A mechanism for DNA-PK activation requiring unique contributions from each strand of a DNA terminus and implications for microhomology-mediated nonhomologous DNA end joining

Katherine S. Pawelczak[1] and John J. Turchi[1,2,*]

[1]Department of Biochemistry and Molecular Biology and [2]Department of Medicine, Indiana University School of Medicine, Indianapolis, IN 46220, USA

ABSTRACT

DNA-dependent protein kinase (DNA-PK) is an essential component of the nonhomologous end joining pathway (NHEJ), responsible for the repair of DNA double-strand breaks. Ku binds a DSB and recruits the catalytic subunit, DNA-PKcs, where it is activated once the kinase is bound to the DSB. The precise mechanism by which DNA activates DNA-PK remains unknown. We have investigated the effect of DNA structure on DNA-PK activation and results demonstrate that in Ku-dependent DNA-PKcs reactions, DNA-PK activation with DNA effectors containing two unannealed ends was identical to activation observed with fully duplex DNA effectors of the same length. The presence of a 6-base single-stranded extension resulted in decreased activation compared to the fully duplex DNA. DNA-PK activation using DNA effectors with compatible termini displayed increased activity compared to effectors with non-compatible termini. A strand orientation preference was observed in these reactions and suggests a model where the 3′ strand of the terminus is responsible for annealing and the 5′ strand is involved in activation of DNA-PK. These results demonstrate the influence of DNA structure and orientation on DNA-PK activation and provide a molecular mechanism of activation resulting from compatible termini, an essential step in microhomology-mediated NHEJ.

INTRODUCTION

DNA double-strand breaks (DSB) can be induced by ionizing radiation (IR), certain chemical compounds and collapsed replication forks. If not repaired, these breaks can lead to genomic instability, chromosomal abnormalities and cell death. Nonhomologous end joining (NHEJ) is the pathway largely responsible for the repair of DSBs induced by IR (1,2). The DNA-PK holoenzyme comprised of the Ku DNA-binding domain and the catalytic subunit is the main complex initiating the NHEJ process. Ku first binds to the DNA termini, whereby the ring-like structure of Ku encircles the DNA end (3). This structure can also account for the ability of Ku to translocate along the duplex DNA (4–6). DNA-PKcs then binds to the DNA terminus in a Ku-dependent manner. Formation of this complex on DNA results in activation of the serine/threonine protein kinase enzymatic activity. DNA-PK has been demonstrated to phosphorylate a number of proteins in the NHEJ pathway (7–13). Importantly, only phosphorylation of Artemis and autophosphorylation of DNA-PKcs have been demonstrated to alter biological activity and NHEJ catalyzed repair (14,15).

Structural analysis of DNA-PK has revealed an open region within the kinase that upon interaction with DNA induces a conformational change in the protein that may play a role in activation of the kinase (16). Further work modeled a passage way through the DNA–PK complex within the catalytic subunit that can accommodate DNA, and upon DNA threading through this channel, may serve to protect the free termini. Following DNA binding, the formation of a DNA-PK dimer has been proposed to create a synaptic region responsible for bringing the two

DNA ends to be ligated in proximity of each other (17). Such a complex may facilitate recruitment of subsequent proteins and final ligation of the DNA.

The terminus resulting from an IR-induced DNA DSB can vary in structure, size, and chemistry. In this study, the activation of DNA-PK has been assessed using a variety of DNA molecules differing in structure, chemistry and sequence. It has been shown that DNA-PK is activated by full duplex DNA but not by hairpin structures or supercoiled plasmids (18). Additionally, it has been shown that DNA-PK is preferentially activated by DNA with 3′ pyrimidine-rich termini (19), but activity is severely inhibited by cisplatin-DNA adducts (20). Interestingly, chemical structures attached to DNA such as biotin do not inhibit kinase activity (21). Analysis of Ku-independent DNA-PK activation has shown that DNA-PKcs is highly activated by DNA with single-strand overhangs (21). Despite this collection of data, it remains unclear what aspects of DNA are important for activation of the kinase.

It has been suggested that melting of the DNA terminus may be necessary for DNA-PK to bind in a stable complex with the DNA (22). Melting results in two single-strand ends of DNA, though the role that these ends may play in DNA-PK activation has yet to be elucidated. A model for DNA threading through the kinase and separating of the DNA ends has been proposed, with single strand termini entering an active site on the kinase following synapsis of two DNA bound DNA-PKcs proteins (23). The authors demonstrated formation of a synaptic complex in the absence of sequence homology using blunt-end substrates and cooperative activation of the kinase suggesting that the synaptic complex displays greater catalytic activity. The role of DNA structure and sequence on synaptic complex formation and whether activation requires *cis* or *trans* interactions is not known. Our previous results show that DNA sequence bias as a function of strand orientation significantly influences kinase activation (19), suggesting a unique role for each strand of the DNA terminus in DNA-PKcs activation. To investigate the effect of different DNA structures on DNA-PK activation, a collection of DNA effectors with different structures and sequences was designed. Our results reveal a unique role of each strand of the termini in synaptic activation, which is independent of complex formation. These results have been used to develop a mechanistic model for DNA-PK activation as a function of the DNA termini sequence homology and structure.

