DNA Gyrase-mediated Wrapping of the DNA Strand Is Required for the Replication Fork Arrest by the DNA Gyrase-Quinolone-DNA Ternary Complex

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The ability of DNA gyrase (Gyr) to wrap the DNA strand around itself allows Gyr to introduce negative supercoils into DNA molecules. It has been demonstrated that the deletion of the C-terminal DNA-binding domain of the GyrA subunit abolishes the ability of Gyr to wrap the DNA strand and catalyze the supercoiling reaction (Rampranis, S. C., and Maxwell, A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 14416–14421). By using this mutant Gyr, Gyr (A59), we have studied effects of Gyr-mediated wrapping of the DNA strand on its replicative function and its interaction with the quinolone antibacterial drugs. We find that Gyr (A59) can support oriC DNA replication in vitro. However, Gyr (A59)-catalyzed decatenation activity is not efficient enough to complete the decatenation of replicating daughter DNA molecules. As is the case with topoisomerase IV, the active cleavage and reunion activity of Gyr is required for the formation of the ternary complex that can arrest replication fork progression in vitro. Although the quinolone drugs stimulate the covalent Gyr (A59)-DNA complex formation, the Gyr (A59)-quinolone-DNA ternary complexes do not arrest the progression of replication forks. Thus, the quinolone-induced covalent topoisomerase-DNA complex formation is necessary but not sufficient to cause the inhibition of DNA replication. We also assess the stability of ternary complexes formed with Gyr (A59), the wild type Gyr, or topoisomerase IV. The ternary complexes formed with Gyr (A59) are more sensitive to salt than those formed with either the wild type Gyr or topoisomerase IV. Furthermore, a competition experiment demonstrates that the ternary complexes formed with Gyr (A59) readily disassociate from the DNA, whereas the ternary complexes formed with either the wild type Gyr or topoisomerase IV remain stably bound. Thus, Gyr-mediated wrapping of the DNA strand is required for the formation of the stable Gyr-quinolone-DNA ternary complex that can arrest replication fork progression.

Topoisomerases are ubiquitous enzymes that alter the linking number of DNA. As such, they play essential roles in every aspect of DNA metabolism (1). Type II topoisomerases are well conserved throughout the evolution and form a large protein family. DNA gyrase (Gyr) is unique among the type II topoisomerases. Gyr wraps the DNA strand around itself when it binds to the DNA, and this unique mode of DNA binding allows Gyr to introduce the negative supercoils into DNA molecules (1).

Kampranis and Maxwell (2) have recently demonstrated that Gyr can be converted into a conventional type II topoisomerase by deleting the C-terminal of the DNA binding domain of the GyrA subunit. This mutant Gyr, Gyr (A59), no longer wraps the DNA strand around itself and cannot catalyze the supercoiling reaction. However, Gyr (A59) is still capable of catalyzing decatenation and relaxation reactions. In addition, Gyr (A59) can partially complement the phenotype of a parC™ mutant (2). It is suggested that Gyr (A59) could substitute for the cellular function of topoisomerase IV (Topo IV).

Topoisomerases are the cellular targets for clinically important antibacterial and anticancer drugs (3–5). These topoisomerase inhibitors convert topoisomerases into poisons by trapping the covalent topoisomerase-DNA complex (also called the “cleavable complex”) as a topoisomerase-drug-DNA ternary complex. In the ternary complex, the DNA helix is broken, and the topoisomerase bridge maintains the linear integrity of the DNA. Although the ternary complex formation is critical for the cytotoxicity of these topoisomerase inhibitors, ternary complexes are normally reversible. It has been proposed that an active DNA transaction, such as the passage of replication forks, is required for the disruption of ternary complexes and the generation of nonreversible, cytotoxic DNA lesions (3–5).

We have studied the molecular events during the collision between a replication fork and a Topo IV-norfloxacin (Norf)-DNA ternary complex in vitro, using the oriC replication system reconstituted with purified proteins (6). The active strand cleavage and reunion activity of Topo IV are required for the formation of the ternary complex that can arrest replication fork progression. Interestingly, the collision between a replication fork and a Topo IV-Norf-DNA ternary complex converts the ternary complex to a nonreversible form but does not generate a double-strand break (DSB). An additional step is required for the generation of DSBs. Thus, the cytotoxicity associated with this class of topoisomerase inhibitors results from a two-step process as follows: the conversion of a topoisomerase-quinolone-DNA ternary complex to a nonreversible form and the generation of a DSB by subsequent denaturation of the topoisomerase in the dead-end complex (6). To investigate further the molecular mechanism of replication fork arrest by the topoisomerase-quinolone-DNA ternary complex, DNA gyrase (Gyr) is unique among the type II topoisomerases. Gyr wraps the DNA strand around itself when it binds to the DNA, and this unique mode of DNA binding allows Gyr to introduce the negative supercoils into DNA molecules (1).

