Simian Virus 40 DNA Replication Is Dependent on an Interaction between Topoisomerase I and the C-Terminal End of T Antigen

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Topoisomerase I (topo I) is needed for efficient initiation of simian virus 40 (SV40) DNA replication and for the formation of completed DNA molecules. Two distinct binding sites for topo I have been previously mapped to the N-terminal (residues 83 to 160) and C-terminal (residues 602 to 708) regions of T antigen. By mutational analysis, we identified a cluster of six residues on the surface of the helicase domain at the C-terminal binding site that are necessary for efficient binding to topo I in enzyme-linked immunosorbent assay and Far-Western blot assays. Mutant T antigens with single substitutions of these residues were unable to participate normally in SV40 DNA replication. Some mutants were completely defective in supporting DNA replication, and replication was not enhanced in the presence of added topo I. The same mutants were the ones that were severely compromised in binding topo I. Other mutants demonstrated intermediate levels of activity in the DNA replication assay and were correspondingly only partially defective in binding topo I. Mutations of nearby residues outside this cluster had no effect on DNA replication or on the ability to bind topo I. These results strongly indicate that the association of topo I with these six residues in T antigen is essential for DNA replication. These residues are located on the back edges of the T-antigen double hexamer. We propose that topo I binds to one site on each hexamer to permit the initiation of SV40 DNA replication.

The replication of simian virus 40 (SV40) DNA has been extensively investigated as a model to understand the replication of eukaryotic DNA in detail (4, 13, 23, 46). SV40 DNA has a well-defined origin, and it encodes an initiator protein called large T antigen that functions as a bidirectional helicase and, in coordination with host proteins, promotes bidirectional replication from the viral origin (28, 54, 65, 66). The use of cell-free SV40 DNA replication systems has identified more than 10 host proteins that are essential for this process (31, 53, 62, 67, 68). These proteins have been purified and investigated for their role in initiation of DNA synthesis and elongation of DNA (21, 26, 33, 36, 43, 56, 69). Among them, only three proteins, DNA polymerase α-primase (pol/prim), replication protein A (RPA), and topoisomerase I (topo I), are required for initiation of DNA chains by forming an active initiation complex with T antigen (8, 12, 18, 20, 35, 55).

In order to initiate replication, T antigen binds to the core origin in a defined manner (9, 10, 25, 32, 39, 64). In the presence of ATP, T antigen assembles at the core origin from individual monomers into a double hexamer or as a preformed hexamer followed by cooperative association with another hexamer (9, 58). The double hexamer is the functionally active form of T antigen that untwists the AT-rich tract, melts the early palindrome, and begins to unwind DNA bidirectionally using its helicase activity (1–3, 11). The three cellular initiator proteins are then recruited to the origin, but the dynamics of this process are not clearly understood. In a recent study (47), we showed evidence that pol/prim must be present in order for topo I and RPA to bind, suggesting that it binds first. That study also suggested that topo I is recruited next, followed by RPA. The function of topo I is to continually relieve the superhelical tension generated during the DNA-unwinding process (69). We have assumed that this activity is tightly coordinated with DNA unwinding when topo I and T antigen interact with one another (48–50). RPA is required to stabilize the unwound single strand and to stimulate pol/prim activity (16, 24). The primase subunit of pol/prim triggers the formation of small RNA primers on the exposed single-stranded template, and these are extended into short stretches of DNA by polymerase α (38, 57). All three cellular proteins can directly bind with T antigen, and these interactions appear to be essential to effectively initiate DNA synthesis (12, 19, 35, 37, 48). However, exactly how all four proteins associate with one another to form an initiation machine is still not clearly understood.

Topo I can bind to T antigen in vitro, and multiple studies have provided evidence that this association is functionally important (18, 42, 48–50, 55). Topo I significantly enhances T-antigen-mediated DNA replication and induces the formation of much larger amounts of completed molecules (50, 55). The enzyme is needed from the point of initiation (18, 55) and cannot stimulate replication if added after the reaction has started (55). It makes the T antigen unwinding reaction origin specific when tested with a linear DNA fragment (50). Under DNA replication assay conditions, topo I binds preferentially to a double-hexameric structure of T antigen associated with origin DNA and cannot readily interact with smaller structures (14). The stoichiometry of this complex is one molecule of topo I per T-antigen hexamer (14, 47).

