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Non-homologous End Joining Requires That the DNA-PK Complex Undergo an Autophosphorylation-dependent Rearrangement at DNA Ends*

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Repair of chromosome breaks by non-homologous end joining requires the XRCC4-ligase IV complex, Ku, and the DNA-dependent protein kinase catalytic subunit (DNA-PKcs). DNA-PKcs must also retain kinase activity and undergo autophosphorylation at six closely linked sites (ABCDE sites). We describe here an end-joining assay using only purified components that reflects cellular requirements for both Ku and kinase-active DNA-PKcs and investigate the mechanistic basis for these requirements. A need for DNA-PKcs autophosphorylation is sufficient to explain the requirement for kinase activity, in part because autophosphorylation is generally required for end-joining factors to access DNA ends. However, DNA-PKcs with all six ABCDE autophosphorylation sites mutated to alanine allows access to ends through autophosphorylation of other sites, yet our *in vitro* end-joining assay still reflects the defectiveness of this mutant in cellular end joining. In contrast, mutation of ABCDE sites to aspartate, a phosphorylation mimic, supports high levels of end joining that is now independent of kinase activity. This is likely because DNA-PKcs with aspartate substitutions at ABCDE sites allow access to DNA ends while retaining affinity for Ku-bound ends and stabilizing recruitment of the XRCC4-ligase IV complex. Autophosphorylation at ABCDE sites thus apparently directs a rearrangement of the DNA-PK complex that ensures access to broken ends and joining steps are coupled together within a synaptic complex, making repair more accurate.

The mammalian non-homologous end-joining pathway has an important role in all cell types for repair of double strand breaks (DSBs)[1] caused by DNA damage (*e.g.* after ionizing radiation). End joining is also essential for efficient resolution of DSB intermediates in V(D)J recombination, a lymphocyte-specific process required for assembly of the antigen-specific receptors of the immune system. Defective end joining thus results in radiation sensitivity, an increased incidence of cancer, and severe immunodeficiency (1).

Repair of broken ends by this pathway requires 1) recognition of broken ends and recruitment of end-joining factors, 2) alignment or synapsis of a pair of ends, and 3) processing by polymerases or nucleases as needed to generate compatible ends 4) followed by ligation. End joining uses the XRCC4-ligase IV complex (LX) for the ligation step (2, 3), whereas the processing step uses at least the Artemis nuclease (4, 5) and polymerase μ (6, 7). The DNA-dependent protein kinase (DNA-PK) has been linked to early steps in end joining, and consists of the Ku heterodimer (Ku70 and Ku80) and a 460-kDa serine/threonine DNA-PK catalytic subunit (DNA-PKcs). Ku is required for end recognition and, once loaded on ends, acts as a scaffold for subsequent recruitment of DNA-PKcs (8), the LX complex (9–11), polymerase μ (6), and probably other factors as well.

The role of DNA-PKcs is not well understood. Several observations, including its ability to associate with Artemis and activate the endonuclease activity of Artemis (4), suggest a specific role for DNA-PKcs in directing end-processing events; however, DNA-PKcs deficiency affects cellular end joining even when processing is not required (see Ref. 12 and references therein). A more general role for DNA-PKcs in end joining is also consistent with *in vitro* evidence indicating that it helps Ku recruit the LX complex to ends (10, 13) and promotes the intermolecular synapsis of two DNA ends (14). Together, these functions of DNA-PKcs may explain why LX activity is necessary *in vitro* when using substrates that do not require processing (10, 15).

Recent studies indicate that cellular end joining requires DNA-PKcs to undergo autophosphorylation (16–18). Six autophosphorylation sites are clustered within a 40-amino acid region ("ABCDE" sites) (see Fig. 2*A*), although at least two other sites are also autophosphorylated (19). Individual mutation of ABCDE sites has mild or negligible effects, but a mutant with all six ABCDE sites converted to alanine (ABCDE(ala)) is unable to complement DNA-PKcs-deficient cells (17). Autophosphorylation in general had previously been shown *in vitro* to trigger a dissociation of DNA-PKcs from Ku-bound ends (20, 21) and is required to make ends accessible to exogenous factors (*e.g.* exonucleases) (22, 23) and probably end-joining factors as well (24). Together, this evidence suggests that the cellular defects of the ABCDE(ala) mutant could simply be explained by its inability to be autophosphorylated and then

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[1] The abbreviations used are: DSB, double strand break; LX, XRCC4-ligase IV complex; PKcs, protein kinase catalytic subunit; DNA-PK, DNA-dependent protein kinase.

dissociate, because its continued presence would prevent access to DNA ends by other end-joining factors.

