Mutations of Vaccinia Virus DNA Topoisomerase I That Stabilize the Cleavage Complex*

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Two mutations in vaccinia virus topoisomerase I, K167D and G226N, have been characterized. SOS induction was observed in Escherichia coli expressing vaccinia topoisomerase I with either one of these mutations. The mutant enzymes were purified to homogeneity and compared with the wild type enzyme for relaxation activity and the partial activities of substrate binding, site-specific DNA cleavage and DNA religation to determine the mechanism of SOS induction. The K167D mutant enzyme had reduced binding affinity for the DNA substrate with a $K_{app}$ that was 10-fold higher than wild type. Nevertheless, in reactions with high enzyme concentration, its substrate cleavage activity was 90% that of wild type. The G226N mutant enzyme had virtually wild type binding and cleavage activities. However, intermolecular religation by these two mutants were observed to be significantly reduced. The cleavage complexes formed with the K167D and G226N mutants were more stable to high salt than the wild type cleavable complex. We propose that these mutants in vivo induce the SOS response in E. coli due to the shift of topoisomerase cleavage-religation equilibrium towards cleavage and increased stability of the cleavage complex. The mutation thus has a similar effect as the topoisomerase-targeting inhibitors that turn topoisomerases into DNA damaging agents.

DNA topoisomerases are enzymes that catalyze the interconversion of DNA topological forms by cleavage of DNA followed by DNA strand passage, and then rejoining of the cleaved DNA (reviewed in Refs. 1-4). DNA cleavage is due to the nucleophile attack of DNA phosphodiester bond by the hydroxyl group of a tyrosine residue in the topoisomerase active site. A phosphotyrosine linkage is formed in the resulting covalent complex. After strand passage or swivel action at the cleavage site leading to topological change, nucleophilic attack on the phosphotyrosine linkage by the DNA hydroxyl group displaced previously rejoins the DNA phosphodiester backbone. DNA cleavage-rejoining is highly concerted. To isolate the covalent cleavage complex with duplex DNA, denaturation of enzyme or unusual substrates that abolish certain protein-DNA interactions are required (2, 5-8). A large number of clinically important antimicrobial and anti-cancer drugs have been shown to target against topoisomerases (9-13). Many of these drugs act by enhancing the formation of the covalent topoisomerase-DNA cleavage complexes either by increasing the DNA cleavage rate or inhibition of DNA religation rate. For example, the anti-cancer drug camptothecin has been shown to inhibit the relaxation activity and enhance the cleavage complex of eukaryotic type I DNA topoisomerases from tumor, yeast, Drosophila, wheat germ, Tetrahymena, and calf thymus (14-18). Unlike these eukaryotic type I topoisomerases, vaccinia virus topoisomerase I is resistant to camptothecin (19). We have demonstrated previously that the single mutation D221V can convert the vaccinia topoisomerase I to camptothecin sensitive (20). It therefore has a potential interaction site for camptothecin despite its small size (32,000 $M_r$) compared to the other eukaryotic type I topoisomerases (90,000-100,000 $M_r$).

The relatively small size of vaccinia topoisomerase I facilitates structure and function analysis. We have been attempting to isolate and characterize topoisomerase mutations that mimic the action of the topoisomerase poisons in inhibiting the religation of topoisomerase-mediated DNA cleavage. Cells expressing such mutants are expected to have increased accumulation of the topoisomerase-DNA covalent cleavage complex. In yeast, topoisomerase inhibitors targeting against both topoisomerase I and topoisomerase II have been shown to induce $\beta$-galactosidase production from chromosomal DIN3::lacZ fusion (18). The DIN3 gene is a DNA damage-inducible gene that is probably involved in repair of topoisomerase-mediated lesion. In bacteria, gyrase inhibitors are also known to induce the SOS response (21). The recA and dinD1 genes of Escherichia coli are two of the genes induced in the SOS repair pathway in response to DNA damage (22-24). E. coli strains with chromosomal dinD1::lacZ fusion have been utilized to characterize cleavage of DNA by restriction enzymes in vivo (25, 26). A $\lambda$::lacZ fusion strain has also been used to isolate IS10 transposable mutants that produce a transposition intermediate and induce the SOS response but are unable to complete transposition (27). Our screening for in vivo SOS induction by vaccinia topoisomerase I mutants using E. coli with dinD1::lacZ or recA::lacZ (28) fusions has identified two mutants, K167D and G226N. The lacZ fusion strains expressing these mutant enzymes formed smaller and blue colonies on indicator plates containing X-gal,1 a chromogenic substrate of $\beta$-galactosidase. Cells expressing the wild type vaccinia topoisomerase I formed larger and white colonies. In vitro characterization of DNA relaxation and partial enzyme activities confirmed that these mutations have affected the DNA cleavage-religation equilibrium and stabilized the enzyme cleavage complex.

