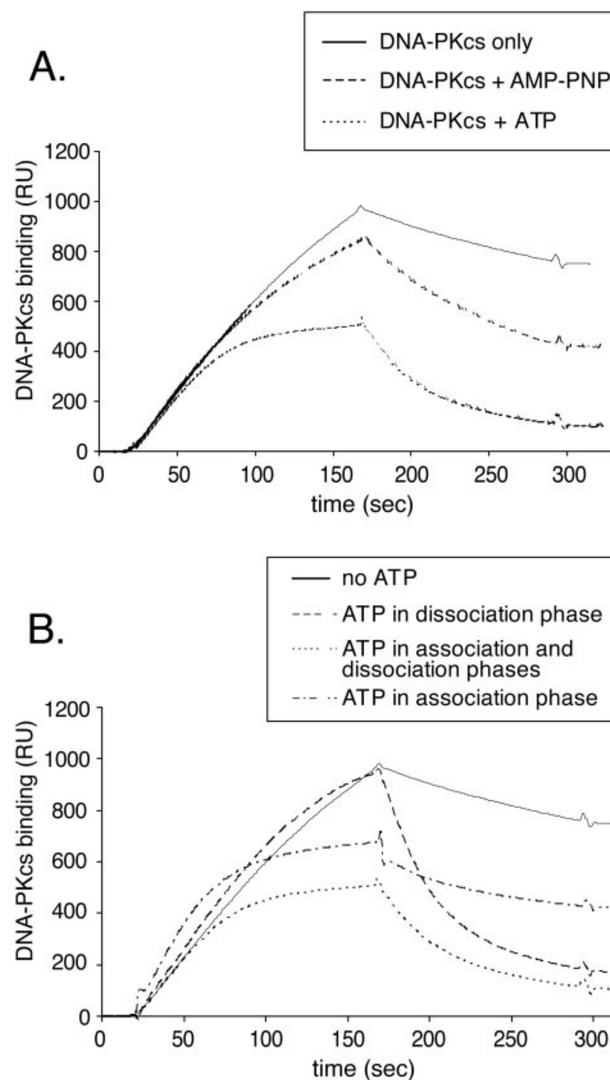


Figure 3. Effect of ATP on binding of DNA-PKcs to different DNAs. Binding was performed identically as in Figure 2, using the unpaired DNA (h3B-27-2T3) sensor chip at 37°C. (**A**) ATP or AMP-PNP (100 μM) was included in both the association and dissociation phases for the indicated binding curves. (**B**) Effect of ATP on DNA-PKcs association and dissociation measured separately. As indicated, ATP (100 μM) was included in the DNA-PKcs sample only (present during association phase) or in the wash buffer only (present prior to sample injection, absent during association phase, present during dissociation phase). A small effect on association (paradoxical because ATP is nominally absent during the association phase) probably reflects slight carry-through of ATP from the initial wash buffer. To facilitate comparison, binding curves in the absence of ATP, and with ATP present during both association and dissociation phases, are replotted from (A).

that ATP and AMP-PNP both affected binding indicates that the effect of adenosine nucleotides is attributable, in part, to an allosteric mechanism, but do not exclude an additional effect of protein autophosphorylation.

To further investigate the mechanism of the ATP affect, we performed an experiment in which ATP was present during the association phase of the binding reaction but withdrawn during the dissociation phase. An allosteric effect should be reversible, whereas an autophosphorylation effect should be irreversible. Complexes formed in the presence of ATP, which was then removed, were at least as stable as those formed in the absence of nucleotide (Figure 3B). The reversibility of the ATP effect favors the allosteric model.

We also performed the reciprocal experiment, where ATP was present in the dissociation phase and absent from the association phase. Under these conditions, ATP dramatically decreased the stability of the initial complexes, consistent with the pattern seen when ATP was present continuously (Figure 3B). The decrease in stability occurred immediately, with no apparent lag. This is significant because, as will be shown subsequently, autophosphorylation occurs with much slower kinetics under similar conditions.

