Simultaneous Interactions of Bacteriophage T4 DNA Replication Proteins gp59 and gp32 with Single-stranded (ss) DNA

CO-MODULATION OF ssDNA BINDING ACTIVITIES IN A DNA HELICASE ASSEMBLY INTERMEDIATE

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The T4 gp59 protein is the major accessory protein of the phage’s replicative DNA helicase, gp41. gp59 helps load gp41 at DNA replication forks by promoting its assembly onto single-stranded (ss) DNA covered with cooperatively bound molecules of gp32, the T4 single-strand DNA binding protein (ssb). A gp59-gp32-ssDNA ternary complex is an obligatory intermediate in this helicase loading mechanism. Here, we characterize the properties of gp59-gp32-ssDNA complexes and reveal some of the biochemical interactions that occur within them. Our results indicate the following: (i) gp59 is able to co-occupy ssDNA pre-saturated with either gp32 or gp32-A (a truncated gp32 species lacking interactions with gp59); (ii) gp59 destabilizes both gp32-ssDNA and (gp32-A)-ssDNA interactions; (iii) interactions of gp59 with the A-domain of gp32 alter the ssDNA-binding properties of gp59; and (iv) gp59 organizes gp32-ssDNA versus (gp32-A)-ssDNA into morphologically distinct complexes. Our results support a model in which gp59-gp32 interactions are non-essential for the co-occupancy of both proteins on ssDNA but are essential for the formation of structures competent for helicase assembly. The data argue that specific “cross-talk” between gp59 and gp32, involving conformational changes in both, is a key feature of the gp41 helicase assembly pathway.

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§ The abbreviations used are: gp59, gp32, gp41, the T4 gene 59, gene 32, and gene 41 proteins, respectively; ssDNA, single-stranded DNA; cDNA, etheno-modified M13mp19 ssDNA; poly(deA); gp32-A, a truncated gp32 species containing amino acid residues 1–253; A-domain, a truncated gp32 species containing amino acid residues 213–301; gp32 sp., gp32 species (generic).
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The gene 59 protein (gp59)1 plays critical roles in the coupled DNA replication and recombination systems of bacteriophage T4. gp59 is essential for the recombination-dependent pathway of DNA replication initiation in T4 (1), which is the major DNA synthesis pathway during late stages of T4 infection in Escherichia coli cells. Mutations in gene 59 disrupt this pathway, causing arrested DNA synthesis leading to reduced phage burst size (2). In addition to their DNA-arrest phenotypes, 59 mutants are characterized by recombination deficiency, UV sensitivity, and sensitivities to chemical mutagens and etoposide drugs (3, 4), consistent with direct roles of gp59 in T4 genetic recombination and recombinational DNA repair processes. Biochemical studies have established that gp59 is a major accessory protein of the T4 replicative DNA helicase, gp41 (5–12). gp59 interacts specifically with gp41 and alters its enzymatic activities including ssDNA-stimulated ATP and GTP hydrolysis (7). gp59 promotes the assembly of gp41 onto ssDNA and is nearly essential for the assembly of the helicase onto ssDNA molecules coated with cooperatively bound gp32, the T4 ssDNA-binding protein (5, 7, 8, 12). This helicase-loading function of gp59 (a) allows gp59 to accelerate the rate at which nascent DNA replication forks acquire the gp41 helicase (5), (b) is essential for the branch migration activity of gp41 demonstrated during certain DNA strand exchange reactions initiated by the T4 UvsX recombinase (11), and (c) is essential for the helicase and primosome functions of gp41 during T4 recombination-dependent DNA synthesis reactions reconstituted in vitro (1, 13).2

gp59 is a basic (pI = 10.18) 26-kDa protein with intrinsic binding activity toward single-stranded DNA. Hydrodynamic studies employing sucrose density gradients (10) and analytical ultracentrifugation3 have established that gp59 exists predominantly as a monomer in solution. The oligomeric structure of gp59 in its ssDNA-bound state is currently unknown. However, Lefebvre and Morrical (14) rigorously characterized the thermodynamics of gp59 interactions with single-stranded polynucleotides including native ssDNA and demonstrated that gp59 exhibits at least two distinct ssDNA-binding modes dependent on salt and/or lattice effects. One of these modes is characterized by extremely high cooperativity (ω ~ 2500) but occurs at relatively low salt concentrations (14). Moderate to low cooperativity is seen at higher salt. These results indicate that gp59 is capable of forming clusters on ssDNA, gp59 binds to all single-stranded polynucleotides with a binding site size of n = 9–10 nucleotide residues (14).