However, the purified ABCDE(ala) mutant protein also dissociates after autophosphorylation (17). Autophosphorylation of sites outside of the ABCDE cluster is therefore sufficient for dissociation and end access, and the mechanism by which ABCDE autophosphorylation promotes end joining remains unclear.

We therefore developed an *in vitro* assay using only purified Ku, DNA-PKcs, and the LX complex that reflects the cellular requirement for autophosphorylation at the ABCDE sites. We show that autophosphorylation at ABCDE sites uniquely makes ends accessible while still allowing DNA-PKcs to persist at ends and retain LX within a complex at ends.

EXPERIMENTAL PROCEDURES

Proteins—Recombinant Ku heterodimer and LX were purified from baculovirus-infected cells as described previously (9). DNA-PKcs was purified from 50 g of human placenta ("native" DNA-PKcs) or 5 liters of V3 cells stably expressing the various constructs (recombinant), also as described previously (17), with the following exception. Instead of using polyethyleneimine cellulose, nucleic acids were depleted by addition of polyethyleneimine to the extract to 0.2% v/v, centrifugation at 15,000 × *g* for 15 min, and further clarification of the extract by the addition of phosphocellulose to 10% v/v and filtration.

Approximate concentrations of proteins were calculated by Bradford assay using bovine serum albumin as a standard, and quantitative Western analyses, used to confirm these estimates, were accurate within 20% when comparing different mutants.

When indicated, kinase activity of DNA-PKcs was blocked by preincubation of a 100 nM protein stock with 10 μM wortmannin (Sigma) (dissolved in dimethyl sulfoxide) for 10 min on ice; thus, assays typically contain less than 1 μM wortmannin and 1% dimethyl sulfoxide. All experiments compare activity of wortmannin-pretreated DNA-PKcs with mock-treated DNA-PKcs (*i.e.* pretreated in the same way with the appropriate amount of dimethyl sulfoxide).

Assays—All reactions contained 25 mM TRIS-HCl, pH 7.5, 50 μg/ml bovine serum albumin, 45 mM KCl, 105 mM NaCl, 0.05% Triton X-100, 0.1 mM EDTA, and 2 mM dithiothreitol.

End-joining assays used a 754-bp TaqI fragment of pUC18. Significant LX activity with this substrate required Ku as long as LX concentration was not in great excess over DNA ends. Additional requirement for DNA-PKcs was observed when the concentration of Ku also did not greatly exceed the concentration of DNA ends and when concentrations of all components, including DNA ends, were reduced. Reactions were supplemented with 10% polyethylene glycol (v/v) and, except as noted for Fig. 1*C*, preincubated with Ku and DNA-PKcs for 10 min at 25 °C before the addition of LX, 5 mM $MgCl_2$, and 100 μM ATP and transfer to 30 °C. Fig. 1*C* shows wortmannin (10 μM final concentration) added to reactions at the noted times during a 10-min preincubation, performed with $MgCl_2$ and ATP but without LX. Reactions shown in Fig. 1*C* were then started by the addition of LX and transfer to 30 °C. All reactions were stopped by the addition of an equal volume of a solution containing 25 mM TRIS-HCl, pH 8.0, 5 mM EDTA, 0.2% SDS, and 1 mg/ml proteinase K, incubated at 55 °C for 1 h, and extracted with an equal volume of phenol and chloroform, before electrophoresis on a 0.8% agarose gel. Products were visualized by direct DNA staining with SYBR green (Sigma) and by Southern analysis using the 5′-^{32}P-labeled oligonucleotide YR11 (5′-GCGTTTCGGTGATGACGGTGAAAAACCTCTG-3′) as a probe. The amount of product species was determined using a PhosphorImager (Amersham Biosciences) and ImagequaNT software.

