DNA Ligase III Is Recruited to DNA Strand Breaks by a Zinc Finger Motif Homologous to That of Poly(ADP-ribose) Polymerase

IDENTIFICATION OF TWO FUNCTIONALLY DISTINCT DNA BINDING REGIONS WITHIN DNA LIGASE III*

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Mammalian DNA ligases are composed of a conserved catalytic domain flanked by unrelated sequences. At the C-terminal end of the catalytic domain, there is a 16-amino acid sequence, known as the conserved peptide, whose role in the ligation reaction is unknown. Here we show that conserved positively charged residues at the C-terminal end of this motif are required for enzyme-AMP formation. These residues probably interact with the triphosphate tail of ATP, positioning it for nucleophilic attack by the active site lysine. Amino acid residues within the sequence RFPR, which is invariant in the conserved peptide of mammalian DNA ligases, play critical roles in the subsequent nucleotidyl transfer reaction that produces the DNA-adenylate intermediate. DNA binding by the N-terminal zinc finger of DNA ligase III, which is homologous with the two zinc fingers of poly(ADP-ribose) polymerase, is not required for DNA ligase activity in vitro or in vivo. However, this zinc finger enables DNA ligase III to interact with and ligate nicked DNA at physiological salt concentrations. We suggest that in vivo the DNA ligase III zinc finger may displace poly(ADP-ribose) polymerase from DNA strand breaks, allowing repair to occur.

Three human genes encoding DNA ligases, LIG1, LIG3, and LIG4, have been isolated (1, 2). The enzymes encoded by these genes and all other eukaryotic DNA ligases utilize ATP as a co-factor in the DNA joining reaction. In this regard, eukaryotic DNA ligases are similar to the DNA ligases encoded by the bacteriophages T4 and T7. The formation of a covalent enzyme-NMP reaction intermediate, in which the NMP moiety is linked to a lysine residue via a phosphoramidite bond, is a property shared by DNA ligases, RNA ligases, and mRNA capped enzymes (3). In DNA ligases, the active site lysine was first identified in bovine DNA ligase I (4). By comparing the amino acid sequence of the adenylated peptide from bovine DNA ligase I with other DNA ligases, an active site motif, KXXDGR, that is diagnostic for DNA ligases was defined (4).

A second peptide sequence, known as the conserved peptide, was initially revealed by a comparison of vaccinia DNA ligase with the DNA ligases encoded by the CDC9 and CDC17 genes of Saccharomyces cerevisiae and Schizosaccharomyces pombe, respectively (5). Subsequently, sequences exhibiting homology with the conserved peptide have been detected in all eukaryotic and pox virus DNA ligases (1, 2) except for the putative DNA ligase encoded by African swine fever virus (6). At the present time, the role of the conserved peptide in the DNA joining reaction is not known. Since the sequences and the number of amino acids between the active site and conserved peptide motif are similar in all eukaryotic DNA ligases, it appears that the conserved motifs define a minimal catalytic domain (1, 2, 7). The generation of catalytically active fragments of mammalian DNA ligase I containing the conserved motifs by limited proteolysis (8) and the complementation of the conditional lethal phenotype of a yeast cdc9 DNA ligase mutant by fragments of human DNA ligase I cDNA that encode the putative catalytic domain (9) support this notion.

Alignment of DNA ligases, mRNA capping enzymes, and RNA ligases led to the identification of another four conserved motifs in addition to the active site and conserved peptide (3). In the crystal structure of T7 DNA ligase, the active site lysine residue is at the bottom of a cleft between two domains (10). All of the other motifs, except for the conserved peptide, form the protein surfaces that surround the active site lysine residue (10). Thus, it seems likely that this structural organization occurs in all the enzymes that form a covalent enzyme-NMP reaction intermediate.

Considerably less is known about how DNA ligase interacts with nicked DNA in the latter steps of the ligation reaction. Although the catalytic domain must contain amino acid residues that recognize and interact with nicks in duplex DNA, the molecular mechanisms by which the AMP moiety is transferred to the 5′-phosphate terminus at a nick in duplex DNA and by which the phosphodiester bond is then formed from the DNA-adenylate intermediate have not been defined. Interestingly, DNA ligase IIIα and DNA ligase IIIβ, which are generated by alternative splicing of the mammalian LIG3 gene transcript, have an amino-terminal sequence that is homologous with the zinc fingers of poly(ADP-ribose) polymerase (PARP)1 that interact with DNA strand breaks (7, 11, 12). This has led to the suggestion that the zinc finger of DNA ligase III may function as the nick sensor for this enzyme (13).

In this study, we demonstrate that the DNA ligase III zinc

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1 The abbreviations used are: PARP, poly(ADP-ribose) polymerase; DTT, dithiothreitol; MES, 2-(N-morpholino)ethanesulfonic acid; PCR, polymerase chain reaction; Δzf, zinc finger-deleted; bp, base pairs.
finger does indeed bind specifically to DNA single-strand breaks, enabling this enzyme to catalyze the joining of nicks at salt concentrations that inhibit other eukaryotic DNA ligases. However, deletion of this DNA binding motif does not abolish either DNA joining activity or the ability of this enzyme to complement the conditional lethal phenotype of an Escherichia coli lig mutant, indicating that there are other residues that interact with the nicked DNA substrate during the ligation reaction. Using site-directed mutagenesis, we have identified residues within the conserved peptide that play critical roles in the interaction of DNA ligase III and presumably other eukaryotic DNA ligases with nicked DNA during the latter stages of the DNA ligation reaction.