In addition to its interactions with gp41 and ssDNA, gp59 exhibits very strong protein-protein interactions with gp32 (5, 7, 8, 10). Previous work in our laboratory demonstrated that gp59 interacts specifically with determinants in the acidic, C-terminal “A-domain” of gp32 and that the assembly of gp41 onto gp32-ssDNA complexes requires direct contacts between gp59 molecules and the A-domains of ssDNA-bound gp32 molecules (8). These and other observations suggested a model of gp59 action in which a gp59-gp32-ssDNA complex of defined structure and stoichiometry serves as an obligatory intermediate in the gp59-dependent helicase assembly pathway. In ad-

2 J. E. Barry, M. L. Wong, and B. M. Alberts, manuscript in preparation.
3 S. D. Lefebvre, M. L. Wong, and S. W. Morrical, unpublished results.
Interactions of gp59 with gp32-ssDNA Complexes

This procedure generally leads to greater than 70% modification of adenine and cytosine residues (21). The integrity of the eDNA was verified by agarose gel electrophoresis. The eDNA had a slightly reduced electrophoretic mobility compared with unmodified M13mp19 ssDNA, consistent with previous reports (14, 20), and no fragmentation of the eDNA was observed. The eDNA was then used in a 5′-xanthine polynucleotide kinase (poly(dA)) reaction. The concentrations of eDNA and poly(dA) were determined by the phosphate method (14, 19) as described above for the unmodified polynucleotides. All of the experiments reported in this paper were repeated with at least two independent preparations of eDNA or poly(dA), with essentially identical results.

EXPERIMENTAL PROCEDURES

Reagents, Resins, and Buffers—Chemicals, biochemicals, and commercial enzymes were purchased from standard suppliers and were used as received, unless otherwise noted.

Circular single-stranded DNA from the bacteriophage M13mp19 was isolated by extraction from purified phage particles (17, 18). Poly(dA) was synthesized from 300 nucleotide residues (average chain length 500 nucleotide residues) was purchased from Amersham Pharmacia Biotech. The ssDNA concentrations are expressed in units of micromoles of nucleotide residues per liter. The 60-mer was 5′-end-labeled with 32P using T4 polynucleotide kinase (New England Biolabs) according to the manufacturer’s instructions. Circular single-stranded DNA from the bacteriophage M13mp19 was isolated by extraction from purified phage particles (17, 18). Poly(dA) (average chain length ≈ 500 nucleotide residues) was purchased from Amersham Pharmacia Biotech. The ssDNA and poly(dA) were determined by phosphate analysis (14, 19).

A portion of the M13mp19 ssDNA was used to make etheno-DNA (eDNA) by modification with chloroacetalddehyde (obtained from Aldrich) according to the method of Menestesi and Kowalczykowski (20).

All ssDNA concentrations are expressed in units of micromoles of nucleotide residues per liter. All ssDNA concentrations are expressed in units of micromoles of nucleotide residues per liter. The ssDNA or is one maintained solely through protein-protein interactions? As a corollary to this question, how do gp59 and gp32 affect the ssDNA-binding properties of each other? A key result obtained by N. P. (8) was that ssDNA molecules saturated with gp32-A, a truncated form of gp32 lacking the A-domain, are completely refractory to helicase assembly, even in the presence of gp59. One possible explanation for this result is that gp59 maintains contact with gp32-ssDNA complexes solely through protein-protein interactions with gp32. This would predict that gp59 is incapable of binding to (gp32-A)-saturated complexes. Alternatively, gp59 may bind to (gp32-A)-ssDNA complexes via contacts with the ssDNA itself but fails to load helicase due to the absence of essential gp59-gp32 contacts. To address these alternative models, we have investigated the interactions of gp59 with both gp32-ssDNA and (gp32-A)-ssDNA complexes and with ssDNA in the presence of the isolated A-domain fragment of gp32. By using methods including ssDNA-cellulose affinity chromatography, eDNA fluorescence enhancement, hydroxy radical protection, and electron microscopy, we find the following: (i) gp59 is able to co-occupy ssDNA pre-saturated with either gp32 or gp32-A; (ii) gp59 destabilizes both gp32-ssDNA and (gp32-A)-ssDNA interactions; (iii) interactions of gp59 with the A-domain of gp32 alter the ssDNA-binding properties of gp59; and (iv) gp59 organizes gp32-ssDNA versus (gp32-A)-ssDNA into morphologically distinct complexes. Our results support a model in which gp59-gp32 interactions are non-essential for the co-occupancy of both proteins on ssDNA but are essential for the formation of structures competent for helicase assembly. The data argue that specific “cross-talk” between gp59 and gp32, involving conformational changes in both, is a key feature of the gp41 helicase assembly pathway.
Bradford assay and SDS-polyacrylamide gel electrophoresis. Following each experiment the ssDNA-cellulose resin was treated with DNase I according to the method of Kowalczykowski et al. (23), and the released nucleotides were quantitated by the phosphate method (14, 19) in order to determine the number of protein-accessible nucleotide residues on the resin.

