The Gene 59 Protein of Bacteriophage T4

CHARACTERIZATION OF PROTEIN-PROTEIN INTERACTIONS WITH GENE 32 PROTEIN, THE T4 SINGLE-STRANDED DNA BINDING PROTEIN*

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The gene 59 protein (gp59) of bacteriophage T4 stimulates the activities of gene 41 protein (gp41), the T4 replicative DNA helicase, by promoting the assembly of gp41 onto single-stranded (ss)-DNA molecules that are covered with cooperatively bound gene 32 protein (gp32). This helicase-ssDNA assembly process, which is important for the reconstitution of the primosome component of the T4 DNA replication fork, appears to require both gp59-gp41 and gp59-gp32 protein-protein interactions. In this study we characterize the physical and functional interactions of gp59 with gp32, the T4 ssDNA-binding protein. Experimental results presented herein indicate: 1) that gp59 binds specifically to both free and ssDNA-bound gp32 molecules; and 2) that in both cases binding involves contacts between gp59 and the acidic C-terminal domain of gp32 (the so-called “A-domain”). We further show that single-stranded DNA molecules coated with (gp32-A), a truncated form of gp32 lacking the A-domain, are refractory to gp59-dependent helicase assembly. The data indicate that specific contacts between gp59 molecules and the A-domains of gp32 molecules are essential for gp59-dependent assembly of gp41 onto gp32-ssDNA complexes. Our results are consistent with a model in which gp59 binds to gp32 molecules within the gp32-ssDNA complex and therein forms a target site for helicase-ssDNA assembly.

The gene 59 protein (gp59) is an important component of the coupled DNA replication and recombination systems of bacteriophage T4 (Mosig, 1994; Kreuzer and Morrical, 1994). Mutations in gene 59 are characterized by a DNA arrest phenotype, indicating that the gene product is essential for the late, recombination-dependent pathway of replication initiation in T4 (reviewed in Kreuzer and Morrical (1994)). Although bacteriophage T4 is capable of growing in the absence of functional gp59, phage burst size is reduced, resulting in a small plaque phenotype (Cunningham and Berger, 1977, 1978). While gp59 is not essential for T4 DNA growth and reproduction, T4 59- double mutant strains are non-viable (Doherty and Gauss, 1982; Gauss et al., 1983). The dna gene encodes one of two known phage-encoded DNA helicase enzymes, the other being the product of gene 41. The dna helicase is non-essential for T4 growth, but may be involved in T4's early, origin-dependent pathway of replication initiation based on the DNA delay phenotype of certain dna mutants (Gauss et al., 1994). Conversely, the product of gene 41 is essential for T4 growth, and the gene 41 protein (gp41) is known to be the major replicative DNA helicase of the bacteriophage, as well as an essential component of the phage primosome (Alberts, 1987; Nossal, 1994). The non-viability of the 59 dna double mutants suggests that gp59 may modulate the replication activities of the gp41 helicase in vivo, a hypothesis that is supported strongly by biochemical data (see below). A number of other genetic traits of T4 59 mutants indicate that this protein is intimately involved in homologous recombination and recombinational DNA repair processes in T4, including interchromosomal phage recombination, multiplicity reactivation, post-replication recombinational repair, and double-strand break repair (Mosig, 1994; Kreuzer and Drake, 1994).

Biochemical characterization of gp59 has been facilitated by its cloning and purification (Barry and Alberts, 1994a; Spacipolli and Nossal, 1994; Yonesaki, 1994). Purified gp59 exhibits no enzymatic activities of its own, however, it binds to single-stranded DNA, and it exhibits strong, specific protein-protein interactions with both gp41 and gp32, the T4-encoded ssDNA-binding protein (Barry and Alberts, 1994a; Morrical et al., 1994; Yonesaki, 1994). The interaction with gp32 is particularly strong; elution of gp59 from gp32-agarose affinity columns requires on the order of 2 M NaCl. gp59 appears to be capable of binding simultaneously to both gp32 and gp41, suggesting that this protein may act as an adapter molecule between the helicase and ssDNA-binding protein (Morrical et al., 1994).