The effect of ATP on binding of DNA-PKcs to the other DNAs was also tested. The effect on the blunt-end probe was similar to the effect on the unpaired probe but less dramatic (data not shown). ATP had little or no measurable effect on binding to the hairpin probe. Although binding to the hairpin probe was too weak overall to be conclusive, the data suggest that the effect of ATP might be specific for the 'open complex' formed with the unpaired and blunt probes.

Autophosphorylation of initial complexes

It was of interest to compare the kinetics of ATP-dependent dissociation with the kinetics of DNA-PKcs autophosphorylation as measured directly under similar conditions. Our SPR instrument did not permit the recovery of analyte for chemical analysis. We therefore adapted our assay to a multiwell plate format where DNA was surface-immobilized, as in the SPR assays. We have previously characterized the assembly of DNA-PKcs complexes on these plates and demonstrated that binding is DNA-dependent (46). After blocking the plates to reduce nonspecific DNA-PKcs binding, as described in the earlier study, we pre-incubated DNA-PKcs with DNA to form initial complexes, added [γ-^{32}P]ATP and analyzed the incorporation of radiolabel into DNA-PKcs by SDS–PAGE and PhosphorImager analysis.

We first compared the three DNA probes. The level of phosphate incorporation was greatest with the unpaired DNA, less with the blunt DNA and negligible above background with hairpin DNA (Figure 4A). Thus, the rank order of activity correlated with avidity of binding in the SPR assay. Because of the higher level of signal, we focused on the unpaired DNA in remaining experiments.

We examined the time course of phosphate incorporation. To obtain the most information, we analyzed free DNA-PKcs and DNA-PKcs bound to the plate separately. The results are shown in Figure 4B, with quantification in Figure 4C. Incorporation of radiolabel into the free DNA-PKcs pool, which accounted for the majority of the phosphorylation at all time points, proceeded linearly for at least 30 min. Incorporation into the bound pool was much lower and occurred with somewhat different kinetics, approaching a plateau after about 4 min.

Whether we consider the free, bound or total population, it is clear that the rate of autophosphorylation is slow, compared with the essentially instantaneous effect of ATP on dissociation in the SPR assays (Figure 3). It is therefore unlikely that ATP-dependent dissociation in these assays can be explained solely by an autophosphorylation mechanism.

We have not investigated the exact mechanism underlying the slow continuous buildup of autophosphorylated DNA-PKcs in the free pool. Under the conditions used (2 nM DNA-PKcs, ATP and excess DNA) we expect there to be dynamic exchange between bound and free populations of nonphosphorylated DNA-PKcs. The overall rate of the autophosphorylation reaction may thus be controlled by the slow, continuous, diffusion of free DNA-PKcs, to the enzymatically active complexes bound to DNA on the surface of the plate. Depending on the site(s) of phosphorylation, autophosphorylated DNA-PKcs may then irreversibly enter the free pool.

