The Glu-84 of the ParC Subunit Plays Critical Roles in Both Topoisomerase IV−Quinolone and Topoisomerase IV−DNA Interactions†

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ABSTRACT: DNA gyrase and topoisomerase IV (Topo IV) are cellular targets of quinolone antibacterial drugs. The Ser-80 and the Glu-84 of the ParC subunit have been identified as mutational hotspots for quinolone resistance. Mutant Topo IV proteins containing a quinolone resistance-conferring mutation have been constructed, and the effects of these mutations on Topo IV are assessed. Both S80L and E84K mutations abolish the ability of quinolones to trap covalent Topo IV−DNA complexes, demonstrating that both the Ser-80 and the Glu-84 of ParC are essential for Topo IV−quinolone interaction. In addition, the E84K mutation greatly reduces the catalytic activity of Topo IV. Covalent Topo IV−DNA complexes formed with Topo IV containing the E84K mutation are more stable than those formed with the wild-type protein. Interestingly, the E84P mutation confers quinolone resistance to Topo IV without affecting its catalytic activity. The E84P mutation inhibits the formation of covalent Topo IV−DNA complexes when Mg^{2+}, but not Ca^{2+}, is used as a cofactor. These results show that the Glu-84 plays an important role in Topo IV−DNA interaction. Thus, the Glu-84 of ParC is critical for the interactions of Topo IV with both the quinolone drug and the DNA in topoisomerase−quinolone−DNA ternary complexes.

Type II topoisomerases are the targets of many clinically important antibacterial (e.g., ciprofloxacin (Cipro)[1]) and anticancer (e.g., etoposide) drugs (*1*−*3*). These topoisomerase inhibitors convert essential enzymes into cellular poisons by trapping a covalent topoisomerase−DNA complex, causing the inhibition of DNA replication and leading to the generation of double-strand breaks and subsequent cell death. Although the formation of topoisomerase−drug−DNA ternary complexes is essential for the cytotoxicity of these drugs, ternary complexes are completely reversible, and the DNA strands can be religated. It has been proposed that an active DNA transaction, such as the passage of a replication fork, is required for the disruption of a ternary complex to generate a nonreversible, cytotoxic DNA lesion. Because of their unique mode of action, these topoisomerase inhibitors are often referred to as "topoisomerase poisons" (*1*−*3*).

DNA gyrase and topoisomerase IV (Topo IV) are the bacterial type II topoisomerases (*4*, *5*) and the cellular targets of quinolone antibacterial drugs (*6*−*8*). Quinolone resistance-conferring mutations arise rapidly, and they are particularly clustered within a small region (between residues 67 and 106 of GyrA, often referred to as the quinolone resistance-determining region) of the *gyrA* gene in *E. coli* (*1*). Both the Ser-83 and the Asp-87 of *E. coli* GyrA are mutational hotspots for quinolone resistance, and these amino acids are thought to directly interact with the quinolone drug in the gyrase−quinolone−DNA ternary complex (*1*, *9*, *10*). However, the exact quinolone binding site has not been determined. Homologous mutations in *parC*, including those at two mutational hotspots Ser-80 and Glu-84, are identified as quinolone resistance-conferring mutations as well (*8*, *11*). Recent structural studies have revealed that these mutational hotspots for quinolone resistance locate in the α4 helix of the helix−turn−helix region, which locates close to the active site Tyr, of either the GyrA or ParC subunit (*12*, *13*).

We have used the wild-type Topo IV and a quinolone-resistant mutant Topo IV, ParC S80L Topo IV, to show that Topo IV−quinolone−DNA ternary complexes formed with ParC S80L Topo IV, unlike those formed with the wild-type Topo IV, could not arrest replication fork progression (*14*). In addition, the S80L mutation abolished the ability of Topo IV to stimulate the quinolone-induced structural perturbation of DNA (*15*).

I have continued our studies on the effects of quinolone resistance-conferring mutations on Topo IV using a series of quinolone-resistant Topo IV proteins. Both S80L and E84K mutations in ParC conferred quinolone resistance to Topo IV and abolished the ability of quinolones to stimulate the formation of covalent Topo IV−DNA complexes. The E84K mutation also affected the catalytic activity of Topo IV. In contrast, the E84P mutation conferred quinolone resistance to Topo IV without affecting its catalytic activity. The E84P seemed to shift the cleavage−religation equilibrium to inhibit the formation of covalent Topo IV−DNA complexes when Mg^{2+}, but not Ca^{2+}, was used as a cofactor. Topo IV−DNA complexes formed with ParC E84K Topo IV were more stable than those formed with the wild-type Topo IV, ParC S80L Topo IV, or ParC E84P Topo IV. These results suggested that the Glu-84 of ParC played critical roles in both Topo IV−quinolone and Topo IV−DNA interactions.

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[1] Abbreviations: BSA, bovine serum albumin; Cipro, ciprofloxacin; DTT, dithiothreitol; form I, covalently closed, negatively supercoiled circular; kDNA, kinetoplast DNA; K−Glu, potassium glutamate; $MgOAc_2$, magnesium acetate; Topo, topoisomerase.

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EXPERIMENTAL PROCEDURES

DNAs and Proteins. pBR322 form I (negatively supercoiled circular) DNA and kinetoplast DNA (kDNA) were purchased from New England Biolabs and Topogen, respectively.

Site-directed mutagenesis of *parC* was accomplished using the splicing by overlap extension polymerase chain reaction technique (*16*). The ParC S80L open reading frame was described previously (*14*). ParC E84K and ParC E84P open reading frames were engineered in a similar manner as the ParC S80L open reading frame. pET21-*parC* (*14*) was used as the template for the polymerase chain reaction. DNA sequences of open reading frames were confirmed by dideoxy DNA sequencing.

