End-joining of Free Radical-mediated DNA Double-strand Breaks in Vitro Is Blocked by the Kinase Inhibitor Wortmannin at a Step Preceding Removal of Damaged 3’ Termini*

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Both mammalian cells and Xenopus eggs possess activities for the joining of nonhomologous DNA ends, and such activities may play a major role in double-strand break repair. In order to dissect the biochemical processing of breaks with oxidatively modified ends, vectors containing various site-specific double-strand breaks with 3’-phosphoglycolate termini were constructed and treated with Xenopus egg extracts. These vectors were rejoined by the extracts at rates 30–100 times slower than comparable 3’-hydroxyl vectors. Vectors with blunt or cohesive 3’-phosphoglycolate ends yielded single repair products corresponding to simple phosphoglycolate removal followed by ligation, while a vector with mismatched ends was also rejoined but yielded a mixture of products. Addition of the kinase inhibitors wortmannin and dimethylaminopurine not only blocked rejoining, but also suppressed phosphoglycolate removal, implying an early, essential, kinase-dependent restriction point in the pathway. The results suggest that double-strand breaks with oxidatively modified ends are repaired in Xenopus eggs by a highly conservative and stringently regulated end-joining pathway, in which all biochemical processing of the breaks is contingent on both end alignment and a specific phosphorylation event. Several lines of indirect evidence suggest DNA-dependent protein kinase as a likely candidate for effecting this phosphorylation.

Although most if not all organisms are able to rejoin DNA double-strand breaks by homologous recombination with an intact copy of the damaged sequence (1–3), mammalian cells (4–6), Xenopus eggs (7–9), and to a lesser extent yeast (10) and bacteria (11), also possess “end-joining” activities which are capable of rejoining broken DNA ends by mechanisms which do not involve homologous recombination and do not require a duplicate copy of the sequence. In the case of free radical-mediated double-strand breaks, such end-joining is complicated by the presence of fragmented sugars and damaged or missing bases at the termini. All of these damaged DNA moieties must be removed and replaced with normal nucleotides, and the specific manner in which they are processed will likely have important consequences for the fidelity of repair. Here we describe an in vitro system for examining such processing, and present evidence that a wortmannin-sensitive kinase plays a key role in regulating this end-joining pathway.

EXPERIMENTAL PROCEDURES

Materials—The preparation of 3’-phosphoglycolate-terminated plasmids will be described in detail elsewhere (12). Briefly, 5’-3’-end-labeled 3’-phosphoglycolate-terminated single-strand fragments 8–12 bases in length were generated by bleomycin treatment of short partial DNA duplexes (13, 14), and were ligated into 10- or 11-base 3’-recessed ends which had been generated in a restriction enzyme-linearized plasmid (pSV56, a 5504-bp derivative of pZ189 (15)) by limited exonuclease digestion with T4 DNA polymerase (16). Plasmid molecules which failed to incorporate the 3’-phosphoglycolate oligomers were eliminated by treatment with T7 DNA polymerase, which acts as a 3’→5’ exonuclease only for 3’-hydroxyl-terminated DNA. The remaining 3’-phosphoglycolate-terminated full-length double-stranded DNA was isolated on an agarose gel.

Eggs were collected from gonadotropin-induced Xenopus laevis females (NASCO). Following activation with calcium ionophore A23187, extracts were prepared by ultracentrifugation (17) and frozen in small aliquots in liquid N2. Kinase inhibitors and okadaic acid were from Sigma.

End-joining Assays—The 3’-phosphoglycolate repair substrates (20 ng) were added to a 20-mg reaction containing 15 µl of egg extract and incubated, usually for 4 h at 13 °C. The reaction was terminated by treatment with proteinase K (300 µg/ml, 55 °C, 3 h). Samples were extracted with phenol and then with chloroform and ethanol-precipitated. The samples were then treated with either DpnI or with XhoI plus BstXI and electrophoresed on denaturing 22% polyacrylamide gels. Wet gels were subjected to quantitative phosphorimage analysis. In some cases, an aliquot of uncult DNA was transfected into WM1100 (reoA) cells by electroporation, and the sequences of the repair joints were determined (18).