Our previous studies (44, 48) have demonstrated the existence of two binding sites for topo I on T antigen, one at the N-terminal region (residues 83 to 160) and the other at the C-terminal region (residues 602 to 708). We also showed that the cap domain of topo I interacts with both sites (44). Electron microscopic analysis...
of T-antigen double hexamers assembled over the core origin revealed that the two hexamers communicate through the N-terminal region that includes the origin binding domain (residues 131 to 260) (59–61). X-ray crystallography of origin binding domain dimers identified the putative sites of interaction between adjacent hexamers (34), in agreement with earlier genetic data (64). One topo I binding site must therefore be located close to the hexamer-hexamer interface, and the other must be near the distal ends of the double hexamer (44). Since the recruitment of topo I to T-antigen double hexamers is also dependent on an association with DNA (47), this suggests that topo I is recruited to the C-terminal site (where the DNA is exposed) to participate in initiation of DNA replication. Separation of the duplex DNA is thought to take place very close to the central channel at the back end of the double hexamer (15, 51), and by associating close to that site, topo I might work together with T antigen as a swivelase, relaxing the DNA as it becomes unwound (14, 44). The role of the N-terminal topo I binding site on T antigen is not known, but since it is located close to the binding site for RPA (63), it is possible that the topo I enzyme there might be involved more directly in DNA synthesis as opposed to DNA unwinding.

In the present study, we sought to more precisely identify the topo I binding site at the C-terminal end of T antigen and investigate its role in SV40 DNA replication. For this purpose, we generated a number of T-antigen mutants with substitutions within this region and tested them for their ability to bind topo I and to support DNA replication. We identified a cluster of amino acids on the surface of the T-antigen helicase domain that is needed for binding to topo I and for promoting DNA replication. The results strongly indicate that an interaction of that region of T antigen with topo I is required for the proper functioning of the DNA replication machine.

MATERIALS AND METHODS

Site-directed mutagenesis. Single point mutations were introduced into SV40 T antigen cDNA using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s recommendations. Mutations were generated in a recombinant plasmid consisting of the baculovirus transfer vector p1393 (BD Pharmingen) with an SV40 T-antigen cDNA insert (6). Briefly, two synthetic oligonucleotide primers containing the desired mutation were annealed using Turbo polymerase in a PCR. Nonmutated parent DNA was cleaved by restriction digestion with DpnI for 1 h at 37°C, and the DNA was introduced into XL-Gold competent cells (Stratagene). The DNA was purified from individual clones and sequenced using appropriate primers to confirm the mutation. The same mutations were generated in p1393 with a cDNA insert that expresses only amino acids 246 to 708 of T antigen (48). Some mutations were introduced into pBS/KS(+) (Stratagene) according to the manufacturer’s recommendations. 25-bp-long oligonucleotides were synthesized and annealed to the denatured plasmid DNA template, and the primers were extended by using Pfu Turbo polymerase in a PCR. Nonmutated parent DNA was cleaved by digestion with DpnI for 1 h at 37°C, and the DNA was introduced into XL-Gold competent cells (Stratagene). The DNA was purified from individual clones and sequenced using appropriate primers to confirm the mutation. The same mutations were generated in p1393 with a cDNA insert that expresses only amino acids 246 to 708 of T antigen (48). Some mutations were introduced into pBS/KS(+) (Stratagene).

Generation of recombinant baculoviruses. Sf9 (Spodoptera frugiperda) cells were cotransfected with p1393–T-antigen constructs and BaculoSapphire DNA inserted into the BamHI site of pSK(+) (32). T-antigen fragments (Tfs) consisting of residues 246 to 708 were purified by first binding to Pab101 beads, followed by high pH elution, as described by Simanis and Lane (45). All full-length as well as fragment T antigens were dialyzed overnight against a solution containing 0.01 M Tris (pH 8.0), 0.1 M NaCl, 0.001 M EDTA, 0.001 M dithiothreitol, and 50% glycerol and stored at −20°C. Protein concentrations were estimated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis using bovine serum albumin (BSA) as a standard.

Topo I was purified from infected High 5 insect cells as previously described (52).