METHODS

DNA effectors

Single-strand oligonucleotides were purchased from Integrated DNA Technology (IDT, Coralville, IA, USA) and are presented in Table 1. Oligonucleotides were synthesized with a 5′-biotin molecule by IDT, as indicated. The single-strand oligonucleotides were gel-purified by preparative denaturing 15% polyacrylamide gel electrophoresis, quantified and annealed to complementary oligonucleotides, as follows. The 24-bp full duplex DNA

Table 1. Oligonucleotide sequences

Name	Sequence*
3′24	biotin-TAGATGGACTATCGCAGCACTGTA
3′30	biotin-TAGATGGACTATCGCAGCACTGTACGGCTT
3′24 + 6	biotin-TAGATGGACTATCGCAGCACTGTAGCCGAA
3′24 + 6A	biotin-TAGATGGACTATCGCAGCACTGTAAAAAAA
3′24 + 6T	biotin-TAGATGGACTATCGCAGCACTGTATTTTTT
3′24 + 6mix	biotin-TAGATGGACTATCGCAGCACTGTAAAGCCG
5′24	TACAGTGCTGCGATAGTCCATCTA
5′30	AAGCCGTACAGTGCTGCGATAGTCCATCTA
5′24 + 6	TTCGGCTACAGTGCTGCGATAGTCCATCTA
5′24 + 6A	AAAAAATACAGTGCTGCGATAGTCCATCTA
5′24 + 6T	TTTTTTTACAGTGCTGCGATAGTCCATCTA

*All sequences are 5′ to 3′ with biotin modifications as indicatetd.

effector was created by annealing 3′24 to 5′24, and the 30-bp full duplex DNA created by annealing oligonucleotides 3′30 and 5′30. The two Y-shaped effectors were prepared by annealing 3′24 + 6 to 5′30 and 3′30 to 5′24 + 6. The 3′ overhang effector was prepared by annealing oligonucleotide 3′30 to 5′24. To prepare effectors with 3′ overhangs for microhomology studies oligonucleotide 3′24 + 6T, 3′24 + 6A or 3′24 + 6mix were annealed to 5′24 to make the 3′ overhang effectors containing homopolymeric Ts, As or with compatible sequences, respectively. Additionally, the 3′24 oligonucleotide was annealed to 5′30 to make the 5′ overhang effector, and 3′24 was annealed 5′24 + 6T or 5′24 + 6A to make the 5′ overhang effectors for microhomology studies. Duplex DNA was gel-purified by native polyacrylamide gel electrophoresis to ensure homogeneity, eluted from gel, precipitated and quantified. DNA-PK was purified from HeLa cells, and a series of titrations of both DNA and DNA-PK were performed to determine the appropriate concentrations to use in kinase assays to accurately assess binding and activation.

Protein purification

Cell-free extracts were prepared from 12 L of HeLa cells as described previously (24) and DNA-PK was purified as previously described (20). Briefly, extracts were fractionated on a 50-ml cisplatin-damaged DNA-Sepharose column, heparin-Sepharose column and Q-Sepharose column. DNA-PK containing fractions were identified by Coomassie staining of SDS gels, DNA-PK kinase activity and Ku-binding activity. The fractions were pooled, dialyzed, aliquoted and frozen at $-70°$.

DNA-PK kinase assays

Kinase assays containing a single effector per reaction were performed at $37°C$ in a final volume of $20\,\mu l$ containing $20\,mM$ HEPES, pH 7.5, $8\,mM$ $MgCl_2$, $1\,mM$ DTT, 5% glycerol, $125\,\mu M$ ATP, $[\gamma\text{-}^{32}P]$ ATP ($0.5\,\mu Ci$), $6.3\,nM$ DNA-PK, $5\,nM$ DNA and $500\,\mu M$ p53 synthetic peptide. Taking into account the buffer in which the DNA-PK preparations were dialyzed, the final KCl concentration of the reactions ranged from $20\,mM$ to $70\,mM$. In addition, to determine the effect of ionic strength, a series of DNA-PK activation experiments were performed at varying KCl concentrations and the results demonstrated

no significant effect over the range of concentrations tested (Supplementary Data). Biotinylated duplex DNA was incubated with 1 ng streptavidin (SA)/fmol DNA and incubated on ice for 5 min. DNA-PK and reaction buffer containing peptide were added to DNA and incubated on ice for 5 min. Reactions were initiated with addition of ATP, incubated at 37°C for 15 or 30 min, as indicated, and terminated by addition of 20 μl of 30% acetic acid. Reaction products were spotted on P81 phosphocellulose filter paper. The paper was washed five times, 5 min. each, in 15% acetic acid, once in 100% methanol and allowed to dry. Samples were quantified by PhosphorImager® analysis using ImageQuant software (Molecular Dynamics). Each DNA effector used in the assays to analyze dimeric activation was bound with SA then DNA-PK was added to the DNA and allowed to incubate for 5 min on ice. Each effector was then added alone to a tube or mixed with the complementary prebound effector and allowed to incubate on ice for 5 min. Reaction buffer and peptide were then added to the DNA-DNA-PK mix, the reaction was initiated with ATP and the assay was completed as described above.

DNA-PK autophosphorylation assay

Kinase assays were performed as described above with the following modifications. Reactions contained 20 nM DNA-PK and 1.0 μCi [γ-^{32}P] ATP. Where indicated, reactions were performed in a time-dependent manner (Supplementary Data). Reactions were terminated with addition of SDS buffer and loaded onto an 8% SDS–PAGE gel. Following electrophoresis, the gel was dried and visualized by PhosphorImager® analysis.