RESULTS

Specificity and Kinetics of End-joining—Incubation of either blunt- or cohesive-end 3’-phosphoglycolate substrates (see Fig. 1) in Xenopus egg extracts resulted in a 5- to 20-fold increase in the number of transfectants obtained upon electroporation into Echerichia coli (data not shown), suggesting that the plasmids were recircularized during incubation in the extract. Sequence analysis revealed that nearly all (34/38) of the clones derived from extract-treated blunt-ended molecules retained all bases from both DNA ends, to give a repair joint with a (CG)$_n$ sequence, suggesting a mechanism involving simple phosphoglycolate removal followed by blunt-end ligation (Fig. 1). Nearly all clones (37/39) derived from the cohesive-end substrate had a 2-base-shorter (CG)$_n$ repair joint, consistent with a mechanism involving annealing of the cohesive ends, phosphoglycolate removal, and ligation. Analysis of radiolabeled plasmid by agarose gel electrophoresis confirmed that a small fraction of the blunt-ended 3’-phosphoglycolate substrate, generally...
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**Substrates:**

A Blunt  
- CTCGAGAACTGGCTGAC  
- GAGCTCTTGCGCP

B Cohesive  
- CTCGAGAACTGGCTGAC  
- GAGCTCTTGCGCP

C Mixed  
- CTCGAGAACTGGCTGAC  
- GAGCTCTTGCGCP

**Products:**

Recircularized:

D (CG)$_4$  
- CTCGAGAACTGGCTGAC-CTCGCTGAC-N$_{10}$-TACAG--  
- GAGCTCTTGCGCP-N$_{10}$-ATGGTC--

E (CG)$_3$  
- CTCGAGAACTGGCTGAC-CTCGCTGAC-N$_{10}$-TACAG--  
- GAGCTCTTGCGCP-N$_{10}$-ATGGTC--

Intermolecular:

F (CG)$_3$  
- CTCGAGAACTGGCTGAC-CTCGCTGAC-N$_{10}$-TACAG--  
- GAGCTCTTGCGCP-N$_{10}$-ATGGTC--

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**Fig. 1.** Terminally blocked end-joining substrates and repair products. The substrates are linearized 5.5-kilobase plasmids which have been modified by addition of phosphoglycolate ends (●) and labeled to high specific activity at the 5'-phosphate of the nucleotide shown (●). The blunt- and cohesive-ended substrates each yielded almost exclusively (CG)$_4$ and (CG)$_3$ repair products, respectively, while the mixed-end substrate yielded a mixture of the two. The cohesive and mixed-end substrates also yielded significant amounts of an intermolecular cohesive-end-joining product (F).

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In order to examine more directly the processing of a 3'-phosphoglycolate-terminated DNA strand, a substrate was prepared in which one strand was labeled to high specific activity near the 3'-phosphoglycolate terminus (see Fig. 1). By cleaving the repair substrates and products with XhoI and BstXI following incubation in the extracts, it was possible to follow phosphoglycolate removal and subsequent ligation separately. XhoI releases the unprocessed 3'-phosphoglycolate end as part of a 10- or 12-base fragment; phosphoglycolate removal can then be detected as a slight decrease in the mobility of this fragment on a sequencing gel, and fill-in of the recessed end as conversion of the 10-base fragment to 11- and 12-base fragments. Recircularization of the plasmid produces a 39-base XhoI/BstXI fragment for blunt-end ligation (Fig. 1D) or a 37-base fragment for cohesive-end ligation (Fig. 1E).

These experiments (Fig. 2) showed unequivocally that a fraction of the initially 3'-phosphoglycolate-terminated DNA strands in each of the vectors was ligated to the opposite end of the break, but with much lower efficiency than a strand with a 3'-hydroxyl terminus. The cohesive- and blunt-ended 3'-phosphoglycolate vectors each yielded predominantly a single 37- or 39-base recircularization product, as predicted from sequencing studies. The cohesive-end vector also yielded some products of an 18-base fragment, presumably resulting from intermolecular "head-to-head" joins (Fig. 1F). For both substrates, phosphoglycolate termini were surprisingly persistent, with >85% remaining unaltered after 4 h of incubation in the extracts (Figs. 2 and 3). Phosphoglycolates on single-strand break ends were quantitatively removed within 6 h; data not shown.)

