Specific Function of DNA Ligase I in Simian Virus 40 DNA Replication by Human Cell-free Extracts Is Mediated by the Amino-terminal Non-catalytic Domain*

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The joining of Okazaki fragments during lagging strand DNA replication in mammalian cells is believed to be due to DNA ligase I. This enzyme is composed of a 78-kDa carboxyl-terminal catalytic domain and a 24-kDa amino-terminal region that is not required for ligation activity in vitro. Extracts of the human cell line 46BR.1G1, in which DNA ligase I is mutationally altered, supported aberrant in vitro SV40 DNA replication; the joining of Okazaki fragments was defective, and unligated intermediates were unstable. Human DNA ligase I, but not DNA ligase III or bacteriophage T4 DNA ligase, complemented both defects in 46BR.1G1 extracts. The catalytic domain of DNA ligase I was 10-fold less effective in complementation experiments than the full-length protein, indicating that the amino-terminal region of the enzyme is required for efficient lagging strand DNA replication. Moreover, in vitro SV40 DNA replication in normal human cell extracts was inhibited by an excess of either full-length DNA ligase I or the amino-terminal region of the protein, but not by the catalytic domain. This inhibition may be mediated by the interaction of the amino-terminal region of DNA ligase I with other replication proteins.

Several distinct DNA ligases have been identified in mammalian cells, DNA ligase I being a major activity in proliferating cells (1, 2). Cytostaining experiments with antibodies against DNA ligase I showed that the enzyme is specifically localized in the nucleus with the same granular staining pattern as DNA polymerase α, implicating DNA ligase I in DNA replication (3). DNA ligase I (in conjunction with DNA polymerase α, δ, or ε; RNase H1; and the 5′-nuclease DNase IV/FEN-1) is able to complete lagging strand DNA replication in vitro on a synthetic DNA substrate (4), and DNA ligase I activity also functions to generate closed circular DNA during SV40 DNA replication reconstituted with SV40 large T antigen and purified mammalian proteins (5, 6).

The human DNA ligase I cDNA encodes a 102-kDa polypeptide (7). A 78-kDa carboxyl-terminal domain shows significant amino acid sequence homology to the CDC9 and cdc17* gene products of Saccharomyces cerevisiae and Schizosaccharomyces pombe as well as to the human DNA ligase III and IV cDNAs. This domain is catalytically active in vitro in the absence of the amino-terminal region and is able to complement a conditional lethal DNA ligase mutant of Escherichia coli (8). The 24-kDa amino-terminal portion of human DNA ligase I is a protease-sensitive hydrophilic region that has no counterpart in other mammalian DNA ligases or the yeast DNA ligases. Although this amino-terminal region is not required for activity of DNA ligase I in standard in vitro DNA joining assays, it is essential in vivo to counteract the lethal effect of knocking out DNA ligase I in mouse embryonic stem cells by the ectopic expression of DNA ligase I (9). A functional role for the amino-terminal portion of DNA ligase I during DNA replication has not been directly demonstrated, but this region may serve to localize the protein to sites of DNA replication by specific contacts with other replication factors (10).

The human cell line 46BR, derived from an individual exhibiting retarded growth, severe immunodeficiency, and lymphoma, has been shown to have an inactivating Glu-566 → Lys mutation in one allele of the DNA ligase I gene and an Arg-771 → Trp mutation in the other (11). Both of these amino acid changes are within the catalytic domain of the protein. The SV40-transformed subline 46BR.1G1 is either homozygous or hemizygous for the mutation at Arg-771 and shows the same physiological defects as the primary cell line, including the accumulation of low molecular size DNA species during DNA replication (12). 46BR.1G1 cells contain normal levels of DNA ligase I protein, but the protein exhibits only ~5% of normal DNA joining activity, and the cells show delayed joining of Okazaki fragments during DNA replication in permeabilized, synchronized cells (13).

In this study, we have used the in vitro SV40 DNA replication system to monitor aberrant DNA replication in 46BR.1G1 cell-free extracts. This permeable system allowed us to specifically study the function of DNA ligase I and the amino-terminal region of the enzyme in DNA replication. We show that DNA ligase I, but not DNA ligase III or bacteriophage T4 DNA ligase, complements the replication defect in 46BR.1G1 cell-free extracts and that the amino terminus of DNA ligase I is required for efficient complementation. Furthermore, the addition of DNA ligase I to normal cell extracts, in >10-fold excess over the amount required for complementation of 46BR.1G1 extracts, inhibits their ability to perform in vitro SV40 DNA replication, and this inhibition appears to be mediated by the amino terminus of the protein.