ParC S80L, ParC E84K, and ParC E84P were expressed in *E. coli* BL21(DE3) (*17*) and purified according to Hiasa et al. (*14*). The final preparations of mutant ParC proteins were greater than 96% homogeneous for a single band on SDS-polyacrylamide gel electrophoresis (data not shown). The wild-type and mutant ParCs were mixed with the wild-type ParE to reconstitute Topo IV as described previously (*7*, *14*).

Decatenation of kDNA. Reaction mixtures (20 μL) containing 40 mM HEPES−KOH (pH7.6), 10 mM magnesium acetate ($MgOAc_2$), 100 mM potassium glutamate (K−Glu), 10 mM dithiothreitol (DTT), 50 μg/mL bovine serum albumin (BSA), 1 mM ATP, 4 μg/mL tRNA, 0.6 μg kDNA, and the indicated amounts (as tetramer) of either the wild-type or mutant Topo IV were incubated at 30 °C for 30 min. Various concentrations of Cipro were included when indicated. Reactions were terminated by adding EDTA to 25 mM and incubating at 37 °C for 5 min. The DNA products were analyzed by electrophoresis through vertical 1.2% agarose gels (14 × 10 × 0.3 cm) at 2 V/cm for 12 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 buffer). Gels were stained with ethidium bromide and photographed using an Eagle Eye II system (Stratagene).

Relaxation of Superhelical Plasmid DNA. Reaction mixtures (20 μL) containing 40 mM HEPES−KOH (pH7.6), 10 mM $MgOAc_2$, 100 mM K−Glu, 10 mM DTT, 50 μg/mL BSA, 1 mM ATP, 4 μg/mL tRNA, 0.58 μg (0.2 pmol as molecule) pB*R*322 form I DNA, and the indicated amounts (as tetramer) of either the wild-type or mutant Topo IV were incubated at 30 °C for 30 min. Various concentrations of Cipro were included when indicated. Reactions were terminated and the DNA products were analyzed as described in the previous section.

DNA Cleavage Reaction. pBR322 DNA was linearized by digestion with *Eco*RI and 3′-end-labeled by incorporation of two residues of [^{32}P]dAMP with Klenow enzyme. The labeled DNA was digested with *Pst*I, and then a 0.75-kb fragment, uniquely labeled at one end, was recovered after electrophoresis through a 1.4% SeaPlaque agarose (BioWhittaker Molecular Applications) gel. This DNA fragment was used as a substrate in the cleavage assay.

Reaction mixtures (20 μL) containing 50 mM Tris−HCl (pH 7.5 at 23 °C), 10 mM of either $MgCl_2$ or $CaCl_2$, 10 mM DTT, 50 μg/mL BSA, 1 mM ATP, 20 fmol (as molecule) DNA substrate, indicated amounts (as tetramer) of either the wild-type or mutant Topo IV, and indicated concentrations of either Cipro or K−Glu were incubated at 30 °C for 10 min. SDS was added to 1%, and the reaction mixtures were 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 8% polyacrylamide (19:1, acrylamide to bisacrylamide) gels (140 × 160 × 1.2 mm) at 15 V/cm for 2 h using 50 mM Tris-borate (pH 8.3) and 1 mM EDTA as the electrophoresis buffer (TBE buffer). Gels were dried under vacuum onto DE81 papers (Whatman) and autoradiographed with Hyperfilm MP films (Amersham Biosciences).

RESULTS

Quinolone Resistance-Conferring Mutation E84K, but not S80L, in ParC Affects the Catalytic Activity of Topo IV. Mapping of quinolone resistance-conferring mutations in *E. coli* has revealed the quinolone resistance-determining region of the *gyrA* gene and two major mutational hotspots, Ser-83 and Asp-87 (*1*, *9*, *10*). The Ser-83 is conserved among type II topoisomerases, and homologous mutations in the ParC subunit and yeast topoisomerase II (Topo II) at the conserved Ser confer quinolone resistance to Topo IV and Topo II, respectively (*8*, *14*, *18*). The Glu-84 of ParC, which corresponds to the Asp-87 of GyrA, also turns out to be a mutational hotspot for quinolone resistance (*8*, *11*). Recent studies on mutant gyrase proteins have indicated that both the Ser-83 and the Asp-87 of GyrA interact with C-8 position of quinolone drugs (*9*, *10*). Here, a series of mutant Topo IV proteins were constructed and characterized to determine the effects of quinolone resistance-conferring mutations on Topo IV.

First, decatenation and relaxation assays were performed to determine the catalytic activities and drug sensitivities of ParC S80L Topo IV and ParC E84K Topo IV. Specific activity of ParC S80L Topo IV during either the decatenation of kDNA (Figure 1A) or the relaxation of superhelical plasmid DNA (Figure 1B) was similar to that of the wild-type enzyme. In contrast, the E84K mutation affected the catalytic activity of Topo IV. Apparent specific activities of ParC E84K Topo IV during the decatenation (Figure 1A) and the relaxation (Figure 1B) reactions were roughly 20−30-fold and 5−10-fold lower than those of either the wild-type Topo IV or ParC S80L Topo IV, respectively. The reduced decatenation activity of ParC E84K Topo IV might contribute to the slow growth rate of an *E. coli* strain carrying the E84K mutation in the *parC* gene (*8*). Thus, quinolone resistance-conferring mutations at the Ser-80 and the Glu-84 of the ParC subunit affected the catalytic activity of Topo IV differently.

Because S80L and E84K mutations were identified as quinolone resistant-conferring mutations (*8*, *10*, *11*), ParC S80L Topo IV and ParC E84K Topo IV were expected to be resistant to quinolone drugs. In fact, both ParC S80L Topo IV- and ParC E84K Topo IV-catalyzed decatenation and relaxation reactions were strongly resistant to Cipro, a model quinolone drug (Figure 2). These results demonstrated that both S80L and E84K mutations conferred high levels of quinolone resistance to Topo IV.