DNA-PK pull-down assay

DNA synaptic complex formation was determined by incubating 500 fmol of each biotinylated DNA effector with 10 μl slurry of SA magnetic beads (Promega) in 500 μl of 10 mM Tris-pH 7.5, 1 mM EDTA, 5% glycerol, and 0.1% BSA for 30 min to allow for binding to occur. SA-DNA magnetic beads were washed three times to remove excess DNA. DNA-PK was bound to the SA- DNA as well as to a nonbiotinylated DNA effector with a [γ-^{32}P] ATP 5′ label in a 20 μl reaction containing 20 mM HEPES, pH 7.5, 8 mM MgCl$_2$, 1 mM DTT and 5% glycerol. Following a 5-min incubation period, the DNA-PK-5′ labeled DNA effector complex was added to the SA-DNA-DNA-PK bead mixture and allowed to incubate at room temperature for an additional 5 min. The beads were collected in a magnetic separator and the supernatant was removed, beads were gently washed two times in reaction buffer, and the bound DNA quantified via scintillation counting.
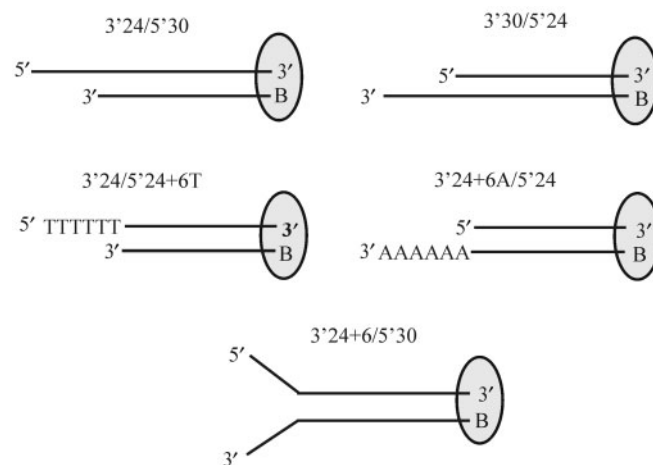
RESULTS

DNA effectors used to assess the influence of DNA structure on activation of DNA-PK

We have previously demonstrated that DNA-PK is differentially activated by 3′ pyrimidine and 5′ purine-rich DNA termini, suggesting that one strand terminus may be more important for activation than the other. Therefore, we have designed a series of DNA effectors with different termini to determine how each strand, 3′ or 5′, contributes to activation of DNA-PK. DNA effectors were designed with one strand containing a biotinylated 5′ terminus so that when incubated with SA, only one termini from each duplex effector is rendered accessible for DNA-PK binding (Figure 1). Effectors were designed as fully duplex, or contained either a 3′ or 5′ overhang strand, or Y-shaped termini, all located on the accessible end (Figure 1). Using these DNA effectors and purified DNA-PK, we were able to conduct a series of *in vitro* reactions to determine how these different DNA structures influence activation of DNA-PKcs in Ku-dependent reactions.

Effect of DNA termini on activation of DNA-PK

It has previously been shown that when the DNA-PKcs is purified away from Ku, kinase activity is dramatically stimulated when DNA effectors have single-stranded overhangs compared to full duplex effectors (21). However, the effect of single-strand overhang ends on activation of the heterotrimeric complex, Ku and DNA-PKcs, has not been investigated. DNA effectors described in Figure 1 and Table 1 were utilized, and all experiments included a 30-bp control to ensure that differences were not a result of experimental variation. Results show that although DNA-PK is activated by a 24-bp duplex effector, a 30-bp duplex effector results in a greater degree of activation (Figure 2). This result was expected, as previous publications have demonstrated that DNA-PK activity is length dependent, with longer DNA effectors resulting in greater kinase activity (5,21). Analysis of a DNA effector comprised of 24 bp of duplex DNA with an additional 6 base overhang DNA on either the 3′ or the 5′ termini resulted in no greater activity than that from a full duplex DNA effector with 24 bp (Figure 2A), and was in fact

Figure 1. DNA effectors used in this study. Duplex DNA effectors were prepared from gel purified single strand oligonucleotides. The addition of streptavidin, as indicated by the oval, results in blocking of the 5′ biotinylated terminus, indicated by the B. The effectors generated have either a 3′ overhang, 5′ overhang or Y-shaped structure at the DNA-PK accessible terminus.
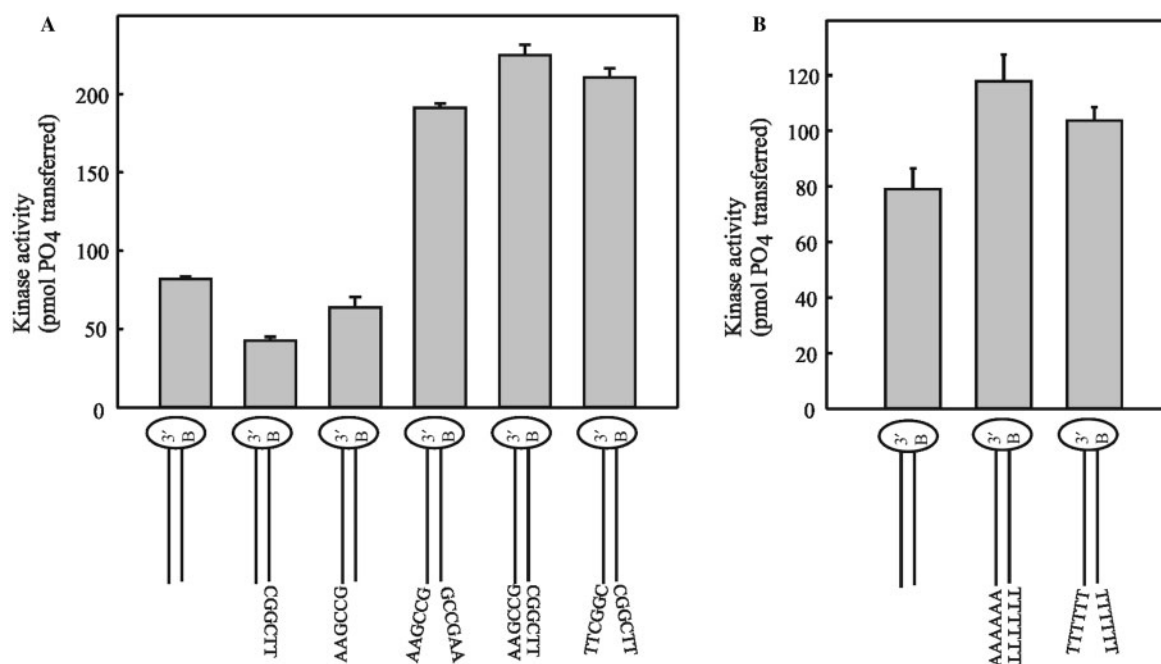
Figure 2. Effect of DNA overhangs on DNA-PK activation. DNA-PK kinase assays were conducted with annealed DNA effectors, as described in 'Materials and methods' section. Briefly, 24- and 30-bp full duplex DNA effectors, 3′ and 5′ 6-bp overhang effectors and Y-shaped effectors were assayed. Reactions contained 6.3 nM DNA-PK and 5 nM SA-DNA, as indicated. Results are presented as the picomoles of ^{32}P transferred to the synthetic p53 peptide in a 30-min reaction. Reactions were performed in triplicate and the mean and standard deviation are presented.

consistently reduced compared to the 24-bp duplex. These results suggest that in the presence of Ku, DNA-PKcs does not require DNA with a single-strand overhang for maximal activation.