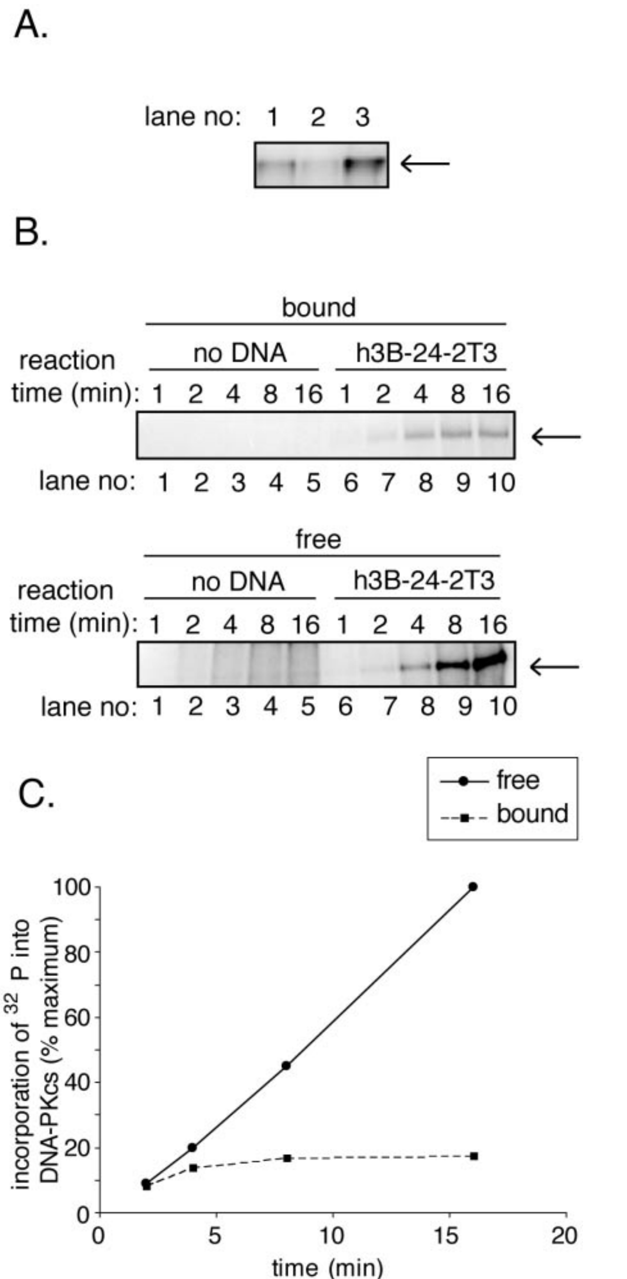


Figure 4. DNA-PKcs autophosphorylation assay. (**A**) h3B-24-2T3, h3B-27 and h3B-27cp (100 pmol) were pre-incubated in wells of a Reacti-Bind streptavidin-coated 96-well plate as described in Materials and Methods. After washing, DNA-PKcs (85 fmol, 2 nM) was added and incubated 5 min at 37°C in kinase buffer (without ATP). [γ-^{32}P]ATP was added (12.5 μM, ~20 Ci/mmol), and incubation was continued for 30 min. Products were resolved by 6% SDS–PAGE and visualized by PhosphorImager. Arrow denotes 470 kDa DNA-PKcs polypeptide. (**A**) Six percent SDS–PAGE analysis of radiolabeled reaction products, visualized by PhosphorImager. Lane 1, h3B-27; lane 2, h3B-27cp; lane 3, h3B-24-2T3. (**B**) Time course of DNA-PKcs autophosphorylation in the presence of h3B-24-2T3. Bound and free fractions were analyzed separately as indicated. (**C**) Quantification of results in (B).

DISCUSSION

The question, 'Why is the DNA-dependent protein kinase DNA-dependent?' is central to understanding the function of this enzyme in DSB repair. Prior studies have provided an important clue with the observation that single-stranded DNA termini activate DNA-PKcs exceptionally well in solution-phase assays (33,41). These steady-state activity assays are limited, however, because the measured activity reflects a composite of individual steps leading to enzyme activation. For a protein as complex as DNA-PKcs, these steps may be many and varied, including initial DNA binding, formation of a synaptic complex and progressive

autophosphorylation at various sites. Our goal in the present study is to establish a framework for understanding the contribution of individual steps to enzyme activation, with an emphasis on early steps that precede synapsis. We showed that the ability to discriminate between different DNA structures is first manifested at the level of initial complex formation. This ability to discriminate between DNAs is an autonomous property of DNA-PKcs bound to a solitary DNA end and does not require contact with a second DNA. The preference for single-stranded ends, and the inability to form a stable complex with hairpin termini, suggests that separation, or melting, of the DNA termini may be needed for stable DNA-PKcs binding. The temperature dependence of binding to the fully base-paired substrate, reminiscent of requirements for formation of an 'open complex' of RNA polymerase and promoter DNA (36), reinforces this conclusion.