Gp59 has been shown to stimulate the activities of the gp41 helicase enzyme in a variety of biochemical assays. Gp59 stimulates the intrinsic and ssDNA-stimulated ATPase and GTPase activities of gp41, by lowering the enzyme's apparent Km values for the nucleotide substrates (Morrical et al., 1994). In strand displacement DNA synthesis reactions, gp59 increases the rate at which nascent DNA replication forks acquire helicase (Barry and Alberts, 1994a, 1994b). The de novo initiation of DNA synthesis on M13 ssDNA circles, requiring the combined activities of the T4 primosome (gp41 helicase + gp61 primase), is stimulated by gp59 (Barry and Alberts, 1994a). The stimulation is most dramatic when the ssDNA is covered with cooperatively bound gp32 molecules, suggesting that an important function of gp59 is to facilitate the loading of the primosome onto gp32-ssDNA complexes. This suggestion is borne out by studies of the effects of gp32 and gp59 on the ssDNA-stimulated ATPase activity of the gp41 helicase:
ssDNA-saturating concentrations of gp32 strongly inhibit gp41’s ssDNA-stimulated ATPase activity, but activity is restored and further stimulated by stoichiometric concentrations (with respect to gp41) of gp59 (Morrical et al., 1994). A similar stimulatory effect of gp59 is observed in DNA unwinding reactions catalyzed by gp41; gp32 inhibits the unwinding of tailed duplex molecules by the helicase, but stoichiometric gp59 (with respect to helicase) restores and further stimulates unwinding activity (Tarumi and Yonesaki, 1995). In addition, gp59 is essential for gp41 helicase and primosome functions in the presence of the T4-encoded homologous recombination proteins, uvsX and uvsY. gp59 is required along with gp41 to promote the branch migration phase of DNA strand exchange reactions initiated by uvsX, uvsY, and gp32 (Salinas and Kodadak, 1995). Furthermore, gp59 is essential for gp41 helicase and primosome activation in an in vitro system for the T4 recombination-dependent DNA synthesis pathway (reviewed in Kreuzer and Morrical (1994)).

Several observations suggest that interactions with gp32 and/or with gp32-ssDNA complexes are of central importance in the biochemical and biological functions of gp59. First, in the presence of the uvsX and uvsY recombination proteins, de novo initiation of DNA synthesis on M13 ssDNA circles by the T4 primosome requires not only gp59, but gp32 as well, suggesting that gp59 targets helicase/primosome assembly to patches of ssDNA-bound gp32 within a gp32-uvsX-uvsY-ssDNA mixed filament. Second, gp59-dependent assembly of gp41 onto gp32-ssDNA complexes, as measured by activation of the helicase’s ssDNA-stimulated nucleotidease activity, is inhibited by excess free gp32, suggesting that gp59 must contact ssDNA-bound gp32 molecules in order to promote helicase-ssDNA assembly (Morrical et al., 1994). Third, in DNA strand-exchange reactions initiated by T4 recombination proteins, gp59/gp41-promoted DNA branch migration is inhibited by (gp32-A), a truncated form of gp32 lacking the C-terminal “A-domain” (Salinas and Kodadak, 1995). The latter observation suggests that specific contacts between gp59 and the C terminus of gp32 are important for function.

In this article, we present a detailed investigation of the nature and importance of the strong protein-protein interactions that occur between gp59 and gp32. Data presented herein demonstrate: 1) that gp59 interacts with gp32 specifically via the C-terminal A-domain of the latter protein, 2) that gp59 co-localizes on ssDNA with gp32, forming gp59-gp32-ssDNA complexes, and 3) that contacts between gp59 molecules and the A-domains of ssDNA-bound gp32 molecules are essential for the gp59-dependent assembly of the gp41 helicase onto gp32-ssDNA complexes. Our findings clearly establish the critical importance of gp59-gp32 physical and functional interactions in the T4 DNA replication and recombination systems.

MATERIALS AND METHODS

Reagents and Resins—Ribonucleoside triphosphates were purchased from Pharmacia Biotech Inc. Other chemicals, biochemicals, and commercial enzymes were purchased from Sigma, unless specifically noted in the text. Affi-Gel-10 and Affi-Gel-15 activated agarose beads were purchased from Bio-Rad. Single-stranded DNA cellulose affinity resin was synthesized according to the procedure of Alberts and Herrick (1971). Circular single-stranded DNA from bacteriophage M13mp19 was isolated from purified phage particles according to published procedures (Yamamoto et al., 1970; Miller, 1987). The concentration of ssDNA in stock solutions was determined by the absorbance at 260 nm, using an extinction coefficient of 36 μg/mlA260. All solution concentrations of ssDNA are expressed in units of μg/ml nucleotide residues.