Etheno-DNA (ssDNA) Fluorescence Enhancement Experiments. The interactions of gp59 (+/− the A-domain fragment of gp32) with fluorescent etheno-DNA lattices were carried out essentially as described by Lefebvre and Morrical (14). All fluorescence measurements were performed using an SLM 8000 spectrofluorimeter, in the ratiometric mode, with an external circulating water bath used to keep the sample chamber at a constant 25°C. Sample cells containing a total of 2 ml of Chloride Buffer (+ NaCl as indicated) and a starting concentration of nucleic acid and/or protein depending upon the experiment were preincubated for 5 min prior to taking the first reading to allow for thermal equilibration. The samples were also magnetically stirred for rapid equilibration following titrant addition. During titrations, each data point was recorded 1 min after titrant addition to ensure complete equilibration. Etheno-DNA fluorescence was monitored by exciting the sample at 300 nm and measuring the emissions at 405 nm. The slits were set at 4 nm, and the photomultiplier voltage was kept constant in all experiments. Corrections of the data for dilution, inner-filter effects, protein fluorescence, and/or the fluorescence of free etheno-DNA were made as appropriate to the experiment, using procedures described by Lefebvre and Morrical (14). The corrections performed for each experiment are summarized in the figure legends. No corrections were made for photobleaching effects since the exposure of either εDNA or poly(dεA) to UV light for extended periods resulted in negligible decreases in fluorescence.

Salt-back titrations were performed by forming complexes between gp59 (0.75 μM) and random-sequence (M13mp19-derived) εDNA (7.5 μM) in the presence and absence of A-domain (1.0 μM) in 2 ml of Chloride Buffer with no NaCl present. Reactions lacking A-domain contained an equivalent volume of A-domain storage buffer to maintain constant initial salt conditions. Small aliquots of a concentrated NaCl solution were added sequentially to the cuvette. Decreases in εDNA fluorescence were recorded as the protein(s) were progressively dissociated from the εDNA by increasing salt concentration.

Forward titrations of poly(dεA) with gp59 were performed as described (14), in the presence and absence of A-domain. A fixed amount of poly(dεA) (7.5 μM) was incubated in 2 ml of Chloride Buffer + 50 mM NaCl either in the presence of 1.0 μM A-domain or in the absence of A-domain but with an equivalent volume of A-domain storage buffer. Small aliquots of gp59 solution were titrated into the cuvette while monitoring the increase in poly(dεA) fluorescence.

Hydroxy Radical Fingerprinting—gp59, gp32, and gp32-A, either alone or in combination as indicated in the figure, were incubated with the 5-32P-labeled ssDNA 60-mer (see above) for 10 min in R1 at 25°C. In reactions containing two protein species, either gp32 or gp32-A was added to the oligonucleotide first, incubated for 10 min at 25°C, then gp59 was added, and a second 10-min, 25°C incubation ensued. Subsequent to the incubation period(s), the protein-ssDNA complexes were set at 4 nm, and the photomultiplier voltage was kept constant in all experiments. Corrections of the data for dilution, inner-filter effects, were set at 4 nm, and the photomultiplier voltage was kept constant in all experiments. Corrections of the data for dilution, inner-filter effects, protein fluorescence, and/or the fluorescence of free etheno-DNA were made as appropriate to the experiment, using procedures described by Lefebvre and Morrical (14). The corrections performed for each experiment are summarized in the figure legends. No corrections were made for photobleaching effects since the exposure of either εDNA or poly(dεA) to UV light for extended periods resulted in negligible decreases in fluorescence.

RESULTS

Destabilization of gp32-ssDNA Interactions by gp59—Morrical et al. (8) demonstrated that a ssDNA-cellulose affinity resin will simultaneously retain stoichiometric amounts of both gp59 and gp32 proteins. The experiments in Fig. 1 extend this method to examine the salt stabilities of both protein species within the immobilized gp59-gp32-ssDNA complex and the role of gp59-gp32 protein-protein interactions in the formation of the complex. Recently, similar methods were used successfully to analyze protein-ssDNA and protein-protein interactions withinUvsY-gp32-ssDNA complexes (30). By using the batch style method described under “Experimental Procedures,” we examined the salt-dependent retention of the following combinations of proteins by 50-μl mini-beds of ssDNA-cellulose: gp59 alone, gp32 alone, gp32-A alone, gp59 + gp32, gp59 + gp32-A, and gp59 + A-domain. Results are shown in Fig. 1, and Table I contains information on the binding capacities of the ssDNA-cellulose used in these experiments for the gp59, gp32, and gp32-A protein species, alone and in combination. The empirically determined binding capacities of the ssDNA-cellulose resin for gp59, gp32, and gp32-A are similar (Table I, part A), consistent with the approximately equal binding site sizes of the three species on ssDNA (14, 26), and each is capable of saturating at least 95% of the protein-accessible binding sites in the resin (data not shown). The A-domain fragment of gp59 does not bind to ssDNA-cellulose (data not shown) since it lacks the ssDNA binding domain of gp32 (27).

ssDNA-cellulose saturated with gp59 alone was eluted sequentially with buffers containing 0.2, 0.6, and 2.0 M NaCl with the following results (Fig. 1, lanes 1, 7, and 13). Approximately equal amounts of gp59 eluted in both the 0.2 and 0.6 M NaCl fractions, consistent with previous results (5, 9). Identical treatments of ssDNA-cellulose saturated with either gp32 alone (Fig. 1, lanes 2, 8, and 14) or gp32-A alone (Fig. 1, lanes 3, 9, and 15) revealed that both proteins elute exclusively in the 2.0 M NaCl fraction.