The 60-bp double-stranded DNA substrate used in electrophoretic mobility shift analysis assays has been described previously (9). Reactions were assembled in standard buffer and incubated for 10 min at room temperature before cross-linking with 0.06% glutaraldehyde. Fixing with glutaraldehyde was not required for detection of DNA-protein complexes, but this treatment reproducibly improved the definition of retarded species. Complexes were resolved on a 3.5% polyacrylamide gel in 90 mM TRIS borate, pH 8.2, and 1 mM EDTA (1× TBE) at 18 volts/cm.

For exonuclease assays, we used the 754-bp TaqI substrate described above and 0.05 unit of exonuclease V (Amersham Biosciences). This concentration was determined by serial dilution of enzyme to be the minimum concentration of enzyme necessary to degrade ~95% of the full-length substrate in 5 min in the absence of other added proteins. DNA-protein complexes were formed by preincubation at room temperature for 5 min, after which exonuclease V, 5 mM $MgCl_2$, and 500 μM ATP were added. Reactions were then incubated at 30 °C before being stopped and deproteinized at the indicated times also as described for ligation assays. Samples were subjected to electrophoresis on an 8% polyacrylamide gel, and products were visualized with SYBR green DNA stain (Sigma).

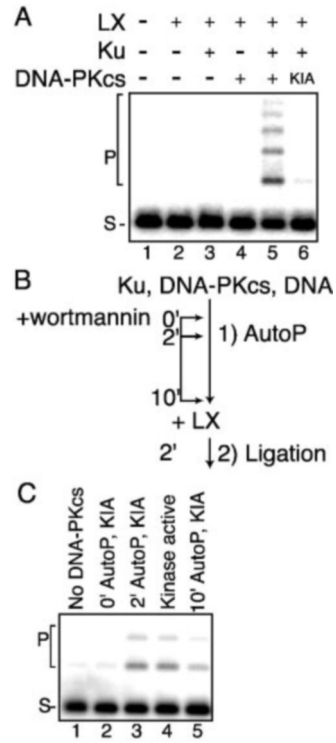

FIG. 1. **Requirement for kinase-active DNA-PK in *in vitro* end joining.** *A*, the 754-bp substrate (*S*) and concatemer ligation products (*P*) were detected by Southern analysis. All reactions contained 1.25 nM substrate and, as indicated by +, 1 nM LX, 2.5 nM Ku, and 5 nM DNA-PKcs. Reactions were stopped 10 min after addition of LX. DNA-PKcs was mock-treated (+) or wortmannin-treated to block kinase activity prior to addition. *B* and *C*, reactions for Fig. 1*C* were performed as in *A*, except with a 10-min preincubation at 25 °C in the absence of LX, and end joining was assessed only 2 min after addition of LX. Kinase activity (*KIA*) was blocked by wortmannin addition at the start of preincubation (*lane 2*, 0′ *AutoP*, *KIA*), or 2 min (*lane 3*, 2′ *AutoP*, *KIA*) and 10 min (*lane 5*, 10′ *AutoP*, *KIA*) after the start of preincubation. In *lane 4*, DNA-PKcs was mock-treated, and ATP and Mg^{2+} were withheld until addition of LX (kinase active). *AutoP*, autophosphorylation.

RESULTS

Requirement for Kinase-active DNA-PK in in Vitro End Joining—We addressed the role of Ku and DNA-PKcs in *in vitro* end joining by the LX complex using a 754-bp linear duplex DNA substrate with 2-bp complementary overhangs. Both DNA-PK subunits are required for significant LX activity when using the conditions described here (Fig. 1*A*, compare *lanes 2–5*; see also "Experimental Procedures"). Importantly, end joining is reduced more than 10-fold when the kinase activity of DNA-PKcs is blocked by pretreatment with wortmannin (Fig. 1*A*, compare *lanes 5* and *6*).