Bacteriophage T4 Prodrins—Purification and storage conditions for the T4 gp32 (34 kDa), gp41 (58 kDa), and gp59 (26 kDa) proteins were as described by Morrical et al. (1994). gp32 storage buffer contained 20 mM Tris-HCl, pH 8.1, 0.5 mM dithiothreitol, 1 mM EDTA, 100 mM NaCl, and 20% (w/v) glycerol. The gp41 storage buffer contained 20 mM Tris-HCl, pH 8.1, 1 mM 2-mercaptoethanol, 1 mM EDTA, 3 mM magnesium chloride, 67 mM NaCl, and 62.5% (w/v) glycerol. Purified (gp32-A) protein (28 kDa, gp32 amino acids 1–253) was a generous gift from Dr. David Giedroc. (gp32-A) was stored at −80°C in (gp32-A) storage buffer (10 mM Tris-HCl, pH 8.1, 100 mM NaCl, 50% (w/v) glycerol). A protein fragment containing the A-domain of gp32 (9 kDa, gp32 amino acids 213–301) was purified from Escherichia coli strain BL21(DE3)/pMM1, which overexpresses the protein fragment, according to the procedure of Hurley et al. (1993). The purified A-domain polypeptide was stored at −20°C in gp32 storage buffer. All T4 protein stock solutions used in this study were judged to be nuclease free by these criteria.

Protein Affinity Chromatography—Affinity columns containing immobilized gp59 or gp32, and control columns containing immobilized BSA, were constructed by covalently coupling the proteins to Bio-Rad Affi-Gel-10 activated agarose beads (Formosa et al., 1983). A similar procedure was used to immobilize the purified A-domain of gp32 on Bio-Rad Affi-Gel-15 beads (Hurley et al., 1993). All protein affinity chromatography experiments were conducted at a temperature of 4°C. Columns (1.5–2.0 ml bed volume, each containing 0.5–1.0 mg of immobilized protein/ml of bed volume) were equilibrated in column buffer CB-50 (20 mM Tris-HCl, pH 8.1, 1 mM EDTA, 1 mM 2-mercaptoethanol, 5 mM magnesium chloride, 10% (w/v) glycerol, and 50 mM NaCl). Protein samples to be loaded on affinity columns were dialyzed into CB-50 buffer and applied to columns at a flow rate of 2.0 ml/h. Subsequent to sample loading, the columns were washed with 5 ml of CB-50 buffer, then eluted sequentially with 4-ml steps of buffers CB-200, CB-600, and CB-2000 (numerals representing NaCl concentrations in millimoles/liter; all other components identical to CB-50 buffer). Washing and elution steps were performed at a flow rate of 2.75 ml/h, and 0.5-ml fractions were collected, except where otherwise noted. Column fractions were analyzed for protein content by Bradford assays and by analyses of Coomassie Blue-stained SDS-polyacrylamide gels. The NaCl concentrations of various column fractions were determined by measuring their relative conductivities on a VWR Scientific Model 604 conductivity meter. ssDNA-Cellulose Affinity Experiments—(This was used to detect co-localization of gp32 and gp59 on immobilized ssDNA molecules.) All experiments were carried out at 25°C unless otherwise noted. Each experiment, 100 μl (bed volume) of pre-swollen single-stranded DNA cellulose resin was placed in a 1.5-ml Eppendorf microcentrifuge tube, and equilibrated in Reaction Buffer RB (20 mM Tris acetate, pH 7.4, 80 mM potassium acetate, 10 mM magnesium acetate). All equilibration, protein addition, washing, and elution steps were carried out batch style. Each batch step involved the addition of a 1.0-ml volume of solution to the 100 μl of resin. The suspension was then spun for 5 min while the tube was slowly rotated. The suspension was then spun for 3 min at 2000 rpm in a microcentrifuge, after which the supernatant was removed. This procedure was repeated for each subsequent batch step.