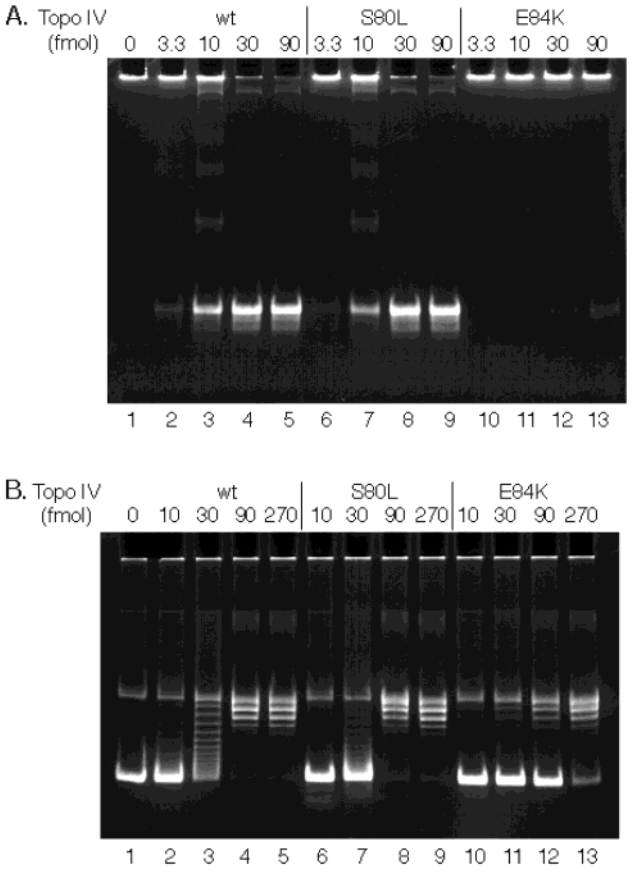

FIGURE 1: Quinolone resistant-conferring mutant E84K affects the catalytic activity of Topo IV. Decatenation (panel A) and relaxation (panel B) activities of ParC S80L Topo IV and ParC E84K Topo IV were assessed using kDNA and pBR322 form I DNA as a substrate, respectively. Assays were performed as described under Experimental Procedures. wt, the wild-type Topo IV; S80L, ParC S80L Topo IV; E84K, ParC E84K Topo IV.

Quinolone Resistance-Conferring Mutation E84K Alters the Cleavage Site Specificity of Topo IV. Next, effect of Cipro on Topo IV-catalyzed cleavage was assessed using the wild type and two quinolone-resistant mutant Topo IV proteins. As described previously (*7*, *19*), equilibrium levels of DNA cleavage mediated by Topo IV in the absence of a quinolone drug are considerably higher than those mediated by either DNA gyrase or eukaryotic type II topoisomerases. Neither ATP binding nor ATP hydrolysis affects DNA binding affinity of Topo IV (*15*), and the absence of ATP did not alter cleavage site specificity of Topo IV (data not shown). Cipro stimulated the wild-type Topo IV-catalyzed cleavage of DNA (Figure 3). In contrast, ParC S80L Topo IV- and ParC E84K Topo IV-catalyzed cleavages were only slightly stimulated by Cipro. Similar results were obtained when norfloxacin, another quinolone drug, was used (data not shown). These results demonstrated that quinolone resistance-conferring mutations greatly reduce the ability of quinolone drugs to stimulate the formation of covalent Topo IV−DNA complexes.

During the experiments shown in Figure 3, I noted that, in the absence of Cipro, the cleavage site specificity (Figure 3, lane 10) was somewhat different from that of either the wild-type Topo IV− (Figure 3, lane 2) or ParC S80L Topo IV-catalyzed (Figure 3, lane 6) cleavages. To examine the effects of these quinolone resistance-conferring mutations on Topo IV−DNA interaction, the cleavage assay was performed in the absence of Cipro and the presence of either Mg^{2+} or Ca^{2+} (Figure 4). The wild type and two mutant Topo IV proteins cleaved the DNA to a similar extent. Although these proteins bound to the same sites, their cleavage site specificities were different. ParC S80L Topo IV (Figure 4, lanes 3 and 7) exhibited an identical cleavage site specificity to the wild-type Topo IV (Figure 4, lanes 2 and 6), whereas the cleavage site specificity of ParC E84K Topo IV (Figure 4, lanes 4 and 8) was different from that of the wild-type protein. These results showed that the E84K mutation altered Topo IV−DNA interaction.

ParC E84P Topo IV Is a Catalytically Active, Quinolone-Resistant Mutant Topo IV. Both the Ser-80 and the Glu-84 of the ParC subunit are likely to interact with the quinolone drug in Topo IV−quinolone−DNA ternary complexes (*8*−*11*). Results presented here demonstrated different effects of these quinolone resistance-conferring mutations, S80L and E84K, on Topo IV. Namely, the E84K mutation not only abolished the ability of quinolones to stabilize covalent Topo IV−DNA complexes but also greatly reduced the catalytic activity of Topo IV. The question was: Is there any amino acid substitution at the Glu-84 of ParC that confers quinolone resistance to Topo IV without affecting its catalytic activity? The E84P mutation, the substitution of Glu-84 with Pro, turned out to be such a mutation.

The catalytic activity of ParC E84P Topo IV (Figure 5) was at least 10-fold higher than that of ParC E84K Topo IV (Figure 1). The specific activities of ParC E84P Topo IV during decatenation (Figure 5A) and relaxation (Figure 5B) reactions were 30−40% and 80−100% of those of the wild-type Topo IV (Figure 1), respectively. ParC E84P Topo IV-catalyzed reactions (Figure 5C,D) were resistant to Cipro to a similar extent to those catalyzed by ParC S80L Topo IV (Figure 2B,D). Thus, the E84P mutation conferred quinolone resistance to Topo IV without affecting its catalytic activity.

