Implication of DNA-Dependent Protein Kinase in an Early, Essential, Local Phosphorylation Event during End-Joining of DNA Double-Strand Breaks in Vitro†

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ABSTRACT: Previous work with *Xenopus* egg extracts suggested that a wortmannin-sensitive protein phosphorylation event precedes both the removal of modified termini from DNA double-strand break ends and the joining of unmodified ends. To assess the possible role of DNA-dependent protein kinase in effecting this phosphorylation, both DNA end-joining and DNA-stimulated phosphorylation were examined in the presence of various inhibitors. Linear but not supercoiled DNA stimulated the phosphorylation of several endogenous proteins in the extracts, including species of approximately 48, 87, and 96 kDa. This phosphorylation was selectively suppressed by the kinase inhibitors wortmannin, dimethylaminopurine, and LY294002, with a dose response that in each case paralleled the inhibition of DNA end-joining. If wortmannin was added while the end-joining reaction was in progress, end-joining of DNA already present in the reaction continued for some time, but newly added DNA was not joined or processed at all. Ends with 3′-hydroxyl termini were joined much faster than those with 3′-phosphoglycolate termini, although both were equally effective in stimulating protein phosphorylation. The results support a role for DNA-dependent protein kinase in regulating end-joining in vitro, and suggest that at least one of the necessary phosphorylations involves a protein bound at or near the DNA end to be joined. In contrast, nuclear extracts from human cells joined double-strand breaks with normal but not modified termini, and the joining was unaffected by kinase inhibitors, suggesting that the dominant mechanism of end-joining in these extracts did not involve DNA-PK.

DNA-dependent protein kinase (DNA-PK)[1] is one of a family of protein serine-threonine kinases that have stronger homology to phosphatidylinositol 3-kinase (PIK) than to other protein kinases (*1*). DNA-PK consists of the catalytic subunit DNA-PK_{CS} (465 kDa) plus the two subunits (70 and 86 kDa) of the DNA end-binding heterodimer Ku (*2*, *3*). Binding of Ku to DNA ends activates DNA-PK, whose in vitro substrates include a wide array of DNA-binding proteins (*4*).

Genetic and biochemical data have strongly implicated DNA-PK in double-strand break repair in mammalian cells (reviewed in refs *5* and *6*), but its precise function in repair and the critical target proteins involved are not known. Cytogenetic data, however, have indicated that in *scid* cells [which express a mutant DNA-PK protein lacking kinase activity (*7*, *8*)] there is less overall rejoining of radiation-induced double-strand breaks, but more apparent misjoining of exchanged ends, than in normal cells (*9*). These data suggest a possible regulatory role for DNA-PK in matching correct ends to be joined.

DNA ends formed as a result of oxidative double-strand cleavage by radiation or radiomimetic drugs usually bear modified termini, most commonly 3′-phosphoglycolates (PGs), 3′-phosphates, and in a few cases 5′-aldehydes (*10*−*13*). These ends therefore require additional processing beyond simple religation. In this respect, it is intriguing that murine *scid* cells, which have only a small carboxy-terminal truncation in DNA-PK_{CS}, show a specific deficiency in V(D)J recombination in that they are able to form signal joints, which result from the joining of normal blunt ends, but are unable to form coding joints, which require the resolution and joining of covalently closed hairpin ends (*5*). By contrast, equine *scid* cells, which lack the entire kinase domain of DNA-PK_{CS}, can form neither signal nor coding joints (*14*). These results suggest that DNA-PK may play multiple roles in end-joining, including a specific role in the processing of modified double-strand break termini.

Although DNA end-joining has been detected in various mammalian cell extracts (*15*−*18*), there is as yet no convincing evidence that these systems reflect DNA-PK-mediated double-strand break repair. However, in *Xenopus* egg extracts, not only DNA end-joining but also processing of damaged DNA ends prior to joining is suppressed by DNA-PK inhibitors (*19*). Here, we present evidence that this suppression is indeed a consequence of the inhibition of DNA-PK, and that at least one of the critical phosphorylations is a local event, specific to the DNA end to be joined.

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[1] Abbreviations: DNA-PK, DNA-dependent protein kinase; PIK, phosphatidylinositol 3-kinase; PG, phosphoglycolate.

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EXPERIMENTAL PROCEDURES

Extracts. *Xenopus laevis* eggs were collected from gonadotropin-induced females (NASCO). Following activation of the eggs with calcium ionophore A23187 (Sigma), extracts were prepared by ultracentrifugation (*20*) and frozen in small aliquots in liquid N_2.

Nonadherent human REH lymphoblastoid cells (generous gift of Dr. Steven Grant) were grown in 20 × 100-mm bacterial culture dishes in RPMI1640 medium supplemented with 10% fetal bovine serum to a density of 8×10^5/mL, and a nuclear extract was prepared from 500 mL of cells as described by North et al. (*16*). Nuclear extracts from human A549 lung carcinoma cells, MO59K glioma cells, and the matched DNA-PK-deficient line MO59J (*21*, *22*) were prepared by the procedure of Olnes and Kurl (*23*). MO59J and MO59K cells were generously provided by Dr. Joan Turner.

DNA Substrates. Linear plasmid substrates bearing 3′-PG termini were prepared by ligation of isolated 3′-PG oligomers into appropriately modified plasmids, as described (*24*) but with several modifications. The initial 3′-PG oligomers were generated by treatment of the end-labeled partial DNA duplexes with Fe(III)•bleomycin for 1 h at 4 °C in the presence of 1 mM H_2O_2, as described by Suh et al. (*25*). Following addition of EDTA to 20 mM and formamide to 50% v/v, the sample was loaded onto a 22% denaturing polyacrylamide gel (20 × 60 × 0.08 cm) which was electrophoresed for 20 h at 2200 V. Under these conditions, there is very little breakdown of bleomycin-induced abasic sites to form 3′-phosphate-terminated breaks, and the 3′-PG fragments resulting from direct sequence-specific strand breakage are well-separated from other minor cleavage products. These fragments were visualized by autoradiography, cut from the gel, eluted by diffusion, and purified by HPLC on a Rainin Microsorb 5 μm, 4.6 × 250 mm C_{18} column, using a 30 min gradient of 5−50% acetonitrile in water, containing 0.1 M triethylammonium acetate, pH 7. The labeled oligomers were detected in the HPLC eluate by their ^{32}P radioactivity using an in-line Geiger counter, and the concentration was determined from the peak area on the absorbance monitor (260 nm). The oligomers usually eluted within a volume of 1 mL, and were evaporated to dryness and dissolved in H_2O at a concentration of 0.5−5 μM.