The ssDNA-cellulose used in these experiments contained 1.0 mg of immobilized, denatured calf thymus DNA/ml of bed volume, as determined by the recovery of DNA from boiled samples (Alberts and Herrick, 1971). The protein-binding capacity of the resin was determined independently for the gp32 and gp59 proteins. This procedure involved repeated batch style washings of the resin (100 μl bed volume) with saturating amounts of gp32 in RB, followed by two RB washing steps to remove unbound protein, followed finally by elution of the bound gp32 with RB + 2.0 M NaCl. The amount of gp32 present in the high-salt supernatant was quantitated by Bradford assay, and used to calculate the binding capacity. An identical procedure was followed with the gp59 protein. Using this method, we determined that the ssDNA-cellulose used in these studies had approximately equal binding capacities for
gp32 and gp59; the resin retained approximately 10 mg of either protein per milliliter of bed volume at saturation (see also Table I, "Results").

Partial Trypsinysis—Partial trypsinolysis reactions were carried out according to a modification of the procedure of Williams and Konigsberg (1978). Limited trypsin digests of gp32, (gp32-A), and/or gp59 proteins were performed in 25-μl reaction volumes at 20°C. Reaction mixtures contained Reaction Buffer RB, plus the following concentrations of macromolecules: 0 or 500 μg/ml gp32; 0 or 390 μg/ml (gp32-A); 0 or 760 μg/ml gp59; 0 or 190 μg/ml (nucleotides) M13mp19 ssDNA. Proteins were added to a solution containing buffer = ssDNA, and the mixtures were preincubated for 5 min at 20°C. Constant salt conditions were maintained between reactions through the addition of appropriate amounts of gp32, (gp32-A), and gp59 storage buffers. Trypsin (Sigma type XI-I-S) was added to a final weight ratio of 1 μg of trypsin/25 μg of total protein, and the reaction mixtures were incubated for 10 min at 20°C. Five-microliter aliquots were removed at various times; each sample was immediately added to 2 μl of concentrated stopping buffer solution (300 mM Tris-Cl, pH 6.8, 600 mM β-mercaptoethanol, 0.6% bromphenol blue, 12% SDS, 60% glycerol, 35 mM benzamidine-HCl, 3.5 mM phenylmethylsulfonyl fluoride) and placed in a boiling water bath for 5 min. Each stopped reaction mixture was then electrophoresed on a 15% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue R-250.

ATPase Assays—Coupled spectrophotometric assays for gp41-catalyzed ATP hydrolysis were carried out as described by Morrical et al. (1994). All measurements were made on a Hitachi U-2000 spectrophotometer. A constant cuvette temperature of 37 ± 0.5°C was maintained during all reactions. Reaction mixtures contained (final concentrations): 20 mM Tris-acetate, pH 7.4, 80 mM potassium acetate, 10 mM magnesium acetate, 6 units/ml pyruvate kinase, 6 units/ml lactate dehydrogenase, 2.3 mM phosphoenolpyruvate, 0.46 mM NADH, 2.5 mM ATP, and 5 μg/ml gp41, plus variable concentrations of M13mp19 ssDNA, gp59, gp32, and (gp32-A). Constant salt conditions were maintained between reactions through the addition of appropriate amounts of gp32, (gp32-A), and gp59 storage buffers. Reactions were initiated by the addition of gp41 to preincubated mixtures containing all other reaction components. Preincubations lasted 5 min or longer to insure temperature equilibration. ATPase reaction velocities were determined from the time-dependent changes in NADH absorbance at 380 nm, using an extinction coefficient of ε380 = 1.3 mM⁻¹ cm⁻¹ for NADH (Morrical et al., 1994). The background rate in each cuvette was determined prior to the addition of gp41. The background rate was negligible for all of the assays reported in this study.

RESULTS

gp59-Agarose Affinity Chromatography Experiments—A gp59-agarose affinity column was constructed and run as described under "Materials and Methods." Fig. 1, A-C, shows results of affinity chromatography experiments performed with this gp59-agarose column, in which either native gp32, gp32 A-domain, or (gp32-A) was loaded onto the column. Results are also summarized in Table I. As shown in the top panel, native gp32 bound quantitatively to the gp59-agarose column in buffer containing 0.05 M NaCl (Fig. 1A). The column was washed successively with buffers containing 0.2, 0.6, and 2.0 M NaCl. gp32 was observed to elute in the 0.2 and 0.6 M NaCl steps (Fig. 1A). gp32 did not bind to a BSA-agarose control column (data not shown; results summarized in Table I). These results confirm the result of Yonesaki (1994), who showed that gp32 is retained specifically by a gp59-agarose column, and serve as a positive control for the gp32 binding activity of the immobilized gp59 contained in the affinity column used in our studies.