To determine if this inhibition was due to the presence of a single stranded terminus, effectors were designed with 24 bases of complementary DNA, and an additional 6 bases of noncomplementary sequence on each strand at the terminus (Y-effectors). Analysis of kinase activation revealed that maximal DNA-PK activation was nearly completely restored with Y-effectors, suggesting that single-stranded ends *per se* are not inhibitory (Figure 2A). In order to rule out any potential differences in DNA-PK activation resulting from sequence or orientation dependent activation, we created the identical structure but varied the sequence on each strand. These results again demonstrated DNA-PK activation by the Y-DNA effectors is comparable to the fully duplex DNA effectors and any minor differences can be attributed to strand orientation-dependent sequence bias. To fully establish this point, similar effectors with homopolymeric 6-base extensions were constructed. The analysis of these effectors is presented in Figure 2B and again demonstrates that Y-shaped effectors to not result in greater activation of DNA-PK compared do fully duplex DNA effectors of identical length. Together, these results demonstrate that in the presence of Ku, DNA-PKcs does not require single-strand termini for maximal activation.

The results from Figure 2 suggest that DNA-PK is not preferentially activated by single-strand overhang effectors compared to full duplex effectors of the same length and in fact display reduced activation. Statistical analysis of those results revealed statistically significant difference

with a *P*-value <0.05 in comparisons with each set of effectors. We hypothesized that if there were truly relevant differences in activation with the varying overhang effectors these may become accentuated as a function of DNA concentration. In our standard kinase assay reaction with fully duplex DNA effectors, the 5-nM DNA concentration is saturating (data not shown). DNA-PK activity was assayed with increasing concentrations of the 3′ overhang effector or the 5′ overhang effector (Figure 3) and a substantial difference in activity over the entire range of effector concentrations was observed. These results confirm that DNA-PK exhibits lower activity from a 3′ overhang DNA effector compared to the 5′ overhang effector. The finding that increased DNA concentrations of the overhang effectors were required to reach maximal activity suggests a decrease in affinity of the DNA–PK complex for these effectors compared to full duplex (data not shown).

Influence of DNA effectors on autophosphorylation of DNA-PK

It has been shown that DNA-PK activity is modulated by autophosphorylation. Upon autophosphorylation, the kinase is believed to dissociate from the DNA effector, thus lowering overall kinase activity of DNA-PK (25). To investigate if the level of DNA-PK autophosphorylation was responsible for the differential peptide phosphorylation observed in Figure 2, kinase assays were performed as described above, with streptavidin bound effectors having either 3′ or 5′ overhang ends, Y-shaped termini, or blunt termini accessible for DNA-PK binding. Following incubation, the samples were separated on
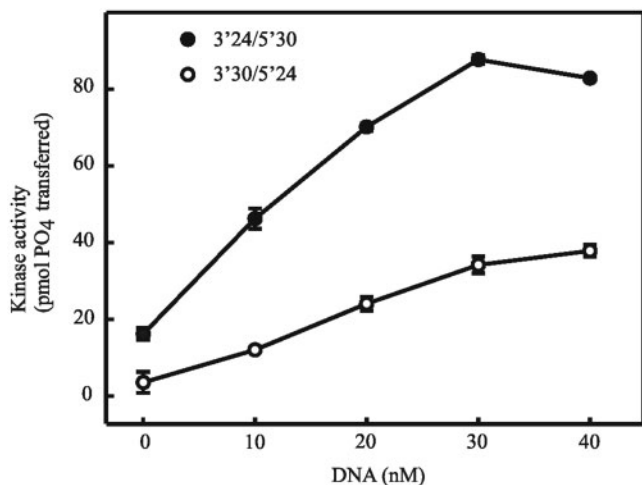
Figure 3. Titration of DNA effectors containing 3′ and 5′ overhangs. DNA with a 3′ or 5′ 6-base overhang, as described in 'Methods' section, were assayed for DNA-PK kinase activation. Reactions were incubated with increasing concentrations of each effector. Results are presented as the picomoles of ^{32}P transferred to the synthetic p53 peptide in a 30-min reaction. Reactions were performed in triplicate and the mean and standard deviation are presented.
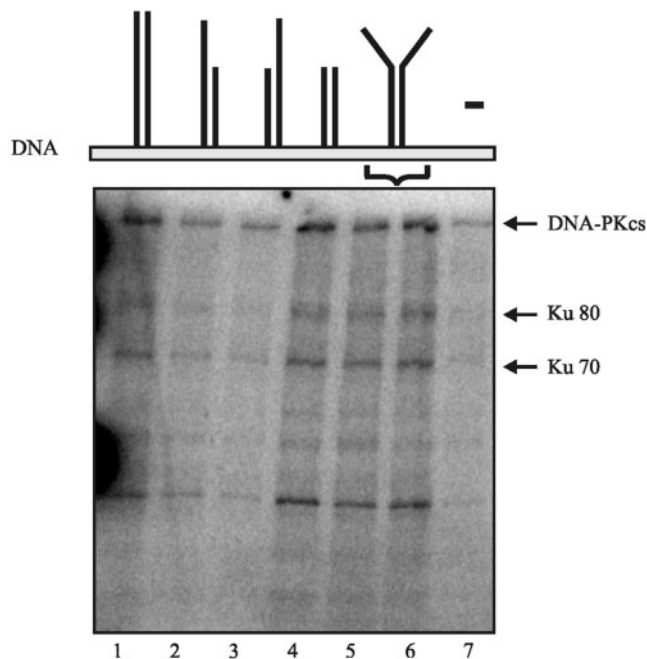