To obtain very high specific activity 3′-PG oligomers, 0.3 nmol of the initial 17-mer (*24*) was labeled with 1 mCi of [γ-^{32}P]ATP (6000 Ci/mmol, New England Nuclear). Annealing, cleavage, and fragment isolation were performed as described (*25*), except that the bleomycin concentration was reduced proportionately.

Preparation of plasmids with 10- and 11-base 3′-recessed ends, and ligation of the 3′-PG oligomers into the ends, was performed as previously described (*24*), except that after the ligation reaction and phenol and chloroform extractions the substrate was ethanol-precipitated at 22 °C in the presence of 2 M ammonium acetate and then again in the presence of 0.3 M sodium acetate, before the second ligation step or treatment with T7 polymerase. These precipitation steps consistently gave much higher substrate recovery than ultrafiltration but were equally effective in removing unligated oligomer.

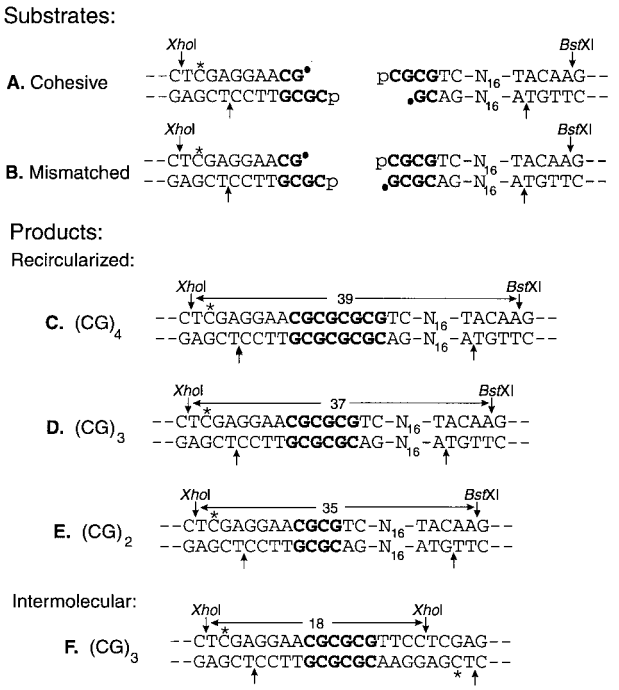

FIGURE 1: End-joining substrates and repair products. The two ends of the linearized plasmid substrates are shown, with "•" indicating a 3′-PG terminus and "*" indicating the position of ^{32}P label. The 3′-PG substrates were prepared by ligation of 3′-PG oligonucleotides into 5′ overhangs generated in *Mlu*I-cut pSV56 (*24*). A comparable 3′-hydroxyl substrate with a 5′-CG overhang (not shown) was prepared by cleavage of pSV56 with *Bsa*HI.

To obtain a vector similar to the cohesive-end construct (Figure 1A), but with 3′-hydroxyl termini, pSV56 (*24*) was treated with *Bsa*HI, which cuts at two sites 382 bp apart in the *bla* gene and leaves 2-base 5′ CG overhangs. Following treatment with calf intestinal phosphatase and 5′-end-labeling with polynucleotide kinase (*26*), the longer 5.1-kb fragment was purified by agarose gel electrophoresis.

End-Joining Assays. Reactions contained 16 μL of egg extract plus linear DNA (usually 10 ng) and inhibitors in a total volume of 20 μL. Wortmannin (Sigma) and LY294002 [2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; Calbiochem] were dissolved and diluted in DMSO, and 1 μL of an appropriate dilution was added to each sample. Dimethylaminopurine was dissolved in water at 60 °C at a concentration of 50 mM. Reactions were incubated at 13 °C for various times, and terminated by quickly freezing in liquid nitrogen. Each sample was then diluted into 280 μL of 1 mg/mL proteinase K/20 mM Tris (pH 7.6)/0.3 M NaCl/10 mM EDTA/1% SDS and incubated at 65 °C for 3 h. Following phenol and chloroform extractions and ethanol precipitation, samples were digested with the appropriate restriction enzymes. For the PG-terminated constructs, each sample was treated with 10 units of *Bst*XI for 3 h at 55 °C, ethanol-precipitated, treated with 100 units of *Xho*I for 16 h at 37 °C, ethanol-precipitated, and dissolved in 10 μL of formamide loading solution. For the hydroxyl-terminated (*Bsa*HI-cut) vector, digestion was with *Nla*III (10 units, 3 h). All digestions were in 50 μL of the buffer provided by the vendor (New England Biolabs). Bovine serum albumin at 100 μg/mL was added to the *Xho*I digestion. Robust treatment with *Xho*I was required, probably due to the proximity of the cleavage site to the DNA end.

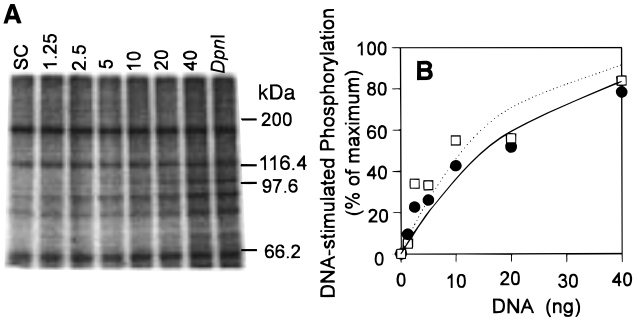

FIGURE 2: Stimulation of protein phosphorylation by DNA ends in *Xenopus* egg extracts. (A) Extracts were incubated for 10 min in the presence of [γ-^{32}P]ATP plus various amounts (in ng, as indicated) of a linear, cohesive-end DNA substrate bearing 3′-hydroxyl termini. Proteins in the extracts were separated by polyacrylamide gel electrophoresis, and phosphorylation was detected by phosphorimaging. *Dpn*I indicates incubation in the presence of 10 ng of *Dpn*I-cut pSV56, giving 5 nM DNA ends, and SC indicates incubation in the presence of 10 ng of supercoiled DNA, which gave a pattern indistinguishable from that seen with no DNA (not shown). 40 ng of linear plasmid corresponds to 1 nM DNA ends. (B) Quantitative analysis of DNA-stimulated phosphorylation. Following incubation in the presence of [γ-^{32}P]-ATP plus various amounts of linear DNA bearing 3′-hydroxyl (●—●) or 3′-PG (□···□) termini, the intensity of the 96-kDa protein band was determined and normalized to that for a sample containing 10 ng of *Dpn*I-cut DNA.