The abbreviations used are: Gyr, DNA gyrase; DSB, double-strand break; DTT, dithiothreitol; ERI, early replicative intermediates; form II, nicked or gapped DNA molecule(s); LRI, late replicative intermediate; Norf, norfloxacin; Topo, topoisomerase.

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complex, we modeled the collision between a replication fork and a Gyr-Norf-DNA ternary complex, using the wild type and two mutant Gyr proteins, Gyr (A59) and Gyr (A, Y122F). Gyr (A59) cannot wrap the DNA strand around itself (2) and Gyr (A, Y122F) is a catalytically inactive mutant. Norf stimulated both the wild type Gyr- and Gyr (A59)-catalyzed DNA cleavages. No DNA cleavage was detected when Gyr (A, Y122F) was used. The ternary complexes formed with either Gyr (A, Y122F) or Gyr (A59) did not block the progression of replication forks in vitro. These results demonstrated that the formation of the quinolone-induced covalent Gyr-DNA complex was necessary but not sufficient to arrest the replication fork progression.

We also assessed the stability of ternary complexes formed with either Gyr (A59), the wild type Gyr, or Topo IV. We found that Gyr (A59)-Norf-DNA ternary complexes were more sensitive to salt than Gyr-Norf-DNA and Topo IV-Norf-DNA ternary complexes. A competition experiment showed that the Gyr (A59)-Norf-DNA ternary complexes readily disassociated from the DNA, whereas both Gyr-Norf-DNA and Topo IV-Norf-DNA ternary complexes remained stably bound. These results demonstrated that the ternary complexes formed with Gyr (A59) were less stable than those formed with either the wild type Gyr or Topo IV. Thus, Gyr-mediated wrapping of the DNA strand was required for the formation of stable Gyr-quinolone-DNA ternary complexes that could arrest the progression of replication forks. These results indicated that the stability of the topoisomerase-quinolone-DNA ternary complex might determine if the collision between a replication fork and a topoisomerase-quinolone-DNA ternary complex would result in the inhibition of DNA replication.

MATERIALS AND METHODS

Replication Proteins—Escherichia coli DNA replication proteins, generous gifts of Kenneth Marians (Memorial Sloan-Kettering Cancer Center), were as described previously (7–9).

A truncated GyrA subunit, GyrA(59) (2), was overexpressed and purified according to the procedure of She and Hiasa (10) with a slight modification. Briefly, after the chromatography on Q-Sepharose FF (Amersham Pharmacia Biotech) and DEAE-cellulose DE52 (Whatman), the protein fraction was loaded onto a Superose 6 HR column (Amersham Pharmacia Biotech) and eluted with Buffer A (25 mM Tris-HCl, pH 7.5 (4 °C), 0.5 mM DTT, 0.1 mM EDTA, 40% NaCl). The peak of the 59-kDa protein was pooled and dialyzed against Buffer B (50 mM Tris-HCl, pH 7.5 (4 °C), 5 mM DTT, 1 mM EDTA, 40% NaCl). The peak of the 59-kDa protein was pooled and dialyzed against Buffer B (50 mM Tris-HCl, pH 7.5 (4 °C), 5 mM DTT, 1 mM EDTA, 40% NaCl). The peak of the 59-kDa protein was pooled and dialyzed against Buffer B (50 mM Tris-HCl, pH 7.5 (4 °C), 5 mM DTT, 1 mM EDTA, 40% NaCl).

The active site Tyr of the GyrA subunit was replaced with Phe by the site-directed mutagenesis of the cloned gyrA gene using the overlap extension polymerase chain reaction technique.2 The GyrA (Y122F) protein was overexpressed and purified according to the same protocol used for the purification of GyrA(59). The final preparation of GyrA (Y122F) was greater than 95% homogeneous for a single band on SDS-polyacrylamide gel electrophoresis (data not shown).

GyrA(59) and GyrA (Y122F) were mixed with the wild type GyrB to reconstitute Gyr (A59) and Gyr (A, Y122F), respectively. The wild type Gyr was also prepared by mixing the wild type GyrA and GyrB proteins.