Results help discriminate between two different ideas about the role of single-stranded DNA that are prevalent in the current literature. In one model, a single-stranded terminus interacts with an allosteric site in DNA-PKcs that is bound in *cis* to the same DNA fragment (Figure 5, Model A), and in the other, the unpaired terminal DNA interacts in *trans* with DNA-PKcs bound to a second DNA molecule (Figure 5, Model B). Our data, suggesting that single-stranded termini interact in *cis* with DNA-PKcs, do not rule out an additional role for interactions in *trans*, but suggest that these are not necessary for the initial step of stable DNA-PKcs•DNA complex formation.

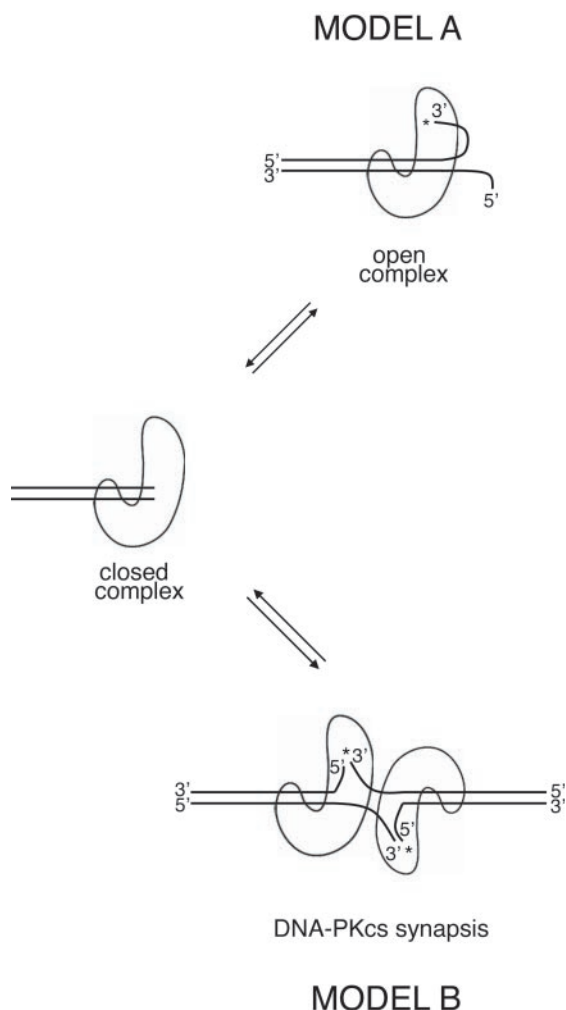


Figure 5. Two models for interaction of unpaired DNA tails with DNA-PKcs. Model A is styled after ref. (5) and Model B after ref (33).

A potential concern with this interpretation is that immobilization of DNA at low density on a solid support may not completely prevent interactions between the ends of different DNA fragments. That is, tethered DNAs might retain sufficient mobility to encounter one another and form a synaptic complex. Although this is difficult to exclude altogether, we believe that it is unlikely to explain our data. If synapsis were occurring, binding should be very sensitive to the density of DNA loaded onto the SPR chips. However, relative avidity of binding to different DNAs was qualitatively unchanged when the amount of DNA loaded on the SPR chips was increased 3-fold or decreased 2.5-fold (data not shown).

In separate experiments, we attempted to force synapsis by flowing additional DNA or an additional DNA-PKcs•DNA mixture over preformed initial complexes. No additional binding was detected (data not shown). SPR is limited in its ability to detect very weak or transient interactions, and the inability to detect synaptic complexes in this assay does not necessarily conflict with observations that such complexes can be visualized by electron microscopy (47). Synapsis may provide an important mechanism for bringing protein substrates into the proximity of the active initial complex, thus influencing protein substrate preference. However, investigation of this additional level of regulation was beyond the scope of the present study.