EXPERIMENTAL PROCEDURES

Materials—DNA oligonucleotides used as primers for site-directed mutagenesis were synthesized in an Applied Biosystems synthesizer and used without purification. Oligonucleotide substrates for the enzyme were purified by high performance liquid chromatography. Phagemid pKS-top was constructed by cloning the BamHI-PatI fragment containing the entire vaccinia topoisomerase I coding sequence from pl940 (19) into the corresponding sites in phagemid pKS+ from Stragene.

1 The abbreviation used is: X-gal, 5-Bromo-4-chloro-3-indolyl $\beta$-galactosidase.
Negatively supercoiled plasmid DNA used in relaxation assay was purified by alkaline lysis (29) and CsCl centrifugation. E. coli strains GE94 (28) and JH140 (26) were kindly provided by Dr. G. M. Weinstock of the University of Texas Medical School, Houston, and Dr. J. Heitman of Duke University, respectively.

Site-directed Mutagenesis—The Mutagen-Gene oligonucleotide-directed in vitro mutagenesis kit from Bio-Rad was used. Single-stranded phagemid pKVT DNA containing part of the vaccinia topoisomerase coding sequence (20) was used as template. The G226N mutagenesis primer has the sequence 5'-CTGATAATTTGCAATATACGTCCG-3'. The K167D mutagenesis primer has the sequence 5'-ACTTTGT- CATCCTCCTAACA-3'. The restriction sites of pA9topo and pKS-top, respectively.

Expression of Mutant Topoisomerases—Mutant vaccinia topoisomerase I sequence was excised in a NsiI-BamHI restriction fragment from the BamHI restriction sites of pA9topo (19). The noncoding region between the BamHI restriction sites of pA9topo is absent in the resulting clone. That has no apparent effect on enzyme expression. The induction of expression and enzyme purification were carried out as described previously (19). Wild type and mutant vaccinia topoisomerase I preparations appeared to be homogeneous in SDS-polyacrylamide gel electrophoresis analysis. Protein concentrations were determined by the Bio-Rad protein assay.

Oligonucleotide Cleavage Assay—The 32-nucleotide length J6P (5'-AACATTCCTCTGTGCCCTTATTCCCTTTT-3') described in Ref. 30. It was a strong binding and cleavage site for vaccinia topoisomerase I. It was labeled with 32P at the 5'-end by T4 polynucleotide kinase. Five pmol of end-labeled J6P was hybridized to 5.5 pmol of unlabeled J6P that has the complementary nucleotide sequence (3'-TTGTAAAAAGCGACCGGAAATAGGGG-5') by heating to 65 °C for 2 min in 20 μl of 0.25 mM NaCl, then slowly cooled to room temperature. Binding reaction mixtures (10 μl) containing 50 μM Tris-HCl, pH 7.5, 100 fmol of J6P/J6P, and topoisomerase were incubated for 5 min at 37 °C and then spotted on nitrocellulose filters soaked previously in TE buffer (50 mM Tris-acetate, pH 8, 2 mM EDTA). The DNA was visualized by ethidium bromide staining and photographed over UV light.