EXPERIMENTAL PROCEDURES

Plasmids—Removal of a HindIII fragment (nucleotides 21–219) of PARP cDNA, which encodes the two zinc fingers of the human PARP (residues 1–234) from pTG PARP (11), followed by religation, produced pTG PARP ΔFIIΔIII. cDNA sequences encoding each of the zinc fingers were amplified by the polymerase chain reaction (PCR) and then reintroduced into pTG PARP ΔFIIΔIII to produce pTG PARP ΔFII and pTG PARP ΔIII, which encode PARP proteins with deletions of zinc finger I and zinc finger II, respectively. A 1095-bp PstI fragment of DNA ligase III cDNA, which encodes residues 1–360 of DNA ligase III (7, 14) was subcloned into the prokaryotic expression vector pTG 161 (15) to generate the plasmid pTG N-ter Lig III (Fig. 1). After removal of the 3' overhangs, the same fragment was subcloned into the mammalian expression vector pcDNA3 (16) so that the DNA ligase III open reading frame was expressed as a glutathione S-transferase fusion protein.

Plasmids encoding oligohistidine (His)-tagged versions of DNA ligase IIIβ and polypeptides were constructed as follows. For full-length DNA ligase IIIβ with an N-terminal His tag, the DNA ligase IIIβ open reading frame was amplified from the glutathione S-transferase-DNA ligase IIIβ plasmid (17) by the PCR using PvoII polymerase (Roche Molecular Biochemicals). The PCR product was digested with BamHI and SacI and subcloned into the same restriction sites of pQE32 (Qiagen) to generate pHis Lig IIIβ (Fig. 1). For His-tagged versions of DNA ligase III that lack the N-terminal zinc finger motif (ΔZII), a 2.4-kilobase pair ApoISalI fragment of DNA ligase III cDNA encoding residues 49–862 was subcloned into pBluescript II KS. The 2.4-kilobase pair fragment was released from pBluescript II KS by digestion with KpnI and SalI and subcloned into pQE32 to generate the plasmid pHis ΔZII Lig IIIβ (Fig. 1). Constructs encoding His-tagged ΔZII DNA ligase IIIβ fusion proteins with single amino acid changes in the conserved peptide were generated by replacing the 600-bp EcoRI/SalI fragment from the wild type DNA ligase III cDNA with mutated 600-bp EcoRI/SalI fragments (Fig. 1).

Mutagenesis of DNA Ligase III cDNA—PCR-based mutagenesis (18) was performed using DNA ligase IIIβ cDNA (14) as the template. Amplification was carried out in a 50-μl volume containing 200 ng of plasmid DNA, 50 μM dNTPs, 200 nM primers, 1× Pfu buffer (Stratagen), and 2.5 units of Pfu DNA polymerase. An initial cycle of 94 °C for 2 min, 56 °C for 1 min, and 72 °C for 1 s was performed, followed by 13 cycles of 94 °C for 30 s, 56 °C for 1 min, and 72 °C for 1 s. A 2-kilobase pair fragment was released from pBluescript II KS by digestion with KpnI and SalI and subcloned into pQE32 to generate the plasmid pHis ΔZII Lig IIIβ (Fig. 1). Constructs encoding His-tagged ΔZII DNA ligase IIIβ fusion proteins with single amino acid changes in the conserved peptide were generated by replacing the 600-bp EcoRI/SalI fragment from the wild type DNA ligase III cDNA with mutated 600-bp EcoRI/SalI fragments (Fig. 1).

Analysis of the DNA Ligase III β Zinc Finger by Immunoblotting, Southernwestern Blotting, and DNase I Footprinting—Crude extracts of plasmid-containing TGE900 bacteria were prepared as described previously (11). Briefly, cells from a 1.5-ml culture were collected by centrifugation and resuspended in 100 μl of 25 mM Tris-HCl (pH 8.0), 50 mM KCl, 10 mM EDTA, and 100 μl of sample buffer (50 mM Tris-HCl (pH 6.8), 6 μg/μl, 6% mercaptoethanol, 3% SDS, 0.003% bromophenol blue, 0.15% sonication buffer, bacterial proteins were separated by electrophoresis through a 10% SDS-polyacrylamide gel (19) and then either stained with Coomassie blue or transferred to a nitrocellulose membrane (BAS 83; Schleicher & Schuell). Immunoblotting experiments with rabbit antibodies against the human PARP zinc finger FI (20) or zinc finger FI (21) were carried out as described previously (22).