We tested whether autophosphorylation of DNA-PK was sufficient to explain the requirement for kinase activity by separating the reaction into distinct autophosphorylation and ligation stages (Fig. 1*B*). DNA-PK was autophosphorylated to varying degrees in the first stage by addition of wortmannin at the beginning (Fig. 1*C*, *lane 2, 0′ AutoP*), after 2 min (Fig. 1*C*, *lane 3*, *2′ AutoP*), or after 10 min (Fig. 1*C*, *lane 5*,*10′ AutoP*) of preincubation containing everything except LX. In the second stage, LX was added to assess how well autophosphorylated

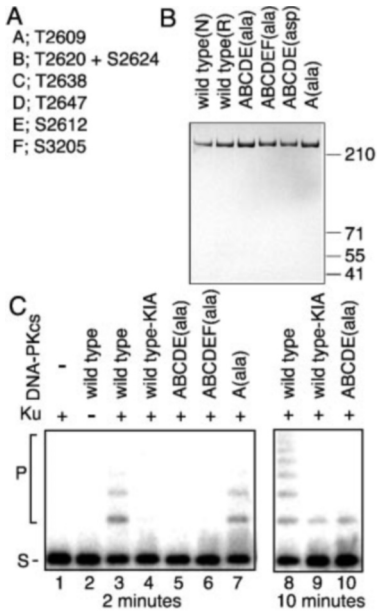

FIG. 2. ***In vitro* end-joining activity of autophosphorylation mutants.** *A*, sites of autophosphorylated serine (*S*) and threonine (*T*) in human DNA-PKcs (identified as in Ref. 17). *B*, Coomassie Blue-stained SDS-PAGE of ~1 μg of purified DNA-PKcs proteins. Native wild type DNA-PKcs (*N*) was purified from human placenta, whereas recombinant wild type DNA-PKcs (*R*) and mutants were purified from DNA-PKcs-deficient cell lines stably expressing appropriate constructs. *C*, reactions were performed as in Fig. 1*A* and stopped 2 min (*lanes 1–7*) or 10 min (*lanes 8–10*) after addition of LX. All DNA-PKcs preparations were wortmannin-treated to block kinase activity (*KIA*) or mock-treated (+) prior to addition.