Vectors from which the 3'-phosphoglycolates had been removed by treatment with human abortive endonuclease (gift of B. Temple and D. M. Wilson) (19) were much more efficiently rejoined (70% recircularized within 30 min; data not shown), indicating that the low efficiency of end-joining was due solely to the presence of blocked 3' ends.

For a vector having mixed incompatible 3'-phosphoglycolate ends, one blunt and one with a 2-base 5'-overhang (Fig. 1C), significant amounts of both the 37- and 39-base recircularization products, as well as of the 18-base intermolecular cohesive-end-joining product, were formed (Fig. 2C). Formation of the 39-base product implies that the recessed 3' end had been filled in prior to ligation, while formation of the 37-base product implies that the 5' overhang either had been removed or had displaced two bases of the 3' terminus on the blunt end of the break. Most strikingly, however, the intermediate processing of the recessed 3'-phosphoglycolate terminus was much different for the mixed-end than for the cohesive-end vector (Figs. 2, B...
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Fig. 3. Quantitative analysis of the effect of kinase inhibitors on phosphoglycolate removal and end-joining. Data in A through D are derived from phosphorimage analysis of the experiments in Fig. 2 using blunt- (A), cohesive- (B), or mixed-end (C) 3'-phosphoglycolate vectors, or a blunt 3'-hydroxyl vector (D). E is a replicate experiment with the mixed-end vector. F, G, and H represent additional experiments with the blunt 3'-hydroxyl vector, with incubation for 10, 30, and 90 min, respectively, at 13°C. I and J represent similar experiments (90-min incubations) with vectors having 3'-hydroxyl ends and either 2-base (CG) (I) or 4-base (CGCG) (J) cohesive 5' overhangs. These were generated by cleavage of pSV56 with BamHI or MluI, respectively, followed by 5'-end-labeling; analysis was as in Fig. 2. The dimethylaminopurine concentration was 2.5 mM in all cases. The wortmannin concentration was 2 μM or 10 μM (*). In all cases, the fraction rejoined represents the sum of all end-joined products divided by the total radiolabel in each lane. Since removal of 3'-phosphoglycolates must necessarily precede rejoining, the percent phosphoglycolate removal is calculated as the sum of all free 3'-hydroxyl ends plus the sum of all end-joined products, divided by the total radiolabel in the lane. DMAP = dimethylaminopurine; ND = not done.

The effect of kinase inhibitors on DNA end-joining was investigated. Wortmannin, a broad-specificity kinase inhibitor, inhibited phosphatase activity, with the extent of 3'-phosphoglycolate removal significantly greater for the mixed-end vector. Moreover, the mixed-end vector allowed both the accumulation of unligated 3'-hydroxyl intermediates, and partial fill-in of the 5' overhang, while the cohesive-end vector did not. These marked differences in processing of the same recessed 3'-phosphoglycolate end suggest that alignment of the opposite ends of the break must have preceded biochemical modification of the termini.

Kinetic studies (Fig. 4A) showed that the joining of terminally blocked substrates was 50-100 times slower than joining of a substrate with normal 3'-hydroxyl termini. Nevertheless, extended incubation resulted in ligation of a significant fraction (up to 25%) of the terminally blocked DNA strands, implying that end-joining was not restricted to some minor contaminant in the preparation.

Effect of Kinase and Phosphatase Inhibitors—To assess the possible role of protein phosphorylation in regulation of end-joining, reactions were performed in the presence of kinase and phosphatase inhibitors. Wortmannin inhibits phosphatidylinositol 3-kinase (PI3K), and certain PI3K-related protein serine-threonine kinases (20, 21). I-(5-Isoquinolinylsulfonyl)-2-methyl piperazine (H7), inhibits protein kinase C and related serine-threonine kinases (22), but does not affect PI3K (23).