For DNA binding and DNase I footprinting assays, the membranes were washed with 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% Nonidet P-40 at room temperature and then preincubated for 10 min at 0 °C in binding buffer (20 mM Tris-HCl (pH 8.0), 0.1 mM NaCl, 2 mM MgCl₂, 2 mM dithiothreitol (DTT), 0.1% Nonidet P-40). After incubation for 1 h with 20 ng of a 32P-end-labeled 66-bp duplex DNA (either with or without a single nick) in the DNA binding buffer (23), the membranes were washed three times with binding buffer at 0 °C and then either dried and subjected to autoradiography to visualize the protein-DNA complexes or were autoradiographed wet for 1 h so that the filter-bound protein-DNA complex could be excised and used for DNase I footprinting assays as described previously (23).
mm EDTA, 0.5 mm DTT, 1 mm phenylmethylsulfonyl fluoride, 1 mm benzamidine-HCl. The dialysate was then fractionated by P11 phosphocellulose (Whatman) chromatography. Eluted fractions were assayed for protein by the method of Bradford (24) and for DNA ligase by detecting formation of a labeled enzyme-adenylate intermediate (see below). Bound proteins were eluted by fractionated by FPLC Resource S chromatography and then by gel filtration chromatography using a Superdex 200 column (Amersham Pharmacia Biotech). The peak fractions from the gel filtration column were then aliquoted, flash-frozen in liquid nitrogen, and stored at −80 °C.

Purification of His-tagged DNA Ligase IIIb—The E. coli strain M15 was transformed with plasmids encoding DNA ligase IIIb. Cultures (1 liter) were grown at 37 °C in LB medium containing 100 μg/ml ampicillin and 50 μg/ml kanamycin. When the culture reached an A600 of 0.6, isopropyl thiogalactoside was added to a final concentration of 1 mM, and incubation was continued for 6 h. Bacterial cells were collected by centrifugation and then resuspended in 50 ml of ice-cold 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine-HCl. After washing with buffer A, bound proteins were eluted stepwise with buffer A containing 0.2 M and then 0.5 M NaCl. Eluted fractions were assayed for protein by the method of Bradford (24) and for DNA ligase by detecting formation of a labeled enzyme-adenylate intermediate (see below). Fractions containing DNA ligase IIIb, which was present in the 0.5 M eluate, were pooled. After the addition of imidazole to a final concentration of 10 mM, the sample was incubated by constant rotation at 4 °C for 3 h with nickel beads (1 ml of beads per 10 mg of protein) that had been pre-equilibrated with buffer A, bound proteins were eluted stepwise with buffer A containing 0.2 M and then 0.5 M NaCl. Eluted fractions were assayed for protein by the method of Bradford (24) and for DNA ligase IIIb by detecting formation of a labeled enzyme-adenylate intermediate (see below). Bound proteins were batch-eluted from the beads with buffer A containing 250 mM imidazole and 50 mM EDTA. After dialysis against buffer A, the dialysate was aliquoted, flash-frozen in liquid nitrogen, and stored at −80 °C.

Electrophoretic Mobility Shift Assay—A 38-mer duplex containing a single nick (25) was constructed by annealing oligonucleotides and used as the substrate for electrophoretic mobility shift assays. The 38-mer (8 pmol) was end-labeled with [γ-32P]ATP (Amersham Pharmacia Biotech) to a specific activity of 10^6 cpm/μg and then annealed with two complementary oligonucleotides (8 pmol of each) to generate a labeled 38-mer duplex with a specific activity of 10^6 cpm/μg and then annealed with two complementary oligonucleotides (8 pmol of each) to generate a labeled 38-mer duplex with a specific activity of 10^6 cpm/μg and then annealed with two complementary oligonucleotides (8 pmol of each) to generate a labeled 38-mer duplex with a specific activity of 10^6 cpm/μg. Reaction mixtures contained 5% glycerol, 0.1 mM ZnCl2, and 600 mM bovine serum albumin with 32 ng of linearized pBluescript and KCl concentrations ranging from 100 to 400 mM. Similar assays were carried out in the presence of 32, 96, and 320 ng of cold 38-mer duplex, either intact or with a single nick, at 100 mM KCl. After 30 min, samples were loaded onto nondenaturing 5% polyacrylamide gels. Bound proteins were visualized by autoradiography.

Ligation Assay—A 38-mer oligonucleotide containing a single nick was used as the substrate in DNA ligation assays (25). The 5’ end of the 20-mer, which forms one of the nick termini, was end-labeled with polynucleotide kinase (New England Biolabs) and [γ-32P]ATP (Amersham Pharmacia Biotech) to a specific activity of 10^6 cpm/μg. Reaction mixtures (60 μl), which contained 60 mM Tris-HCl (pH 8.0), 10 mM MgCl2, 5 mM DTT, 1 mM ATP, 50 μg/ml bovine serum albumin, DNA substrate (60,000 cpm) and DNA ligase, were incubated at 16 °C for 30 min. After the addition of 10 μl of formamidase dye solution (Amersham Pharmacia Biotech), samples were heated at 85 °C for 3 min. Aliquotes (3 μl) were separated by denaturing gel electrophoresis (24). After fixing and drying, the gel was exposed to x-ray film. DNA joining was detected by conversion of the labeled 20-mer to a labeled 38-mer and quantitated by PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA) analysis.