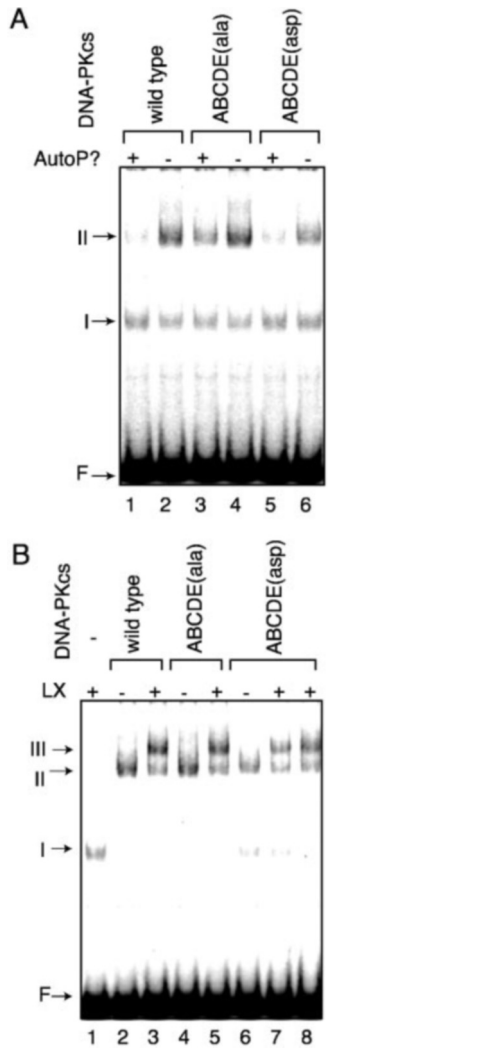

FIG. 3. **DNA-PKcs protein-DNA complexes.** *A*, reactions were incubated for 10 min with 100 nM ^{32}P-labeled 60-bp DNA duplex, 5 nM Ku, 5 mM $MgCl_2$, and 200 μM ATP, and autophosphorylation was controlled by prior mock treatment (*AutoP*+) or wortmannin treatment (*AutoP*−) of 10 nM DNA-PKcs. *F*, free DNA; *I*, Ku-bound DNA; *II*, Ku and DNA-PKcs bound DNA. No complexes are observed without Ku. *B*, complexes were formed as in *A* except without autophosphorylation (no preincubation, $MgCl_2$, or ATP). 10 nM LX and 10 nM various DNA-PKcs preparations are included as noted, except *lane 8* where 25 nM ABCDE(asp) was added. Species *III* contained Ku, DNA-PKcs, and LX. A species with mobility intermediate to *II* and *III*, containing Ku and LX but not DNA-PKcs, was detected only at a reduced salt concentration (75 mM). *AutoP*, autophosphorylation.

DNA-PKcs promoted end joining but now without concurrent kinase activity. As a standard for comparison, we allowed autophosphorylation and ligation to proceed in parallel (essentially as in Fig. 1*A*, *lane 5*) by omitting wortmannin and withholding ATP and Mg^{2+} until the addition of LX (Fig. 1*C*, *lane 4*). Importantly, 2 min of autophosphorylation was sufficient to make wild type DNA-PKcs independent of concurrent kinase activity in the end-joining assay (Fig. 1*C*, compare *lanes 3* and *4*). Autophosphorylation of DNA-PK is thus sufficient to explain the role of kinase activity in this assay, and autophosphorylation need not occur in parallel with end joining to be effective. More exhaustive autophosphorylation (10 min) still promoted significant end-joining activity, but levels were reduced ~2-fold relative to the 2-min autophosphorylation (Fig. 1*C*, compare *lanes 3* and *5*). Therefore, although at least some autophosphorylation is required for end joining, the partial autophosphorylation achieved after 2 min is most effective.

We next purified four DNA-PKcs variants with different mutations at sites of autophosphorylation (Fig. 2, *A* and *B*) and assessed their activity in this assay (Fig. 2*C*). Importantly, ligation activity relative to wild type DNA-PKcs is reduced 10-fold when using a mutant in which autophosphorylation of ABCDE serines and threonines is blocked by alanine substitutions (ABCDE(ala)) (Fig. 2*C*, compare *lanes 3* and *5*). Similar results were obtained with several independent preparations of ABCDE(ala) and also after prolonged incubation (Fig. 2*C*, *lanes 8–10*). Previously, a phosphorylation site outside the ABCDE cluster (serine 3205) was identified (19). Mutants of this site in conjunction with ABCDE sites (ABCDEF(ala)) also behave similarly to ABCDE(ala), both in cellular assays[2] and *in vitro* (Fig. 2*C*, *lane 6*). In contrast, mutation of single sites in the ABCDE cluster (A(ala), C(ala)) did not significantly reduce end joining *in vitro* (Fig. 2, *lane 7*),[3] consistent with the near wild type ability of these mutants to promote radio resistance or V(D)J recombination in cells (17). The requirements of this *in vitro* end-joining assay for autophosphorylation within ABCDE are, thus, identical to requirements previously revealed in cellular end-joining assays (17).

Role of ABCDE Autophosphorylation—Levels of end joining seen with the ABCDE(ala) mutant are similar to those seen when kinase activity of wild type DNA-PKcs is blocked, indicating that autophosphorylation of ABCDE sites is sufficient to explain the role of kinase activity in this assay. However, ABCDE(ala) is remarkably indistinguishable from wild type DNA-PKcs in most other respects. This mutant retains wild type levels of kinase activity as measured using p53 peptide, Artemis (17), or Ku as targets.[2] Electrophoretic mobility shift

[2] K. Meek and Q. Ding, unpublished data.

[3] Y. V. R. Reddy and D. A. Ramsden, unpublished data.