For end-joining assays in mammalian cell extracts, 50-μL reactions contained 5−20% v/v of nuclear extracts (giving a final protein concentration between 40 and 100 μg/mL) and 20 ng of labeled DNA in either buffer A [70 mM Tris-HCl (pH 7.5)/10 mM $MgCl_2$/1 mM ATP/10 mM dithiothreitol] or buffer B [20 mM Tris-HCl (pH 7.9)/3 mM $MgCl_2$/1 mM ATP/10 mM dithiothreitol]. Buffer A was previously shown to promote end-joining of restriction enzyme-induced DNA breaks in mammalian cell extracts (*16*), but was found to strongly suppress end-joining in *Xenopus* egg extracts; buffer B is essentially the same as the buffer in the egg extracts (*20*). Following incubation, usually for 4 h at 17 °C, samples were deproteinized and analyzed as above.

Protein Phosphorylation Assays. Reactions in *Xenopus* egg extracts (20 μL) were performed as above, and included either defined DNA substrates or fragments generated by *Dpn*I cleavage of pSV56, plus 10 μCi of [γ-^{32}P]ATP (6000 Ci/mmol, New England Nuclear). Following incubation for 10 min at 13 °C, an equal volume of 0.5 M Tris-HCl (pH 6.8)/4% sodium dodecyl sulfate/2% glycerol/1% β-mercaptoethanol/0.5% bromophenol blue was added, and proteins were denatured at 90 °C for 3 min. Aliquots (5 μL), along with appropriate molecular weight markers (Bio-Rad), were loaded onto 8 × 5.5 × 0.08 cm denaturing gels [containing 7.5% polyacrylamide (37.5:1 cross-linked)/0.1% SDS/0.375 M Tris-HCl, pH 8.8] overlayered with a stacking gel (1.5 cm, containing 4% polyacrylamide/0.1% SDS/0.125 M Tris-HCl, pH 6.8). Gels were electrophoresed for 45 min at 200 V at 22 °C in a running buffer of 0.025 M Tris-HCl (pH 8.3)/0.192 M glycine/0.1% SDS. One gel plate had been coated with γ-methacryloxypropyltrimethoxysilane (Sigma; 2% in ethanol) so that the gel would adhere to the plate, and after electrophoresis, the gel was stained with Coomassie blue, rinsed extensively with water, and dried on the plate. ^{32}P incorporation was quantitated by phosphorimaging of the gels, with exposure usually for 16 h. The intensity of a DNA-dependent band migrating at ~96 kDa was determined and normalized to the total ^{32}P incorporation in each lane, and this intensity was taken as a measure of DNA-dependent protein phosphorylation.

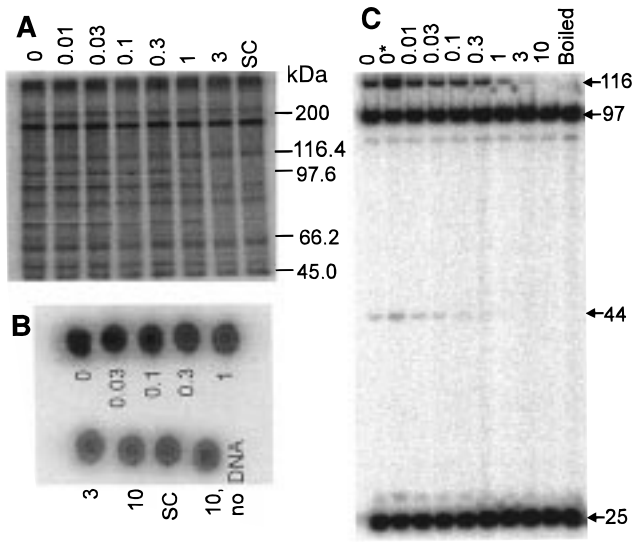

FIGURE 3: Effect of various concentrations of wortmannin (in μM, as indicated) on DNA-stimulated phosphorylation (A, B) and on DNA end-joining (C). *Dpn*I-cut pSV56 (10 ng) was added to all samples in (A) and (B), except for a control which contained 10 ng of supercoiled pSV56 ("SC"). All samples except that marked "0*" in (C) contained 5% DMSO (the solvent for wortmannin). In (A), which shows phosphorylation of endogenous proteins, note the gradual loss of the 87- and 96-kDa protein bands as the concentration of wortmannin is increased. Phosphorylation of the DNA-PK-specific peptide EPPLSQEAFADLWKK was similarly inhibited (B). The end-joining substrate in (C) was 10 ng of a 5′-end-labeled linear DNA with cohesive ends and normal 3′-hydroxyl termini. Following incubation in the extracts for 10 min, the substrate was cut with *Nla*III, to release the two labeled ends as 25- and 97-base fragments. Joining was calculated as the sum of the 116-base fragment (resulting from recircularization of the plasmid) and the 44-base fragment (resulting from head-to-head intermolecular joining), expressed as a percent of total end-labeled fragments. (Fragment lengths are not additive due to the differences in the stagger of restriction cuts.)

Phosphorylation of the biotinylated peptide EPPLSQEAF-ADLWKK (a modification of the phosphorylation site in p53) was determined using a DNA-PK assay kit (Promega). Reactions (25 μL) contained DNA (10 ng of *Dpn*I-cut pSV56), 10 μCi of [γ-^{32}P]ATP, inhibitor, and 20 μL of extract. Following incubation for 10 min at 13 °C, a 10-μL aliquot was applied to a streptavidin-coated membrane, which was washed according to the manufacturer's instructions and analyzed by phosphorimaging.