For exploratory purposes, we attempted to fit our SPR binding data to a quantitative model. We assumed 1:1 stoichiometry of interaction between DNA-PKcs and DNA, with mass transfer limitation. We performed analysis of binding to the unpaired probe (h3B-24-2T3) at 37°C, using both the data in Figure 2 and additional data collected at 2.5-fold lower DNA density (data not shown). Association rate constants were in the range 4×10^7–1.5×10^8 M^{-1} s^{-1}, and dissociation rate constants were 10^{-2}–10^{-3} s^{-1}. Derived equilibrium dissociation constants were 10^{-11}–3×10^{-10} M^{-1}. These are in the range expected for high affinity macromolecular interactions and are in general agreement with estimates from other authors (35).

Our results point to an interesting analogy between DNA-PKcs and RNA polymerases, in that stable binding of both proteins appears to be dependent on induction of localized DNA melting. The temperature-dependent binding of RNA polymerase to promoter DNA was a seminal observation that led to this 'open complex' model for promoter recognition (36). For RNA polymerases, the open complex model has been refined and validated by a large body of biochemical and structural studies, culminating in a detailed structural model [see (48) and references therein]. Clearly, analogous studies will be needed with DNA-PKcs to confirm whether the 'open complex' analogy is valid. We note, however, that as with RNA polymerases, there is a strong biological rationale for formation of an open complex as an essential and early step in the reaction. For RNA polymerases, the open complex allows

access to the template DNA strand by initiating nucleotides. For DNA-PKcs, the open complex may facilitate access to damaged DNA bases by processing enzymes as well as facilitating microhomology-based DNA synapsis.

Because present studies are based on measurement of binding, rather than kinase activity, it was possible to investigate the effect of ATP separately from other parameters. We found that the effect of ATP was unexpectedly complex. Although it is well established that incubation with ATP can influence DNA-PKcs binding and activity by an autophosphorylation mechanism, several lines of evidence suggest that ATP also has an allosteric effect. The effect of ATP on complex stability was partially mimicked by a nonhydrolyzable analog. It was also more rapid than could be explained by autophosphorylation and was reversible when ATP was removed. These data imply that the effect of ATP on DNA-PKcs•DNA interaction is more complex than previously appreciated. Although several clusters of autophosphorylation sites have been identified, knowledge of critical sites for promoting ATP-dependent dissociation remains elusive. The possibility of an allosteric effect, in addition to autophosphorylation-driven dissociation, needs to be taken into account in any general model. It may be, for example, that ATP influences the ability of the kinase domain and the N-terminal domain to 'clamp' together in the presence of DNA (22).

It is important to recognize that interactions with additional repair proteins, which were not present in our system, may augment and build upon the intrinsic specificity of DNA-PKcs revealed here (49,50). It will be particularly interesting to characterize the ability of Artemis to modify the behavior of the minimal system. Although DNA-PKcs displayed very little affinity for hairpin ends under the conditions used here, cooperative interactions between DNA-PKcs and the Artemis protein might stabilize assembly of a complex at such ends *in vivo*, enabling processing of V(D)J coding joints.

Although caution is warranted in extrapolating from *in vitro* studies with isolated DNA-PKcs and DNA, the ability of DNA-PKcs to recognize and respond to the structure of solitary DNA ends might have significant biological implications. Unrepaired free DNA ends are highly recombinogenic and thus destabilizing to the genome. If DNA-PKcs were activated *in vivo* by forming a stable complex with solitary DNA ends, this would enable the enzyme, in effect, to broadcast an alarm signal declaring the presence of a persistent DSB. Consistent with this, DNA-PKcs is required for DNA damage-dependent apoptosis in some cell types (51,52). An exposed $3'$ tail is necessary to initiate strand invasion and recombination [reviewed in (53)] whereas a hairpin end is presumably inert in this respect. Thus, it is of interest that unpaired DNAs have the greatest ability to bind and activate DNA-PKcs, whereas hairpin ends had little, if any ability to do so. Providing a signal indicating the presence of a dangerously recombinogenic free DNA end could be an important function of DNA-PKcs in addition to its direct role in repair. An analogous ability of single-stranded DNA to activate ATM kinase, which is related to DNA-PKcs and has overlapping functions in DNA damage recognition, has been cited as evidence for a central role of single-stranded DNA as an evolutionarily conserved signal for DNA damage (54).

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