The E84P Mutation Inhibits Mg^{++}-Induced, but not Ca^{++}-Induced Cleavage of DNA. A unique effect of the E84P mutation on Topo IV became evident when ParC E84P Topo IV-catalyzed cleavage was assessed. ParC E84P Topo IV-catalyzed cleavage was not detected when Mg^{2+} was used as a cofactor, although Ca^{2+} could induce ParC E84P Topo IV-catalyzed cleavage (Figure 6A). The cleavage site specificity of ParC E84P Topo IV was identical to that of ParC E84K Topo IV (Figure 4) and different from that of the wild-type Topo IV. These results suggested that structural alterations caused by the introduction of Pro in the middle of the α4 helix of ParC affected Topo IV−divalent cation interaction. The steady-state level of the covalent topoisomerase−DNA complex depends on the cleavage−religation equilibrium. The E84P mutation seemed to shift the equilibrium, particularly in the presence of Mg^{2+}, to reduce the level of covalent Topo IV−DNA complexes.

Cipro slightly stimulated Mg^{2+}-dependent, ParC E84P Topo IV-catalyzed cleavage (Figure 6B). Apparent quinolone resistance of ParC E84P Topo IV (Figure 5C and 5D) could be due to not only a reduced Topo IV−quinolone interaction but also a shift of the cleavage−religation equilibrium.

Covalent Topo IV−DNA Complexes Formed with ParC E84K Topo IV Are More Stable than Those Formed with the Wild-Type Topo IV, ParC S80L Topo IV, or ParC E84P Topo IV. Salt sensitivity of Ca^{2+}-induced covalent Topo IV−

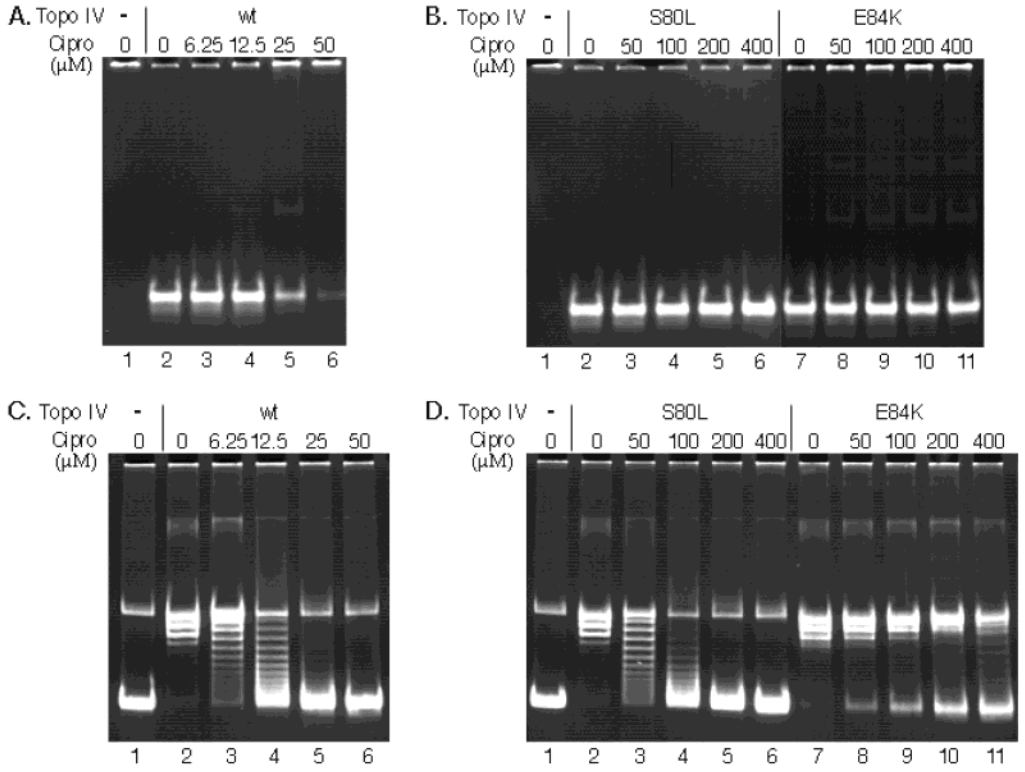

FIGURE 2: Both S80L and E84K mutations confer quinolone resistance to Topo IV. Both decatenation reaction mixtures containing 50 fmol (as tetramer) of the wild-type Topo IV (panel A), 50 fmol (as tetramer) of ParC S80L Topo IV, or 1 pmol (as tetramer) of ParC E84K Topo IV (panel B) and indicated concentrations of Cipro and relaxation reaction mixtures containing 100 fmol (as tetramer) of the wild-type Topo IV (panel C), 100 fmol (as tetramer) of ParC S80L Topo IV, or 500 fmol (as tetramer) of ParC E84K Topo IV (panel D) and indicated concentrations of Cipro were incubated, processed, and analyzed as described under Experimental Procedures. Abbreviations are the same as those in the legend to Figure 1.

DNA complexes was measured to compare the stabilities of Topo IV−DNA complexes formed with either the wild-type or mutant Topo IV (Figure 7). Formations of covalent Topo IV−DNA, ParC S80L Topo IV−DNA, and ParC E84P Topo IV DNA complexes were significantly reduced by 100 mM (Figure 7, lanes 4, 9, and 19) and completely inhibited when 200 mM K−Glu was present (Figure 7, lanes 5, 10, and 20). In contrast, ParC E84K Topo IV−DNA complexes were less sensitive to salt and 400 mM K−Glu was required for the inhibition of covalent ParC E84K Topo IV−DNA complex formation (Figure 7, lane 16). These results demonstrated that ParC E84K Topo IV−DNA complexes were more stable than Topo IV−DNA, ParC S80L Topo IV−DNA, and ParC E84P Topo IV−DNA complexes. Since the covalent Topo IV−DNA complex is a reaction intermediate during topoisomerization, a higher stability of the covalent ParC E84K Topo IV−DNA complex might contribute to the reduction of its catalytic activity.