DNAs—Two types of oriC plasmids, pBROTB353 type I (11) and pBROTB353 type I (12), were prepared according to Hiasa and Marians (13).

oriC DNA Replication—The standard oriC DNA replication assay was performed as described previously (11, 13), except that phospho-creatine and creatine kinase were omitted from the reaction mixtures. Reaction mixtures (12.5 μl) containing the oriC plasmid pBROTB353 type I DNA, DnaA, DnaB, DnaC, DnaG, HU protein, single-stranded DNA-binding protein, the DNA polymerase III holoenzyme, and topoisomerase were incubated at 30 °C for 10 min. After terminating the reaction by adding EDTA to 25 mM, nucleotide incorporation was measured, and the replication products were analyzed by the native agarose gel electrophoresis as described by Hiasa and Marians (11).


does not show significant changes in the reaction conditions are indicated in the figure legends.

Staged Nascent Chain Elongation during oriC DNA Replication—The modified pulse-chase protocol was performed, using pBROTB353 type I DNA as the DNA template, as described previously (6, 14). Any changes in the reaction conditions are indicated in the figure legends.

Topoisomerase-catalyzed DNA Cleavages—pBROTB353 type I DNA was linearized by digesting with the EcoRI restriction endonuclease and 3′-and 5′-end-labeled by incorporation of 2 residues of [32P]dAMP with Klenow enzyme. This 3′-end-labeled linear plasmid DNA was used as a substrate.

Standard reaction mixtures (2 μl) containing 40 mM Hepes-KOH (pH 7.6), 10 mM MgOAc2, 10 mM DTT, 5 mM ATP, 20 fmol (as molecule) of [32P]-labeled linear pBROTB353 type I DNA, the indicated concentrations of Norf, and the indicated amounts of either the wild type or a mutant Gyr were incubated at 37 °C for 10 min. SDS was added to 1% to terminate the reactions, and the reaction mixtures were further incubated at 37 °C for 5 min. EDTA and proteinase K were then added to 25 mM and 100 μg/ml, respectively, and the incubation was continued for an additional 15 min. The DNA products were purified by extraction of the reaction mixtures with phenol/chloroform (1:1, v/v) and then analyzed by electrophoresis through vertical 1% agarose (SeaKem ME, FMC) gels (14 × 10 × 0.3 cm) at 4 V/cm for 4 h or at 2 V/cm for 8 h in a running buffer of 50 mM Tris-HCl (pH 7.9 at 23 °C), 40 mM sodium acetate, and 1 mM EDTA (TAE). Gels were dried onto GF plates (Whatman) and autoradiographed with Hyperfilm MP films (Amersham Pharmacia Biotech). Amounts of cleaved DNA were quantitated by scanning images by a STORM 840 PhosphorImager (Molecular Dynamics).

Stability Assay for the Topoisomerase-Quinolone-DNA Ternary Complex—Reaction mixtures were assembled as described in the previous section, and 300 fmol (as tetramer) of Gyr, Gyr (A59), or Topo IV was bound to 20 fmol (as molecule) of [32P]-labeled linear pBROTB353 type I DNA in the presence of 100 μM Norf during the first stage of incubation at 37 °C for 10 min. Then, various concentrations of NaCl were added to the reaction mixtures, and the reaction mixtures were incubated at 37 °C for 5 min. Reactions were terminated by adding SDS to 1% and incubating at 37 °C for 5 min. EDTA and proteinase K were added to a final concentration of 25 mM and 100 μg/ml, respectively, and the incubation was further continued at 37 °C for an additional 15 min. The DNA products were purified and analyzed as described in the previous section.

Decay Assay for the Topoisomerase-Quinolone-DNA Ternary Complex—Reaction mixtures (120 μl) containing 40 mM Hepes-KOH (pH 7.6), 10 mM MgOAc2, 10 mM DTT, 50 μg/ml bovine serum albumin, 2 mM ATP, 120 fmol (as molecule) of [32P]-labeled linear pBROTB353 type I DNA, 1.2 pmol (as tetramer) of the wild type Gyr, Gyr (A59), or Topo IV and 100 μM Norf were incubated at 37 °C for 10 min. A cold competitor, 9.6 μg (2.4 pmol as molecule) of pBROTB353 type I DNA, was added to the reaction mixtures, and the incubation was continued at 37 °C. Portions (18 μl each) of the reaction mixtures were withdrawn at indicated times, and SDS was added to 1% to terminate the reactions. The reaction mixtures were further incubated at 37 °C for 5 min. EDTA and proteinase K were then added to 25 mM and 100 μg/ml, respectively, and the incubation was continued for an additional 15 min. The DNA products were purified and then analyzed as described in the previous section.