Complementation of the Temperature-sensitive Phenotype of an E. coli lig Mutant by Expression of DNA Ligase IIIb Polypeptides—The E. coli strain AK76 lig ts7 was transformed with DNA ligase IIIβ plus mids. Overnight cultures of transformants, which were grown at 30 °C, were streaked onto LB agar plates containing 50 μg/ml ampicillin and 40 μg/ml isopropyl thiogalactoside. The plates were then incubated at either 30 or 40 °C for 24 h.

Formation of DNA-Adenylate—Reaction mixtures contained 60 mM Tris-HCl (pH 8.0), 10 mM MgCl2, 5 mM DTT, 50 μg/ml bovine serum albumin, DNA ligase III, and 0.5 μCi of [γ-32P]ATP (3000 Ci/mmol, Amersham Pharmacia Biotech). After incubation, reactions were stopped by the addition of SDS sample buffer, heated at 90 °C for 5 min, and then electrophoresed through a SDS-polyacrylamide gel. Labeled polypeptides were detected in the dried gel by either autoradiography.

RESULTS

The Putative Zinc Finger of DNA Ligase III Is Immunologically Related to Zinc Finger 1 of PARP and Binds Specifically to DNA Single Strand Breaks—The N-terminal region of PARP contains two homologous sequences that correspond to two zinc fingers, finger 1 (residues 1–97) and finger II (residues 106–207), that are encoded by the first four exons of the PARP gene (26). Analysis of the open reading frame encoded by human DNA ligase III cdNA revealed that this polypeptide has a putative zinc finger structure at its N terminus (7) that exhibits homology with the PARP zinc fingers (26). A fragment of the DNA ligase III cDNA open reading frame encompassing the putative zinc finger of DNA ligase III (residues 1–360) was subcloned, and the polypeptide was overexpressed in E. coli. This polypeptide cross-reacted with an antibody that specifically recognizes finger I of PARP but did not cross-react with an antibody that specifically recognizes finger II of PARP (Fig. 2B). Thus, the putative zinc finger of DNA ligase III is antigenically related to PARP finger I.

A characteristic feature of the PARP zinc fingers is their ability to bind to DNA breaks, in particular single strand breaks (11, 12). Like PARP, the N-terminal fragment of DNA ligase III forms a specific complex with a labeled, nicked DNA probe in Southwestern blotting assays (Fig. 3A). Further analysis of these DNA-protein complexes by DNase I footprinting revealed that PARP and the N-terminal fragment of DNA ligase III bind to a similar region of the DNA probe that encompasses the nick at position 33 in probe 2 (Fig. 3B). To demonstrate that the DNase I protection is caused by the DNA ligase III zinc finger binding specifically to the nick, we have constructed DNA duplexes that either lack a nick (probe 1) or contain a single nick at position 22 (probe 3). In these experiments, DNA ligase IIIβ purified from E. coli (Fig. 4A) was immobilized on nitrocellulose and incubated with the DNA probes, and the resultant DNA-protein complexes were treated with DNase I. The pattern of the DNase I digests from the DNA-protein complexes (Fig. 3D) was compared with the patterns generated by digestion of the DNA substrates alone (Fig. 3C). As expected, no region of protection was observed with the intact duplex, whereas a footprint that was dependent upon the position of the nick was detected in the presence of nicked DNA probes (Fig. 3D). These results demonstrate that the N-terminal zinc finger of DNA ligase III binds to DNA single strand breaks.

Effect of the DNA Ligase III Zinc Finger on DNA Binding and DNA Joining in Vitro—We have chosen to further examine the biochemical properties of the DNA ligase III zinc finger in DNA
The membranes were incubated with polyclonal antibodies specific for either PARP zinc finger domain I (N-terminal 360 residues of DNA ligase III) or PARP zinc finger domain II (N-terminal 360 residues of DNA ligase III) as indicated. The positions of molecular mass standards are shown on the left.

Identification of Amino Acids That Are Required for the Interaction of DNA Ligase IIIβ with Nicks during the Ligation Reaction—To identify amino acids that are required for DNA joining, in particular for transferring the AMP group from the DNA ligase to the 5'-phosphate terminus of a nick and the subsequent formation of a phosphodiester bond, we focused on the 16-amino acid sequence that is conserved within the catalytic domain of ligase IIIβ, since this form of the enzyme, which is generated by tissue and cell type alternative splicing (17), is more amenable to overexpression and purification than the α form and does not interact with Xrc1 (17). Full length and zinc finger-deleted (∆zf) versions of DNA ligase IIIβ, each with an N-terminal His tag, were purified to near homogeneity from E. coli (Fig. 4A). In addition, untagged full-length DNA ligase III was purified to near homogeneity from baculovirus-infected insect cells (Fig. 4A). As expected, there was no significant difference in the ability of these polypeptides to form a labeled enzyme-AMP complex (Fig. 4C), and both full-length versions but not the zinc finger-deleted version of DNA ligase IIIβ cross-reacted with the PARP finger I antibody (Fig. 4B).