Orign DNA unwinding assays. 32P-labeled origin-containing 64-bp DNA was generated from the following two oligonucleotides as described by Jiao and Simanis (32): top strand, 5’-CAGACTACCTTCGAGAATATGCAGACGGCCGCCTCGAAGCGCGTCTCCAGGAGGCCGCTTCGCGGCTCTGAATTTTTTTTATTATTGAGCCGGCGCTCGCCGTCTGA GCTATTCCGAAGATGTGGCTATTTTTTTTATTTATGCAGAGGCCGAGGCCGCCTCGGCCTCTGCATAAATAAAAAAAATTA 3’; bottom strand, 5’-TAATTTTTTTTTTTTTACCTTCTGGAATAGCTCAGAGGCCGAGGCCGCCTCGGCCTCTGA GCTATTCCGAAGATGTGGCTATTTTTTTTATTTATGCAGAGGCCGAGGCCGCCTCGGCCTCTGCATAAATAAAAAAAATTA 3’. For the unwinding assay, 400 ng of full-length T antigen was incubated with 2 ng of radiolabeled 64-bp DNA for 1 h at 37°C in unwinding buffer (30 mM HEPES [pH 7.5], 7 mM MgCl2, 40 mM creatine phosphate, 4 mM ATP, 1 mM dithiothreitol, and 0.1 mg/ml BSA) supplemented with 50 μg of creatine phosphokinase per ml and 2.8 μg of Escherichia coli single-stranded DNA binding protein (Pharmacia) per ml. The reactions were terminated by addition of stop buffer (0.02 M EDTA, 0.4% SDS, and 0.25 mg proteinase K per ml [final concentrations]), and the mixtures were further incubated for 30 min at 37°C. The reaction mixtures were subjected to electrophoresis on nondenaturing 7% acrylamide gels for 3 h at 110 V. The gels were dried and exposed to phosphor screens overnight, and the labeled DNA was visualized and quantitated with a Molecular Dynamics PhosphorImager.

In vitro DNA replication assays. SV40 DNA replication assays were performed in topo I-deficient Ad293 cell extracts as previously described (54, 55). Topo I-deficient extracts were identified by their responsiveness to added purified topo I. The reaction mixtures contained 236 ng of origin-containing pSV0 + 11, 200 to 300 μg of cell extracts, and 1 μg of full-length T antigen in the presence or absence of 100 ng purified human topo I. DNA synthesis was terminated by incubation with stop buffer (0.02 M EDTA, 0.4% SDS, 0.25 μg of proteinase K per ml [final concentrations]) for 30 min at 37°C. The labeled DNA was purified by phenol chloroform extraction followed by passage through a Spin column (Princeton Separation). The DNA was separated on 1.5% agarose gels (in Tris-borate-EDTA) by electrophoresis for 5 h at 100 V. The gels were dried and exposed, and the labeled DNA products were quantified with a PhosphorImager.

ELISAs. Enzyme linked immunosorbent assays (ELISAs) were performed as described elsewhere (48). In brief, 100 ng of topo I was allowed to attach to wells of microtiter plates. After blocking with phosphate-buffered saline containing 1% gelatin, 0 ng to 200 ng of T antigen (residues 246 to 708) was added and incubated for 2 h at room temperature. After the unbound protein was washed off, an antibody specific to the C-terminal end of T antigen (Pab101) was added to all wells, followed by the addition of horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG). Peroxidase substrate (ortho-phenylenediamine) was then added and left for a few minutes, and the reaction was terminated by the addition of 50 μl of 2.5 M H2SO4. The absorbance of each well was measured at 490 nm using a Dynex plate reader. The absorbance was plotted against the corresponding concentration of the T antigen, and the data were analyzed using the Graph Pad Prism 5 program (Graph Pad Prism Software Inc., San Diego, CA).

Far-Western blotting assays. Far-Western blotting assays were carried out as described by Simmons et al. (48). Six hundred nanograms of topo I was loaded in each well of an SDS–10% acrylamide gel and subjected to electrophoresis for 50 min at 200 V, followed by transfer to a nitrocellulose membrane for 1 h at 100 V. The membrane was cut into strips corresponding to each lane, and the membrane strips were blocked with milk buffer (3.3% dry milk in Tris-buffered saline containing 0.3% Tween 20). Each strip was incubated with 1.2 μg of T antigen consisting of residues 246 to 708 in 2 ml of milk buffer for 2 h at room temperature. After the excess protein was washed off, the membrane strips were incubated with Pab101 antibody and ECL reagent (Amersham) according to the manufacturer’s recommendations. As a negative control, BSA was used instead of T antigen. Bands were visualized by chemiluminescence and quantified using a FluorChem 8800 instrument (Alpha Innotech).