RESULTS

DNA Ends Stimulate Protein Kinase Activity in Xenopus Egg Extracts. Stimulation of protein phosphorylation by the presence of DNA ends, the hallmark of DNA-PK, has been detected in extracts from a variety of sources, including *Xenopus* eggs (*27*). Moreover, loss of this activity in egg extracts was shown to be associated with cleavage of an apparent *Xenopus* homologue of DNA-PK_{CS} (*28*), a segment of which was recently cloned and found to have 65% identity with the human protein (*29*). To determine whether this activity was expressed under the conditions of DNA end-joining assays, incorporation of ^{32}P radioactivity from

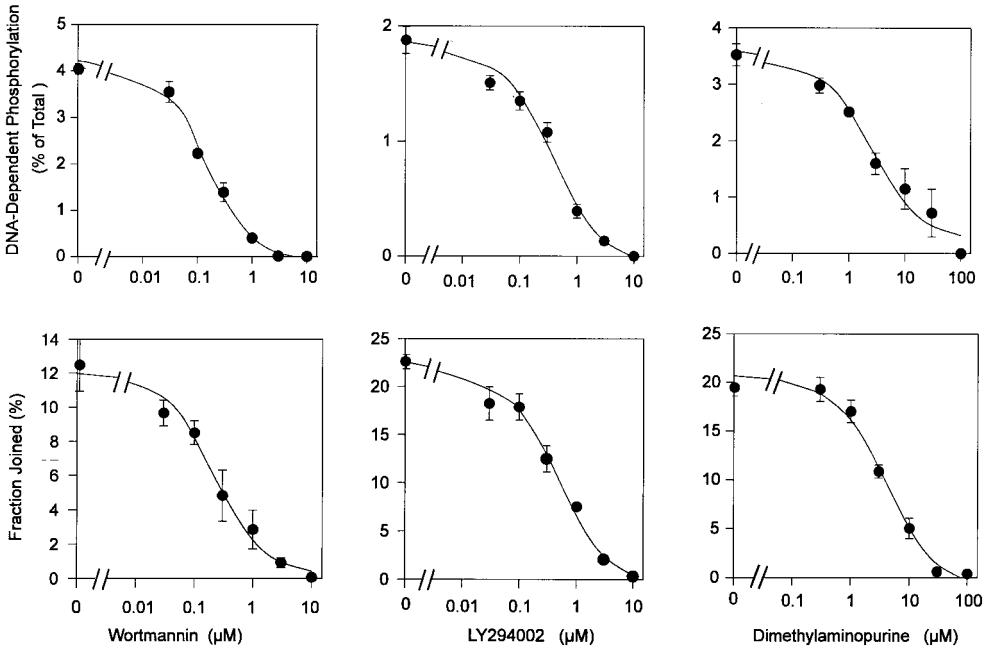

FIGURE 4: Dose responses for inhibition of DNA-stimulated phosphorylation of endogenous proteins (top panels) and on DNA end-joining (bottom panels), by wortmannin, LY294002, and dimethylaminopurine. Reaction conditions are as in Figure 3. Data shown are the mean ± SD of 3 independent experiments with each inhibitor. The control values for fraction joined and fraction of total phosphorylation are different for each inhibitor because different batches of extract were used.

[γ-^{32}P]ATP into endogenous proteins in the extracts was assessed.

Addition of 10 ng of *Dpn*I-cut plasmid pSV56 (5 nM DNA ends) induced the phosphorylation of a number of specific endogenous proteins in the extracts, while addition of the same amount of supercoiled DNA did not stimulate phosphorylation at all (Figures 2 and 3A). Although the pattern of protein phosphorylation varied considerably among different batches of extract, the most prominent phosphorylation targets usually were species of approximately 48, 87, and 96 kDa. Double-stranded DNA linked to cellulose beads likewise stimulated phosphorylation of these proteins; however, the labeled proteins were recovered mainly in the supernatant (data not shown), suggesting that the proteins were not tightly bound to DNA, at least in their phosphorylated forms. The 96-kDa species showed the strongest and most consistent dependence of phosphorylation on DNA ends, and so was used for quantifying kinase activity in subsequent experiments. Phosphorylation of the peptide EPPLSQEAFADLWKK [considered to be a relatively specific substrate of DNA-PK (*30*)] was also stimulated by *Dpn*I-cut but not supercoiled pSV56 (Figure 3B).

We previously showed that linear DNA substrates with 3′-PG termini were joined 30−100 times more slowly than comparable substrates with normal 3′-hydroxyl ends (*19*). To determine whether this difference was due to an intrinsic inability of the PG-terminated ends to efficiently stimulate kinase activity, parallel phosphorylation assays were performed using various amounts of cohesive-ended substrates bearing either 3′-PG or 3′-hydroxyl termini. As shown in Figure 2, phosphorylation of the 96-kDa protein was stimulated to an equal extent by linear DNA with either type of terminus, approaching a plateau level at 40 ng of plasmid, or 1 nM DNA ends. Similar results have been obtained using purified human DNA-PK in a peptide phosphorylation assay (*31*). Thus, although the results are not necessarily applicable to all targets of DNA-dependent phosphorylation, it appears that in general the degree of stimulation is not strongly dependent on the chemical structure of the DNA termini.

These results are consistent with the proposal that there is a *Xenopus* homologue of human DNA-PK, and that it is active in the egg extracts used for end-joining.

Inhibition of DNA-Dependent Kinase Activity Parallels Inhibition of End-Joining. We previously found that the concentration of wortmannin required to inhibit end-joining in *Xenopus* egg extracts (*19*) was approximately the same as that required to inhibit the phosphorylation of synthetic peptides by purified human DNA-PK (*2*). However, there are several sources of uncertainty in this correlation, including differences in incubation temperature and ATP concentration [which can alter the kinetics of inhibition (*32*)] and possible intrinsic differences between the human and *Xenopus* homologues. To compare DNA-stimulated kinase activity and end-joining activity more directly, each was measured in the same extracts under as nearly identical conditions as possible (Figure 3). For these assays, samples were harvested after 10 min of incubation, when <25% of the DNA had been joined and end-joining was still proceeding approximately linearly with time. A dose response for inhibition by each of three DNA-PK antagonists, wortmannin (*2*), LY294002 (*33*), and dimethylaminopurine (*34*), was obtained.