As shown in Table I, part B, addition of gp59 to gp32-saturated ssDNA-cellulose resulted in retention of saturating amounts of both proteins. Note that both proteins were retained at close to their normal individual binding capacities on the ssDNA-cellulose resin (Table I, part B), confirming the earlier results and conclusions of Morrical et al. (8) that the two proteins form an integrated, stoichiometric complex on ssDNA. In subsequent salt elution steps, both proteins eluted quantitatively in the 0.6 M NaCl fraction (Fig. 1, lane 10). (The gp32 band in lane 10 appears more intense than that in lane 14 (the experiment with gp32 alone) due to the following two factors:

FIG. 1. ssDNA-cellulose assays for co-retention of gp59 plus gp32 species on single-stranded DNA. Affinity chromatography experiments were carried out batch style as described under “Experimental Procedures.” Proteins were loaded onto the ssDNA-cellulose matrix in DC-100 buffer (100 mM NaCl) and then eluted sequentially with DC-200 buffer (200 mM NaCl; lanes 1–6), DC-600 buffer (600 mM NaCl; lanes 7–12), and DC-2000 buffer (2000 mM NaCl; lanes 13–18). Separate batch reactions contained either gp59 alone (lanes 1, 7, and 13), gp32 alone (lanes 2, 8, and 14), gp32-A alone (lanes 3, 9, and 15), gp59 plus gp32 (lanes 4, 10, and 16), gp59 plus gp32-A (lanes 5, 11, and 17), or gp59 plus A-domain (lanes 6, 12, and 18). See text for details.

Electron Microscopy—gp59, gp32, and gp32-A alone or in combination were incubated with M13mp19 ssDNA in R11 for 10 min at room temperature as indicated in the figure. A final concentration of 1% (v/v) of glutaraldehyde was added to each sample. Incubation continued for 15 min at room temperature followed by gel filtration through a Sepharose CL-2B (Amersham Pharmacia Biotech) column to remove unbound protein. The samples were spread directly onto grids for electron microscopy. After platinum shadowing at an angle of 8° and carbon coating, the samples were examined in a Phillips EM400 electron microscope.
Quantitative binding and recovery data for gp59 and gp32 species chromatographed on ssDNA-cellulose affinity resin

| Protein(s) | Loaded\(^a\) | Bound\(^b\) | Eluted ([NaCl]) | Total protein recovered |
|------------|-------------|-------------|------------------|------------------------|
| A. Determination of binding capacities for gp59, gp32, and gp32-A | | | | |
| gp59       | 1.0         | 0.5         | 0.5 (2.0 \(M\)) | 0.5                    |
| gp32-A     | 1.0         | 0.5         | 0.5 (2.0 \(M\)) | 0.5                    |
| B. Simultaneous retention of gp59 and gp32 | | | | |
| gp32       | 1.0         | 0.5         | ND\(^c\) (0.6 \(M\)) | 1.0\(^d\)            |
| gp59       | 1.0         | 0.5         | ND\(^c\) (0.6 \(M\)) | 1.0\(^d\)            |
| C. Simultaneous retention of gp59 and gp32-A | | | | |
| gp32-A     | 1.0         | 0.5         | ND\(^e\) (0.6 > 0.2 \(M\)) | 0.95\(^f\)          |
| gp59       | 1.0         | 0.45        | ND\(^e\) (0.2 > 0.6 \(M\)) | 0.95\(^f\)          |

\(^a\) Proteins in DC-100 buffer were loaded batch style onto 50-\(\mu\)l bed volumes of ssDNA-cellulose as described under “Experimental Procedures.”
\(^b\) The amount of protein that remained bound to the ssDNA-cellulose after repeated washing with DC-100 buffer was calculated by measuring the difference in protein present in supernatants (as determined by Bradford assay) before and after batch adsorption and centrifugation.
\(^c\) For binding capacity determinations (see A above), bound protein was recovered from ssDNA-cellulose by resuspending the saturated matrix in DC-2000 buffer as described under “Experimental Procedures.”
\(^d\) For experiments involving co-retention of gp59 and gp32, both protein species were co-eluted in DC-600 buffer. ND, indicates not determined, since amounts of individual proteins in the two-protein mixture could not be quantitated directly. Instead, the total amount of both proteins recovered in elution steps with DC-600 buffer was measured by Bradford assay and is listed in the rightmost column. Comparisons with Fig. 1 and with calculations of protein bound to the ssDNA-cellulose, above, indicated that approximately equal amounts of gp59 and gp32 were eluted from the matrix in 0.6 \(M\) NaCl.
\(^e\) For experiments involving co-retention of gp59 and gp32-A, both protein species were eluted in both the DC-200 and DC-600 buffer fractions. ND indicates not determined, since amounts of individual proteins in the two-protein mixture could not be quantitated directly. Instead, the total amounts of both proteins recovered in elution steps with the DC-200 and DC-600 buffers were measured by Bradford assay, added, and the sum is listed in the rightmost column. Comparisons with Fig. 1 and with calculations of protein bound to the ssDNA-cellulose, above, indicated that approximately equal amounts of gp59 and gp32 were eluted from the matrix in the 0.2–0.6 \(M\) NaCl fractions.