DISCUSSION

Quinolone antibacterial drugs target both DNA gyrase and Topo IV and convert these essential enzymes into cellular poisons (*6*−*8*). Poisoning of topoisomerases is mediated by the trapping of a covalent topoisomerase−DNA complex as a topoisomerase−quinolone−DNA ternary complex. Topoisomerase−quinolone−DNA ternary complexes cause the inhibition of DNA replication and subsequent generation of double-strand breaks, which ultimately lead to cell death (*1*−*3*). Some of anticancer drugs poison human topoisomerases in a similar manner (*20*). On the basis of the unique mode of action, these topoisomerase inhibitors are often referred to as "topoisomerase poisons" (*1*−*3*).

Despite the clinical importance of quinolone antibacterial drugs, the quinolone binding site has not been determined. Mapping of quinolone resistance-conferring mutations provides useful insights to the location of the quinolone binding site and the molecular basis of topoisomerase−quinolone interaction. The majority of quinolone resistance-conferring mutations have been mapped in the quinolone resistance-determining region of either *gyrA* or *parC* (*1*). It has been suggested that amino acids at two mutational hotspots for quinolone resistance, the Ser-83 and the Asp-87 of *E. coli* GyrA and the Ser-80 and the Glu-84 of *E. coli* ParC, directly interact with the quinolone drug in topoisomerase−quinolone−DNA ternary complexes (*9*, *10*). Here, two quinolone-resistant mutant Topo IV, ParC S80L Topo IV and ParC E84K Topo IV, were constructed and used to assess the effects of quinolone resistance-conferring mutations on Topo IV. Both S80L and E84K mutations greatly reduced the ability of a quinolone drug to stimulate the formation of covalent Topo IV−DNA complexes (Figure 3). Barnard and Maxwell have recently demonstrated that substitutions of the Ser-83 and/or the Asp-87 of *E. coli* GyrA with Ala confer quinolone resistance to DNA gyrase and reduce the stimulation of gyrase-catalyzed cleavage by quinolone drugs (*9*). These results strongly support the model that the Ser-83 and the Asp-87 of the GyrA subunit as well as the Ser-80 and the Glu-84 of the ParC subunit are critical for topoisomerase−quinolone interaction. Future studies on the structure of the ternary complex are necessary to determine

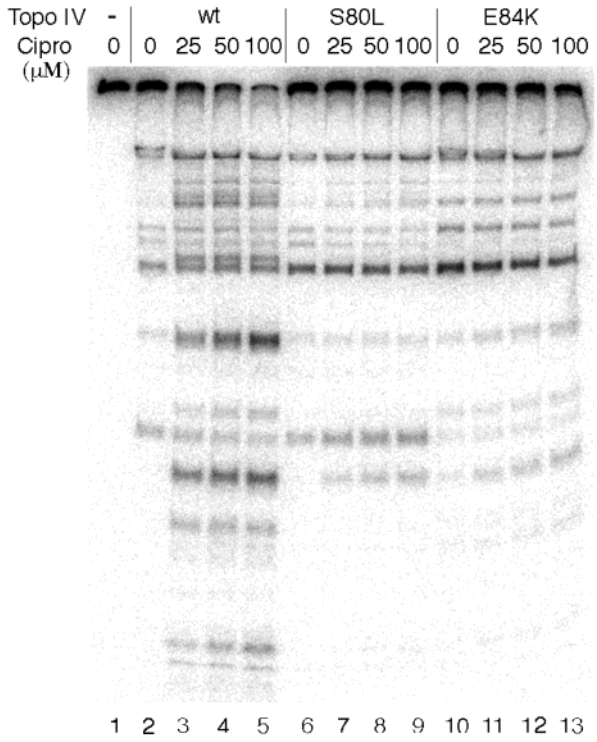

FIGURE 3: Both S80L and E84K mutations abolish the stimulation of Topo IV-catalyzed cleavage by Cipro. Eighty fmol (as tetramer) of the wild-type Topo IV, ParC S80L Topo IV, or ParC E84K Topo IV was incubated with 20 fmol (as molecule) of a 750-base-pair DNA fragment in the presence of indicated concentrations of Cipro. Reaction mixtures were incubated, processed, and analyzed as described under Experimental Procedures. Abbreviations are the same as those in the legend to Figure 1.

the exact quinolone binding site and the molecular basis of topoisomerase−quinolone interaction.

Mutations that do not affect the topoisomerase−quinolone interaction could change the drug sensitivity of a topoisomerase. Poisoning of topoisomerases is mediated by trapping of the covalent topoisomerase−DNA complex. During the normal catalytic cycle, the steady-state level of the covalent topoisomerase−DNA complex depends on the cleavage-religation equilibrium. If a mutation affects the catalytic cycle and shift the equilibrium to either stimulate strand cleavage or inhibit religation, this mutation could affect the apparent drug sensitivity of the topoisomerase. In fact, recent studies have demonstrated that some mutations alter drug sensitivities of topoisomerases by affecting their catalytic cycles (*21*−*24*). Quinolone resistance of ParC E84P Topo IV (Figure 5) could be due, at least in part, to a shift of the cleavage-religation equilibrium.