For all three inhibitors, dose responses for inhibition of end-joining of cohesive 3′-hydroxyl ends paralleled those for inhibition of DNA-stimulated protein kinase activity, as judged by the extent of DNA-stimulated phosphorylation of the endogenous 96-kDa substrate (Figure 4). Half-maximal inhibition required 0.2 μM wortmannin, 0.3 μM LY294002, or 3 μM dimethylaminopurine. Moreover, these antagonists also inhibited phosphorylation of the peptide EPPLSQEAFADLWKK, with similar dose response curves, and high concentrations of this peptide (>1 mM) partially but specif-

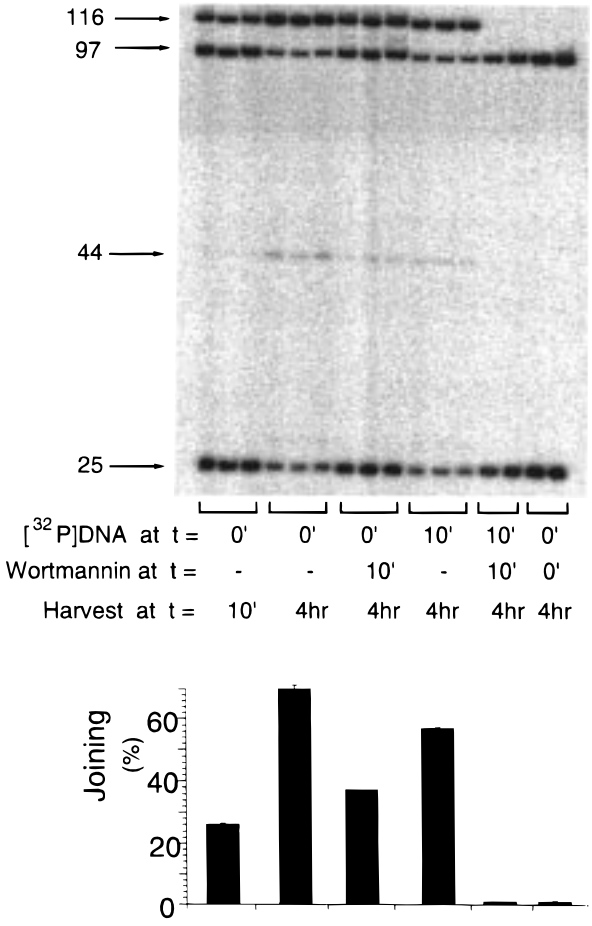

FIGURE 5: Continued end-joining of a 3′-hydroxyl substrate following wortmannin addition. The cohesive 3′-hydroxyl substrate (10 ng, either labeled or unlabeled) was added to *Xenopus* egg extract. After 10 min, an additional 5 ng of either labeled or unlabeled substrate was added, and the samples were incubated for 4 h. Wortmannin was also added at the times indicated; for the samples with no wortmannin, DMSO was added at 10 min to give the same concentration as in the wortmannin-containing samples (5%). End-joining was assessed as in Figure 3. Error bars show mean ± SD for the three replicate samples for each condition.

ically inhibited DNA-dependent protein phosphorylation, as well as DNA end-joining (Figure 3B and data not shown). These results provide additional evidence that DNA-PK is responsible for the putative wortmannin-sensitive phosphorylation step in the end-joining pathway. The results at least implicate some PIK-related protein kinase, rather than PIK itself, since wortmannin inhibits PIK at 100-fold lower concentrations (*35*).

Kinetic Data Suggest That a Local Phosphorylation Event Precedes End-Joining. The suppression of end-joining by DNA-PK inhibitors could reflect either a requirement for an increase in the overall phosphorylation level of one or more protein(s) in the extract, or a requirement for phosphorylation of some specific protein stably bound to DNA in each end-joining complex. Kinetic studies were performed in an attempt to distinguish between these possibilities (Figure 5). Following a 10-min incubation of a labeled or unlabeled 3′-hydroxyl cohesive-end substrate in the extract, wortmannin was added at a concentration sufficient to suppress end-joining completely; additional labeled or unlabeled substrate was then added and the reaction continued for 4 h. Thus, all reactions contained precisely the same amounts of substrate, and differed only in the timing of addition of wortmannin and of *labeled* substrate.

When labeled substrate was present from the beginning of the reaction, joining was consistently greater for samples incubated for 4 h after wortmannin addition than for samples harvested immediately after the initial 10-min incubation (kinetic studies indicated that most of this joining occurred within 15 min of wortmannin addition; data not shown). However, there was no detectable joining of labeled substrate added at the same time as wortmannin, even when extracts were preincubated for 10 min in the presence of an identical, unlabeled substrate. This lack of joining was not due to saturation of end-joining activity by the initially added substrate, since in the absence of wortmannin, labeled substrate added after the initial 10-min incubation was efficiently joined. Thus, following addition of wortmannin, DNA substrates that had been preincubated in the extracts continued to be joined, but any newly added substrates were not joined. This result implies that there was some local, wortmannin-sensitive event which occurred at each DNA end and was essential to its subsequent religation. Since there is no evidence for chemical modification of either the 5′-phosphate or the 3′-hydroxyl termini prior to ligation, the most likely candidate for this local event is a modification (such as phosphorylation by DNA-PK) of some protein in an end-bound complex.

For 3′-PG Substrates, a Wortmannin-Sensitive Event Is Rate-Limiting. We previously showed that end-joining of 3′-PG substrates by *Xenopus* egg extracts was 30−100 times slower than that of comparable 3′-hydroxyl substrates, and that this difference was primarily due to very slow removal of the 3′-PG termini (*19*). However, since a wortmannin-sensitive phosphorylation step apparently precedes 3′-PG removal, either the putative wortmannin-sensitive phosphorylation step or the subsequent PG removal could be rate-limiting. If the protein phosphorylation is rapid, but 3′-PG removal is slow, then there should be an accumulation of phosphorylated repair complexes which would continue to undergo slow 3′-PG removal and end-joining after DNA-PK inhibitors are added.