RESULTS

Generation of T-antigen mutants defective in binding topo I. The C-terminal topo I binding site in T antigen is located...
between residues 602 and 708 (44). This C-terminal region is believed to lie on the face of the distal surfaces of double hexamers that form over the SV40 origin (59, 60) and appears to attract one topo I molecule per hexamer (14). In this study, we mutagenized certain residues within this region to more narrowly map the site of interaction and to determine the significance of the binding of topo I to this region for SV40 DNA replication. We chose to mutagenize certain residues within a smaller region (residues 602 to 627) based on the fact that deletion of residues beyond residue 627 had no influence on DNA replication activity (29, 41). In the first round of mutagenesis, the residues that were mutated were determined by sequence conservation among various polyomavirus T antigens (Fig. 1A) as well as by their exposure on the surface of

FIG. 1. Sequence and structural analysis of the C-terminal topo I binding site of SV40 T antigen. (A) Sequences of various T antigens from five different polyomaviruses, i.e., monkey SV40, human BK virus (BKV) and JC virus (JCV), monkey lymphotropic papovavirus (LPV), and mouse polyomavirus (PyV) from residues 602 to 627 (SV40 numbering) and residues 550 and 552 (as compiled by James M. Pipas). Mutated residues are shown in bold, and the substitutions are indicated in bold italic at the bottom. (B) Close-up view of the T-antigen–topo I interaction site predicted by a Z-dock model of the trimeric T-antigen helicase domain–topo I complex. A T-antigen monomer (blue, red, and green) is associated with the cap domain of topo I (residues 215 to 433) (yellow). The residues of T antigen mutated in this study are shown in red and green. The green residue corresponds to K616.
the helicase domain (27). Only one mutant in this series (K616R) was found to be defective in binding topo I (Table 1). A second series of mutants was then generated based on the modeling of T-antigen–topo I complexes using the docking program Z-dock (http://zlab.bu.edu/zlab/people.shtml). Multiple hits were obtained, but we selected the thermodynamically most stable model that predicted the involvement of residue K616 in T antigen and the cap region of topo I. This model is illustrated in Fig. 1B. All T-antigen residues in close proximity to and facing topo I in the model as well as other exposed residues were selected for mutagenesis (Fig. 1B and Table 1). The list includes two residues (K550 and Y552) (Fig. 1A) that map to the same face on the protein but lie outside of region from residue 602 to 627.

The specific substitutions that were made at these various residues were determined in part by sequence conservation (Fig. 1A), in part by the expected impact of various substitutions on T-antigen structure, and in part by the prediction that a certain substitution would alter topo I binding. In most cases, conservative mutations were selected; however, in some cases, more dramatic changes were made in order to prevent possible interactions between the two proteins. We made several substitutions at residues 616 and 621 (Fig. 1A and Table 1). Mutations were generated in two baculovirus transfer vectors: one that contains the cDNA for full-length T antigen and another that expresses a truncated T antigen (residues 246 to 708) missing the N-terminal domain. Recombinant baculoviruses were made, and the mutant proteins were expressed and purified from virus-infected insect cells. Yields of all mutant full-length and fragment proteins were equivalent to those obtained with the WT proteins.

**Origin-unwinding activity of mutant T antigens.** Since all the mutations we made were at residues that were exposed on the surface of the helicase domain, we considered it unlikely that the mutations would significantly alter the overall structure of the protein or interfere with its major function as an origin-specific helicase. To check this, however, we tested the mutant proteins for their ability to unwind a synthetic 64-bp origin-containing DNA. The separated labeled “top” strand of this DNA was detected and quantitated by gel electrophoresis (Fig. 2A). All mutant proteins were able to unwind the origin DNA like WT T antigen (Fig. 2B and Table 1). Most mutants possessed 80 to 100% of the normal activity, although some of them (e.g., K605Q, N618S, and G622A) appeared to be slightly more active than WT T antigen. Therefore, all mutant proteins possessed at least normal origin-unwinding activity. This conclusion was reinforced by assays with “mnimal” origin-containing DNA performed as previously described (14). In this assay, the formation of underwound 388-bp circular DNA is dependent on both T antigen and topo I. All mutants were tested and found to possess normal activity (data not shown).

**In vitro DNA replication activity of mutant T antigens.** Topo I is required for efficient SV40 DNA replication (49, 55, 65, 68, 69). Previous reports (49, 50, 55) have shown that the addition of topo I to T-antigen-mediated replication stimulates replication and greatly promotes the completion of DNA chains. The enzyme acts at the point of initiation and is a component of the initiation complex (18, 47, 55). To check whether the T-antigen mutants can promote DNA replication and whether topo I stimulates replication, we performed SV40 DNA replication assays using topo I-deficient 293 cell extracts. In this assay, topo I (100 ng) stimulated total DNA replication in the presence of WT T antigen by about 2.5-fold, although the synthesis of covalently closed form I molecules was stimulated by 7- to 8-fold (Fig. 3 and Table 1), in agreement with earlier results (55). Mutant T antigens promoted DNA replication to various degrees (Fig. 3 and Table 1). Some mutants, (e.g., Q613R and N618S), behaved similarly to the WT (Fig. 3B and C). Others, (e.g., K616M and M621I), demonstrated a partial defect in DNA replication, but replication was only slightly stimulated by topo I. A third category of mutants (e.g., Y612N and K616R), supported virtually no DNA replication, and added topo I had no effect (Fig. 3B and C). Different substitutions at the same site had different effects. Replacement of Lys 616 with