Figure 4. Autophosphorylation of DNA-PK by overhang and Y-shaped effectors. DNA-PK autophosphorylation was measured with 24- and 30-bp full duplex DNA effectors, 3′ and 5′ overhang effectors and Y-shaped effectors. Reactions were performed as described in Figure 1, initiated with 1.0 μCi [γ-^{32}P] ATP, and stopped with SDS. Reaction products were separated by 8% SDS–PAGE. The gel was dried and exposed to a PhosphorImager.

a SDS denaturing gel and ^{32}P-labeled DNA-PK was visualized with a PhosphoImager®. DNA-PK autophosphorylation resulting from the overhang effectors, both 3′ and 5′, was significantly reduced when compared with the full duplex 30-bp effector (Figure 4).

Furthermore, the kinetics of autophosphorylation of DNA-PK was determined with the 30-bp effector and the 3′ overhang effector (Supplementary Data). The results confirm the decreased autophosphorylation activity obtained with the 3′ overhang effector compared to the full duplex DNA (Figure 4). The level of autophosphorylation presented in Figure 4 comparing the overhang effectors and the 30-bp duplex effector mimics the level of peptide phosphorylation observed in peptide-based kinase assays (Figure 2). Interestingly, the level of autophosphorylation resulting from the 24-bp effector is the same as that from the 30-bp effector (Figure 4). If DNA-PK autophosphorylation resulting from the full duplex sets of DNA were mimicking the peptide phosphorylation observed in the kinase assays, a lower amount of autophosphorylation from the 24-bp effector would be expected. Importantly, there is no difference in autophosphorylation between the 3′ overhang and 5′ overhang DNA effectors. These results demonstrate that the lower activity resulting from the 3′ overhang compared to the 5′ overhang effector is not due to a disparity in autophosphorylation. In contrast, the increase in autophosphorylation resulting from the 24 bp may account for the decrease in peptide phosphorylation from the 24-bp DNA compared to the 30-bp duplex effector (Figure 2). To ensure these results were consistent with the peptide kinase assays, reactions were performed in the presence and absence of the peptide substrate and similar results were obtained under both conditions (data not shown). Thus, length dependence may play a role in influencing autophosphorylation.

Influence of DNA effectors containing regions of microhomology

After determining that a single-strand overhang on a duplex DNA effector does not increase DNA-PK activity over a full duplex effector, we designed a set of DNA effectors with homopolymeric overhang strands to determine if sequence bias on a single-strand overhang differentially activates DNA-PK. One series of effectors contained 3′-6 base extensions of either Ts or As (Figure 5A). The second series were identical except the extensions were on the 5′ strand of the accessible terminus (Figure 5B). Assays were performed at two DNA concentrations as indicated in the figure. Results show that DNA-PK activity with the 3′A extension was slightly decreased compared to the activity observed in reactions containing the 3′A extended effector, an effect observed at both DNA concentrations (Figure 5A). These results are similar to our previously published results regarding sequence bias and DNA-PK activation. The difference in activation between the two is not as dramatic as we observed with full duplex effectors containing 3′T or 3′A, but the general pattern is consistent with our previous results (19). Analysis of the 5′ extended DNA effectors also revealed a decreased activation with the A-containing effector compared to the T-containing effector (Figure 5B). Consistent with our previous results, activation with the 5-extensions is greater compared to the 3′-extension, independent of sequence. Interestingly, the
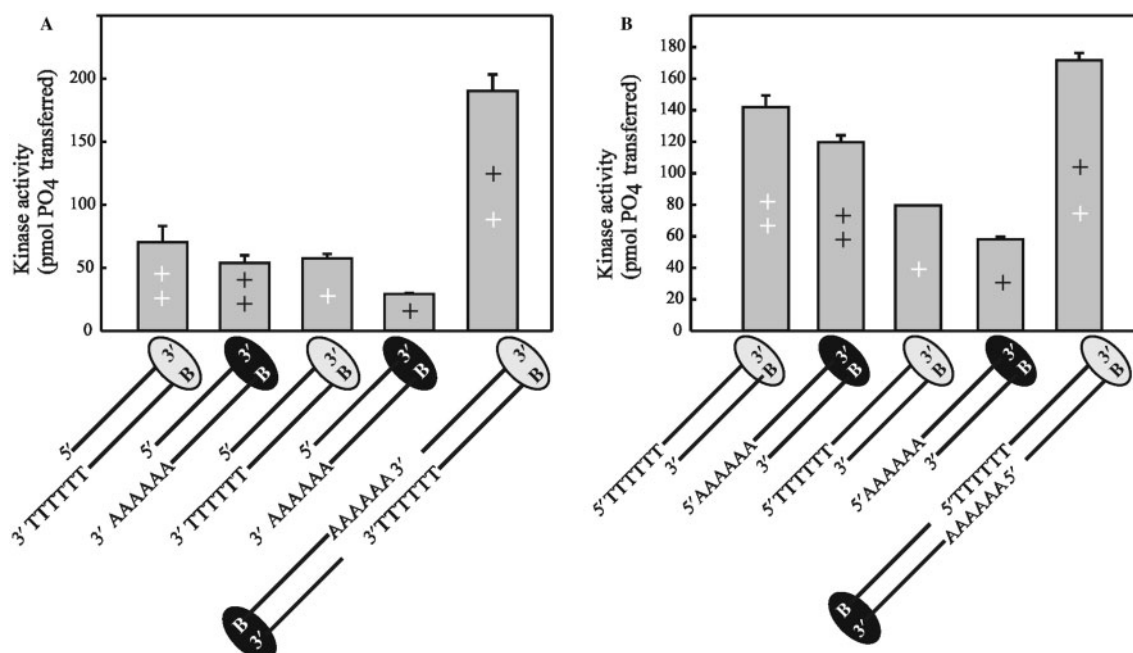
Figure 5. Dimeric activation of DNA-PK from effectors containing compatible homopolymeric overhang ends. (**A**) Effectors with either a 6-base poly-T 3′ overhang or a 6-base poly-A 3′ overhang were prebound with heterotrimeric DNA-PK and assayed for DNA-PK kinase activation. Assays were conducted with either 1 or 0.5 pmol of each effector per reaction, as indicated, or 0.5 pmol of each effector was incubated together in a single reaction. 0.5 pmol of the poly-T effector is indicated by a white plus symbol, and half a picomole of the poly-A effector is indicated by the black plus symbol. Results are presented as the picomoles of ^{32}P transferred to the synthetic p53 peptide in a 30-min reaction. Reactions were performed in triplicate and the mean and standard deviation are presented. (**B**) Effectors with either a 6-base poly-T 5′ overhang or a 6-base poly-A 5′ overhang were assayed for DNA-PK kinase activation as described above. 0.5 pmol of the poly-T effector is indicated by a white plus symbol, and half a pmol of the poly-A effector is indicated by the black plus symbol.