However, Figure 6 shows that this clearly was not the case. Although end-joining of both cohesive and mismatched-end 3′-PG substrates was slow and inefficient, joining abruptly stopped upon addition of wortmannin. Moreover, although 3′-PG groups were removed from a substantial fraction (up to 10%) of the mismatched ends, PG removal also stopped abruptly. Thus, there was little or no accumulation of the putative phosphorylated protein required for end-processing, either locally or in the extract as a whole. Rather, continued end-joining was contingent on continued DNA-PK activity. Addition of excess cohesive-end 3′-hydroxyl substrate appeared to stimulate end-processing somewhat, yet the majority of 3′-PG ends remained unprocessed, confirming that the slow processing of these ends was not due to insufficient activation of DNA-PK in the extract as a whole (see Figure 2).

Human Cell Extracts Lack Detectable DNA-PK-Dependent End-Joining Activity. While *Xenopus* egg extracts may provide an in vitro model for DNA-PK-based rejoining of DNA double-strand breaks, a mammalian model would be potentially more informative, since there may be significant interspecies differences in end-joining pathways, and the

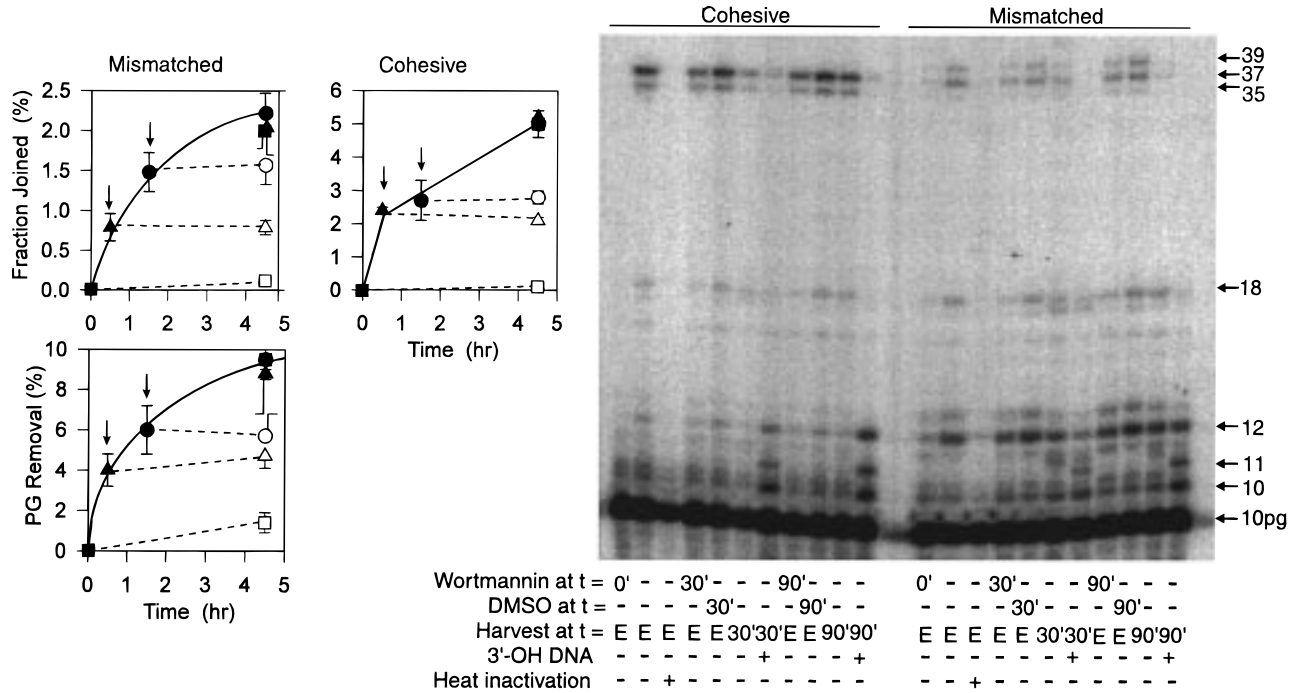

FIGURE 6: Arrest of processing of 3′-PG substrates upon addition of wortmannin. The gel shows an experiment in which either the mismatched or the cohesive 3′-PG substrate (10 ng; see Figure 1) was incubated in egg extract (or heat-inactivated extract). Wortmannin or DMSO (solvent control) was added at various times. Samples were harvested at the times indicated; "E" denotes harvest at the end of the experiment, a total incubation time of 4.5 h. In some cases, 3′-hydroxyl DNA (40 ng of an unlabeled 5.1-kb *Bsa*HI fragment bearing the same 2-base 5′ overhang) was also added to stimulate DNA-PK. All samples were then cleaved with *Xho*I and *Bst*XI, and analyzed on sequencing gels. The 10 pg fragment is the unaltered 3′-PG terminus, and the 10-, 11-, and 12-mer fragments are 3′-hydroxyl intermediates (the 11- and 12-mers resulting from fill-in of the 5′-overhang). The 35-, 37-, and 39-mer fragments are recircularization products, and the 18-mer is a head-to-head intermolecular ligation product (see Figure 1). Graphs show quantitative analysis of these and similar end-joining and PG removal data (mean ± SD of 3−4 experiments). At 0, 30, or 90 min (arrows), samples were either harvested immediately or subjected to continued incubation in the presence of wortmannin (dotted lines, open symbols) or DMSO (solid lines, closed symbols). End-joining was calculated as the total of the 35-, 37-, 39-, and 18-mer bands, expressed as a fraction of the total label in the lane. PG removal was calculated as the sum of all 3′-hydroxyl intermediates plus all end-joining products, as a fraction of the total. Because of the low levels of 3′-hydroxyl intermediates with the cohesive substrate, total PG removal was very similar to total end-joining and so is not shown.

genetics of DNA-PK-based repair have been developed almost exclusively in mammalian systems. A variety of human cell extracts were therefore screened for wortmannin-sensitive end-joining activity, using both 3′-PG and 3′-hydroxyl-terminated substrates. Nuclear extracts from REH cells (Figure 7) and from several other human cell lines (data not shown) efficiently joined ends bearing 2-base cohesive 5′ overhangs and 3′-hydroxyl termini, but this joining was not sensitive to wortmannin, and occurred even in extracts of MO59J cells (Figure 8), which lack detectable DNA-PK activity (*21*, *22*). None of the extracts showed detectable joining of cohesive-end 3′-PG substrates, and there was little or no removal of the PGs. Since these extracts could remove 3′-PGs from internal single-strand breaks (data not shown), the lack of removal from double-strand break ends is presumably due to intrinsic resistance of double-strand break end 3′-phosphodiesterase(s) (*25*) and/or sequestration of the DNA ends by end-binding proteins such as Ku.