RESULTS

The ability of Gyr to introduce negative superhelical into DNA molecules distinguishes this enzyme from other topoisomerases (1). Recently, it has been demonstrated that Gyr can be converted into a conventional type II topoisomerase, an enzyme similar to Topo IV, by deleting the C terminus of the DNA binding domain of the GyrA subunit (2). This mutant protein, Gyr (A59), unlike the wild type Gyr, does not wrap the DNA strand and cannot catalyze the supercoiling reaction. By using our preparations of the wild type Gyr and Gyr (A59), we assessed biochemical activities of these enzymes. Gyr (A59) could catalyze decatenation and relaxation reactions, but no supercoiling activity was detected. The specific activity of Gyr (A59) was identical to that of the wild type Gyr when the decatenation activity of these enzymes was measured using kinetoplast DNA as a substrate (data not shown). Here, we investigated effects of Gyr-mediated wrapping of the DNA strand on its functional activities during DNA replication and...
its interaction with the quinolone antibacterial drugs. Gyr (A59) Can Support Nascent Chain Elongation, but Not Decatenation of Replicating Daughter DNA Molecules, during oriC DNA Replication in Vitro—The wild type Gyr is incapable of decatenating replicating daughter DNA molecules (15). In the oriC replication system reconstituted with purified proteins, little monomer product is generated when only Gyr is present as a topoisomerase. Topoisomerase III or Topo IV is required for the production of the final monomer product (15, 16). Kampranis and Maxwell (2) have demonstrated that gyrA/Y122F) cannot decatenate replicating daughter DNA molecules during oriC DNA replication in vitro. A standard oriC DNA replication reactions containing 140 fmol (as tetramer) of the wild type Gyr and the indicated amounts (as tetramer) of either Gyr (A59) or Topo IV were incubated at 30 °C for 10 min, and the DNA products were analyzed by the electrophoresis through 0.8% native agarose gels (11). Total DNA synthesis (as nucleotides): lane 1, 186 pmol; lane 2, 218 pmol; lane 3, 201 pmol; lane 4, 220 pmol; lane 5, 180 pmol; lane 6, 235 pmol; lane 7, 200 pmol. B, standard oriC DNA replication reactions were incubated in the presence of the indicated amounts (as tetramer) of either the wild type Gyr or Gyr (A59). The replication products were analyzed by native agarose gel electrophoresis (11). Total DNA synthesis (as nucleotides): lane 1, 186 pmol; lane 2, 175 pmol; lane 3, 220 pmol; lane 4, 262 pmol; lane 5, 236 pmol; lane 6, 76 pmol; lane 7, 115 pmol; lane 8, 164 pmol; lane 9, 234 pmol. wt, the wild type Gyr; A59, Gyr (A59); IV, Topo IV; LRI, late replicative intermediates; ERI, early replicative intermediates; II/III, form II-form II DNA dimers.

Next, we assessed the effect of the quinolone drugs on Gyr (A59). By using a 3’-end-labeled linear plasmid DNA as a substrate, we measured the stimulation of Gyr (A59)-catalyzed DNA cleavages by Norf (Fig. 2). Norf stimulated both the wild type Gyr- and Gyr (A59)-catalyzed cleavages in a concentration-dependent manner. However, Gyr (A59) was less sensitive to Norf than the wild type protein. The [Norf]1/2 for Gyr (A59)-catalyzed and the wild type Gyr-catalyzed DNA cleavages were 4–5 and 0.1–0.2 μM, respectively (Fig. 2B; data not shown).

Both enzymes cleaved greater than 90% of the plasmid DNA when Norf was present at high concentrations (>50 μM). These results showed that, under these conditions, the plasmid DNA was occupied by at least one ternary complex formed with either the wild type Gyr or Gyr (A59).

The Active Strand Cleavage and Reunion Activity of Gyr Is Required for the Formation of the Gyr-Norf-DNA Ternary Complex That Can Arrest Replication Fork Progression—The active strand cleavage and reunion activity of Topo IV are required for the formation of the Topo IV-Norf-DNA ternary complexes that can arrest the progression of replication forks in vitro (6). We examined if this was also the case with Gyr, by using a catalytically inactive mutant Gyr (A, Y122F). First, the standard DNA cleavage assay was performed and showed no Gyr (A, Y122F)-catalyzed DNA cleavage (Fig. 3A). These results confirmed that the substitution of the active site Tyr with Phe abolished the strand cleavage activity of Gyr.