Fig. 1B shows results of an affinity chromatography experiment in which the purified A-domain-containing fragment of gp32 (henceforth called A-domain) was loaded onto the gp59-agarose column (the same column used above). The majority (approximately three-fourths) of the A-domain bound to the column, while the remainder appeared in the 0.05 M NaCl flow-through fractions. The bound A-domain eluted entirely in

![Image](331x440 to 548x736)

Fig. 1. Protein affinity chromatography of gp32 and derivatives on gp59-agarose. The gp59-agarose column (2.0-ml bed volume, containing 2 mg of immobilized gp59) was prepared and run as described under "Materials and Methods." The same column was used for each of the experiments described in panels A-C. A, chromatography of a gp32 sample on gp59-agarose. 300 μg of gp32 in CB-50 buffer was loaded onto the column while collecting 0.5-ml fractions. The column was washed successively with buffers containing 0.05, 0.2, 0.6, and 2.0 M NaCl as described under "Materials and Methods." The breakthrough fractions for each salt step were determined by conductivity. For each salt step, 10-μl samples from the first 4–5 fractions after the breakthrough fraction were electrophoresed on a 15% SDS-polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue R-250 and photographed. Lane 1, gp32 and gp59 markers. Lanes 2–6, 0.05 M NaCl flow-through (FT) fractions. Lanes 7–11, 0.2 M NaCl fractions. Lanes 12–16, 0.6 M NaCl fractions. Lanes 17–20, 2.0 M NaCl fractions. B, chromatography of gp32 A-domain on gp59-agarose. The procedure was identical to that in panel A, except that 200 μg of A-domain was loaded onto the column. Lane 1, gp59 and A-domain markers. Lanes 2–6, 0.05 M NaCl flow-through (FT) fractions. Lanes 7–11, 0.2 M NaCl fractions. Lanes 12–15, 0.6 M NaCl fractions. Lanes 16–19, 2.0 M NaCl fractions. C, chromatography of (gp32-A) on gp59-agarose. The procedure was identical to that in panel A, except that 300 μg of (gp32-A) was loaded onto the column. Lane 1, gp59 and (gp32-A) markers. Lanes 2–6, 0.05 M NaCl flow-through (FT) fractions. Lanes 7–11, 0.2 M NaCl fractions. Lanes 12–16, 0.6 M NaCl fractions. Lanes 17–20, 2.0 M NaCl fractions.

the 0.2 M NaCl fractions (Fig. 1B). The A-domain protein did not bind to a BSA-agarose control column (data not shown; results summarized in Table I). Therefore the A-domain of gp32 by itself is capable of binding specifically to gp59, albeit with some loss of apparent affinity with respect to native gp32 (as evidenced by its elution at slightly lower [NaCl] with respect to native gp32).

Fig. 1C shows results of an affinity chromatography experiment in which (gp32-A), a truncated form of gp32 lacking the to facilitate visualization of this small fragment by Coomassie Blue staining. The unbound A-domain in flow-through fractions was later loaded onto the same gp59-agarose column after re-equilibration, and it all bound to the column (S. Morrical, unpublished results). Thus, the presence of some A-domain in the original flow-through fractions was due to overloading, not to partial inactivity of the A-domain preparation.

The column was overloaded with A-domain (a 2.6-fold molar excess relative to the amount of native gp32 applied to the column in Fig. 1A).
A-domain, was loaded onto the same gp59-agarose column. In this experiment, virtually all of the (gp32-A) passed through the column in the 0.05 M NaCl-containing column buffer (Fig. 1C). A trace amount of (gp32-A), barely visible on the gel photograph in Fig. 1C, lanes 8 and 9, appeared in the 0.2 M NaCl fractions. No protein was detected in either the 0.6 or 2.0 M NaCl fractions. (gp32-A) did not bind to a BSA-agarose control column (data not shown; results summarized in Table I). These results, combined with the data from the experiment in Fig. 1B, suggest that the A-domain of gp32 is essential for the normal, strong protein-protein interactions between gp32 and gp59. However, there may be some residual, very weak interactions between gp59 and the (gp32-A) fragment, as indicated by the trace amounts of (gp32-A) in the 0.2 M NaCl fractions.