Thus, while the observed end-joining in human cell extracts may or may not be related to double-strand break repair as it occurs in intact cells, the joining appears not to involve DNA-PK.

DISCUSSION

The proposal that DNA end-joining as detected in *Xenopus* eggs and egg extracts might be related to rejoining of double-strand breaks in mammalian cells was initially prompted by certain similarities in specificity. In particular, when plasmids containing site-specific double-strand breaks with noncomplementary overhangs were either treated with *Xenopus* egg extracts (*36*) or transfected into mammalian cells (*37*), the plasmids were recircularized, and the junction sequences at the double-strand break sites were similar in the two systems. For example, both systems could apparently join two ends, one with a 3′- and one with a 5′-overhang, without losing a single base from either overhang. This phenomenon was initially referred to as "single-strand ligation" (*37*), but based on additional experiments in the *Xenopus* system (*38*), it was later suggested that it occurred as a result of templated polymerization across the discontinuity, presumably while the two overhangs were held in juxtaposition by some "alignment protein". Similarly, the junctions derived from joining of ends with partially complementary overhangs suggested that both systems could use complementarities of as little as a single base pair to align the overhangs, and could then trim and patch the overhangs as necessary to fully restitute a continuous DNA duplex. These results likewise suggested that there must have been an alignment factor which maintained the juxtapositioning and pairing of the ends during the putative trimming and patching steps.

More recently, we extended the comparison between *Xenopus* and mammalian systems to the end-joining of free radical-mediated double-strand breaks bearing blocked 3′

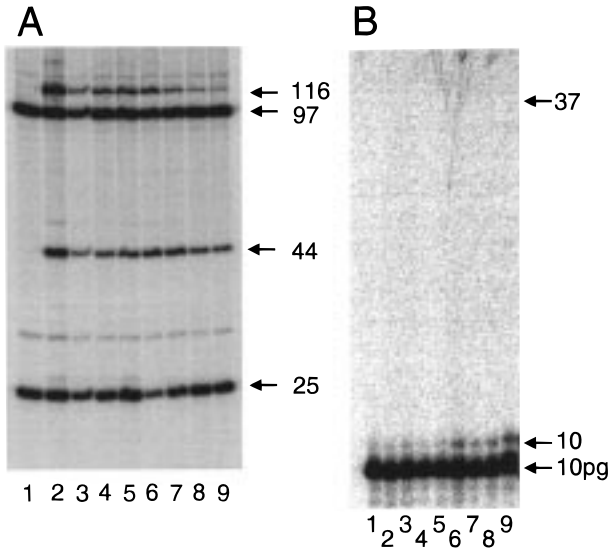

FIGURE 7: End-joining of 3′-hydroxyl but not 3′-PG substrates by nuclear extracts of human REH lymphoblastoid cells. A cohesive-end 3′-hydroxyl (A) or 3′-PG (B) substrate (40 ng; see Figure 1A) was treated for 4 h at 17 °C with either 50 μg/mL (lanes 2, 3 and 6, 7) or 100 μg/mL (lanes 4, 5 and 8, 9) of a nuclear protein extract in buffer A (lanes 2−5) or buffer B (lanes 6−9) in the presence (lanes 2, 4, 6, 8) or absence (lanes 3, 5, 7, 9) of 10 μM wortmannin. Lane 1 is untreated substrate. The DNA was then treated with *Nla*III (A) or with *Xho*I plus *Bst*XI (B) to release terminal fragments. In (A), the 116- and 44-base fragments represent end-joined products (see Figure 3). In (B), the predominant end-joined product would be expected to be 37 bases in length (see Figures 1 and 6).

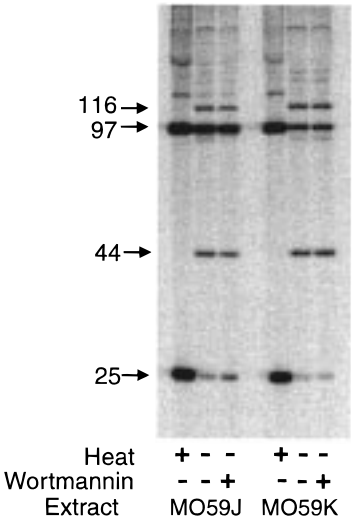

FIGURE 8: Joining of 3′-hydroxyl ends by extracts of MO59K (DNA-PK-proficient) and MO59J (DNA-PK-deficient) glioma cells. Incubation was for 4 h at 17 °C with 40 μg/mL nuclear extract and 40 ng of DNA substrate. The 116- and 44-base fragments represent end-joined products; see Figure 3).

termini. Specifically, we detected, among bleomycin-induced *aprt* mutations in CHO cells, both small deletions (*39*) and reciprocal translocations (*40*), and we showed that both events occurred almost exclusively at expected sites of bleomycin-induced double-strand cleavage, suggesting that they arose by misjoining of the breaks. These events were highly conservative, with putative blunt 3′-PG ends, in particular, virtually always being preserved intact. Using defined substrates, we also found that similar, highly conservative joining of 3′-PG-terminated double-strand breaks occurred in *Xenopus* egg extracts (*19*), with terminal

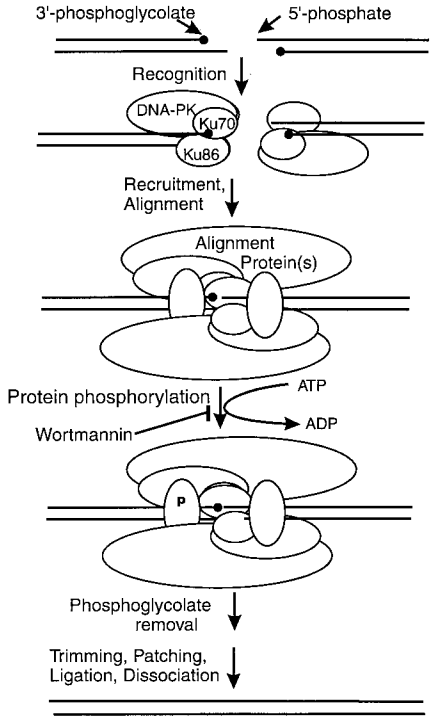

FIGURE 9: Model for end-joining in *Xenopus* egg extracts.