The modified oriC pulse-chase protocol (6, 14) was employed to assess the ability of the ternary complex formed with Gyr (A, Y122F) to arrest replication fork progression in vitro (Fig. 3B). ERI were formed and labeled, and then the paused replication forks were released by linearizing the DNA template with the SmaI restriction endonuclease, which digested the DNA template once at oriC. Linearization of the DNA template was sufficient to release the paused replication forks and generate the full-length product as a result of the run-off DNA replication (Fig. 3B, lane 1). Because no topoisomerase was required to relieve topological constraint, this reaction was insensitive to high concentrations. These results showed that the decatenation activity of Gyr (A59) was not sufficient enough to complete the decatenation of replicating daughter DNA molecules and produce the final monomer product.

We further examined if Gyr (A59) alone could support oriC DNA replication. Standard oriC replication reactions were incubated in the presence of various amounts of either the wild type Gyr or Gyr (A59). Analysis of the replication products revealed the types of DNA molecules generated during the Gyr- and Gyr (A59)-supported oriC DNA replication (Fig. 1B). In the absence of any topoisomerase, only ERIs were accumulated (Fig. 1B, lane 1). ERI are molecules on which initiation has occurred but no extensive nascent chain elongation has ensued (6, 14). As described above, LRI and form II-form II DNA dimers were accumulated during the wild type Gyr-supported oriC DNA replication (Fig. 1B, lanes 2–5). The same patterns of the replication products were generated when Gyr (A59) was used (Fig. 1B, lanes 6–9). We also performed the product analysis by alkaline-denaturing agarose gel electrophoresis, and we confirmed that both leading and lagging strands were synthesized during the oriC DNA replication supported by either the wild type Gyr or Gyr (A59) (data not shown).

These results demonstrated that Gyr (A59) was capable of supporting the nascent chain elongation but not decatenating daughter DNA molecules during the oriC DNA replication in vitro. These results also demonstrated that the supercoiling activity of Gyr per se was not required to support nascent chain elongation.

Gyr (A59) Is Sensitive to the Quinolone Antibacterial Drugs—The active strand cleavage and reunion activity of Topo IV are required for the formation of the ternary complex that can arrest replication forks in vitro (6). We examined if this was also the case with Gyr, by using a catalytically inactive mutant Gyr (A, Y122F). First, the standard DNA cleavage assay was performed and showed no Gyr (A, Y122F)-catalyzed DNA cleavage (Fig. 3A). These results confirmed that the substitution of the active site Tyr with Phe abolished the strand cleavage activity of Gyr.

The modified oriC pulse-chase protocol (6, 14) was employed to assess the ability of the ternary complex formed with Gyr (A, Y122F) to arrest replication fork progression in vitro (Fig. 3B). ERI were formed and labeled, and then the paused replication forks were released by linearizing the DNA template with the SmaI restriction endonuclease, which digested the DNA template once at oriC. Linearization of the DNA template was sufficient to release the paused replication forks and generate the full-length product as a result of the run-off DNA replication (Fig. 3B, lane 1). Because no topoisomerase was required to relieve topological constraint, this reaction was insensitive to
the presence of Norf (Fig. 3B, lane 2). Either the wild type Gyr or Gyr (A, Y122F) was added, together with Norf, to the reaction mixtures prior to the addition of the SmaI restriction enzyme. The subsequent release of replication forks would result in collisions between replication forks and the ternary complexes. The ternary complexes that could arrest replication fork progression would, therefore, manifest themselves in this assay by preventing the appearance of the full-length DNA product.

In the absence of Norf, neither the wild type Gyr nor Gyr (A, Y122F) affected elongation of the nascent chains in the ERI to the full-length product (Fig. 3B, lanes 3 and 5). When Norf was present, replication fork progression was blocked in the presence of the wild type Gyr (Fig. 3B, lane 4) but not in the presence of Gyr (A, Y122F) (Fig. 3B, lane 6). These results demonstrated that the active strand cleavage and reunion activity of Gyr was required for the formation of the ternary complex that could arrest replication fork progression.

The Covalent Gyr-DNA Complex Formation Is Not Sufficient to Arrest Replication Fork Progression in Vitro—We further examined if the formation of covalent Gyr-DNA complexes was not only necessary but also sufficient to arrest replication fork progression. If the covalent Gyr-DNA complex formation is sufficient to inhibit DNA replication, the Gyr (A59)-Norf-DNA ternary complex would block the replication fork progression. The occupancy of the topoisomerase on the DNA is one of the determining factors of the probability of collisions between replication forks and topoisomerase-DNA complexes. The amounts of topoisomerase-catalyzed DNA cleavages represent the formation of the covalent topoisomerase-DNA complexes on the DNA. As shown in Fig. 2, the occupancies of the plasmid DNA template by the ternary complexes formed with either the wild type Gyr or Gyr (A59) were similar when Norf was present at high concentrations. Thus, under these conditions, we expected the collisions between replication forks and the ternary complexes formed with either the wild type Gyr or Gyr (A59) would take place at a similar frequency.