RESULTS

Use of pKS-top for Characterization of Vaccinia Topoisomerase I Mutants in E. coli—To screen for vaccinia topoisomerase I mutants that induced the SOS response in vivo in E. coli, we needed an expression system that would produce a significant amount of vaccinia enzyme, but overexpression of vaccinia topoisomerase I had to be avoided because that would limit the viability of the host (31, 32). We also wanted to avoid SOS induction from overexpression of the wild type enzyme. It was shown that induction of expression of vaccinia topoisomerase I from the very strong T7 promoter caused arrest of growth and recA-dependent lysogenic induction in a λ-lysogen (31). That level of overexpression was therefore not suitable for the SOS-induction assay we have planned to use for identification of mutants deficient in DNA religation. We demonstrated previously (32) that in plasmid p1940 (20), a low level of vaccinia topoisomerase I was expressed at 30 °C, with no apparent ill effect on cell growth. Topoisomerase expression could be induced by shift to 42 °C, and cells would have decreased p1940 copy number and lose viability (32). We constructed a pKS-top phagemid as an expression plasmid for vaccinia topoisomerase I that could be obtained in the single-stranded form. The 5'-flanking sequence for the vaccinia topoisomerase I gene was identical to that found in p1940. We confirmed the expression of vaccinia topoisomerase I DNA by assaying for relaxation activity in cell lysate of E. coli GE94 (Fig. 1) and JH140 (data not shown) transformed with pKS-top. The reaction mixture contained 2.5 mM EDTA and no added divalent ions so the bacterial topoisomerase activities were not active under these conditions and only eukaryotic type I topoisomerase activity could convert the negatively supercoiled DNA to more relaxed forms. We found that these transformants expressed a low level of vaccinia topoisomerase I when grown at 30 °C. A higher level was expressed at 42 °C as found in E. coli expressing p1940 (32). SOS induction due to the presence of the wild type vaccinia topoisomerase I was not detectable in GE94 (recA-λacZ) or JH140 (dinD1-λacZ) colonies after overnight incubation on X-gal indicator plate at 30, 37, or 42 °C. These strains were there-
confirmed that wild type pKS-top appeared as a dimer and pKS-top resulting SOS induction due to DNA damage response. topoisomerase I with stabilized covalent cleavage complex and as SOS-inducing-We had initially hoped to construct mutants suitable for screening for expression of mutant vaccinia topoisomerase I using single-stranded pKS-top as template. Unfortunately the yield of single-stranded pKS-top was low, possibly due to expression of vaccinia topoisomerase I. A number of site-directed mutants in the conserved region of vaccinia topoisomerase I postulated to be important for DNA cleavage-religation were therefore first generated using single-stranded pKT1 phagemid (20) as template. This phagemid lacked the coding sequence for the first 68 amino acids of the enzyme. The mutants were first cloned in the T7 expression plasmid pA9topo for enzyme purification purpose. To determine if these site-directed mutants would induce the SOS response in E. coli, they were cloned via the NsiI-BglII fragment from pA9topo into pKS-top. Ligation using NsiI-BglII fragment with the G226N mutation was mostly in the monomeric form. Lanes 1 and 2, wild type pKS-top. Lanes 3-10, pKS-top with G226N mutation. D, dimeric DNA; M, monomeric DNA.

fore suitable for screening for expression of mutant vaccinia topoisomerase I with stabilized covalent cleavage complex and resulting SOS induction due to DNA damage response. Identification of the K167D and G226N Site-directed Mutant as SOS-inducing—We had initially hoped to construct mutants of vaccinia topoisomerase I using single-stranded pKS-top as template. Unfortunately the yield of single-stranded pKS-top was low, possibly due to expression of vaccinia topoisomerase I. A number of site-directed mutants in the conserved region of vaccinia topoisomerase I postulated to be important for DNA cleavage-religation were therefore first generated using single-stranded pKT1 phagemid (20) as template. This phagemid lacked the coding sequence for the first 68 amino acids of the enzyme. The mutants were first cloned in the T7 expression plasmid pA9topo for enzyme purification purpose. To determine if these site-directed mutants would induce the SOS response in E. coli, they were cloned via the NsiI-BglII fragment from pA9topo into pKS-top. Ligation using NsiI-BglII fragment wild type pA9topo and other site-directed mutan resulted in white transformants of JH140 and GE94 on X-gal indicator plates. In comparison, the ligation using the NsiI-BglII fragment carrying the K167D and G226N mutations yielded a large number of transformants at 30 °C that were blue and had poor growth on X-gal indicator plates. The blue color of the colonies were more intense at 37 and 42 °C, when enzyme expression level was expected to be higher.