The samples were run on different gels, and we always observe less intense Coomassie staining with the high salt fractions. Nevertheless Bradford assays indicate complete protein recovery in both of these fractions (Table I.) Thus, gp59 lowers the salt stability of the gp32-ssDNA complex, resulting in a net destabilization of gp32-ssDNA interactions. At the same time, the salt stability of gp59 on ssDNA-cellulose is increased in the presence of gp32, perhaps due to the strong and highly salt-resistant protein-protein interactions between these two proteins (7, 8).

Is the destabilization of gp32-ssDNA caused by gp59 due to gp59-gp32 protein-protein interactions? To address this question we performed otherwise identical ssDNA-cellulose batch experiments in which gp32-A, which lacks interaction with gp59 (8), replaced full-length gp32. As shown in Table I, part C, addition of gp59 to gp32-A saturated ssDNA-cellulose resulted in retention of saturating amounts of both proteins. A similar result was obtained by employing a gel mobility shift method, in which supershifted complexes of gp59, gp32-A, and 60-mer ssDNA were observed (33). The simplest explanation of these results is that the co-occupation of ssDNA by gp59 and gp32 does not require gp59-gp32 protein-protein interactions. An alternative explanation that binding of gp32-A to ssDNA activates cryptic gp59-(gp32-A) interactions, which are responsible for the retention of gp59, appears to be ruled out by our observation that gp59 produces a strong and nearly identical hydroxy radical protection pattern of ssDNA in the presence versus the absence of gp32-A (see Fig. 4, below).

When the resin containing bound gp59 and gp32-A was eluted with steps of increasing salt, we observed that the presence of gp59 also destabilizes gp32-A, since the latter species now elutes predominantly in the 0.6 \(M\) NaCl fraction (Fig. 1, lane 11). (The intensity of the gp32-A band in lane 11 is greater than that seen in lane 15 (the experiment with gp32-A alone) for the same reasons discussed above for the gp32-containing samples.) Note that the stabilization of gp59 in the complex seen with full-length gp32 is not seen with gp32-A (compare lanes 4 and 10 with 5 and 11). Thus, gp59 elutes from ssDNA-cellulose similarly in the presence and absence of gp32-A, which tends to support the idea raised above that gp59-gp32 protein-protein interactions are responsible for the increased salt stability of gp59 on ssDNA-cellulose in the presence of gp32. Taken together, our results suggest that the gp59 molecules within gp59-(gp32 sp.)-ssDNA complexes are in contact with the ssDNA and that it is these gp59-ssDNA contacts which destabilize the interactions of gp32 species with the ssDNA. Further evidence for gp59-ssDNA contacts within the ternary complexes is presented in a later section.

Destabilization of gp59-ssDNA Interactions by the A-domain Fragment of gp32—The A-domain fragment of gp32 is known to form a very tight and salt-resistant complex with gp59, at least in the absence of ssDNA (8). Could A-domain form a complex with gp59 molecules bound to ssDNA, and could this interaction alter gp59-ssDNA interactions? The results (Fig. 1, lanes 6, 12, and 18) indicate that this is the case. The A-domain fragment, which does not bind to ssDNA-cellulose on its own, is retained quantitatively by the matrix in the presence of saturating gp59, demonstrating the presence of an (A-domain)-gp59-ssDNA complex. Subsequent salt elution steps revealed that both gp59 and A-domain elute exclusively in the 0.2 \(M\) NaCl fraction (Fig. 1, lane 6), whereas gp59 alone is split between the 0.2 and 0.6 \(M\) NaCl fractions (lanes 1 and 7). (Note that the lower staining intensity of gp59 in lane 6 compared with other lanes is most likely due to the slow loss of protein from the ssDNA-cellulose during washing steps, a result of the considerable destabilization of gp59-ssDNA by the A-domain fragment [see also Figs. 2 and 3].) Thus interactions with the A-domain of gp32 impart a significant increase in the salt sensitivity of gp59-ssDNA interactions. A similar result was obtained using a gel mobility shift assay (33). This finding appears contrary to our earlier observation (Fig. 1) that the salt resistance of gp59 on ssDNA-cellulose is enhanced by full-length gp32; however, it may be the case that interactions of gp59 with the A-domain of full-length gp32 weaken gp59-ssDNA interactions, but the weakening is more than compensated for by the stability of gp59-gp32 interactions.
Interactions of gp59 with gp32-ssDNA Complexes