The modified oriC pulse-chase protocol (6, 14) was employed again to model the events during the collision between a replication fork and a Gyr (A59)-Norf-DNA ternary complex (Fig. 4A). The cleavage and reunion activity of Gyr is required for the formation of the Gyr-Norf-DNA ternary complex that can arrest replication fork progression. A, the strand DNA cleavage reaction mixtures containing 20 fmol (as molecule) of 32P-labeled linear pBROTB353 type I DNA, 0.3 pmol (as tetramer) of either the wild type Gyr or Gyr (A59), and the indicated concentrations of Norf. A, the DNA products were analyzed by the electrophoresis through 1% native agarose gels. Abbreviations are the same as in Fig. 1 legend. B, relative amounts of the full-length plasmid DNA were measured. Experiments were repeated twice, and the error range was ±4%. Representative results are shown. C, the wild type Gyr; □, Gyr (A59).
In the absence of Norf, the presence of either the wild type Gyr or Gyr (A59) had no effect on the replication fork progression (Fig. 4, lanes 3 and 5). When the Gyr-Norf-DNA ternary complex was formed, replication fork progression was inhibited (Fig. 4, lane 4). Interestingly, the ternary complexes formed with Gyr (A59) did not arrest the progression of replication forks (Fig. 4, lane 6). We also performed the same assay using ciprofloxacin as a quinolone drug and obtained identical results (data not shown). These results demonstrated that the ternary complexes formed with Gyr (A59) could not arrest replication fork progression. Thus, the quinolone-induced covalent topoisomerase-DNA complex formation was necessary but not sufficient for the inhibition of DNA replication.

The Ternary Complexes Formed with Gyr (A59) Are More Sensitive to Salt Than Those Formed with Either the Wild Type Gyr or Topo IV—It was not clear why the ternary complexes formed with Gyr (A59) could not arrest replication fork progression. One possible explanation was that the ternary complexes formed with Gyr (A59), unlike those formed with either the wild type Gyr or Topo IV, were not stable enough to arrest replication fork progression. To examine this possibility, we assessed salt sensitivity of the ternary complexes formed with either the wild type Gyr or Topo IV—

was recovered as the full-length linear molecule as a result of the reversal of Gyr (A59)-Norf-DNA, Gyr-Norf-DNA, and Topo IV-Norf-DNA ternary complexes, respectively. These results demonstrated that the ternary complexes formed with Gyr (A59) were less stable than those formed with either the wild type Gyr or Topo IV.

Gyr (A59)-Norf-DNA Ternary Complexes Are Less Stable Than Gyr-Norf-DNA and Topo IV-Norf-DNA Ternary Complexes—We also performed a competition experiment to assess the stability of Gyr-Norf-DNA, Gyr (A59)-Norf-DNA, and Topo IV-Norf-DNA ternary complexes. The ternary complexes were formed with 32P-labeled linear pBROTB353 type I DNA, Norf, and the wild type Gyr, Gyr (A59), or Topo IV. After a 10-min incubation at 37 °C, an excess amount of pBROTB353 type I DNA was added to the reaction mixtures as a cold competitor. The incubation was continued at 37 °C, and portions of the reaction mixtures were withdrawn at indicated times. After the denaturation of topoisomerases, the DNA products were purified and analyzed as described under “Material and Methods.”

The amount of the intact linear plasmid DNA in the reaction mixture in the absence of any topoisomerase at 0 min (Fig. 6, lane 1) was used as a standard (100%). After a 60-min incubation in the absence of any topoisomerase, the relative amounts of the intact DNA remained constant (97%; data not shown). At 0 min, the relative amounts of the intact linear plasmid DNA were 3.9, 4.4, and 3.7% in the reaction mixtures containing Gyr-Norf-DNA, Gyr (A59)-Norf-DNA, and Topo IV-Norf-DNA ternary complexes, respectively (Fig. 6, lanes 2, 8, and 14). Relative amounts of the intact DNA remained essentially constant during the period of incubation when either Gyr-Norf-DNA (5.5% at 60 min; Fig. 6, lanes 2–7) or Topo IV-Norf-DNA ternary complexes (5.6% at 60 min; Fig. 6, lanes 14–19) were present in the reaction mixtures, demonstrating that the ternary complexes formed with the wild type Gyr or Topo IV did not dissociate from the DNA. In contrast, the amounts of the intact DNA increased with time when the reaction mixtures contained Gyr (A59)-Norf-DNA ternary complexes (Fig. 6, lanes 8–13), and nearly 40% of the plasmid DNA was recovered as the full-length product at 60 min. These results showed that Gyr (A59)-Norf-DNA ternary complexes readily disassociated from
the DNA, whereas both Gyr-Norf-DNA and Topo IV-Norf-DNA ternary complexes remained stably bound.