Plasmid Copy Number of pKS-top Expressing and K167D and G226N Mutant Was Lowered—When 30 °C overnight cultures of GE94 colonies expressing the G226N mutant were used for plasmid preparation by rapid lysis, we found that the yield of the mutant pKS-top plasmid was drastically reduced compared to the yield of wild type pKS-top plasmid from GE94 (Fig. 2). The expression of the G226N mutant enzyme might have resulted in selection of cells with lowered plasmid copy number, probably due to some damaging effect of the mutant enzyme. Just like plasmid p1940 that also expresses wild type vaccinia topoisomerase I (32), the plasmid pKS-top was isolated as a dimer. The mutant plasmid G226N appeared mostly to be monomeric, as seen previously for insertionally mutated forms of p1940 (32). Transformants of pKS-top with the K167D mutation had even lower copy number when compared to the G226N mutant plasmid (data not shown).

Mutant Topoisomerases Had Reduced Relaxation Activity—
G226N and K167D mutant enzymes were expressed in E. coli strain BL21 from the T7 promoter in pA9topo upon supply of T7 polymerase by phase A CE8 infection. The yields of mutant vaccinia topoisomerase I in the lysate and after purification to apparent homogeneity were about the same as wild type (data not shown). When assayed for relaxation activity using negatively supercoiled plasmid DNA as substrate (Fig. 3), both mutants were found to be less active than wild type. Quantitation of the results from relaxation assay by densitometry scanning showed that the G226N mutant was ~5-10-fold less active than wild type while the K167D mutant was ~30-50-fold less active. At least one of the individual steps involved in relaxation of DNA by enzyme was thus being affected by the mutation.

Noncovalent DNA Binding by Mutant Topoisomerases—Vaccinia topoisomerase I specifically binds to duplex DNA sites with the conserved sequence 5'-CTGCCTT (35). We use a labeled oligonucleotide duplex containing the CCCTT sequence to assay this step of enzyme action by measuring the percentage of substrate DNA that was retained on nitrocellulose filters after incubation with the enzyme (35). There was no contribution of covalent intermediate to overall binding measured in this assay as demonstrated by previous results from the mutant with the active site tyrosine 274 converted to a phenylalanine, eliminating the covalent binding activity (36). The results of our experiments (Fig. 4) showed that the noncovalent binding affinity (determined by concentration needed for half-maximal binding) of the G226N mutant was nearly identical to that of the wild type. The K167D mutant had a binding affinity that was about 10-fold lower than wild type. The amount of maximal binding compared to wild type was 83% for the G226N mutant and 72% for the K167D mutant.

Cleavage of DNA by Mutant Topoisomerases—The ability of the mutant topoisomerases to carry out DNA strand cleavage was determined by quantitation of the percentage of substrate that was cleaved using the labeled oligonucleotide duplex substrate with a specific cleavage site. The results (Fig. 5) for the K167D mutant showed that due to weaker binding, about 10-fold higher K167D mutant enzyme was required for half-maximal cleavage compared to the wild type enzyme and the G226N mutant. The maximal percentage of DNA cleavage for the G226N mutant was 99% of that of the wild type. For the K167D mutant, it was 92% of the maximal percentage of DNA cleavage by wild type. These ratios of mutant to wild type activity were significantly higher than those obtained for noncovalent binding. Since a noncovalent complex had to form first before cleavage can occur, these results suggested that the cleavage-religation equilibrium was shifted towards cleavage for the two mutant enzymes.

The cleavage specificity of the mutant enzymes was compared to the wild type enzyme by using 3'-end-labeled pUC19 DNA (linearized with BsaI) as substrate. The pattern of cleavage products generated by the mutant enzymes were analyzed by electrophoresis in the sequencing gel (35). The sites of cleavage were identical to those of the wild type enzyme (data not shown), indicating that these two mutations did not alter the DNA sequence specificity in binding by the enzyme.