To investigate further the effects of A-domain interactions with gp59 on gp59-ssDNA binding, we employed the etheno-DNA (eDNA) fluorescence enhancement assay used previously to quantitate gp59-ssDNA interactions (14). The A-domain of gp32 cannot bind to eDNA and has no effect on lattice fluorescence (Figs. 2 and 3); thus we were able to add this species to mixtures of gp59 and eDNA while monitoring effects of gp59-eDNA binding exclusively. As shown in Fig. 2, the addition of A-domain to gp59-eDNA causes a significant leftward shift in the salt-back titration profile of the complexes to lower NaCl concentrations, consistent with the A-domain-dependent increase in the salt sensitivity of gp59-ssDNA interactions inferred from the ssDNA-cellulose experiments in Fig. 1. Fig. 2 shows that the midpoint salt concentration for the dissociation of gp59-eDNA is approximately 150 mM NaCl in the absence of A-domain, consistent with previous results (14), whereas the midpoint is lowered to only 100 mM NaCl in the presence of A-domain. Note also that the small region of stoichiometric gp59-eDNA binding (plateau region at lower salt concentrations) seen in the absence of A-domain is abolished in its presence (Fig. 2), consistent with an overall weakening of gp59-eDNA interactions induced by gp59 interactions with the A-domain fragment.

The effects of the A-domain fragment on gp59-ssDNA interactions were also examined in forward titrations of a poly(dA) lattice with gp59 in the presence and absence of A-domain. Results are shown in Fig. 3. In the absence of A-domain, titration of gp59 onto poly(dA) in Chloride Buffer + 50 mM NaCl results in nearly stoichiometric binding with only slight sigmoidicity noticeable in the data (Fig. 3), consistent with previous results (14). Nevertheless, analysis of gp59-poly(dA) binding data at this and slightly higher salt concentrations revealed a very high cooperativity parameter (ω ~ 2500) for the binding reaction (14). When a parallel titration was performed in which gp59 was added incrementally to a solution containing poly(dA) and A-domain in the same buffer, a strikingly different result was obtained as follows: the binding curve was shifted dramatically to the right and exhibited very strong sigmoidicity (Fig. 3). One possible interpretation of these data is that A-domain interactions with gp59 selectively lower the affinity parameter of gp59 for poly(dA) without affecting the high cooperativity parameter. Another possible interpretation is that A-domain competes very strongly with poly(dA) for binding to gp59, so that all of the A-domain in solution (which was present in excess initially) must be titrated out by gp59 before gp59-poly(dA) binding can be detected. However, the latter interpretation would appear to be ruled out by our observations (a) that gp59 produces a large eDNA fluorescence effect in the presence of excess A-domain in the low salt range of Fig. 2, and (b) that both A-domain and gp59 are retained simultaneously on ssDNA-cellulose (Fig. 1).

gp59-ssDNA Contacts in the Presence of gp32 Revealed by Hydroxy Radical Fingerprinting—Results described above suggest that formation of a gp59-gp32-ssDNA complex and destabilization of gp59-ssDNA interactions within this complex require direct gp59-ssDNA contacts. The ability of gp59 to contact the ssDNA within gp59-gp32-ssDNA complexes is supported by the hydroxy radical fingerprinting data shown in Fig. 4. As shown in lanes 7 and 13, saturating levels of either gp32 or gp32-A, respectively, each afford relatively weak protection of a 5'-32P-labeled 60-mer compared with the oligonucleotide-only control reaction shown in lane 19. In contrast, saturating levels of gp59 provide very strong protection of the ssDNA from hydroxy radicals (Fig. 4, lanes 4–6). The gp59 concentration dependence of hydroxy radical protection also shows an interesting pattern (Fig. 4, lanes 2–6). In reactions performed at increasing concentrations of gp59, starting at substoichiometric concentrations equivalent to 50% saturation of ssDNA-binding sites, the data reveal an apparent all-or-none protection pattern consistent with cooperative binding of gp59. As published elsewhere (14), gp59 is capable of extremely high cooperativity (ω ~ 2500) under salt conditions similar to those used in these experiments. A very similar all-or-none protection pattern is observed when variable amounts of gp59 are added to (gp32-A)-saturated 60-mer (Fig. 4, lanes 14–18), indicating that gp59 binds directly and cooperatively to the ssDNA within the gp59-(gp32-A)-ssDNA complex.

The hydroxy radical protection pattern of gp59 on gp32-saturated 60-mer is shown in Fig. 4, lanes 8–12. We observe that gp59 affords additional protection of the ssDNA compared with gp32 alone (lane 7), indicating that gp59 does contact the ssDNA within gp59-gp32-ssDNA complexes. However, the levels of protection observed at saturating gp59 concentration appear weaker than those observed with equivalent concentrations of gp59 alone (compare lanes 4 versus 10). Also note that the cooperativity of gp59 binding is not as evident in the presence of gp32 as it is with gp59 alone or with gp59 + gp32-A. The data suggest that gp59-gp32 protein-protein interactions alter gp59-ssDNA contacts within the gp59-gp32-ssDNA complex even while stabilizing gp59 against salt-induced dissociation from the complex (see Fig. 1). These gp32-dependent alterations of gp59-ssDNA contacts revealed by hydroxy radical protection appear consistent with our observation (Figs. 1–3) that the isolated A-domain fragment of gp32 destabilizes gp59-ssDNA interactions. Whether these alterations result from changes in the affinity or cooperativity parameters of gp59 remains to be determined.