Results described in this section and in previous sections clearly demonstrated that ternary complexes formed with Gyr (A59) were less stable than those formed with the wild type Gyr or Topo IV. Thus, Gyr-mediated wrapping of the DNA strand was required for the formation of stable Gyr-Norf-DNA ternary complexes that could arrest replication fork progression. These results suggested that the stability of the topoisomerase-quinolone-DNA ternary complex would determine if the collision between a replication fork and a ternary complex would result in the inhibition of DNA replication. We also showed that ternary complexes formed with Topo IV were as stable as those formed with Gyr. These results suggested that Gyr and Topo IV interacted differently with the DNA and/or the quinolone drug. Topo IV could form stable ternary complexes without wrapping the DNA strand, whereas Gyr required the wrapping of the DNA strand around itself to form stable ternary complexes.

**DISCUSSION**

Among type II topoisomerases, Gyr is unique because of its ability to supercoil DNA molecules. Gyr wraps the DNA strand around itself upon its binding to the DNA strand and catalyzes the supercoiling reaction (1). It has been demonstrated that the deletion of the C-terminal DNA binding domain of the GyrA subunit converts Gyr into a conventional type II topoisomerase (2). This mutant Gyr, Gyr (A59), does not wrap the DNA strand around itself. Gyr (A59) can catalyze the decatenation and relaxation but not the supercoiling reactions.

Both Gyr and Topo IV contribute to DNA unlinking during DNA replication. However, these topoisomerases function in a distinct manner. Gyr prefers to remove positive supercoils ahead of the advancing replication forks, whereas Topo IV prefers to decatenate precatenanes behind the replication forks (17). Both Gyr and Topo IV can support the elongation of DNA replication but only Topo IV can decatenate replicating daughter DNA molecules. Gyr introduces negative superhelicity into the DNA, which is required for the initiation of DNA replication at oriC (18). It has been thought, however, that the supercoiling activity of Gyr per se is not essential for replication fork progression. In eukaryotes, no topoisomerase can catalyze the supercoiling reaction but topoisomerases complete the DNA unlinking during DNA replication. Here, we assessed the functional activities of Gyr (A59) during oriC DNA replication in vitro. Gyr (A59) alone could support oriC DNA replication (Fig. 1B), demonstrating that the supercoiling activity of Gyr is not required to support the nascent chain elongation. However, Gyr (A59) could not decatenate replicating daughter DNA molecules. As a result, the majority of the replication products were LRI and DNA dimers (Fig. 1A). Thus, the replicative function of Gyr (A59) is similar to that of the wild type Gyr (14, 15), except that Gyr (A59) cannot introduce negative supercoils into DNA molecules (2).

Topo IV is responsible for the decatenation of daughter chromosomes (19, 20). In direct comparison, Topo IV is a much better decatenating enzyme, with a turnover number nearly 100-fold greater than that of Gyr both in vivo and in vitro (17, 21). Kampranis and Maxwell (2) have shown that Gyr (A59) can partially complement the phenotype of a parC

mutant in vivo, indicating that Gyr (A59) can substitute the Topo IV function during chromosome segregation in E. coli. However, we found that Gyr (A59) could not decatenate replicating daughter DNA molecules during the oriC DNA replication in vitro (Fig. 1). It is not clear what causes this apparent paradox. One possible explanation is that the high copy number plasmid-carried gyrA

59 gene used in the in vivo studies (2) provides an elevated level of Gyr (A59) protein, which may be sufficient to support cell growth of the parC

1215 strain at 42 °C. Another possibility is that there might be a protein that interacts with Gyr (A59) in the cell, and this protein-protein interaction could stimulate Gyr (A59)-catalyzed decatenation of daughter chromosomes in vivo. For instance, the dnaX gene, encoding the  and  subunits of DNA polymerase III holoenzyme, has been identified as a high copy suppressor of the phenotype of a parE

mutant (22).