Religation by the G226N and K167D Mutants Was Reduced—To test further if the DNA cleavage-religation equilibrium was altered for the two mutants, we carried out an intermolecular religation assay. A 5'-end-labeled 27-mer (5'-TATTCAACATTTCGTTGTCGCCCTTT-3') was hybridized to an unlabeled 50-mer (3'-ATAAGTTGTAAGGCACAGCGGGAATAAGGAAA- AACCAGCGGTAAACCG5'). The vaccinia topoisomerase I cleaved the 27-mer at two bases from the 3'-end. A 25-mer was then added as acceptor so that religation of the vaccinia topoi-
Fig. 3. Comparison of relaxation activities of K167D and G226N mutant enzymes to wild type. A, 0.4 µg of supercoiled DNA was assayed as described under "Experimental Procedures." Lane 1, control, DNA incubated with no enzyme. Lanes 2–6, 125, 25, 5, and 2.5 ng of wild type vaccinia topoisomerase I. Lanes 7–9, 125, 25, 5, and 2.5 ng of K167D mutant enzyme. Lanes 10–13, 125, 25, 5, and 2.5 ng of G226N mutant enzyme. S, supercoiled DNA; R, relaxed DNA. B, the conversion of supercoiled DNA to relaxed form was quantitated with the Hoefer scanning densitometer. The percentage of DNA that was in the covalently closed relaxed form was plotted against the amount of enzyme present.

We have characterized the effect of two single mutations, K167D and G226N, on the activities of vaccinia DNA topoisomerase I. We are interested in these mutants because when these mutant topoisomerases were expressed in E. coli, the SOS response was induced. They were much less active (<20%) than the wild type enzyme when assayed for relaxation of negatively supercoiled plasmid DNA. When expressed from the same plasmid vector, the wild type enzyme did not induce the SOS response. Therefore it was unlikely that the SOS response was induced by the effect of relaxing activity of the mutant enzymes. We therefore characterized their partial activities that represent the different steps of catalysis. Our initial hypothesis in the screening of these SOS-inducing mutants was that the mutations we would obtain through this screening process...
might be mimicking the action of the drugs that stabilize the cleavable complex of topoisomerases, causing increased cleavage of DNA in vivo. The results from the enzymatic assays we carried out on the two SOS-inducing mutant enzymes, G226N and K167D, have confirmed this hypothesis. After measurement of DNA binding, cleavage, and religation by the G226N mutant enzyme, the only partial activity that was reduced compared to the wild type enzyme mutant was in DNA religation. This mutation is thus unlikely to affect folding of the enzyme to a large extent. We do not have an assay that can measure rate of strand passage or strand swiveling after initial DNA nicking by enzyme. We therefore do not know if that plays a part in the overall 5-fold reduction of relaxing activity in the G226N mutant. Besides affecting DNA religation, the K167D mutation also reduced significantly the binding affinity of the enzyme for the DNA substrate. We postulate that the lower DNA binding affinity of the K167D mutant may be due to loss of a direct interaction which is required for substrate affinity but not for DNA sequence recognition. This mutant still maintained the same maximal DNA cleavage activity and sequence specificity so it is unlikely to have a drastic change in protein folding.

Amino acids 116 through 228 of vaccinia topoisomerase I have about 26% homology to the corresponding region of Saccharomyces cerevisiae, S. pombe, and human enzymes (33, 37). Many lines of evidence have linked this domain to DNA cleavage-religation activity of the enzyme even though it does not contain the active site Tyr(34, 38). Conversion of Asp(221) to Val reduced the cleavage activity of the enzyme, with the religation activity in this mutant becoming sensitive to inhibition by camptothecin even though the wild type enzyme was resistant (20). Mutations of Gly(192) to Asp and Arg(228) to Gln rendered the enzyme inert in formation of covalent complex (36). Mutations of Thr(147) to Ile and Gly(192) to Ser also caused severe defects in covalent complex formation (36). None of these previously described mutations affected the noncovalent DNA binding significantly. The two mutations we characterized here belonged to a different class even though they are found in the same conserved region. DNA cleavage activity was not affected in these two mutants but DNA religation activity was reduced and stability of the cleavable complex increased.