**EXPERIMENTAL PROCEDURES**

Preparation of Cell-free Extracts

The human SV40-transformed fibroblast cell lines MRC5 V1 and 46BR.1G1 (13) were maintained in monolayer culture in E4 medium supplemented with 10% fetal bovine serum. Human HeLa S3 cells, adapted for growth in suspension (14), were grown in RPMI 1640 medium with 5% fetal bovine serum. Cells (1–5 × 10⁶) were harvested in mid-log phase by centrifugation at 1000 × g for 5 min. Extracts were prepared from each cell line according to Cecotti et al. (14).
DNA Ligase I in DNA Replication

Reagent Enzymes

SV40 large T antigen (TAg) was obtained from Molecular Biology Resources, Inc. (Milwaukee, WI), and T4 DNA ligase was from New England Biolabs Inc. Recombinant human DNA ligase III and XRCC1 proteins were produced as described (15).

Subcloning, Expression, and Purification of Recombinant DNA Ligase I Proteins

Hlig I (2–263)—The DNA sequence encoding the aminoo-terminal region of human DNA ligase I was amplified by polymerase chain reaction using a 5′-primer that included an NdeI restriction site and a sequence encoding Met-Gly-His6 immediately upstream of the second residue of the amino-terminal sequence of DNA ligase I. The polymerase chain reaction product was cleaved with NdeI and at an internal BamHI restriction site, cloned between the NdeI and BamHI sites of pET11a, and verified by DNA sequencing. The resultant construct encoded amino acids 2–263 of the full-length protein.

Hlig I (262–919)—Digestion of the DNA ligase I cDNA at the internal BamHI site and at an AvrII site in the 3′-untranslated region generated a fragment that lacked the first 261 amino acids of DNA ligase I and that could be cloned into the BamHI and CsiII restriction sites of pTET6b, using an AvrII-CsiII linker at the 3′-end, to generate an open reading frame encoding Met-Gly-His6-Ser-Gly-His-Ile-Glu-Gly-Arc-His-Met-Leu-Glu from the vector polylinker, followed by the C-terminal domain of DNA ligase I beginning at residue 262.

Hlig I—The AvrII-CsiII linker used above also contained a BamHI site, so that the carboxyl terminus of DNA ligase I could be isolated from Hlig I (262–919) as a BamHI fragment and joined in frame to the amino terminus in pET11a by ligation to BamHI-linearized Hlig I (1–263). This generated a construct encoding histidine-tagged full-length DNA ligase I. Protein expressed from this construct was catalytically active in the absence of post-translational modification, in contrast to DNA ligase I expressed previously as a fusion protein with TAg in the absence of post-translational modification, in contrast to DNA ligase I. Protein expressed from this construct was catalytically active in the absence of post-translational modification, in contrast to DNA ligase I expressed previously as a fusion protein with TAg.

All three constructs were transformed into E. coli strain BL21. Individual colonies were grown to mid-log phase in L-broth supplemented with 600 mM NaCl. All three proteins were dialyzed against a buffer containing 600 mM NaCl, 0.1 mM EDTA, 80 mM Hepes-KOH, pH 7.5, 7 mM MgCl2, 0.5 mM DTT, 4 mM ATP, 40 mM creatine phosphate, 25 mM NaCl, 0.1 mM EDTA, and 10% glycerol and stored at –80 °C.

DNA Ligase Assays

DNA ligation activity was measured by the conversion of [γ-32P]dATP into DNA (see below). The remaining 20 μl was brought to 0.5% SDS and digested with 200 μg/ml proteinase K (Sigma) at 37 °C for 1 h. After extraction once with phenol/chloroform/isoamyl alcohol (24:24:1), the samples were precipitated with 2 volumes of ethanol in the presence of 0.2 M ammonium acetate. After centrifugation, the pellets were washed with 70% ethanol, dried, and resuspended in distilled water supplemented with 20 mM KCl. Samples were electrophoresed through a 1% agarose gel (19) at 2 V/cm for 16 h. Gels were fixed in 7% (w/v) trichloroacetic acid, dried, and autoradiographed.