We showed here, using mutant Gyr proteins, Gyr (A59) and Gyr (A, Y122F), that the formation of the covalent Gyr-DNA complex was necessary but not sufficient to arrest replication fork progression (Figs. 3 and 4). We found that the ternary complexes formed with Gyr (A59) were less stable than those formed with either the wild type Gyr or Topo IV (Figs. 5 and 6). Thus, the Gyr-mediated wrapping of the DNA strand is required for the formation of the stable Gyr-Norf-DNA ternary complex that can arrest replication fork progression. These results suggest that the stability of the quinolone-induced covalent topoisomerase-DNA complex is likely to determine, when a replication fork and a ternary complex collide, if the inhibition of DNA replication occurs.

Gyr and Topo IV are homologous to each other (1). Gyr (A59)
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does not wrap the DNA strand around itself and thus this mutant Gyr seems to bind the DNA in the same manner as Topo IV. However, we showed that Topo IV-Norf-DNA ternary complexes were more stable than Gyr (A59)-Norf-DNA ternary complexes. These results indicate that, despite their similarities, there are critical differences between Gyr and Topo IV in their interactions with the DNA and/or the quinolone drug, which affect the stability of the ternary complexes. Alternatively, it is possible that the deletion of the C-terminal DNA-binding domain of the GyrA subunit not only abolishes the ability of Gyr to wrap the DNA strand but also causes a conformational change of the GyrA subunit, which could reduce the affinity of the Gyr (A59)-DNA interaction. The requirement of high concentrations of Norf for the formation of Gyr (A59)-Norf-DNA ternary complexes (Fig. 2) supports this possibility.

The mechanism by which the Gyr (A59)-quinolone-DNA ternary complex is reversed upon its collision with a replication fork is not clear. It is possible that the replication fork actively forces Gyr (A59) to religate the DNA strands and disassociate from the DNA. Alternatively, replication forks simply pause when they collide with ternary complexes formed with either the wild type Gyr or Gyr (A59). The half-life of Gyr (A59)-Norf-DNA ternary complexes is shorter than that of the paused replication forks, whereas the half-life of Gyr-Norf-DNA and Topo IV-Norf-DNA ternary complexes is longer than that of the replication forks. As a result, replication forks can progress in the presence of the Gyr (A59)-Norf-DNA ternary complexes, but replication fork progression is arrested when the ternary complexes formed with either the wild type Gyr or Topo IV are present.

Quinolone resistance-conferring mutations rapidly arise and are particularly clustered within a small region (between amino acids 67 and 106) of the gyrA gene (often referred to as the “quinolone resistance-determining region”) (23). Homologous mutations in the parC gene also confer quinolone resistance to Topo IV (6, 21). These mutations seem to alter the topoisomerase-quinolone interaction and reduce the probability of the formation of topoisomerase-quinolone-DNA ternary complexes (24). Based on the results presented here, it is interesting to speculate that there is another mechanism of acquiring drug resistance-conferring mutations. Mutations in Gyr and Topo IV could affect the stability of the topoisomerase-drug-DNA ternary complexes without changing the probability of the ternary complex formation. In this scenario, ternary complexes formed with a mutant topoisomerase are not stable enough to arrest replication fork progression. Thus, collisions between topoisomerase-drug-DNA ternary complexes and replication forks do not result in the inhibition of DNA replication. Some of the quinolone resistance-conferring mutations mapped in the regions outside of the quinolone resistance-determining region of the gyrA gene and the gyrB gene could be of this type.

Of course, this possibility needs to be tested.

The covalent topoisomerase-DNA complex is normally a fleeting catalytic intermediate during topoisomerization. Thus, advancing replication forks seldom collide with the covalent topoisomerase-DNA complexes during the chromosomal DNA replication. However, at very low frequencies, collisions do take place. If any covalent topoisomerase-DNA complex can arrest replication fork progression, every collision between an advancing replication fork and a covalent topoisomerase-DNA complex triggers the cytotoxic events. We showed here that the formation of the covalent topoisomerase-DNA complex was necessary but not sufficient to arrest replication fork progression. A certain stability of the topoisomerase-quinolone-DNA ternary complex was required for the replication fork arrest. Thus, it seems reasonable to assume that the normal covalent topoisomerase-DNA complex, a catalytic intermediate, is not stable enough to block the progression of a replication fork. The replication machinery is likely to have an ability to complete the chromosomal replication even in the presence of these unstable topoisomerase-DNA complexes. Thus, the covalent topoisomerase-DNA complex becomes a cellular poison only when it is frozen as a stable topoisomerase-drug-DNA ternary complex by a topoisomerase inhibitor.

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