effect of the extension sequence on DNA-PK activation was similar, with both sets of effectors showing decreased activation with the A extensions.

These effectors were designed such that when individually included in a reaction, the extensions are non-compatible and thus each terminus presumably binds a single DNA-PK. However, if combined, the effectors are capable of annealing, mimicking regions of microhomology that may occur after processing of termini in preparation for ligation. To perform this assay, each DNA effector was pre-incubated with DNA-PK prior to the addition of ^{32}P-ATP to initiate the kinase reaction. In an effort to increase the level of activation resulting from the relatively poor DNA overhang effectors and to control for total DNA in the combined reaction, individual effector reactions were performed at 2 DNA concentrations, one being equal to the DNA concentration in the combined and the other at 1/2 the DNA concentration of the combined reaction. In the combined reactions with the 3′ extended effectors, a significant increase in activation was observed compared to the individual reactions. If kinase activation were simply additive upon combining the effectors, we would have expected kinase activity of ∼85 pmol of phosphate transferred in the combined reaction. If we consider an average of the individual reactions at equal concentrations of total DNA we would have expected ∼62 pmol of phosphate transferred. DNA-PK activity resulting from the effectors with areas of microhomology resulted in close to 190 pmol of phosphate transferred (Figure 5A). This observed synergy is

dependent on the presence of compatible DNA termini and suggests that annealing influences the activation of DNA-PK.

To determine if strand orientation of a microhomologous sequence influences synergistic activation of DNA-PK, we performed analysis of the 5′ extended effectors. Interestingly, while the 5′ overhang effectors with microhomology did produce a small amount of synergistic DNA-PK activity, the level of synergy was not as great as that resulting from the 3′ overhang effectors with complementary regions (Figure 5B). Additive results predicted a value of 137 pmol of phosphate transferred, while averaging the individual reactions provides a value of 130 pmol of phosphate transferred. The combined reactions resulted in synergistic activity, with 171 pmol of phosphate transferred, but the increase of this activity over the additive and averaged values was not as great as that seen with the 3′ compatible overhangs. These results suggest that the 3′ end of the DSB is dictating the aligning and microhomology directed ligation.

The effectors in Figure 5A have complementary overhangs, but because of the homopolymeric sequence, these areas have the potential for annealing in different registers with 2-, 3-, 4-, 5- or 6 bp annealing. To correct and control for this possibility, effectors depicted in Figure 6 were designed with a complementary mixed sequence on the 3′ overhangs. These effectors therefore can only anneal in a single position and thus would contain nicks at each junction following annealing. The kinase assay was conducted as described above, and results show that
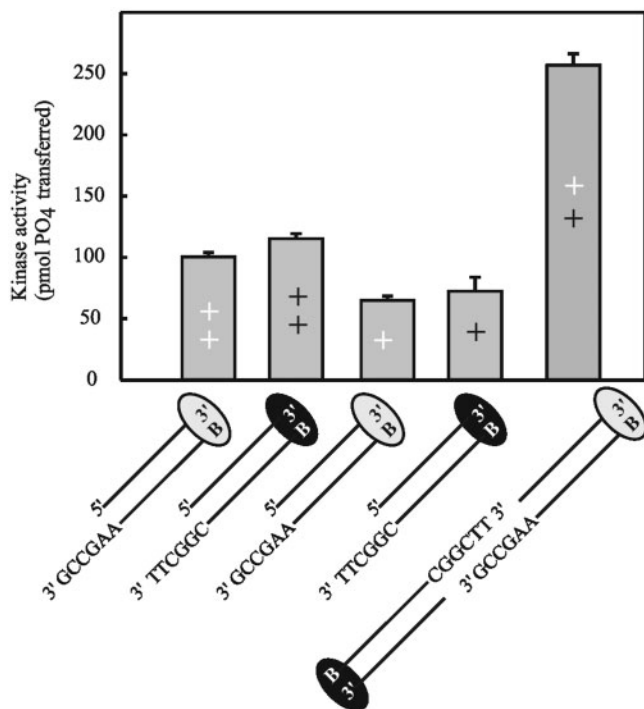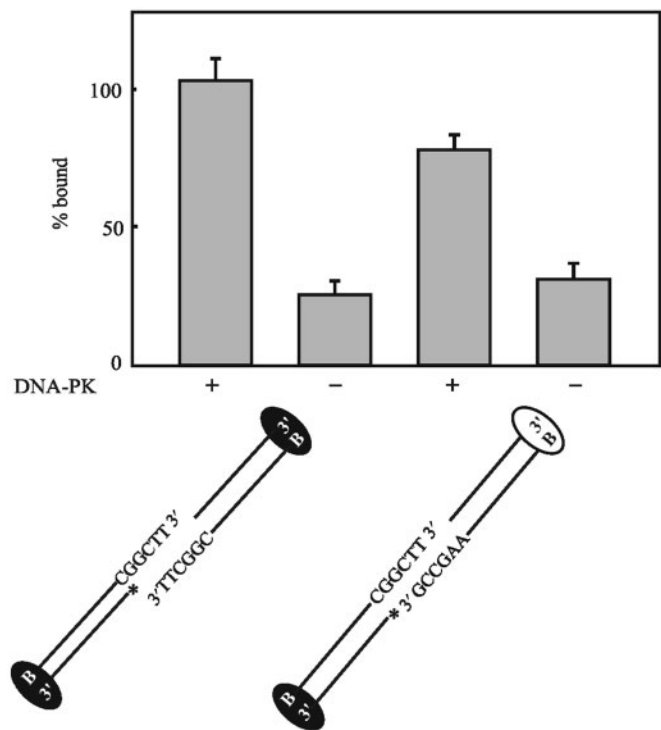
Figure 6. Activation of DNA-PK with DNA effectors containing compatible mixed sequence overhang ends. Effectors with 6-bp 3′ overhang complementary sequences were pre-bound with DNA-PK and assayed for DNA-PK activation. Assays were conducted with 1 pmol or 0.5 pmol of a single DNA effector, or 0.5 pmol of each effector was included together in a reaction, as indicated. The white plus symbol indicates half a pmol of one DNA effector, and the black plus symbol indicates the second effector with a complementary overhang. Results are presented as the picomoles of ^{32}P transferred to the synthetic p53 peptide in a 30-min reaction. Reactions were performed in triplicate and the mean and standard deviation are presented.