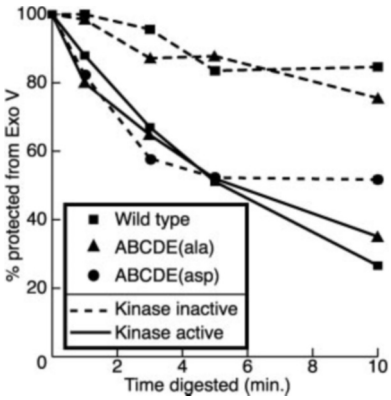

FIG. 4. **Accessibility of DNA ends.** End accessibility was determined by assessing the fraction of full-length substrate remaining over time after the addition of 0.05 unit of exonuclease V to reactions containing 1.25 nM 754-bp substrate, 5 nM Ku, and 10 nM of various DNA-PKcs preparations (*squares*, wild type; *triangles*, ABCDE(ala); *circles*, ABCDE(asp)). DNA-PKcs preparations were mock-treated (*solid lines*, kinase active) or wortmannin-treated to block kinase activity (*dashed lines*, kinase inactive) prior to addition.

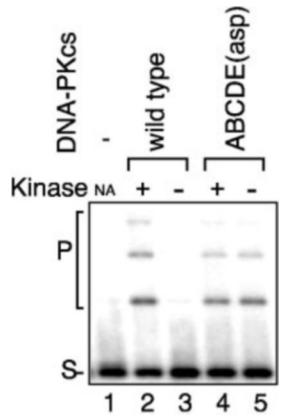

FIG. 5. **Effect of autophosphorylation on end joining.** Reactions were assembled as in Fig. 1*A*. DNA-PKcs was mock-treated (*Kinase +*) or inactivated by wortmannin pretreatment (*Kinase −*). *S*, substrate; *P*, concatemer ligation products.

analysis also indicates that ABCDE(ala) has wild type affinity for Ku-bound DNA ends (17) (Fig. 3*A*, compare *lanes 2* and *4*), and the ability of ABCDE(ala) to promote recruitment of LX to DNA is equivalent to wild type DNA-PKcs (Fig. 3*B*, compare *lanes 3* and *5*).

ABCDE(ala) does not dissociate as fully from Ku-bound ends as does wild type DNA-PKcs after autophosphorylation, but it does dissociate to some extent (17) (Fig. 3*A*, *lanes 1–4*). Autophosphorylation of sites outside the ABCDE cluster is thus largely sufficient for dissociation of DNA-PKcs.

We used the ABCDE(ala) mutant and an exonuclease protection assay to determine whether autophosphorylation of sites outside the ABCDE cluster is sufficient to make DNA ends accessible (Fig. 4). Ku and wild type DNA-PKcs are both required to significantly delay digestion by exonuclease V. Even when both factors are present, linear duplex DNA is well protected only when the kinase is inactivated, consistent with previous studies indicating that autophosphorylation is required for exonucleases to access DNA ends (22, 23) (Fig. 4, compare *solid* with *dashed* lines). Importantly, the ABCDE(ala) mutant is indistinguishable from wild type DNA-PKcs in this assay as well, because autophosphorylation of either protein leads to the parallel progressive digestion of substrate by exonuclease V (Fig. 4, compare *squares* with *triangles*). Wild type DNA-PKcs and ABCDE(ala) thus both allow access to ends on autophosphorylation, but only wild type DNA-PKcs supports high levels of joining by the LX complex. We conclude that simply providing access to ends does not explain why autophosphorylation at ABCDE sites is so critical for cellular end joining.

We suggest instead that ABCDE autophosphorylation may be essential for end-joining activity because it might confer end accessibility, while still allowing DNA-PKcs to remain associated. Persistence of DNA-PKcs at ends would allow it to continue promoting synapsis of ends and stabilizing recruitment of LX, thus enabling LX to take advantage of end synapsis. This hypothesis predicts that DNA-PKcs autophosphorylated only within ABCDE sites should allow access to ends but retain the ability to form a complex with end-joining factors. To test these predictions, we modeled a DNA-PKcs molecule in which autophosphorylation is restricted to ABCDE sites by using a mutant with aspartates (a phosphorylation mimic) substituted at ABCDE sites instead of alanines.