In Vitro SV40 DNA Replication Assay

The assay conditions were slightly modified from the procedure of Li and Kelly (20). Reaction mixtures (50 μl) contained 30 mM Hepes-KOH, pH 7.5, 7 mM MgCl2, 0.5 mM DTT, 4 mM ATP, 40 mM creatine phosphate, 4 mM [γ-32P]dATP, 100 μM dCTP, dGTP, dTTP, 30 μM dATP, 2 μCi of [α-32P]dATP, 40 mM creatine phosphate, 1.2 μg of creatine phosphokinase, 0.3 μg of superhelical replicative form I plasmid DNA containing an SV40 origin of replication, 240 μg of cell extract, and 2 μg of TAg. Reactions were incubated at 37 °C for 2 h, and the extent of DNA replication was monitored by quantifying the incorporation of [α-32P]dATP into DNA (see below). Where assays were supplemented with recombinant DNA ligase I proteins, the protein was preincubated with cell extract for 12 min at 37 °C before the extract was used in the assay.

Quantification of Radiolabel Incorporated from [α-32P]dATP into DNA

Aliquots of reaction mixtures were spotted onto DE81 filters (Whatman) and dried (3 × 15 min) in 200 ml of 0.5 M Na2HPO4 to remove unincorporated [α-32P]dATP, rinsed in water and then in ethanol, and air-dried, and incorporation of radioactive material into DNA was determined by liquid scintillation counting.

RESULTS

Defective Ligation of Okazaki Fragments during in Vitro SV40 DNA Replication in 46BR.1G1 Cell Extracts—The SV40-transformed human fibroblast cell line 46BR.1G1 has ~5% of normal DNA ligase I activity. Previous studies with permeabilized, synchronized cells showed that ligation of Okazaki fragments is retarded in 46BR.1G1 cells (13). Extracts from both 46BR.1G1 cells and a control SV40-transformed fibroblast cell line, MRC5 V1, are able to support in vitro SV40 DNA replication to similar extents, as estimated by incorporation of [α-32P]dATP into plasmid DNA containing an SV40 origin of replication (data not shown). We have used pulse-chase experiments to specifically monitor the maturation of Okazaki fragments during in vitro SV40 DNA replication in these extracts. Origin-containing plasmid DNA was preincubated in a replication reaction mixture containing ATP but lacking other NTPs and dNTPs to allow origin complex formation, prior to pulse labeling with a mixture of rNTPs, dNTPs, and [α-32P]dATP and then chasing for various lengths of time with an excess of unlabeled dATP.

Analysis of replication products on denaturing alkaline agarose gels revealed that during the initial 20-s pulse, the majority of [α-32P]dATP in both MRC5 V1 and 46BR.1G1 extracts was into DNA fragments that migrated between the 125- and 564-nucleotide markers, which corresponds to the expected size of Okazaki fragments (Fig. 1A, lanes 5 and 9). However, in MRC5 V1 extracts, larger DNA molecules were present even at this early time point, and with increasing incubation times after the addition of unlabeled 32P-independent background labeling of contaminating replicative form II DNA and then incubated for 50 min at 37 °C in the presence of TAg. Reactions were pulse-labeled for 20 s by the addition of CTP, GTP, and UTP to 200 μM each; dCTP, dGTP, and dTTP to 100 μM each; and 40 Ci/ml [α-32P]dATP (3000 Ci/μM; Amersham Corp.) to give a final dATP concentration of 0.013 μM. Reactions were chased by the addition of unlabeled dATP to 5 μM, followed by incubation for various lengths of time at 37 °C. 25-μl aliquots were spotted onto ice with the addition of EDTA to 20 mM. 5 μl of each sample was removed for quantification of [32P]radiolabel incorporated from [α-32P]dATP into DNA (see below). The remaining 20 μl was brought to 0.5% SDS and digested with 200 μg/ml proteinase K (Sigma) at 37 °C for 1 h. After extraction once with phenol/chloroform/isoamyl alcohol (24:24:1), the samples were precipitated with 2 volumes of ethanol in the presence of 2.5 M ammonium acetate. After centrifugation, the pellets were washed with 70% ethanol, dried, and resuspended in distilled water supplemented with 20 mM KCl.