sequences of blunt ends again being nearly always preserved. The suppression of this joining by wortmannin suggested the participation of DNA-PK or a similar PIK-related kinase in the end-joining pathway, and thus these results provided a possible biochemical link between end-joining in egg extracts and double-strand break repair in mammalian cells, as the latter is partially dependent on DNA-PK (*5*). The present results provide additional evidence that the wortmannin sensitivity of the pathway in *Xenopus* is due to inhibition of DNA-PK-catalyzed phosphorylation. There are, nevertheless, two alternative interpretations of the data. First, it is possible that inhibitor occupation of the ATP-binding site of DNA-PK blocks not only kinase activity but also some other DNA-PK function (e.g., interaction with other proteins) that is essential for end-joining. Second, despite the demonstrated presence of active DNA-PK in egg extracts (*28*), the kinase essential for end-joining could be a PIK-related kinase other than DNA-PK, but having very similar sensitivity to the various inhibitors. Whatever the nature of the wortmannin-sensitive step in rejoining, however, the kinetic data suggest that it is in some sense a local rather than a global event, and phosphorylation of some protein stably bound to each DNA end to be joined seems the most straightforward possibility.

Assuming that, as suggested by structural similarities (*29*, *41*), *Xenopus* DNA-PK is functionally similar to the mammalian enzyme, a tentative model of the end-joining pathway incorporating DNA-PK-based phosphorylation can be proposed (Figure 9). The salient feature of the model is that both end alignment and a local DNA-PK-catalyzed phosphorylation event precede all biochemical processing of the ends, including removal of any terminal blocking groups, any necessary trimming/patching, and ligation. That end alignment precedes biochemical processing was inferred from the finding that both the nature and the extent of processing of a recessed 3′-PG terminus were dependent on the structure

of the other end of the double-strand break in the plasmid (Figure 6 and ref *19*). It should be noted that the relative order of end alignment vs protein phosphorylation has not been definitively established. The identity of the protein(s) which must be phosphorylated is also unknown, though it is presumably part of an end-joining repair complex. Obvious candidates are either of the Ku subunits of DNA-PK, both of which are known targets of phosphorylation by DNA-PK (*42*). Phosphorylation could have the effect of loosening Ku binding to the DNA ends, allowing or even actively promoting access to the DNA ends by the enzymes required for removal of damaged termini and/or other processing of the ends. The unidentified 48-, 87-, and 96-kDa proteins are also possible candidates, although they appear not to be stably DNA-bound, at least after they become phosphorylated. As originally proposed by Thode (*38*), various features of end processing suggest that the DNA ends must be held in juxtaposition by a presumed "alignment protein". However, recent reports that Ku can promote DNA end-to-end associations (*43*−*45*) raise the possibility that Ku itself may serve this function.

Results presented above (Figure 2A) show that DNA-dependent phosphorylation of endogenous proteins in the egg extracts, like the phosphorylation of a synthetic peptide by purified human DNA-PK (*31*), is stimulated to an equal extent by double-strand break ends with 3′-hydroxyl and 3′-PG termini. It would therefore be predicted that the putative phosphorylation step in the pathway would occur as rapidly for 3′-PG as for 3′-hydroxyl double-strand breaks, and thus would be largely completed within 90 min, a time sufficient to allow joining of the majority of 3′-hydroxyl double-strand break ends (*19*). If the phosphorylation step is rapid, but the subsequent 3′-PG removal is slow, it is expected that there would be some accumulation of intermediates in which the critical protein phosphorylation had occurred, but the 3′-PG had not yet been removed. It is thus surprising that all processing of the 3′-PG double-strand breaks appears to stop abruptly upon addition of wortmannin, implying that there was little if any accumulation of such intermediates.

There are several possible explanations of this unexpected result. First, the putative phosphorylation may be transient, and balanced by a competing dephosphorylation, so that continued DNA-PK activity is needed to maintain a population of complexes in the required phosphorylated state. [It should be noted, however, that addition of the broad-specificity phosphatase inhibitor okadaic acid did not stimulate end-joining of 3′-PG substrates in this system (*19*).] Second, although phosphorylation of some proteins and peptides by DNA-PK is stimulated to an equal degree by 3′-PG and 3′-hydroxyl ends, it is still possible that there may be proteins, particularly DNA end-binding proteins, for which the rate of phosphorylation is more strongly dependent on the structure of the ends. Third, processing 3′-PG ends may require a higher degree of phosphorylation than processing of 3′-hydroxyl ends, or may require phosphorylation of additional proteins. Further experiments will be required to distinguish among these possibilities.

It is well-known that mammalian cells have the capacity to rejoin bleomycin-induced and other terminally blocked double-strand breaks (*13*, *46*), and genetic data suggest that at least one pathway of rejoining involves DNA-PK (*22*, *47*, *48*). Shuttle vectors containing bleomycin-induced, 3′-PG-terminated double-strand breaks are likewise efficiently recircularized when transfected into mammalian cells (*24*, *49*). Thus, the failure of human cell extracts to show end-joining activity toward terminally blocked double-strand breaks must be attributed to the loss of this activity during the preparation of the extracts. The basis of the disparity between these results and a recent report of rejoining of bleomycin-induced double-strand breaks in deproteinized cellular DNA embedded in agarose plugs, by cytoplasmic extracts of HeLa cells (*50*), remains to be explained. Of course, there may well be substantial differences between end-joining in *Xenopus* egg extracts and the end-joining involved in double-strand break repair in mammalian cells. However, the similarity of the repair joints and the apparent involvement of DNA-PK (or a similar PIK-related kinase) in both instances suggest that there is sufficient overlap that studies in the *Xenopus* system can at least provide a useful paradigm from which to approach mammalian end-joining repair pathways.

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