In the presence of ABCDE(asp), ends are sensitive to exonuclease digestion even when kinase activity is blocked by wortmannin (Fig. 4), suggesting that autophosphorylation restricted to ABCDE is sufficient for end access. ABCDE(asp) nevertheless still forms a complex with Ku at DNA ends, although roughly one-third as well as wild type DNA-PKcs (Fig. 3*A*, compare *lanes 4* and *6*). ABCDE(asp) retains kinase activity (17), and autophosphorylation of this mutant at sites outside the ABCDE cluster is required for complete dissociation (Fig. 3*A*, compare *lanes 5* and *6*), as argued previously (17) from the study of the ABCDE(ala) mutant. More significantly, ABCDE(asp) still promotes recruitment of the LX complex (Fig. 3*B*, compare *lane 1* with *lanes 7* and *8*). DNA-PKcs autophosphorylated only within ABCDE thus permits access to ends, maintains affinity for Ku-bound ends, and helps retain LX at ends.

We have shown that at least partial autophosphorylation of DNA-PKcs within ABCDE sites is essential for end-joining activity (Figs. 1 and 2), but the experiments described above suggest it may also be sufficient (*i.e.* autophosphorylation outside ABCDE may be dispensable for end-joining activity in this context). We addressed this possibility by assessing whether ABCDE(asp) was active in the end-joining assay, even when kinase activity was blocked by wortmannin treatment. ABCDE(asp) was only 2-fold less effective than wild type DNA-PKcs in promoting end joining, in contrast to ABCDE(ala) (Fig. 5, compare *lanes 2* and *4*). More importantly, the ability of wild type DNA-PKcs to support end joining requires kinase activity, but ABCDE(asp) does not (Fig. 5, compare *lanes 4* and *5*). This argues that autophosphorylation within ABCDE is both necessary and sufficient for activity of LX *in vitro*.

DISCUSSION

We have developed an *in vitro* end-joining assay using only purified Ku, DNA-PKcs, and LX. These are the factors required for non-homologous end joining in cells, so that our assay reflects the cellular end-joining process. Furthermore, our system depends on the ability of DNA-PKcs to undergo autophosphorylation at ABCDE sites in exact parallel to the situation *in vivo*. Our results using this assay indicate that ABCDE autophosphorylation leads to a destabilization of DNA-PK that is critical for significant end-joining activity. By comparison, autophosphorylation outside the ABCDE cluster has a similar impact on DNA-PK complex stability but does not support end joining. We therefore propose that autophosphorylation within ABCDE is uniquely able to direct a rearrangement of the DNA-PK complex at DNA ends that is essential for end joining both *in vitro* and in cells. This requirement offers important advantages to the end joining repair pathway because it en-

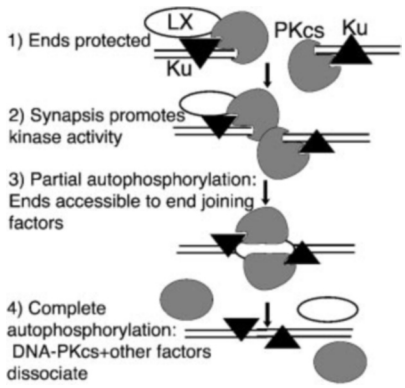

FIG. 6. **Model for the role of DNA-PKcs autophosphorylation in end joining.** *Open ovals*, LX; *filled triangles*, Ku; *gray ovals*, DNA-PKcs.

sures the seamless coupling of each step in end joining to the next as described below (Fig. 6).

Ku and DNA-PKcs initially recruit LX and other end-joining factors to DNA ends, but ends remain inaccessible even to factors within this complex when autophosphorylation is blocked (Fig. 1*A*) (22–24). Autophosphorylation (and kinase activity in general) is stimulated when ends are brought together (synapsis) (Fig. 6, *step 2*) (14, 22). Autophosphorylation thus links end accessibility to synapsis, helping to ensure that only enzymes specific to this pathway, and thus already present within the synaptic complex, can act on ends. The ABCDE(asp) mutant uncouples accessibility from synapsis. This "partially autophosphorylated" DNA-PKcs has only intermediate levels of end joining relative to wild type and the ABCDE(ala) mutant, both *in vitro* and in cellular assays (17). However, reduced effectiveness of the ABCDE(asp) mutant is particularly evident in cells, probably reflective of the greater risk posed by indiscriminate end accessibility in a cellular environment.