A-Domain-Agarose Affinity Chromatography Experiments—To further test the ability of gp59 to recognize and bind the A-domain of gp32, we constructed an A-domain-agarose affinity column, following procedures described by Hurley et al. (1993). As a positive control for the binding activity of the immobilized A-domain molecules, we tested the ability of the A-domain-agarose column to retain gp43, the T4 DNA polymerase. Hurley et al. (1993) showed that gp43 bound specifically to an A-domain-agarose column that they prepared. gp43 bound to our A-domain-agarose column (but not to BSA-agarose) in 0.05 M NaCl, and was eluted by a 0.2 M NaCl wash step (data not shown; results summarized in Table I), a result identical to that of Hurley et al. (1993). Therefore the immobilized A-domain present in our affinity column appeared to be active. We also performed a positive control experiment for the gp32 binding activity of our gp59 protein preparation: the gp59 bound to a gp32-agarose column quantitatively in 0.05 M NaCl-containing buffer, and eluted in the early fractions of the 2.0 M NaCl step (data not shown; results summarized in Table I). gp59 did not bind to a BSA-agarose control column. These results are identical to those obtained in previous studies (Morical et al., 1994; Barry and Alberts, 1994a).

When gp59 from the same stock was applied to the A-domain-agarose column described above, it also bound to the column quantitatively. gp59 eluted from the column in the 2.0 M NaCl fractions, however, in this experiment gp59 eluted from the column only after extensive washing of the column with the 2.0 M NaCl-containing buffer (Fig. 2; results also summarized in Table I). This behavior, indicative of very strong and salt-resistant protein-protein interactions, has sometimes been observed during chromatography of gp59 on gp32-agarose columns (Barry and Alberts, 1994a). The results obtained here contrast in degree with those of the reciprocal experiment (Fig. 1B), where A-domain was observed to elute from gp59-agarose at only 0.2 M NaCl. However, these data strongly support our conclusion, above, that gp59 interacts specifically with the A-domain of gp32. The extremely salt-resistant interaction of gp59 with A-domain-agarose, coupled with the relatively low concentration of gp59 in T4-infected E. coli, may account for the failure of Hurley et al. (1993) to detect gp59 when radiolabeled T4 cell lysates were applied to a similar affinity column.

Partial Proteolysis Experiments—gp32 is organized into three distinct domains, which are separable via limited trypsinolysis (reviewed in Williams et al. (1994)). One trypsin-sensitive site separates the “B-domain” (N-terminal, involved in cooperativity of ssDNA-binding) and “core-domain” (containing ssDNA-binding site and bound Zn\(^{2+}\) atom) of gp32. This site is (relatively) protected from trypsinolysis in the ssDNA-bound form of gp32. A second trypsin-sensitive site appears between

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**TABLE I**

Summary of protein affinity chromatography results

| Protein loaded | A-domain-agarose | gp32-agarose | gp59-agarose | BSA-agarose |
|----------------|------------------|--------------|--------------|-------------|
| A-domain      | ND\(^a\)         | ND           | 0.2 M        | 0.05 M FT\(^b\) |
| (gp32-A)      | ND               | ND           | 0.2–0.6 M    | 0.05 M FT    |
| gp32          | ND               | ND           | 2.0 M        | 0.05 M FT    |
| gp59          | 2.0 M            | 2.0 M        | ND           | 0.05 M FT    |
| gp43          | 0.2 M            | ND           | ND           | 0.05 M FT    |

\(^a\) ND, not determined.

\(^b\) 0.05 M FT denotes proteins that appeared only in the flow-through fractions of a particular column (e.g., did not bind to the column in loading buffer containing 0.05 M NaCl).