In electrophoretic mobility shift assays, full-length DNA ligase IIIβ formed a stable complex with nicked duplex DNA from 100 to 400 mM KCl (Fig. 5A). In contrast, complex formation by the ∆zf version of DNA ligase IIIβ was not detectable under the same reaction conditions. The labeled DNA substrate has two double strand ends in addition to the internal nick. To examine the binding of the DNA ligase III zinc finger to single and double strand interruptions, increasing amounts of unlabeled versions of the substrate, either with or without a nick, were added to the reactions (Fig. 5B). From this experiment, it is apparent that the nicked duplex is a more effective inhibitor of complex formation than the intact duplex. For example, labeled complex formation was 60% inhibited by a 30-fold molar excess of unlaeled nicked duplex, whereas labeled complex formation was only 30% inhibited by the same molar excess of unlabeled intact duplex. These results indicate that the zinc finger of DNA ligase III preferentially binds to DNA single strand breaks.

To examine whether the DNA ligase III zinc finger influences the DNA ligation reaction, the DNA joining activities of ∆zf and full-length versions of DNA ligase IIIβ were compared (Fig. 5C). There was no significant difference in their specific activities when measured at the optimal monovalent ion concentration for each enzyme. However, the DNA joining activity of full-length DNA ligase IIIβ remained relatively constant from 50 to 200 mM KCl (Fig. 5C), whereas the ∆zf version of DNA ligase IIIβ retained only about 20% activity at 200 mM NaCl (Fig. 5C). This degree of inhibition was similar to that of DNA ligase I (Fig. 5C), an enzyme that lacks an obvious DNA binding motif and is probably tethered to DNA substrates in vivo by binding to proliferating cell nuclear antigen (27). Similar results were obtained with NaCl (data not shown). In summary, these in vitro studies demonstrate that the zinc finger of DNA ligase III is not required for DNA joining in vitro, but it does enable this enzyme to bind to and ligate nicked DNA at physiological salt concentrations.

Based on the results described above, we considered the possibility that the DNA ligase III zinc finger may be required for in vivo function. To test this, we compared the ability of DNA ligase IIIβ, with and without the zinc finger, to complement the conditional lethal phenotype of an E. coli lig mutant. Both full-length and zinc finger-deleted versions of DNA ligase III expressed as either His-tagged or glutathione S-transferase fusion proteins enabled the E. coli strain to grow at the non-permissive temperature, whereas no growth was observed when the same strain was transformed with the empty expression vectors. Thus, the DNA ligase III zinc finger is not required for DNA joining in vivo, at least in E. coli.

**Fig. 2.** Alignment of the amino acid sequences of human PARP zinc finger domain I and zinc finger domain II with the putative zinc finger of human DNA ligase III. A, the alignment of PARP finger domain I (residues 1-97), PARP finger domain II (residues 106-207), and the putative zinc finger of human DNA ligase III (residues 1-97) is shown. Spaces (periods) have been introduced to optimize the alignment. Identical amino acids or conservative changes are in boldface uppercase type. Unrelated amino acids are in normal lowercase type. B, extracts of E. coli cells overexpressing full-length PARP (PARP) or PARP deletion mutants lacking zinc finger I (∆FI), finger II (∆FII), or both zinc fingers (∆F) and the N-terminal 360 residues of DNA ligase III (N-Lig III). Proteins were separated through SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated with polyclonal antibodies specific for either PARP zinc finger domain I (anti F I antibody) or PARP zinc finger domain II (anti F II antibody) as indicated.
The membrane was incubated with the indicated 66-bp 32P-end-labeled probe and then harboring a nick at position 33 as described under "Experimental Procedures." Labeled protein-DNA complexes were detected by autoradiography. The positions of molecular mass standards are shown on the left. B, the indicated labeled protein-DNA complexes were excised from the SDS gel and incubated with DNase I as described under "Experimental Procedures." The first lane contains degradation products generated from labeled probe 2 by the guanine-specific reaction. The protected region extending on either side of the nick at position 33 is bracketed. C, DNase I digestion products generated from the labeled probes 1, 2, and 3. D, DNA ligase IIIβ (1 μg) purified from E. coli was spotted onto the nitrocellulose membrane prior to incubation with the indicated labeled probe. D.Nase I digestion products from the labeled DNA-protein complexes are shown. The protected regions extending on either side of the nicks at positions 23 and 33 are bracketed.

lytic domain of eukaryotic DNA ligases and is referred to as the conserved peptide (Fig. 6 A). Notable features of the conserved peptide include a sequence RFPR that is invariant among human DNA ligases and a high proportion of basic amino acids. The C-terminal 10 residues of the conserved motif correspond to motif VI, which was identified based on its conservation in DNA ligases and mRNA capping enzymes (3). Single amino acid changes at positions throughout the conserved peptide of DNA ligase III that are invariant or highly conserved in human DNA ligases (Fig. 6 B) were made in the His-tagged ΔZf version of DNA ligase IIIβ to facilitate purification and analysis of DNA interactions involving the catalytic domain.

Initially, the effects of amino acid changes on the ability of DNA ligase IIIβ to complement the conditional lethal phenotype of an E. coli lig mutant were examined. Replacement of glycine 712 with alanine and proline 718 with either alanine or threonine did not abolish complementation activity (Fig. 6 B). Thus, we conclude that, although these residues are invariant within the conserved peptide of eukaryotic DNA ligases, relatively conservative substitutions do not abolish catalytic activity. Similar results were obtained when arginine 722, a conserved basic amino acid residue within the conserved peptide, was replaced with either glutamine or valine (Fig. 6 B).