It is possible to suggest different possible mechanisms for these mutations to affect the cleavage-religation activity of vac-

FIG. 4. Noncovalent binding by wild type and mutant topoisomerases. The noncovalent binding assay was carried out as described under "Experimental Procedures." The percent of input DNA bound to the filter was corrected for the binding of free DNA to filter (5-17% of input radioactivity). The average of data from three separate experiments were plotted.

FIG. 5. Cleavage of oligonucleotide substrate by wild type and mutant topoisomerases. The oligonucleotide cleavage assay was carried out as described under "Experimental Procedures." The percentage of input substrate cleaved by the enzyme (average of data from three separate experiments) was plotted here.

FIG. 6. Religation by wild type and mutant topoisomerases. The religation assay was carried out as described under "Experimental Procedures." The percentage of input DNA that formed the intermolecular religated product (average of data from three separate experiments) was plotted here.

FIG. 7. Time course of religation by wild type and mutant topoisomerases. Two-hundred fifty fmol of the oligonucleotide hybrid used in the religation assay was incubated with 100 fmol of wild type vaccinia topoisomerase I, 100 fmol of G226N mutant enzyme, or 1000 fmol of K167D mutant enzyme in 50 mm Tris-HCl, pH 7.5, for 5 min at 37 °C. After addition of 0.2 m NaCl, 35 pmol of the 25-mer acceptor was added and further incubated for the time indicated. The reactions were terminated and analyzed as described under "Experimental Procedures."
cinia topoisomerase I. The tyrosine hydroxyl acts as a nucleophile in phosphodiester bond cleavage during the cleaved complex formation. During the relaxation step, the 5'-hydroxyl on the cleaved DNA is now the nucleophile that acts to displace the phosphotyrosine linkage. Basic residues on the enzyme might assist in these catalytic steps by withdrawing a proton from the hydroxyl groups to increase their strength as nucleophiles, similar to the general acid-base catalysis mechanism proposed for DNase I (28). These basic residues if mutated, would affect the corresponding cleavage or relaxation activity of the enzyme severely. Hydrogen bonding or non-ionic interactions between the enzyme and DNA are likely to differ to a certain degree when the noncovalent enzyme-DNA complex undergoes the transition to cleaved complex. Basic residues such as Lys can also participate directly in binding to DNA by charge-charge interaction with the DNA backbone phosphate. The energy of binding for the two kinds of complexes relative to each other will affect the equilibrium between noncovalent and cleaved complexes. A mutation can affect the energy of protein-DNA complexes by either directly being involved in noncovalent interaction with DNA, especially at the region downstream from the cleavage site where the DNA strand containing the 5'-hydroxyl group is not covalently bound to the enzyme, or through steric and protein folding effects. If a mutation has a net effect of lowering the binding energy of the cleaved complex relative to the noncovalent complex, it will result in stabilization of the cleaved complex and shift the equilibrium towards DNA cleavage. Alternatively, if the mutation promotes dissociation of the noncovalently bound DNA from the protein after the cleavage complex is formed, the 5'-hydroxyl will then be out of position for interactions required for reforming the phosphodiester linkage. That would also decrease DNA religation and stabilize the cleavage complex. The latter mechanism is more likely for the K167D mutant which has decreased noncovalent binding affinity for DNA. Further results, preferably structural information on the enzyme, are needed to determine how the G226N and K167D mutations reduce DNA religation and stabilize the cleavage complex with DNA.

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Fig. 8. The mutants form cleavable complexes that were more salt-stable than the wild type cleavable complex. The reaction mixture (20 μl) contained 200 fmol of labeled JSP6/P12 cleavage substrate in 50 mM Tris-HCl, pH 7.5, 50 mM NaCl. 6.7 pmol of wild type or G226N mutant enzyme were used in the individual reaction so that approximately the same amount of cleavable complex would be formed at 50 mM NaCl. After incubation at 37 °C for 5 min, different amounts of 5 mM NaCl solution were added to the reaction mixture to achieve the final NaCl concentration indicated. After incubation for an additional 5 min, the reactions were stopped and the amount of cleavage product and substrate was determined as described under “Experimental Procedures.” The cleavage ratio was first calculated as the percentage of input substrate that remained as cleavage product at each final NaCl concentration and then divided by the percentage of cleavage seen at 50 mM NaCl to obtain the percentage of residual cleavage shown here.