As shown in Figs. 2 and 3, wortmannin (2 μM) as well as the broad-specificity kinase inhibitor dimethylaminopurine (2.5 mM) not only dramatically reduced end-joining of terminally blocked substrates, but also strongly inhibited phosphoglycolate removal. This was most apparent in the blunt and mixed-end vectors, where free 3'-hydroxyl termini (12-mer and 10-mer bands in Fig. 2, A and C) accumulated in control (lanes 1, 7, and 8) but not in inhibitor-containing samples (lanes 2 and 5). While the extent of both end-joining and phosphoglycolate removal differed for different substrates and different batches of extract, the suppression by the kinase inhibitors was quite reproducible (Fig. 3). Dimethylaminopurine and wortmannin also suppressed the joining of both blunt- and cohesive-end breaks bearing 3'-hydroxyl termini (Fig. 3, D and F–J), and this suppression was expressed within 10 min of inhibitor addition. Half-maximal suppression of rejoining required 0.1–0.3 μM wortmannin (Fig. 4, B and C), comparable to values reported for inhibition of wortmannin-sensitive protein kinases (20, 21). H7 had no effect on end-joining of any of the substrates. Thus, the results suggest that the end-joining pathway requires a specific phosphorylation event, catalyzed by a PI3K-related or other wortmannin-sensitive kinase, as a prerequisite to all biochemical processing of double-strand break ends, blocked or unblocked.

Effects of the broad-specificity phosphatase inhibitor okadaic acid were complex and less definitive. It had no effect on joining of a 3'-hydroxyl-terminated vector, but it did partially inhibit joining of phosphoglycolate-terminated vectors.

DISCUSSION

It has long been postulated that double-strand break repair in higher eukaryotes may be carried out at least in part by simple end-joining rather than by homologous recombination (24–25). Recently, support for this hypothesis has come from the discovery that in mammalian cells DNA-dependent protein kinase (DNA-PK, a PI3K-related kinase) and its essential cofactor, the DNA end-binding heterodimer Ku, are required both for efficient repair of radiation- and bleomycin-induced double-strand breaks and for ostensibly similar end-joining events which occur during V(D)J recombination (26–32).

Although the extent of overlap between this putative mammalian end-joining repair system and the end-joining activity detected in Xenopus egg extracts is unknown, the present re-
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Fig. 4. Kinetics of end-joining of 3-phosphoglycolate-termini
denatured double-strand breaks in Xenopus egg extracts. A, the cohesive-end ( ), blunt-end ( ), 3-phosphoglycolate vector ( ). B and C, the cohesive-end 3-phosphoglycolate substrate was incubated in egg extract at 13°C for 4 h and similarly analyzed.

sults show that the Xenopus system, like its mammalian counterpart, involves a wortmannin-sensitive kinase, and is capable of processing DNA breaks with oxidatively modified ends. The apparent presence of DNA-PK activity in Xenopus egg extracts (33), its known association with DNA ends, and its sensitivity to dimethylaminopurine (34), are consistent with its being a protein kinase, and is certainly possible that damage/repair responses (35), and it is certainly possible that the wortmannin sensitivity of end-joining in Xenopus egg extracts may reflect involvement of a kinase(s) other than DNA-PK.

Analysis of end-joining events in both Xenopus egg extracts and mammalian cells has suggested a model wherein DNA ends are first aligned (often on the basis of partial complementarities in 5’ or 3’ overhangs), then trimmed and patched as necessary to produce a ligatable substrate (4, 9). The present results further suggest that at least in the Xenopus system, not only patching and ligation, but also removal of 3’-terminal blocking groups, are all stringently regulated by phosphorylation. Since terminal processing seems to be also dependent on prior completion of the end alignment step (Fig. 2), we speculate that perhaps the wortmannin-sensitive phosphorylation event is contingent on end alignment, and that one of its functions may be to delay biochemical processing of the DNA ends until they have been optimally aligned. Since free radical-mediated double-strand breaks will often have staggered ends with missing bases and damaged termini, the capacity to use partial complementarities for such alignment, along with the capacity to remove and replace fragmented as well as mismatched nucleotides (apparently without disturbing end alignment), makes the Xenopus system seem ideally suited for repair of these complex lesions. Whether end-joining of double-strand breaks in mammalian chromosomal DNA is carried out by a similar mammalian homologue of this system, remains to be determined; however, the finding that the Xenopus pathway can be arrested in vitro, by kinase inhibitors and by modified DNA ends, should greatly improve prospects for isolation of an intact, active end-joining complex, and for definitive identification of its constituents.

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