DNA ligase IIIβ polypeptides encoded by plasmids that failed to complement the temperature sensitivity of the E. coli lig strain were selected for further analysis. After partial purification by metal-chelating chromatography, the ability of the DNA ligase IIIβ polypeptides to form the enzyme-AMP complex was examined. The substitutions serine 714 with isoleucine, arginine 724 with glycine, and lysine 727 with glycine all resulted in polypeptides that were unable to form the covalent enzyme-AMP reaction intermediate (Fig. 6 B). An example of one of these defective polypeptides, K727G, which has been purified to near homogeneity by phosphocellulose and metal-chelating chromatography, is shown in Fig. 7. The failure of these altered versions of DNA ligase IIIβ to form a labeled enzyme-AMP complex was not due to the presence of a high proportion of enzyme-AMP complexes in the purified fraction because preincubation with pyrophosphate, which reverses the first step of the ligation reaction, had no effect on enzyme-AMP formation by these polypeptides (data not shown). Thus, these amino acid changes result in polypeptides that are defective in the first step of the ligation reaction.

Another group of amino acid substitutions, arginine 716 with glycine, phenylalanine 717 with leucine, and arginine 719 with glycine, resulted in polypeptides that were not defective in enzyme-AMP formation but still failed to complement the E. coli lig mutant (Fig. 6 B). Formation of the enzyme-AMP complex by these polypeptides and a polypeptide with the wild type sequence, which have been purified to near homogeneity, is shown in Fig. 7. The lack of functional complementation does not appear to be due to differences in expression levels or protein stability because these polypeptides were expressed at similar levels to the wild type polypeptide and were obtained in similar yields after purification by phosphocellulose and metal-chelating chromatography (data not shown). Therefore, we examined the ability of these enzymes to catalyze phosphodiester bond formation. The DNA joining activities of the R716G and F717L polypeptides were greater than 100-fold less than that of the wild type polypeptide (Fig. 8 A). Surprisingly, the R719G polypeptide had the same or even slightly higher DNA joining activity than the polypeptide with the wild type sequence un-
The apparently normal enzyme—identified as described under “Experimental Procedures.” After separation by SDS-polyacrylamide gel electrophoresis, DNA ligase III polypeptides (200 ng of each) were transferred to a nitrocellulose membrane. The membrane was incubated with polyclonal antibodies specific for PARP (200 ng of each) and then stained with Coomassie Blue. The positions of molecular mass standards are shown on the left. B, after separation by SDS-polyacrylamide gel electrophoresis, DNA ligase IIIβ polypeptides (200 ng of each) were transferred to a nitrocellulose membrane. The membrane was incubated with polyclonal antibodies specific for PARP and then visualized by enhanced chemiluminescence. C, DNA ligase IIIβ polypeptides (200 ng of each) were incubated with [α-32P]ATP as described under “Experimental Procedures.” After separation by SDS-polyacrylamide gel electrophoresis, labeled polypeptides were detected by autoradiography.

under these reaction conditions (Fig. 8A). Studies to investigate the paradoxical in vitro (Fig. 6B) and in vitro (Fig. 8A) results obtained with the R719G polypeptide are described later.

The failure of the R716G and F717L polypeptides to catalyze phosphodiester bond formation could be explained either by a defect in one or both of the latter two steps of the ligation reaction, formation of the DNA-AMP intermediate, and the subsequent phosphodiester bond formation. To address this issue, labeled enzyme-AMP complexes were incubated with an unlabeled duplex DNA containing a single nick. Transfer of the labeled AMP moiety results in the formation of a labeled AMP-DNA intermediate. The consumption of this intermediate in the final step of the ligation reaction, phosphodiester formation, results in the release in the labeled AMP moiety from the DNA substrate. In reactions with the wild type polypeptide, the expected initial accumulation and subsequent consumption of the DNA-adenylate intermediate was observed (Fig. 8B). In contrast, both the R716G and F717L polypeptides were severely impaired in their ability to transfer the AMP moiety to the DNA substrate. Thus, we conclude that arginine 716 and phenylalanine 717 play critical roles in the transfer of the AMP group from the DNA ligase to the 5′-phosphate terminus at a nick in duplex DNA.

The R719G polypeptide had wild type joining activity at both 16 °C (Fig. 8A) and 20 °C (data not shown) but was inactive at 37 °C (Fig. 9A). Next, we investigated the effect of temperature on different stages of the ligation reaction. Formation of the labeled enzyme-adenylate by R719G was reduced about 3-fold at 37 °C compared with 25 °C, whereas the activity of comparable wild type polypeptide was only 1.4-fold lower at the higher temperature (data not shown). Since the adenyllylation reaction is rapid (4), it is possible that adenyllylation occurs prior to thermal inactivation. At 25 °C, enzyme-adenylate formation by both R719G and wild type polypeptides reached a maximum within the first 30 s of incubation (Fig. 9B). When these polypeptides were preincubated at 37 °C prior to incubation at 25 °C, the rate of enzyme-adenylate formation was reduced. Although the R719G polypeptide was more severely affected than the wild type polypeptide, the difference was only 2–3-fold (Fig. 9B). These results suggest that the R719G DNA ligase is more severely impaired in the latter steps of the DNA ligation reaction at higher temperatures.