Once the kinase is activated, autophosphorylation occurs both within the ABCDE cluster and at other sites that are currently being characterized. Our data indicate that ABCDE and other sites are autophosphorylated independently, and either set of autophosphorylations alone is sufficient to make ends accessible (Fig. 4). Therefore, we argue that the primary problem with cells expressing the ABCDE(ala) mutant is not that this mutant blocks the ability of processing factors to access ends. In support of this argument, hairpin intermediates in V(D)J recombination are opened (processed) with similar efficiency in cells expressing either wild type DNA-PKcs or the ABCDE(ala) mutant, but these processed intermediates are not joined in cells expressing ABCDE(ala) (17). Moreover, the ABCDE(ala) mutant is also unable to promote wild type levels of signal end joining in cells, a reaction that does not require processing (17). The primary problem with end joining in cells expressing ABCDE(ala) must therefore be that they are unable to couple synapsis with end access and LX activity (and probably the activity of other end-joining factors as well). This is presumably because ABCDE autophosphorylation of DNA-PKcs is uniquely able to combine end accessibility with its retention at ends (Fig. 6, *step 3*). Although bound to ends in this manner, DNA-PKcs can continue fulfilling its role in promoting end synapsis and the stable recruitment of LX until repair is complete.

Autophosphorylation restricted to either ABCDE sites or outside sites has an equivalent impact on how well DNA-PKcs interacts with Ku-bound DNA ends (Fig. 3*A*, compare *lane 2* with *lanes 3* and *6*). However, our data suggest that autophosphorylation within ABCDE allows access to ends prior to DNA-PKcs dissociation, whereas autophosphorylation of outside sites permits access only when a sufficient number of sites have been phosphorylated so that DNA-PKcs dissociates. This suggests a clear mechanistic difference in how the two types of autophosphorylation affect DNA interactions. It is likely that ABCDE autophosphorylation alters the DNA-PK complex by limiting its impact on the DNA binding activity of DNA-PKcs to the specific disruption of interactions involved in end protection (Fig. 6, *step 3*). In contrast, autophosphorylation at sites outside the ABCDE cluster might disrupt an alternate DNA-binding site not involved in end protection or indirectly impact DNA binding by disrupting interactions with Ku. Consistent with the former idea, biochemistry and electron microscopy support a two-site model for DNA-PKcs interaction with DNA, in which one site interacts with DNA ends, whereas the other independently interacts with the DNA backbone, 15–20 bp removed from the end (25).

Continued autophosphorylation of DNA-PKcs leads to a progressive reduction in DNA-binding activity and eventually complete dissociation (Fig. 6, *step 4*) (20, 21). This may be required for processing events that require a greater degree of access to ends than does ligation. More likely, because DNA-PKcs has a role in stabilizing recruitment of LX (Fig. 3*B*) (10, 13), Artemis (4), and likely other factors as well, dissociation of DNA-PKcs may trigger the general disassembly of the entire end-joining complex, with the possible exception of Ku. Disassembly of the end-joining complex could be important in making the successfully repaired junction accessible for subsequent transactions (*e.g.* transcription and replication). Alternatively, the eventual disassembly of end-joining factors from breaks when repair fails may help cells by making these breaks accessible to other repair pathways. The latter argument is suggested by the ability of a specific inhibitor of DNA-PKcs kinase activity to disrupt homologous recombination-dependent repair of DSBs (26).

DSBs can still be repaired by end joining in the absence of DNA-PK (Ku and DNA-PKcs), but this "backup" pathway is less accurate (27–29) and more prone to cancer-causing translocations (30) than when DNA-PK is present. We suggest that a major reason for this is the ability of ABCDE autophosphorylation to ensure that once ends have been recognized by DNA-PK only the factors appropriate to this pathway, and thus present with DNA-PK in the synaptic complex, can be employed productively. On the other hand, processing events that are not coupled to synapsis will be blocked, explaining how DNA-PK makes end joining more accurate.

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