SV40 DNA Replication in 46BR.1G1 Cell Extracts—Defective ligation of Okazaki fragments in 46BR.1G1 extracts was into DNA fragments that migrated between the 125- and 564-nucleotide markers, which corresponds to the expected size of Okazaki fragments (Fig. 1A, lanes 5 and 9). However, in MRC5 V1 extracts, larger DNA molecules were present even at this early time point, and with increasing incubation times after the addition of unlabeled

The abbreviations used are: TAg, large T antigen; Hlig I, human DNA ligase I; FEN-1, flap endonuclease; PCNA, proliferating cell nuclear antigen; DTT, dithiothreitol.
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Unligated Pulse-labeled Replication Products Are Unstable in 46BR.1G1 Extracts—The amount of radioactivity incorporated into replication products in 46BR.1G1 extracts during the pulse labeling appeared to decrease throughout the subsequent chase period (Fig. IA, lanes 1–4), indicating that partial replication products are unstable in 46BR.1G1 extracts. Incorporation of radiolabeled material into DNA during pulse-chase assays was quantitated by adsorption of samples to DE81 filters, followed by scintillation counting. In MRC5 V1 extracts, as expected, there was no further detectable incorporation of radioactive material into DNA during the chase period (Fig. 1B). However, in 46BR.1G1 extracts, the amount of radioactive material already incorporated into DNA during the pulse actually decreased by >50% during the chase period. Pulse-labeled fragments of decreased size were not detectable during the chase period. In the absence of DNA ligation, nick translation may occur at the junction between adjacent pulse-labeled products and could account for the diminishing signal during the chase period.

Overexpression of Full-length DNA Ligase I and Fragments of the Recombinant Protein—Human DNA ligase I has a 78-kDa carboxyl-terminal catalytic domain and a 24-kDa amino-terminal region that is not required for catalytic activity in vitro (8). To assess the role of the amino-terminal region in DNA replication, we made use of a BamHI restriction site in the cDNA of human DNA ligase I that traverses codon 262 to subclone the individual amino-terminal (Hlig I-(2–263)) and carboxyl-terminal (Hlig I-(262–919)) domains (Fig. 2A). Proteins were overproduced in E. coli with amino-terminal histidine tags to allow rapid affinity purification on Ni<sup>2+</sup>-nitrilotriacetic acid-agarose (Fig. 2B). The ligation activity of full-length DNA ligase I and Hlig I-(262–919) was measured against 1 unit of T4 DNA ligase on a double-stranded substrate of [γ<sup>32</sup>P]dATP into DNA. Ligation activity generates 5<sup>′</sup>-3<sup>′</sup>poly(dT)<sub>16</sub> annealed to poly(dA). Ligation activity generates 5<sup>′</sup>-3<sup>′</sup>polig(dT)<sub>16</sub> multimers, which are resolved on denaturing polyacrylamide gels (Fig. 2C). Full-length DNA ligase I and Hlig I-(262–919) had comparable DNA joining activity on this substrate (1.2 and 1.7 units/pmol, respectively). Previously, up to 249 amino acids had been removed from the amino terminus of DNA ligase I without loss of catalytic activity (8), so Hlig I-(262–919) redenfines the minimal catalytic domain of the protein.

Complementation of the Replication Defect in 46BR.1G1 Extracts with Recombinant DNA Ligase I Is Promoted by the Amino-terminal Region of the Protein—Pulse-chase assays in 46BR.1G1 extracts were supplemented with full-length recombinant DNA ligase I in an attempt to complement the replication defect. In pulse-labeling experiments with chase periods of 0 and 5 min, 0.02 units/μl full-length DNA ligase I was found to restore joining of Okazaki fragments in 46BR.1G1 extracts to a level comparable to that seen in unsupplemented MRC5 V1 extracts (Fig. 3A, lanes 5–8). The addition of up to 0.2 units/μl enzyme did not further increase the rate of ligation in 46BR.1G1 extracts (Fig. 3A, lanes 9 and 10) and had no effect on the incorporation of Okazaki fragments into DNA.
on replication in MRC5 V1 extracts (lanes 1–4), suggesting that DNA ligase I is not limiting in MRC5 V1 extracts. Supplementing 46BR.1G1 extracts with DNA ligase I not only restored the chasing of Okazaki fragments into larger DNA species, but also completely prevented instability of the pulse-labeled DNA fragments during the chase period (data not shown). Complementation also increased the level of incorporation into form I DNA observed after the 5-min chase (Fig. 3A, lanes 8 and 10). This appears to correspond to the synthesis of mature replication products, as background incorporation of radiolabel into forms I and II of the plasmid by nick translation activity was reduced in complemented 46BR.1G1 extracts (Fig. 3A, lanes 7 and 9).