Unique Morphologies of gp59-gp32-ssDNA Versus gp59-(gp32-A)-ssDNA Complexes—The structural morphologies of gp59-ssDNA, gp32-ssDNA, (gp32-A)-ssDNA, gp59-gp32-ssDNA, and gp59-(gp32-A)-ssDNA complexes were compared by electron microscopy (Fig. 5). gp59-ssDNA complexes were observed to form aggregates (not shown); however a significant number of individual M13mp19 ssDNA circles covered with gp59 were resolvable by EM. Examples of the isolated gp59-ssDNA com-

FIG. 2. Salt-back titrations of gp59-eDNA complexes in the presence and absence of the A-domain fragment of gp32. Preformed complexes were titrated with NaCl while monitoring eDNA fluorescence as described under “Experimental Procedures.” Preincubation reaction mixtures contained 7.5 μM eDNA plus either 0.75 μM gp59 (■), 0.75 μM gp59 and 1.0 μM A-domain (●), or no protein (○). Data were corrected for dilution, and the signal due to protein fluorescence was subtracted. Residual gp59 fluorescence was found to be essentially independent of salt concentration, whereas A-domain fluorescence was negligible at these wavelengths under all salt conditions. A-domain alone has no effect on eDNA fluorescence at any salt concentration (data not shown).
plexes are shown in Fig. 5A. The circularity of the underlying ssDNA molecule is evident in these complexes, which appear as relatively smooth and uninterrupted filaments of gp59 bound to the lattice. In fact, the gp59-ssDNA complexes (Fig. 5A) appear quite similar in morphology to the gp32-ssDNA (Fig. 5B) and (gp32-A)-ssDNA (Fig. 5C) complexes. Thus gp59-ssDNA complexes have a morphology consistent with a cooperatively bound protein.

Electron micrographs of gp59-gp32-ssDNA show predominantly individual complexes as opposed to aggregates (Fig. 5D); however their morphology differs dramatically from complexes formed with either gp59 or gp32 as the sole protein component. The underlying circular structure of the ssDNA is not evident in the gp59-gp32-ssDNA complexes; instead, the complexes appear highly condensed and coarsely beaded in structure (Fig. 5D). In control experiments performed on mixtures of gp59 and gp32 lacking ssDNA, no large structures were observable by EM (data not shown). This plus the uniform size of structures
seen in Fig. 5D indicate that the complexes seen in Fig. 5D still contain the M13mp19 ssDNA species. Clearly, the interaction of gp59 with gp32-ssDNA brings about a dramatic structural remodeling of the protein-ssDNA complex.

EM characterization of gp59-(gp32-A)-ssDNA complexes revealed a relatively homogeneous population of completely condensed structures (Fig. 5E). These complexes appear as simple blobs with none of the underlying ssDNA circularity visible and also lack the beaded morphology of the gp59-gp32-ssDNA complexes. Again, control experiments performed on mixtures of gp59 and gp32 lacking ssDNA revealed no similar structures (data not shown), indicating that the blobs seen in Fig. 5E are in fact protein-ssDNA complexes. The difference in morphology between gp59-(gp32-A)-ssDNA and gp59-gp32-ssDNA complexes presumably results from the absence of gp59-gp32 protein-protein interactions in the former and/or from differences in the degree and nature of gp59-ssDNA contacts as revealed in Fig. 4. It is intriguing to speculate that the failure to form a specific beaded structure may account for the inability of gp59 to load the gp41 helicase onto gp32-A covered ssDNA (8).

**DISCUSSION**

Our studies of gp59 interactions with ssDNA in the presence of gp32, gp32-A, and the A-domain fragment of gp32 have led us to the following major conclusions: (i) gp59 is able to co-occupy ssDNA pre-saturated with either gp32 or gp32-A, and thus co-occupation appears to be independent of gp59-gp32 protein-protein interactions; (ii) gp59 destabilizes both gp32-ssDNA and (gp32-A)-ssDNA interactions; (iii) interactions of gp59 with the A-domain of gp32 alter the ssDNA-binding properties of gp59; and (iv) gp59 organizes gp32-ssDNA versus (gp32-A)-ssDNA into morphologically distinct complexes. Our results resolve several outstanding issues on the nature of gp59-gp32-ssDNA complexes and provide some intriguing insights into the probable mechanism of gp59-dependent helicase loading, which we discuss below.