The E84K mutation affected the catalytic activity of Topo IV, whereas neither the S80L nor E84P mutation did (Figures 1 and 5). In addition, ParC E84K Topo IV seemed to catalyze the relaxation reaction more processively than either the wild-type Topo IV or ParC S80L Topo IV did (Figure 1B). Covalent Topo IV−DNA complexes formed with ParC E84K Topo IV were more stable than those formed with the wild-type Topo IV, ParC S80L Topo IV, or ParC E84P Topo IV (Figure 7). It seems reasonable to assume that the stability of a covalent topoisomerase−DNA complex, which is a reaction intermediate, would affect the catalytic activity of the enzyme. Thus, both the reduced catalytic activity and the increased processivity of ParC E84K Topo IV may be

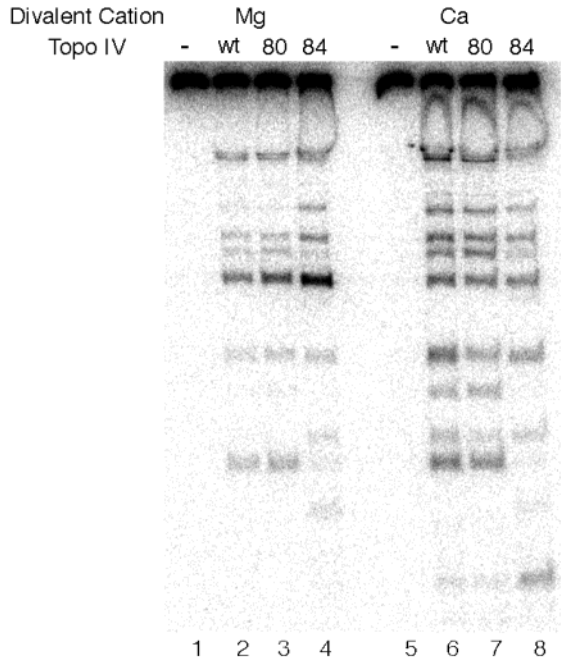

FIGURE 4: E84K mutation alters the cleavage site specificity of Topo IV. Forty fmol (as tetramer) of the wild-type Topo IV, ParC S80L Topo IV, or ParC E84K Topo IV was incubated with 20 fmol (as molecule) of a 750-base-pair DNA fragment in the presence of either 10 mM Mg^{2+} or 10 mM Ca^{2+}. Reaction mixtures were incubated, processed, and analyzed as described under Experimental Procedures. wt, the wild-type Topo IV; 80, ParC S80L Topo IV; 84, ParC E84K Topo IV.

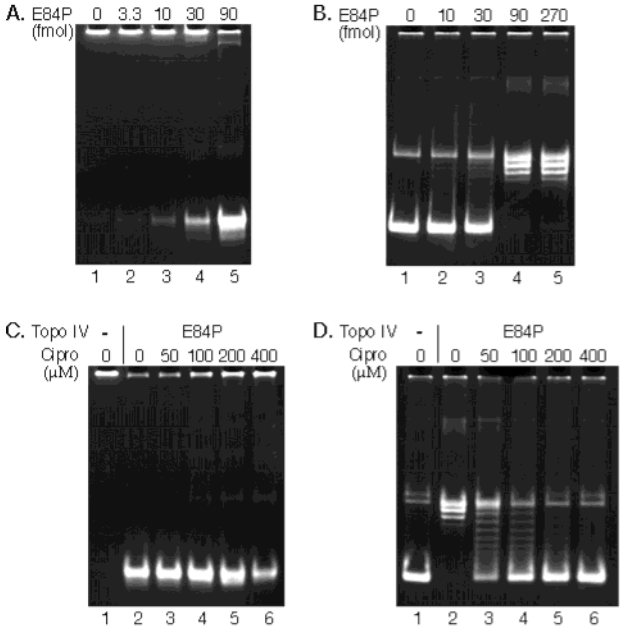

FIGURE 5: ParC E84P Topo IV is a catalytically active, quinolone-resistant mutant Topo IV. Decatenation (panel A) and relaxation (panel B) activities of ParC E84P Topo IV were assessed using kDNA and pBR322 form I DNA as a substrate, respectively. Assays were performed as described under Experimental Procedures. Both decatenation reaction mixtures (Panel C) containing 50 fmol (as tetramer) of ParC E84P Topo IV and indicated concentrations of Cipro and relaxation reaction mixtures (Panel D) containing 100 fmol (as tetramer) of ParC E84P Topo IV and indicated concentrations of Cipro were incubated, processed, and analyzed as described under Experimental Procedures. E84P, ParC E84P Topo IV.

due to the increased stability of covalent ParC E84K Topo IV−DNA complexes. The diminished ability of ParC E84K