Figure 7. DNA-PK binding with DNA effectors containing compatible overhang ends. Five hundred femtomoles of each unlabeled DNA effector was bound to streptavidin magnetic beads. ^{32}P -labeled DNA (indicated by asterisk) and the SA bound unlabeled DNA was incubated with DNA-PK or buffer as indicated, and labeled DNA was incubated in a separate tube with DNA-PK or buffer for 5 min. The labeled DNA effector prebound with DNA-PK or buffer was added to the tubes containing SA-unlabeled DNA and DNA-PK or buffer, incubated for 5 min, washed, and counts were read in a scintillation counter. Results are presented as percent of total DNA-DNA-PK complex bound to SA–DNA–DNA–PK complex.

effectors with 3′ overhangs containing a specific annealing sequence also induce high levels of synergistic activity, 256 pmol of phosphate transferred (Figure 6). This level of activity is much higher than the projected additive and average values, 136 and 108 pmol of phosphate transferred. These results support the conclusion that the 3′ end is crucial for microhomology-associated annealing and solidifies the hypothesis that microhomology is important, as the complementary sequence in these effectors is significantly more specific than the DNA in Figure 5A. This indicates that the dimeric activation observed results from two effectors having the ability to successfully anneal, which requires greater than one complementary base.

Synaptic complex formation with DNA effectors containing single-strand overhangs

To determine if the greater than additive kinase activity observed with the DNA effectors containing 3′ overhangs with regions of microhomology was due to an increase in formation of a synaptic complex between the two DNA effectors and two DNA-PK molecules, we designed a pull-down assay utilizing radiolabeled DNA. The results from this assay (Figure 7) revealed that a synaptic complex is formed in the presence of DNA overhang effectors,

as shown by the increase in activity in the reactions containing samples with prebound DNA-DNA-PK complexes compared to the reactions that were absent of DNA-PK. Interestingly, DNA overhang effectors formed synaptic complexes regardless of the presence of a region of microhomology within the overhang. This result differs from the kinase activity, where activity was increased with DNA overhang effectors containing regions of microhomology. These results demonstrate that homology driven dimeric activation seen in our kinase assays is a catalytic effect, and does not result from increased binding and formation of a synaptic complexes.

DISCUSSION

DNA-PK is a unique protein kinase with a novel mechanism of activation. Precisely how DNA serves to activate DNA-PK is not known. Modeling studies suggest that DNA may thread through the catalytic subunit and enzymatic analyses suggest that single-strand ends may be important for kinase activation (16,23,26). The results presented here in Ku-dependent DNA-PK kinase assay show that Y-shaped effectors with preformed single-strand ends, result in activity similar to that observed

from full duplex DNA effectors. However, the presence of single-strand overhangs results in significantly less activity. This finding is contrary to results obtained in a Ku-independent DNA-PKcs activation assay where a significant increase in activity was observed when DNA with a single-strand overhang were used as effectors (21). While important mechanistically, elegant *in vivo* studies show that DNA-PKcs recruitment to the site of a DSB is dependent on Ku, highlighting the importance of studying the mechanism of holoenzyme activity (27). The differential activation observed with unannealed ends may be explained in part by an energetic argument. The presence of Ku at the termini alleviates the need for preseparated strands. Thus, Ku may promote strand opening in conjunction with DNA-PKcs. While Ku has been reported to possess strand separation activity (28) kinetic analysis brings the catalytic nature of this activity into question (29). In any event, Ku facilitation of unwinding in conjunction with DNA-PKcs to generate the required single-strand ends could explain the differential activation as a function of single strand ends.