To confirm this, the labeled enzyme intermediate was formed at 20 °C and then incubated with an unlabeled DNA substrate containing a nick. Formation of a labeled DNA adenylate and the subsequent consumption of this reaction intermediate in the last step of the ligation reaction was monitored as a function of time at both 20 and 37 °C. With the wild type enzyme, the accumulation and decline of the DNA adenylate intermediate was observed at both temperatures (Fig. 9C). A similar profile was observed with R719G at 20 °C, but no significant formation of the DNA adenylate was detected at 37 °C (Fig. 9C). Thus, we conclude that the R719G polypeptide is a heat-labile enzyme that is unable to transfer the AMP moiety from itself to the 5′-phosphate terminus of a nick in DNA at the nonpermissive temperature. Interestingly, thermal inactivation of R719G DNA ligase activity is reversible. Formation of the DNA adenylate intermediate was observed after the labeled enzyme-adenylate intermediate was preincubated at 37 °C prior to incubation with the nicked DNA substrate at 20 °C (Fig. 9D).

**DISCUSSION**

Three mammalian genes encoding DNA ligases have been identified (1, 2). Although these enzymes have distinct cellular functions, they utilize the same basic reaction mechanism. Thus, it seems reasonable to assume that once recruited to sites of discontinuity in the phosphodiester backbone, the same key residues within the conserved catalytic domain of these enzymes will mediate transfer of the AMP group from the DNA ligase to the DNA substrate followed by phosphodiester bond formation. In this study, we have investigated the role of amino acids within the conserved peptide motif (which includes motif VI), a 16-amino acid sequence that was originally identified in vaccinia DNA ligase (1–3, 5). Amino acid substitutions at three residues, serine 714, arginine 724, and lysine 727, within the conserved peptide of DNA ligase IIIβ abolished enzyme-AMP formation. The positively charged residues correspond to arginine 285 and lysine 288 within motif VI of Chlorella virus mRNA capping enzyme, which is also a nucleotidyl transferase (28). In the closed conformation of the mRNA capping enzyme, these residues appear to contact the triphosphate tail of the GTP (29). Thus, the inability of the mutant DNA ligases to form an enzyme-AMP complex supports the notion that these residues play a critical role in positioning the nucleoside triphosphate for attack by the nucleophilic lysine (residue 421 in DNA ligase III) in nucleotidyl transferases (29).

The sequence RFPR is invariant within the conserved peptide of human DNA ligases (1, 2). None of these residues was required for enzyme-AMP formation, but replacement of either
the first arginine with glycine or the adjacent phenylanine residue with leucine resulted in polypeptides that were defective in transferring the AMP moiety to the DNA substrate. Furthermore, substitution of the second arginine residue with a glycine residue generated a temperature-sensitive enzyme that was more severely deficient in the nucleotidyl transfer reaction compared with formation of the enzyme-AMP complex. In the crystal structures of the mRNA capping enzyme and T7 DNA ligase (10, 29), the region containing the conserved peptide is relatively unstructured and undergoes a large conformational change between the open and closed forms of the mRNA capping enzyme. Since the thermal inactivation of R719G is reversible, it seems likely that the denaturation at elevated temperatures is localized to a small region encompassing the conserved peptide. Together with the structural studies (10, 29), our enzymatic results suggest that the RFPR motif binds to and positions the nicked DNA substrate for the nucleotidyl transfer reaction that presumably occurs within the closed conformation of the enzyme.

The cellular functions of the mammalian DNA ligases appear to be determined by their recruitment to specific DNA substrates in vivo (1, 2). The binding to different protein partners via the unique amino acid sequences that flank the catalytic domain of these enzymes is one mechanism by which this functional specificity is mediated. For example, the partner of DNA ligase IV, XRCC4 interacts with the heterodimeric complex, Ku, that binds to DNA double strand breaks (30–32), whereas DNA ligase I is tethered to DNA molecules via an interaction with the clamp protein, proliferating cell nuclear antigen (27). DNA ligase IIIα and β are distinct from all other DNA ligases identified to date in that they have a zinc finger motif that binds to DNA (7, 17). Interestingly, this zinc finger, which is located at the N terminus of DNA ligase III, is homologous with the two N-terminal zinc fingers of PARP that bind to DNA strand breaks and activate the polymerase activity (7, 11, 12). Since PARP finger II plays a critical role in the interaction with DNA single strand breaks (11, 12), it was proposed that the zinc finger of DNA ligase III would resemble PARP finger II and would act as a molecular nick sensor (13). In this study, we demonstrate that the DNA ligase III zinc finger is in fact most closely related to PARP finger I. However, the DNA ligase III zinc finger does form a specific complex with a nick in duplex DNA as determined by DNase I footprinting. Furthermore, the DNA ligase III zinc finger allows this enzyme to form a stable complex with and join nicked DNA at physiological salt concentrations.