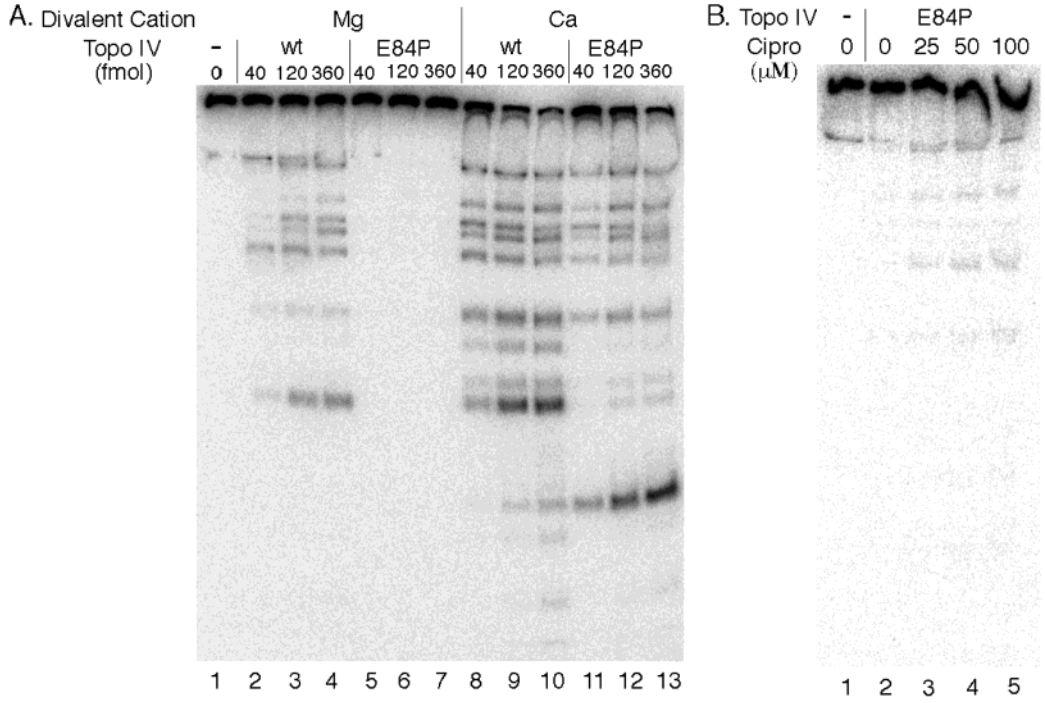

FIGURE 6: E84P mutation abolishes Mg^{2+}-dependent, but not Ca^{2+}-dependent, Topo IV-catalyzed cleavage. Panel A: Indicated amounts (as tetramer) of either the wild-type Topo IV or ParC E84P Topo IV were incubated with 20 fmol (as molecule) of a 750-base-pair DNA fragment in the presence of either 10 mM Mg^{2+} or 10 mM Ca^{2+}. Reaction mixtures were incubated, processed, and analyzed as described under Experimental Procedures. Panel B: Eighty fmol (as tetramer) of ParC E84P Topo IV was incubated with 20 fmol (as molecule) of a 750-base-pair DNA fragment in the presence of indicated concentrations of Cipro. Reaction mixtures were incubated, processed, and analyzed as described under Experimental Procedures. Abbreviations are the same as those in the legends to Figures 4 and 5.

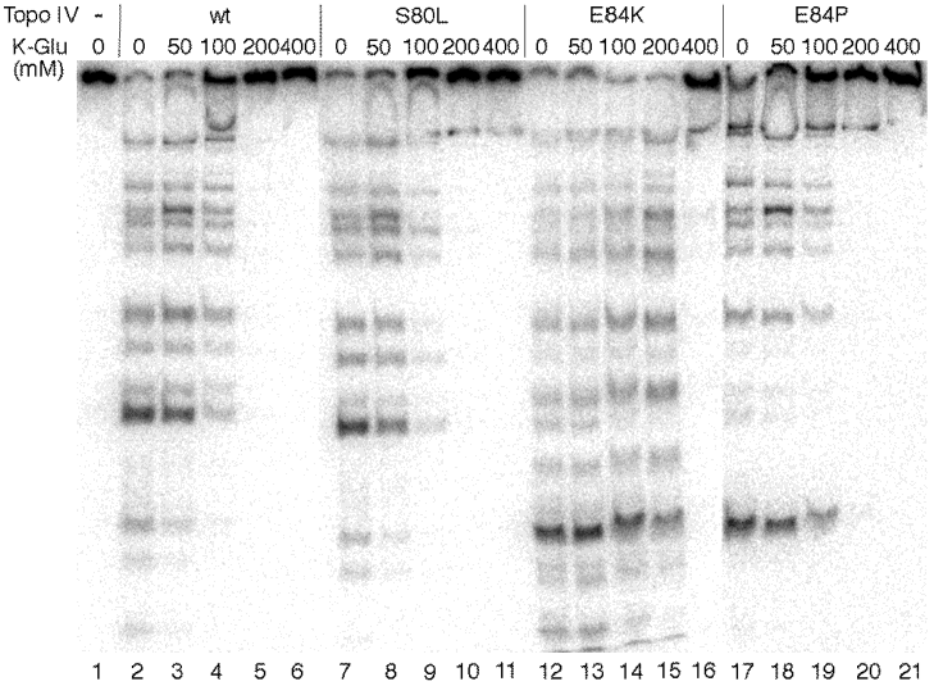

FIGURE 7: Topo IV−DNA complexes formed with ParC E84K Topo IV are more stable than those formed with the wild-type Topo IV, ParC S80L Topo IV, or ParC E84P Topo IV. Cleavage reaction mixtures containing 200 fmol (as tetramer) of either the wild-type or mutant Topo IV, 20 fmol (as molecule) of a 750-base pair DNA fragment, 10 mM Ca^{2+}, and indicated concentrations of K−Glu were incubated, processed, and analyzed as described under Experimental Procedures. Abbreviations are the same as those in the legends to Figures 1 and 5.

Topo IV to decatenate daughter chromosomes may contribute to the slow growth rate of an *E. coli* strain carrying the E84K mutation in the *parC* gene (*8*).

The observations that the E84K mutation, but not the E84P mutation, affected the stability of Topo IV−DNA complexes and the catalytic activity of Topo IV suggest two possibilities. Either the absence of an acidic amino acid (Glu) or the presence of a positively charged amino acid (Lys) at the residue 84 of ParC affects the stability of covalent Topo IV−DNA complexes and the catalytic activity of Topo IV. A

homologous mutant gyrase, GyrA D87A gyrase, also exhibits a reduced catalitic activity (*9*). In addition, both ParC E84K Topo IV and ParC E84P Topo IV exhibit an identical cleavage site specificity, which was different from that of the wild-type Topo IV (Figure 6A). Thus, it seems likely that the absence of an acidic amino acid, but not the presence of a positively charged amino acid, at the residue 84 of ParC affects the stability of covalent Topo IV−DNA complexes and the catalytic activity of Topo IV. These results suggest that the Glu-84 of the ParC subunit plays a critical role in topoisomerase−DNA interaction.

The extent of topoisomerase-catalyzed cleavage of DNA correlates with the cleavage-religation equilibrium, which is equivalent to the ratio of the cleavage and religation rates (*4*, *5*). Divalent cations, a cofactor of type II topoisomerases, affect the cleavage-religation equilibrium. Although Mg^{2+} is the physiological divalent cation, Ca^{2+} and other divalent cations can function as a cofactor (*25*, *26*). In the case of *E. coli* Topo IV, Ca^{2+} induces a higher level of cleavage than Mg^{2+} does (Figure 4). The E84P mutation inhibited Topo IV-catalyzed cleavage when Mg^{2+} was used as a cofactor (Figure 6A). In contrast, Ca^{2+} could still induce ParC E84P Topo IV-catalyzed cleavage. Both ParC S80L Topo IV and ParC E84K Topo IV cleaved DNA as well as the wild-type Topo IV in the presence of either Mg^{2+} or Ca^{2+} (Figure 4). Thus, the E84P mutation, which presumably causes a disruption of the helical structure of the α4 helix (*23*), seems to affect the coordination of the divalent cation in the Topo IV−DNA complex. Because of the absence of a divalent cation in the structures of GyrA and yeast Topo II proteins (*12*, *13*) and the lack of the structure of Topo IV, it is not possible to determine the nature of the coordination of a divalent cation in the topoisomerase−DNA complex.