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**TABLE 1. Summary of biochemical properties of T-antigen mutants**

<table>
<thead>
<tr>
<th>T antigen</th>
<th>% Origin-unwinding activity</th>
<th>Fold stimulation by topo I in:</th>
<th>% Topo I binding activity determined by:</th>
<th>DNA replication</th>
<th>Topo I binding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Overall DNA replication:</td>
<td>ELISA (1/EC50)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Form I</td>
<td>Far-Western blotting</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>100</td>
<td>2.5 ± 0.2</td>
<td>6.9 ± 0.7</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>L603V (Leu→Val)</td>
<td>89 ± 4.5</td>
<td>2.3 ± 0.1</td>
<td>4.8 ± 0.4</td>
<td>70 ± 10</td>
<td>86.6 ± 8.6</td>
</tr>
<tr>
<td>K605Q (Lys→Gln)</td>
<td>115 ± 2.5</td>
<td>1.9 ± 0.3</td>
<td>4.9 ± 0.5</td>
<td>120 ± 3.3</td>
<td>88.6 ± 6.3</td>
</tr>
<tr>
<td>Y612N (Tyr→Asn)</td>
<td>97 ± 13.3</td>
<td>0.9 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>12.3 ± 3</td>
<td>57.6 ± 7.7</td>
</tr>
<tr>
<td>Q613R (Gln→Arg)</td>
<td>84.3 ± 4</td>
<td>3.3 ± 0.5</td>
<td>6.6 ± 1.7</td>
<td>140 ± 7</td>
<td>106 ± 4.5</td>
</tr>
<tr>
<td>K616R (Lys→Arg)</td>
<td>109 ± 4.4</td>
<td>1.0 ± 0.1</td>
<td>0.8 ± 0.2</td>
<td>15 ± 7</td>
<td>25 ± 4.8</td>
</tr>
<tr>
<td>K616Q (Lys→Gln)</td>
<td>87 ± 4.6</td>
<td>1.2 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>27.8 ± 3</td>
<td>37.6 ± 7.3</td>
</tr>
<tr>
<td>K616M (Lys→Met)</td>
<td>94.6 ± 4.7</td>
<td>1.6 ± 0.1</td>
<td>1.7 ± 0.2</td>
<td>49 ± 5</td>
<td>55.5 ± 5.5</td>
</tr>
<tr>
<td>F617N (Phe→Asn)</td>
<td>83.6 ± 9.7</td>
<td>1.1 ± 0.1</td>
<td>1.3 ± 0.2</td>
<td>19.2 ± 6</td>
<td>30 ± 8.1</td>
</tr>
<tr>
<td>N618S (Asn→Ser)</td>
<td>118 ± 6</td>
<td>2.7 ± 0.1</td>
<td>5.0 ± 0.5</td>
<td>98 ± 4</td>
<td>96.6 ± 3.3</td>
</tr>
<tr>
<td>M621I (Met→Ile)</td>
<td>97 ± 19</td>
<td>1.7 ± 0.1</td>
<td>4.5 ± 0.1</td>
<td>51.5 ± 4</td>
<td>43.6 ± 6.2</td>
</tr>
<tr>
<td>M621S (Met→Ser)</td>
<td>84.3 ± 5.9</td>
<td>1.1 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>27.1 ± 6</td>
<td>30 ± 1.6</td>
</tr>
<tr>
<td>G622A (Gly→Ala)</td>
<td>124 ± 10</td>
<td>2.0 ± 0.1</td>
<td>6.0 ± 0.7</td>
<td>97.9 ± 5</td>
<td>81.6 ± 17.3</td>
</tr>
<tr>
<td>I623V (Ile→Val)</td>
<td>115 ± 13</td>
<td>1.9 ± 0.1</td>
<td>6.2 ± 0.1</td>
<td>110 ± 5.6</td>
<td>81.6 ± 15.5</td>
</tr>
<tr>
<td>K550Q (Lys→Gln)</td>
<td>81.3 ± 7</td>
<td>1.1 ± 0.3</td>
<td>1.0 ± 0.1</td>
<td>32.1 ± 9</td>
<td>49 ± 9</td>
</tr>
<tr>
<td>Y552N (Tyr→Asn)</td>
<td>91 ± 9</td>
<td>1.4 ± 0.4</td>
<td>1.1 ± 0.1</td>
<td>47 ± 6</td>
<td>51.6 ± 5.3</td>
</tr>
</tbody>
</table>