To establish whether the amino-terminal region of DNA Ligase I in DNA Replication

FIG. 2. Overexpression and purification of full-length and truncated forms of recombinant human DNA ligase I. A, schematic representation of the 102-kDa human DNA ligase I polypeptide. The position of the active-site lysine residue is indicated within the catalytic domain. A BamHI site traverses the codon for residue 262 and was used to subclone the individual amino- and carboxyl-terminal fragments to generate Hlig I-(2–263) and Hlig I-262-919, respectively. A third construct encoding full-length DNA ligase I (Hlig I) was also generated. B, purified, histidine-tagged, recombinant DNA ligase I proteins. The purified proteins were separated by 8% denaturing polyacrylamide gel electrophoresis and stained with Coomassie Blue. The position of each purified protein is indicated with an arrow. The amino-terminal domain and the full-length enzyme migrated anomalously slowly during denaturing polyacrylamide gel electrophoresis (1). A trace of the carboxyl-terminal catalytic fragment of DNA ligase I, generated by proteolysis of the full-length protein, is also visible in the first lane. C, DNA joining assays. The DNA joining activity of 20 ng of either full-length Hlig I or Hlig I-262-919 (262–919) was estimated by comparison with that of 1 unit of T4 DNA ligase (T4 DNA lig), using a substrate of [5'-32P]oligo(dT)16 annealed to poly(dA), for the incubation times indicated. Reaction products were analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography; the positions of [5'-32P]oligo(dT)16 and multimeric ligation products are indicated.

FIG. 3. Complementation of the DNA replication defect in 46BR.1G1 extracts by full-length Hlig I or Hlig I-262-919. Pulse-chase reactions were carried out as described in the legend to Fig. 1, and samples were taken after the chase times indicated. Reactions were supplemented with full-length DNA ligase I or its catalytic domain as indicated. A, complementation by Hlig I. Lanes 1–4, MRC5 V1 extracts; lanes 5–10, 46BR.1G1 extracts. B, complementation of 46BR.1G1 extracts by Hlig I-262-919 (lanes 5–10, 46BR.1G1 extracts). Reaction products were analyzed by alkaline agarose gel electrophoresis and autoradiography (A and B). Markers were as described in the legend to Fig. 1. C, quantitation of complementation data. The percentage of pulse-labeled replication products smaller than the 564-nucleotide (nt) marker remaining after the 5-min chase was quantitated by PhosphorImager analysis of the data in A and B, together with additional reactions in which Hlig I or Hlig I-262-919 was added in limiting amounts (data not shown). , data from MRC5 V1 extract supplemented with Hlig I; , data from 46BR.1G1 extract supplemented with Hlig I; , data from 46BR.1G1 extract supplemented with Hlig I; , data from 46BR.1G1 extract supplemented with Hlig I-262-919. RF, replicative form.
Pulse-chase reactions using either MRC5 V1 extract (assays with this protein at 0.02 units/µl) or 46BR.1G1 extract (lanes 2–4) were sampled after a 5-min chase, and replication intermediates were analyzed by alkaline agarose gel electrophoresis and autoradiography as described in the legend to Fig. 1. Reactions were supplemented with recombinant protein as follows: lanes 1 and 2, no added protein; lane 3, 0.08 units/µl recombinant human DNA ligase III (Hlig III); lane 4, 0.2 units/µl T4 DNA ligase (T4 lig). Markers were as described in the legend to Fig. 1. nt, nucleotides; RF, replicative form.