Previous work in our laboratory established the following: (a) a gp59-gp32-ssDNA ternary complex is an obligatory intermediate in the gp59-dependent loading of the gp41 DNA helicase onto gp32-saturated ssDNA, and (b) gp59 contacts with the A-domain of gp32 within the ternary complex are essential for helicase loading (8). Left unresolved was the issue of whether both proteins in the complex make contact with the ssDNA. Our current study provides several arguments that both gp59 and gp32 do in fact contact the ssDNA within the co-occupied complex. These arguments include the observation that gp59’s destabilization of gp32 sp.-ssDNA complexes appears to be independent of protein-protein interactions (Fig. 1), indicating that gp59-ssDNA contacts are most likely responsible for this effect. Similarly, the ability of gp59 to protect the ssDNA from hydroxy radical attack occurs in the presence of either gp32 or gp32-A, again consistent with direct gp59-ssDNA contacts. Furthermore, Morrical et al. (8) observed that proteolytic removal of the N-terminal B-domain of gp32 is blocked within a gp59-gp32-ssDNA complex, consistent with gp32 remaining in its ssDNA-bound state (28). Thus it appears that gp59 and gp32 form a co-integrated complex on ssDNA in which both protein species contact the polynucleotide directly. How are two proteins with similar binding site sizes able to make simultaneous contact with ssDNA without competing for binding sites? One clue comes from the crystal structure of the ssDNA-bind-
ing core domain of gp32 (29). The crystal structure shows that within its binding site spanning 7–10 nucleotides, gp32 makes intimate electrostatic contacts only with roughly half of the occluded ssDNA. The other half appears to be bound loosely, and in fact many surfaces of the bound polynucleotide are exposed to solvent throughout the binding site. Therefore, potentially many opportunities exist for other proteins such as gp59 to contact ssDNA within the gp32-ssDNA complex via interactions with exposed surfaces of the ssDNA. Note that the T4 UvsY protein, involved in the assembly of the UvsX recombinase onto gp32-covered ssDNA, also contacts ssDNA within a UvsY-gp32-ssDNA ternary complex while gp32-ssDNA contacts are maintained (16, 30). Thus the structure of the gp32-ssDNA complex appears to be designed so that other proteins in T4 DNA metabolism, and particularly replication and recombination mediator proteins such as gp59 and UvsY, may have access to the ssDNA.

Our observation that gp59 destabilizes gp32-ssDNA complexes confirms our earlier speculation (7) that the local weakening of gp32-ssDNA binding by gp59 is a prerequisite for helicase loading at that site. Surprisingly, our data indicate that this destabilization occurs independently of gp59-gp32 protein-protein interactions, since the destabilization is also observed with gp32-A, the truncated species of gp32 lacking the domain for interactions with gp59 (8). This result argues that changes in local ssDNA structure induced by gp59-ssDNA interactions are responsible for the destabilization of gp32-ssDNA interactions. This situation appears to mirror that of the T4 UvsY recombinase protein, which also destabilizes gp32-ssDNA in a manner independent of protein-protein interactions (30). In the case of UvsY, wrapping of ssDNA around a UvsY hexamer has been proposed as one mechanism to explain the destabilization of gp32-ssDNA, since wrapping would presumably put strain on interactions between neighboring gp32
DNA metabolism and in many systems. The cooperative binding of gp59 to ssDNA could induce kinks or wraps in the ssDNA similar to UvsY hexamer-ssDNA binding, leading to a similar weakening of gp32 cooperativity. The beaded morphology of gp59-gp32-ssDNA complexes as seen by EM (Fig. 5D) is consistent with significant structural distortions introduced in the ssDNA upon gp59 binding to gp32-ssDNA complexes.

The destabilization of gp59-ssDNA interactions by the A-domain of gp32 (Figs. 1–3) represents an interesting twist in an already complicated story, especially since full-length gp32 (containing the A-domain) appears to stabilize gp59 within the gp59-gp32-ssDNA ternary complex (Fig. 1). One explanation of this apparent inconsistency is that gp59-gp32 protein-protein interactions are responsible for the net stabilization of the association of gp59 with the complex, even while gp59-ssDNA interactions are weakened. Our hydroxy radical protection results (Fig. 4) appear to be consistent with the notion that gp59-ssDNA interactions are altered within the gp59-gp32-ssDNA complex. The model in Fig. 6 accommodates this observation along with the observation that gp32-ssDNA interactions are destabilized by gp59. A key feature of the model is that the A-domain of gp32 acts as a conduit for communication and conformational changes between gp59 and gp32, and may ultimately serve as the lever by which gp32 is dislodged from the ssDNA upon entry of gp41 into the gp59-constructed helicase assembly target site (Fig. 6).

It is clear from our data that specific cross-talk between gp59 and gp32, involving conformational changes in both, is a key feature of the gp41 helicase assembly pathway. Although many biochemical and structural features of this process remain to be clarified, it is likely that continued studies of DNA helicase loading in the T4 system will reveal principles common to many protein-DNA assembly processes that occur throughout the DNA metabolism and in many systems.

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