* Mutants that possessed 60% or less of the WT activity were considered to be defective. N, no; Y, yes.
the similarly charged Arg or with polar Gln resulted in a complete replication defect, whereas replacement with the hydrophobic Met resulted in only partial loss of activity (Fig. 3 and Table 1). Likewise, replacement of Met 621 with Ile permitted a modest stimulation of replication by topo I (Fig. 3C and Table 1), whereas replacement with Ser led to the total absence of stimulation. When the fold stimulation was measured only in completed form I molecules, the differences between the activities of certain mutants and WT T antigen were even more striking (Fig. 3D). In this case, mutants clearly fall into only two categories: those that were stimulated normally by topo I and those that were completely refractive to added topo I (Fig. 3D and Table 1). These results strongly support the idea that the interaction of topo I with certain T-antigen residues within this region is required for full DNA replication activity.

**Binding of T-antigen mutants to topo I. (i) ELISAs.** Since some of the mutant T antigens failed to support DNA replication in the presence of topo I, we were interested in testing them for their ability to bind topo I. Because of the presence of another topo I-interacting site near the N-terminal region (residues 83 to 160) (44), we introduced the same mutations in a TF consisting of residues 246 to 708 and missing the N-terminal binding site. These mutant fragments were first tested for their topo I binding activity by ELISAs. Different amounts of Tfs were added to topo I-coated wells, and the bound T antigen was detected with an antibody (PAb101) to the C-terminal end of T antigen (17), horseradish peroxidase-conjugated anti-mouse antibody, and substrate. As previously described (48), binding between the two proteins occurs readily and reaches saturation at about 100 ng of T antigen (Fig. 4A). Several of the mutant fragments bound to topo I less well than the WT Tf, as shown in the representative ELISA in Fig. 4A. To quantitate the signal and compare binding of topo I to various T-antigen mutants, we used the Prism 5 data fitting program (Graph Pad Software Inc., San Diego, CA) to first calculate a 50% effective concentration (EC_{50}) for each curve. This value corresponds to the concentration of the Tf at which the absorbance is half of maximum. We then calculated the reciprocal of this number and normalized each to the value for the WT Tf, which was taken as 100%. These numbers are plotted in Fig. 4B and included in Table 1.

**FIG. 2.** Origin-unwinding assay of WT T and mutant T antigens. Full-length WT or mutant T antigen was incubated with 32P-end-labeled 64-bp origin DNA in the presence of ATP for 1 h at 37°C. The DNA was purified and applied to a 7% acrylamide gel. (A) A representative gel result with WT T antigen and several mutants is shown. The positions of the labeled double-stranded (ds) and separated labeled single-stranded (ss) DNAs are shown. Lane 1 shows the results of a reaction without T antigen, and lane 2 shows the labeled single-stranded DNA obtained after boiling the labeled 64-bp DNA. (B) Bar graph of the origin-unwinding activities of all studied T antigen mutants as measured by the released labeled single-stranded DNA. Activity is expressed as a percentage of that of WT T antigen. Error bars represent standard deviations obtained from three data sets.
poorly to topo I compared to WT Tf (Fig. 4B and Table 1), whereas mutants K616Qf, K616Mf, M621If, M621Sf, K550Qf, and Y552Nf bound a little better to topo I but still significantly less well than WT Tf. Interestingly, the topo I binding-defective mutants were also DNA replication defective, whereas those that bound topo I normally supported replication normally (Table 1). Overall, there was an excellent correlation between the two activities.

(ii) Far-Western blotting assay. To corroborate the ELISA findings, we performed far-Western assays where purified topo I was first subjected to SDS-polyacrylamide gel electrophoresis, transferred to a membrane, and then incubated with T-antigen mutant fragments. Binding was detected by reactivity with monoclonal antibody PAb101 (17), horseradish peroxidase-conjugated anti-mouse antibody, and chemiluminescent substrate. As shown in the representative gel in Fig. 5A, fragments bound to the topo I on the membrane to different extents. The signal was quantitated and plotted as a percentage of that of the WT T fragment (Fig. 5B). In agreement with the ELISA results, the L603Vf, K605Qf, Q613Rf, N618Sf, G622Af, and I623Vf mutants were as competent as WT Tf in binding topo I, whereas mutants K616Rf, F617Nf, and M621Sf were about 30% efficient in binding topo I. These data agreed with the ELISA results for all mutants except Y612Nf, which showed close to 60% binding by far-Western blotting but only 12% by ELISA (Table 1). This is a large difference, but it appears that this mutant T antigen, as well as all other binding-defective mutants listed above, bound better to denatured topo I in the far-Western assay than to native topo I in ELISAs. These differences indicate that the binding site on the denatured topo I is more tolerant of changes in T antigen than is the one on native topo I.