DNA ligase I is required for the replication function of the enzyme, analogous experiments were performed using Hlig I-(262–919), which lacks the amino-terminal part of the protein and encodes only the carboxyl-terminal catalytic domain. Supplementing assays with this protein at 0.02 units/µl had no effect on the joining of Okazaki fragments in 46BR.1G1 extracts, even though this amount of the full-length protein was able to fully complement defective joining (Fig. 3B, lanes 1–4). However, the addition of 0.2 units/µl Hlig I-(262–919) did complement the joining defect in 46BR.1G1 extracts, and again, stabilization of pulse-labeled products was simultaneously achieved (Fig. 3B, lanes 5 and 6). The percentage of labeled replication products smaller than the 564-nucleotide marker that remained after a 5-min chase was calculated from the data in Fig. 3 (A and B) and from further experiments in which full-length DNA ligase I or the catalytic domain was added in limiting amounts to ascertain the minimum necessary to achieve complementation (data not shown). The catalytic domain of DNA ligase I was only effective when added at 10-fold excess over the amount of full-length protein required for complementation (Fig. 3C), suggesting that although the amino-terminal region is not essential for the replication activity of DNA ligase I, it greatly improves the efficiency of the reaction.

Figure 4. Inability of human DNA ligase III or T4 DNA ligase to complement DNA replication-defective 46BR.1G1 extracts. —Inhibition of in Vitro SV40 DNA Replication in Extracts of Normal Human Cells by Addition of DNA Ligase I or Its Amino-terminal Domain—The amino-terminal region of DNA ligase I is implicated in interactions with other replication proteins, so it was of interest to establish whether the addition of Hlig I-(2–263) in excess to competent extracts could perturb DNA replication. In this instance, TAg-dependent dAMP incorporation into DNA during a 2-h SV40 DNA replication assay was estimated. Full-length DNA ligase I was found to inhibit DNA replication in HeLa S3 extracts when added in 10–100-fold excess over the amount of DNA ligase I required to rescue the replication defect in 46BR.1G1 extracts. Synthesis was completely inhibited by the addition of full-length DNA ligase I to a final protein concentration of 5 µM (Fig. 5). The same inhibitory activity was seen with an excess of human DNA ligase I lacking an amino-terminal His6 tag (data not shown). When Hlig I-(2–263) and Hlig I-(262–919) were added separately to the replication assay, the catalytic domain had no significant effect on DNA replication even at 10 µM, whereas Hlig I-(2–263) inhibited the reaction, although to a lesser extent than the full-length protein; the amino-terminal domain alone suppressed DNA synthesis by ~60% when added at 5 µM (Fig. 5). These data indicate that the inhibition of DNA replication by the addition of DNA ligase I to replication-competent normal cell extracts is mediated via the amino-terminal domain of the protein. Analysis of replication products on both neutral and denaturing agarose gels showed no specific effect of DNA ligase I or its amino-terminal domain on the joining of replication products in HeLa S3 extracts, but rather suggested an overall suppression of DNA synthesis (data not shown).

**DISCUSSION**

The SV40-transformed human fibroblast cell line 46BR.1G1 has a mutationally altered DNA ligase I protein with only ~5% of normal DNA joining activity. Studies using permeabilized, synchronized cells indicated that, while most newly synthesized Okazaki fragments were ligated at a normal rate in
46BR.1G1 cells, ~25% remained unligated for extended periods (13), in broad agreement with previous in vivo data (12, 23). In addition, 46BR.1G1 cells are hypersensitive to a wide range of DNA-damaging agents (24), suggesting a role for DNA ligase I in DNA excision repair processes, and cellular hypersensitivity to simple alkylating agents could be normalized by transfection with the wild-type DNA ligase I cDNA (25). Here, we have added recombinant human proteins to solubile extracts supporting in vitro SV40 DNA replication and demonstrated the following: (i) complementation of aberrant DNA replication in 46BR.1G1 cell-free extracts by DNA ligase I, but not by other DNA ligases; (ii) a requirement for the amino-terminal non-catalytic region of DNA ligase I for efficient complementation; and (iii) inhibition of DNA replication in normal cell extracts by excess DNA ligase I or by the amino-terminal fragment of the protein.

During in vitro pulse-chase replication experiments, small DNA products, corresponding to the expected size of Okazaki fragments, were synthesized in both 46BR.1G1 and control MRC5 V1 extracts during the 20-s pulse. In MRC5 V1 extracts, these DNA fragments were rapidly converted into larger species, but in 46BR.1G1 extracts, unligated Okazaki fragments persisted during the chase period. Furthermore, in 46BR.1G1 extracts, but not in control extracts, pulse-labeled replication products appeared unstable, with only ~50% remaining after the 15-min chase. Titration of recombinant DNA ligase I protein into 46BR.1G1 extracts achieved complementation of both replication defects, and the full-length protein was ~10-fold more effective than the carboxy-terminal catalytic domain alone. However, further addition of DNA ligase I protein was detrimental and, in 10–100-fold excess over the amount required to correct aberrant DNA replication in 46BR.1G1 extracts, caused inhibition of DNA replication in normal replication-competent extracts. A similar inhibitory activity was observed with the isolated amino-terminal domain. In contrast, the catalytic domain alone was not inhibitory even at high concentrations.