The inhibition of DNA-PK activity resulting from a 3′ overhang compared to a 5′ overhang DNA effector, combined with the synergistic activity exhibited from complementary overhangs suggests a unique role for each single-strand terminus in DNA-PK activation. These results suggest that the 5′ end is more important for contact with the kinase, thus resulting in a greater degree of activation than the 3′ end. However, each of these strands on their own does not result in maximal activation of DNA-PK, indicating that while each strand plays an important role, both strands are necessary for maximal DNA-PK activation. We have constructed a model for DNA-PK activation, based on existing biochemical and structural analysis of the kinase (17,22,23) in conjunction with the results presented in this paper (Figure 8). Following Ku binding and recruitment of DNA-PKcs to the site of the break, the kinase binds to the DNA terminus. The DNA strand is postulated to protrude through the kinase, with the terminus appearing on the opposite opening of the kinase. Following insertion through the kinase, evidence points to fraying or unwinding of the DNA ends (22). Our data show that a DNA fragment with a 5′ overhang induces greater DNA-PK activation than a DNA fragment with a 3′ overhang. Yet, when two complementary 3′ overhang DNA effectors are incubated together, a synergistic level of DNA-PK activation is observed. When two pieces of DNA with 5′ complementary overhangs are incubated together, the synergistic level of activation is significantly lower. Combined, this data suggest that the 5′ strand is important for activation, while the 3′ strand may be important in scanning for regions of microhomology. The potential role of the 3′ strand in microhomology searching is also consistent with the 5′ to 3′ exonuclease activity of Artemis degrading the 5′ strand. This activity would generate a 3′ single strand at the terminus, which displays relatively low activity until a complementary sequence is identified.

Previous data suggest the possibility that following fraying of the DNA ends after DNA-PK binding, the strands may insert themselves into active sites on the
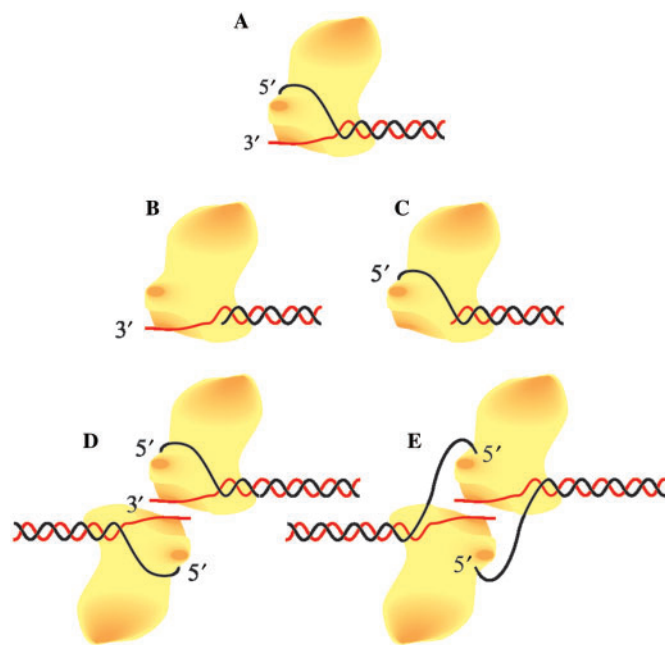
Figure 8. Model for activation of DNA-PK. DNA, depicted in black and orange, threads through DNA-PK, depicted in yellow. (**A**) When DNA of adequate length threads through the kinase, the ends separate and the 5′ end can insert itself into an active site on the periphery of the kinase. (**B**) A 5′ overhang containing effector can activate in the absence of dimerization while a 3′ overhang (**C**) displays less activation. When the 3′ overhang possesses a complementary sequence to the second DNA molecule with a 3′ overhang, the two DNA ends can anneal, thus orienting the two DNA-PK molecules into a synaptic complex. The annealing may allow for more DNA to thread through the kinase, potentially making more of the 5′ strand accessible for kinase activation. This activation could then occur in either a *cis* (**D**) or *trans* (**E**) fashion.

perimeter of the kinase (30). Our data suggest that each DNA termini is bound by DNA-PK in a dimeric complex independent of homology. The DNA is scanned for regions of microhomology, which potentially pulls the DNA further through the kinase upon which once homologous regions are identified, increased kinase activity results. The model predicts that this activation is accompanied by insertion of the 5′ end into the active pocket on the periphery of the kinase (Figure 8). This dimeric activation is expressed as a synergistic level of activity when the two complementary effectors are simultaneously incubated in an assay for DNA-PK activation. Whether the insertion of the 5′ single strand occurs in a *cis* or *trans* fashion is a question that still needs to be answered with future experiments.

The single strand ends with complementary areas have the potential to promote activation of the DNA-PK bound ends. DNA-PKcs has been found to exist in mainly in a monomeric state (31). However, it is thought that when DNA-PKcs is bound to the ends of two DNA molecules at the site of a DSB, the protein kinase is responsible for bringing the two DNA ends into proximity of each other in the form of a synaptic complex. The formation of this synaptic complex is thought to correspond to the dimerization of DNA-PKcs. Furthermore, the dimerization of DNA-PKcs and subsequent formation

of the synaptic complex is thought to result in maximal activation of the kinase (23). Our results are consistent with a model whereby dimerization of DNA-PK is independent of microhomology while kinase activity is greatly influenced by end annealing. There are two models one can envision for the role of DNA in activation of the kinase once these molecules are brought within proximity. One model involves the frayed 5′ DNA end being inserted into the active site of the same DNA-PKcs molecule to which the DNA is bound (19,22). The second model positions the 5′ strand interacting with the DNA-PKcs molecule that is bound to the opposite DNA strand (32). This model is supported by recent data suggesting that DNA-PKcs is autophosphorylated in *trans* (33). An additional caveat is that NHEJ-dependent joining of blunt-end substrates can occur *in vitro* and in cell culture models whereby joining is independent of microhomology. Consistent with this data, we demonstrate that blunt-end DNA effectors are the most effective activators of the kinase. Thus the potential exists for an alternate kinase activation mechanism that may not necessarily include fraying of the termini or nuclease processing. Direct experimental proof of these differing models remains to be obtained and likely will require high resolution mapping of the DNA-protein and protein-protein contacts needed to support DNA-PK activation and NHEJ.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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