In this study, expression of zinc finger-deleted versions of DNA ligase IIIβ complemented the conditional lethal phenotype of *E. coli* lig mutants. Furthermore, vaccinia DNA ligase, which exhibits homology with DNA ligase III over its entire length but lacks an N-terminal zinc finger (5, 7, 14), corrects the temperature-sensitive phenotype of an *S. cerevisiae cdc9*
DNA ligase mutant (33). Thus, the zinc finger is not required for in vivo function in heterologous organisms. Interestingly, the genes encoding PARP, DNA ligase III, and XRCC1 appear to be restricted to multicellular organisms. Furthermore, the sensitivity of cell lines deficient in either PARP or the DNA ligase IIIα-XRCC1 complex to alkylating agents and ionizing radiation is consistent with these enzymes acting in the same DNA repair pathways (34–36). In support of this idea, XRCC1 mutation sensitivity of cell lines deficient in either PARP or the DNA ligase III mutant (33). Thus, the zinc finger is not required for in vivo function in heterologous organisms. Interestingly, the genes encoding PARP, DNA ligase III, and XRCC1 appear to be restricted to multicellular organisms. Furthermore, the sensitivity of cell lines deficient in either PARP or the DNA ligase IIIα-XRCC1 complex to alkylating agents and ionizing radiation is consistent with these enzymes acting in the same DNA repair pathways (34–36). In support of this idea, XRCC1 interacts via different regions with PARP, DNA ligase IIIα, and also DNA polymerase β, suggesting that these proteins may function together as a multiprotein repair complex (17, 37–39). Although the functions of the similar DNA binding domains of PARP and DNA ligase IIIα in the repair reaction mediated by this multiprotein complex have not been defined, there is evidence indicating that the DNA binding properties of the two zinc finger DNA binding domains of PARP are different from those of the DNA ligase III zinc finger. Overexpression of the PARP DNA binding domain has a dominant negative effect on cellular responses to DNA damage (40), whereas overexpression of the DNA ligase III zinc finger does not inhibit activation of poly(ADP-ribosylation) by PARP after DNA damage by hydrogen peroxide (data not shown). One interpretation of this observation is that the PARP DNA binding domain binds to DNA strand breaks with higher affinity and stability than the DNA ligase III zinc finger. This suggests that PARP acts as the sensor of genomic DNA single strand breaks and that the binding of PARP to a single strand break results in the recruitment of the XRCC1-DNA ligase IIIα complex and other repair proteins. A possible role for the DNA ligase III zinc finger in this repair pathway would be to displace the DNA binding domain of automodified PARP, which binds less tightly to nicked DNA, from the single strand break, allowing the DNA ligase and other repair proteins access to the DNA lesion.

In summary, we have identified two functionally distinct regions within mammalian DNA ligase III that interact with DNA ligase mutant (33). Thus, the zinc finger is not required for in vivo function in heterologous organisms. Interestingly, the genes encoding PARP, DNA ligase III, and XRCC1 appear to be restricted to multicellular organisms. Furthermore, the sensitivity of cell lines deficient in either PARP or the DNA ligase IIIα-XRCC1 complex to alkylating agents and ionizing radiation is consistent with these enzymes acting in the same DNA repair pathways (34–36). In support of this idea, XRCC1 interacts via different regions with PARP, DNA ligase IIIα, and also DNA polymerase β, suggesting that these proteins may function together as a multiprotein repair complex (17, 37–39). Although the functions of the similar DNA binding domains of PARP and DNA ligase IIIα in the repair reaction mediated by this multiprotein complex have not been defined, there is evidence indicating that the DNA binding properties of the two zinc finger DNA binding domains of PARP are different from those of the DNA ligase III zinc finger. Overexpression of the PARP DNA binding domain has a dominant negative effect on cellular responses to DNA damage (40), whereas overexpression of the DNA ligase III zinc finger does not inhibit activation of poly(ADP-ribosylation) by PARP after DNA damage by hydrogen peroxide (data not shown). One interpretation of this observation is that the PARP DNA binding domain binds to DNA strand breaks with higher affinity and stability than the DNA ligase III zinc finger. This suggests that PARP acts as the sensor of genomic DNA single strand breaks and that the binding of PARP to a single strand break results in the recruitment of the XRCC1-DNA ligase IIIα complex and other repair proteins. A possible role for the DNA ligase III zinc finger in this repair pathway would be to displace the DNA binding domain of automodified PARP, which binds less tightly to nicked DNA, from the single strand break, allowing the DNA ligase and other repair proteins access to the DNA lesion.

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Recognition of DNA Nicks by DNA Ligase III

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nick DNA. The sequence RFPR, which is present within the catalytic domain, is required for the transfer of the AMP group from the enzyme to the 5'-phosphate terminus at a DNA nick. Since this sequence motif is invariant in mammalian DNA ligases, it is likely to fulfill the same role in the ligation reaction catalyzed by DNA ligases I and IV. In contrast, the zinc finger motif is a unique feature that distinguishes DNA ligase III from other DNA ligases. The DNA ligase III zinc finger targets this enzyme to nicks in duplex DNA, enabling it to form stable complexes with and ligate nicked DNA at physiological salt concentrations in vitro. Further studies will provide insights into the in vivo role of this DNA binding activity in base excision and single strand break repair pathways.