Replication of mutant viruses in vivo. To investigate the significance of the T-antigen–topo I binding reaction for virus replication in vivo, we introduced several of the same mutations in pBS/SV40 (30), a plasmid that contains the entire SV40 genome. BSC-1 monkey cells were transfected with mutant or WT plasmid, and after 4 days, the virus was collected and the titer was determined by plaque assays. DNA replication- and topo I binding-defective mutants Y612N, F617N, and M621S yielded significantly less virus (>2,000-fold-, 14-fold-,
and 5-fold-lower titers, respectively), and the plaques that appeared for M621S were much smaller (about one-third) than normal. On the other hand, DNA replication- and topo I binding-normal mutant L605Q gave rise to normal amounts of virus, and plaque size was not affected. These results indicate that T-antigen mutations that reduce the binding to topo I interfere with normal virus replication in vivo.

**DISCUSSION**

Localization of the C-terminal topo I binding site on T antigen. Our previous mapping studies proposed the existence of two binding sites for topo I on T antigen, one involving amino acids 83 to 160 (N-terminal site) and the other amino acids 602 to 708 (C-terminal site) (44). By a mutagenesis strategy, we identified the specific amino acids within the C-terminal site that are responsible for binding topo I. Mutants that we made with substitutions at residues 612, 616, 617, and 621 were mostly defective in topo I binding, indicating that these residues are important for contact with topo I. Two other mutants with changes at residues 550 and 552 were also defective at binding topo I (Table 1). Although these two residues lie outside of the linear region (residues 602 to 708) previously identified by fragment analysis (44), they map close to the other four residues on the surface of the protein (27). These six residues identify a patch on the edge of the back surface of the helicase domain that is responsible for binding topo I (Fig. 6). This region is highlighted in each of the subunits of the hexamer structure (Fig. 6A). The patch consists of charged, polar, and hydrophobic amino acids; however, based on the substitutions that are more tolerated at residues 616 and 621 (Table 1), it appears that hydrophobic interactions may play a significant role in the binding between these two proteins.
The interaction between topo I and the C-terminal topo I binding site on T antigen is essential for SV40 DNA replication. All full-length mutants possessed normal origin-unwinding activity (Fig. 2) and could form normal amounts of double hexamers at the origin (data not shown), indicating that the mutations did not interfere with T antigen’s major function as a helicase. In spite of this, several mutants were defective in promoting SV40 DNA replication in cell extracts and in responding to added topo I in stimulation of DNA synthesis (Fig. 3). The replication-defective mutants correlated precisely with those that were defective in topo I binding (Fig. 4 and 5 and Table 1). This strongly indicates that the binding of topo I to the C-terminal binding site in T antigen is required for optimal DNA replication.

Mutant T antigens supported SV40 DNA replication to various degrees; however, it was noticed that mutants that were severely compromised in DNA replication activity (e.g., K616R, F617N, and M621S) were also the ones that responded very poorly, if at all, to added topo I (Fig. 3 and Table 1). Mutants that displayed a partial loss of activity, (K616M and M621I) were stimulated slightly by added topo I, and those that had near-normal DNA replication activity were stimulated by topo I just like the WT (Fig. 3B). There was also a correlation between total levels of DNA replication and topo I binding activities of individual mutants. Hence, the mutants severely compromised in topo I binding were the same ones that had near-normal DNA replication activity, and those that had intermediate activity in one assay displayed an intermediate activity in the other (Table 1). These data all point to the conclusion that topo I binding is intimately linked with the

FIG. 6. Locations of the T-antigen residues involved in binding topo I and a model of the topo I–T-antigen complex. (A) Back view of the hexameric structure of the helicase domain of SV40 large T antigen (27), looking into the central channel. Residues K550, Y552 Y612, K616, F617, and M621 are highlighted in dark blue on each monomer. (B) A portion of the structure marked by the rectangle in panel A is expanded, and the highlighted residues are labeled. (C) Side view of a T-antigen hexamer with three front-facing monomers displayed in different colors (red, green, and purple). Residues K550, Y552 Y612, K616, F617, and M621 are shown in blue on these three monomers. In a double hexamer, the highlighted residues would be on the back edges. (D) Trimeric T antigen in the topo I–T-antigen model obtained from the Z docking program (http://zlab .bu.edu/zlab/people.shtml) was modified to contain a complete T-antigen hexamer associated with the cap domain of a single topo I molecule using the Swiss PDB viewer program. Each chain is displayed in a different color. The monomer of T antigen associated with topo I is marked in yellow, and topo I is in cyan. The structure shows the T-antigen central channel (at a slight angle) and the topo I cavity through which DNA passes.
ability of various mutant proteins to support DNA replication. Our in vivo replication data suggest a role of the binding of topo I to this end of T antigen in virus multiplication in culture as well.