ParC E84P Topo IV could catalyze relaxation and decatenation reactions efficiently (Figure 5), although it exhibited drastically reduced the level of covalent Topo IV−DNA complexes (Figure 6). Thus, the E84P mutant seemed to lower the steady-state level of the reaction intermediate without drastically affect the specific activity of Topo IV. It is not clear how this might be possible. Crisona et al. (*27*) have measured the relaxation activity of Topo IV using a single-molecule method. It is noted that there are two distinct states of Topo IV on DNA, a catalytically active state and a paused (catalytically inactive) state. This might explain observations described here. It seems reasonable to speculate that the specific activity of Topo IV is determined by the efficiency of the enzymatic reaction catalyzed by the catalytically active state of Topo IV. If the catalytically inactive state of Topo IV on DNA forms a covalent Topo IV−DNA complex, the steady-state level of covalent Topo IV−DNA complexes is mainly determined by the level of the catalytically inactive state of Topo IV. Thus, changes of the steady-state level of the reaction intermediate may not affect the overall steady-state level of covalent Topo IV−DNA complexes.

Mutational analyses of the helix−turn−helix region of yeast Topo II suggest that differences between prokaryotic and eukaryotic type II topoisomerases in drug sensitivity may be caused by subtle changes in this region (*18*, *23*). Results described here also demonstrated that changes in the helix-turn-helix region altered the catalytic activity and drug sensitivity of Topo IV. Thus, amino acids around the active site Tyr, including those in the α4 helix, of type II topoisomerases are likely to determine the catalytic activity and drug sensitivity of topoisomerases by governing interactions among the topoisomerase, the DNA, the drug, and the cofactor.

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REFERENCES

1. Reece, R. J., and Maxwell, A. (1991) *Crit. Rev. Biochem. Mol. Biol*. *26*, 335−375.
2. Froelich-Ammon, S. J., and Osheroff, N. (1995) *J. Biol. Chem*. *270*, 21429−21432.
3. Li, T.-K., and Liu, L. F. (2001) *Annu. Rev. Pharmacol. Toxicol*. *41*, 53−77.
4. Wang, J. C. (1996) *Annu. Rev. Biochem*. *65*, 635−692.
5. Champoux, J. J. (2001) *Annu. Rev. Biochem*. *70*, 369−413.
6. Kato, J., Suzuki, H., and Ikeda, H. (1992) *J. Biol. Chem*. *267*, 25676−25684.
7. Peng, H., and Marians, K. J. (1993) *J. Biol. Chem*. *268*, 24481−24490.
8. Khodursky, A. B., Zechiedrich, E. L., and Cozzarelli, N. R. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 11801−11805.
9. Barnard, F. M., and Maxwell, A. (2001) *Antimicrob. Agents Chemother*. *45*, 1994−2000.
10. Lu, T., Zhao, X., and Drlica, K. (1999) *Antimicrob. Agents Chemother*., *43*, 2969−2974.
11. Kumagai, Y., Kato, J., Hoshino, K., Akasaka, T., Sato, K., and Ikeda, H. (1996) *Antimicrob. Agents Chemother*. *40*, 710−714.
12. Berger, J. M., Gamblin, S. J., Harrison, S. C., Wang, J. C. (1996) *Nature 379*, 225−232.
13. Morais Cabral, J. H., Jackson, A. P., Smith, C. V., Shikotra, N., Maxwell, A., Liddington, R. C. (1997) *Nature 388*, 903−906.
14. Hiasa, H., Yousef, D. O., and Marians, K. J. (1996) *J. Biol*. *Chem*. *271*, 26424−26429.
15. Marians, K. J., and Hiasa, H. (1997) *J. Biol. Chem*. *272*, 9401−9409.
16. Morton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K., and Pease, L. R. (1989) *Gene 77*, 61−68.
17. Studier, W. F., Rosenberg, A. H., and Dunn, J. J. (1990) *Methods Enzymol*. *185*, 60−89.
18. Hsiung, Y., Elsea S. H., Osheroff, N., and Nitiss, J. L. (1995) *J. Biol. Chem*. *270*, 20359−20364.
19. Anderson, V. E., Gootz, T. D., and Osheroff, N. (1998) *J. Biol. Chem*. *273*, 17879−17885.
20. Tewey, K. M., Rowe, T. C., Yang, L., Halligan, B. D., and Liu, L. F. (1984) *Science 266*, 466−468.
21. Megonigal, M. D., Fertala, J., and Bjornsti, M. A. (1997) *J. Biol. Chem*. *272*, 12801−12808.
22. Mao, Y., Yu, C., Hsieh, T.-S., Nitiss, J. L., Liu, A. A., Wang, H., and Liu, L. F. (1999) *Biochemistry 38*, 10793−10800.
23. Dong, J., Walker, J., and Nitiss, J. L. (2000) *J. Biol. Chem*. *275*, 7980−7987.
24. O'Reilly, E. K., and Kreuzer, K. N. (2002) *Biochemistry 41*, 7989−7997.
25. Osheroff, N., and Zechiedrich, E. L. (1987) *Biochemistry* 26, 4303−4309.
26. Osheroff, N. (1987) *Biochemistry*, *26*, 6402−6406.
27. Crisona, N. J., Strick, T. R., Bensimon, D., Croquette, V., and Cozzarelli, N. R. (2000) *Genes Dev*. *14*, 2881−2892.

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