The identification of human DNA ligase I as a component of a 21 S protein complex that contains the activities required for in vitro SV40 DNA replication (26) indicates that DNA ligase I interacts with other components of the replication machinery. Adding excess DNA ligase I to cell extracts is likely to grossly perturb the stoichiometry governing such protein-protein interactions, and this may be sufficient to inhibit DNA replication. The inhibitory effect of the amino-terminal domain of DNA ligase I alone and the requirement for this domain to achieve efficient complementation of 46BR.1G1 extracts support the notion that the function of DNA ligase I during replication is mediated by specific binding of this amino-terminal non-catalytic domain to other replication protein(s). This concurs with in vivo studies showing that the first 115 amino acids of DNA ligase I are required for colocalization of the enzyme with hromodeoxyuridine at replication foci (10). As the amino-terminal fragment of DNA ligase I is not as potent an inhibitor of DNA replication as the full-length protein, folding of this region of the protein may be somewhat altered in the absence of the carboxy-terminal catalytic domain. In addition, residues in the catalytic domain may be required to stabilize interactions of DNA ligase I with other replication factor(s) and could allow the catalytic domain alone to function weakly during replication in 46BR.1G1 extracts. Bacteriophage T4 DNA ligase and recombinant human DNA ligase III, which have no counterpart of the amino-terminal region of DNA ligase I, could not complement the replication defect in 46BR.1G1 extracts, in agreement with studies of SV40 DNA replication reconstituted with purified proteins (6); DNA ligase I was specifically required, DNA ligase III was unable to substitute, and T4 ligase substituted inefficiently. This is in marked contrast to both base excision repair and nucleotide excision repair in vitro, where no discrimination between different DNA ligases is observed in the joining step of the reconstituted pathways (15, 27).

The requirement for DNA ligase I during mammalian DNA replication suggests that it is an essential enzyme in vivo, and this has been directly demonstrated by gene knockout experiments in embryonic stem cells (9). However, in apparent conflict with these data, DNA ligase I null mouse embryos survived into midterm before dying of acute anemia (28). In this latter study, only the final four exons out of a total of 28 in the mouse DNA ligase I gene were deleted. The resulting truncated mRNA, which still encodes an intact amino-terminal domain and the active site of the enzyme, was reported to be unstable. However, low levels of this mRNA might generate a large fragment of DNA ligase I that, by analogy with a homologous fragment of bacteriophage T7 DNA ligase (29), could have enough residual activity to allow survival of null embryos, at least until very rapid cellular proliferation is required during early erythropoiesis.

Completion of lagging strand DNA synthesis requires RNase H1 to remove the RNA primer, DNase IV/FEN-1 to cleave the final ribonucleotide residue left by RNase H1, a DNA polymerase to fill the gap between adjacent Okazaki fragments, and finally DNA ligase I to seal the nick (4–6). In the absence of DNA ligation, the coordinated activities of the DNase IV/ FEN-1 nuclease and the DNA polymerase would catalyze nick translation in the 5′ → 3′ direction at the junction of two Okazaki fragments (30), and this may account for the observed instability of unligated Okazaki fragments in 46BR.1G1 extracts. Moreover, PCNA has been shown to interact directly with DNase IV/FEN-1 (31), and it has been proposed that PCNA may remain bound to the DNA following Okazaki fragment synthesis to recruit DNase IV/FEN-1 and the gap-filling DNA polymerase, δ or ε (32). In consequence, the ends of unligated Okazaki fragments would not be generally accessible to nuclear proteins, and this model is consistent with the inability of human DNA ligase III or T4 DNA ligase to function in lagging strand DNA replication. Thus, the specificity of DNA ligase I is mediated by a distinct amino-terminal domain, which probably allows access to an otherwise protected nick via interaction with other components of the replication machinery. DNA polymerase δ or ε, PCNA, and DNase IV/FEN-1 are candidates for this role. Identification of the specific protein partner(s) of DNA ligase I will further our understanding of coordinated lagging strand DNA synthesis.

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