According to our working model (47), during DNA replication in the presence of pol/prim, topo I is recruited by T antigen after it has formed a complete double hexamer over the origin (14), and this is followed by the binding of RPA to make an initiation complex (47). We now have evidence, presented in this paper, that topo I binds at least to the C-terminal site to participate in DNA replication. The N-terminal topo I binding site appears to be fully functional in the full-length mutant proteins as determined by topo I binding assays with these proteins (data not shown). However, its role in DNA replication is not known.

The major function of topo I during DNA replication is to relieve the supercoiling strain on unreplicated portions of the DNA (69). We have therefore proposed that topo I associates with the C-terminal binding sites within the context of a double hexamer to coordinate the processes of DNA relaxation and DNA unwinding (48–50). It has been shown (7) that a relative of SV40 T antigen (E1) encoded by papillomavirus 16 stimulates topo I activity. The E1 protein also has two binding sites for topo I, and it is the C-terminal site that most effectively stimulates topo I relaxation activity. It is reasonable to suggest, therefore, that a helicase-topo I association is needed for DNA replication for both virus families. However, in light of the fact that none of our T-antigen mutants were impaired in their ability to generate underwound circular DNA in the presence of topo I (data not shown), our current hypothesis is that this interaction is needed primarily for initiation of new DNA chains and not for relaxing unreplicated supercoiled DNA.

Stoichiometric analysis (14) showed that there are two molecules of topo I per double hexamer in the T-antigen–DNA–topo I complex; hence, one molecule of topo I is situated on each hexamer. In Fig. 6A, we highlight the binding sites on each monomer in the hexamer structure (27), and in Fig. 6B, we show an expanded view of one subunit. Each binding site for topo I consists of a number of residues that cluster to the external edge of each monomer of the hexamer. These sites are widely separated from one another on adjacent subunits (Fig. 6A and C). Hence, in double hexamers, the binding sites map to the distal edges of this complex (Fig. 6A and C). Modeling of the T-antigen hexamer and topo I complex by Z-dock (Fig. 6D) shows that the topo I molecule is located on the side of each hexamer, not in line with the double hexamer as might be assumed from the location of the binding sites. Consequently, the bound topo I molecule is at a large distance from the central channel of the helicase (Fig. 6D). Surprisingly, this channel and the groove through which DNA passes in topo I are nearly parallel to one another. Given the stoichiometry of binding (14, 47), it is most likely that topo I can bind to only one T-antigen subunit at a time. Considering the spatial relationship between the two proteins in this model and the fact that the DNA must be threaded through topo I as a double strand and through the T antigen helicase central channel at least as a single strand, we can infer that the DNA must be significantly bent as it connects the two proteins. This idea is consistent with studies that showed that topo I preferentially interacts with and nicks bent DNA (5, 40). There is no way of predicting whether topo I binds to only one subunit at all times or whether it interacts with different subunits at different times during the process of fork movement. It is certainly conceivable that the topo I enzyme switches from one monomer to another during this process, and if so, the attached DNA would therefore gyrate around as topo I moves from monomer to monomer.

**Assembly of a topo I–T-antigen complex on DNA.** There is good evidence that major structural rearrangements occur in T antigen as it assembles into a double hexamer over the origin (59, 61). Furthermore, complexes consisting of 11 subunits or fewer and origin DNA bind topo I much less well than fully formed double hexamers (14). The initial binding of topo I to T antigen must depend, therefore, on extensive rearrangements of subunits as they assemble into a double hexamer. We have previously proposed that topo I first engages the DNA and then attaches to the ends of the double hexamer (47). The results presented here suggest that as topo I associates with a binding site on one of the T-antigen monomers, the DNA becomes severely bent.

In summary, the evidence presented in this paper clearly points to a dynamic interaction between topo I and the back edges of the T-antigen double-hexamer helicase machine to initiate DNA replication and to promote the synthesis of complete daughter DNA molecules.

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