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**FIG. 2.** Protein affinity chromatography of gp59 on A-domain-agarose. The A-domain-agarose column (1.5-ml bed volume, containing 1 mg of immobilized A-domain) was prepared and run as described under “Materials and Methods.” 200 μg of gp59 in CB-50 buffer was loaded onto the column, and the column was washed successively with buffers containing 0.05, 0.2, 0.6, and 2.0 M NaCl while collecting 0.25-ml fractions. gp59 eluted only after extensive washing of the column with the 2.0 M NaCl-containing buffer, as determined both by Bradford protein assays and by electrophoretic analyses. The data in this figure represent 10 μl samples of alternating fractions from the late portion of the 2.0 M NaCl wash, which were electrophoresed on a 15% SDS-polyacrylamide gel, silver stained, and photographed. Lane 1, gp59 marker. Lanes 2–16, alternating fractions eluted from 10 to 17 ml elution volume with buffer CB-2000, containing 2.0 M NaCl.

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4 The elution of gp59 from gp32-agarose columns also occurs at a higher salt concentration than in the reciprocal experiment (gp32 on gp59-agarose; see Table I), a difference that we have consistently observed while working with several generations of gp59- and gp32-agarose columns (S. Morrical, unpublished results). Other published results are consistent with this observed difference in column performance, e.g., Yonesaki (1994) observed gp32 elution from gp59-agarose at only 0.2 M NaCl. The biochemical basis of the difference is unknown, but could conceivably result from partial occlusion of the gp32-binding site of gp59 caused by the covalent linkage to agarose beads.
and absence of ssDNA. The nearly identical extents of gp59-dependent protease protection seen in the presence and absence of ssDNA suggests that gp59 localizes preferentially with gp32-ssDNA as opposed to naked ssDNA; an observation consistent with the greater salt-stability of gp59-gp32 versus gp59-ssDNA interactions (S. Lefebvre and S. Morrical, unpublished data).

The core-domain and the A-domain (C-terminal, acidic, and where gp59 appears to bind). The latter site becomes relatively hypersensitive to trypsin when gp52 is in the ssDNA-bound state. Since our affinity chromatography experiments pointed to the A-domain of gp32 as the target site for gp59 binding, we reasoned that gp59 might protect the adjacent site from trypsinolysis. Thus we used limited trypsinolysis of gp52 as a probe for the interactions of gp59 with gp52 and with gp52-ssDNA complexes in solution.

Fig. 3A shows results of partial trypsin digests performed on gp52 in the presence and absence of gp59 and in the presence and absence of ssDNA. Fig. 3B shows similar experiments in which purified (gp32-A) (already lacking the A-domain) was treated with trypsin in the presence and absence of gp59 and/or ssDNA. In all experiments, a constant weight ratio of 1/25 trypsin/total protein was used. Note that gp59 was not degraded significantly by trypsin under these conditions in any experiment (Fig. 3A, A and B).

Over the 10-min time courses of the reactions shown, both gp32 (Fig. 3A, left) and (gp32-A) (Fig. 3B, left) undergo extensive proteolysis to the core fragment in the absence of ssDNA and gp59. Generation of the 27-kDa core fragment involves the proteolytic removal both of an A-domain fragment (~6 kDa) and of a B-domain fragment (~1 kDa) from the N and C termini, respectively, of the 34-kDa native gp32 molecule. These two small fragments run off of the gel and cannot be visualized under our experimental conditions. In the case of gp32 trypsinolysis, we observe (gp32-A) generated as an intermediate, with subsequent conversion to core fragment (Fig. 3A, left). Little if any accumulation of gp32-B (lacking only B-domain, predicted to run slightly faster than native gp32) is observed; therefore A-domain cleavage appears to occur more rapidly than B-domain cleavage. This order of cleavage is consistent with previous studies (Moise and Hosada, 1976; Williams and Konigsberg, 1978). With (gp32-A) as the starting material (Fig. 3B, left), core fragment is produced at a similar rate. In both reactions, the (gp32-A) to core conversion appears to occur in two steps, since a discrete band of intermediate size is observed (Figs. 3A, A and B, left). This result differs somewhat from earlier studies (Moise and Hosada, 1976; Williams and Konigsberg, 1978); however, the fact that the same behavior is observed in both the gp32 and (gp32-A) reactions indicates that the two-step reaction is a property either of the trypsin preparation used or of reaction conditions, and is not due to anomalous behavior of our purified gp32 or (gp32-A) stocks.

When gp59 is added to gp32 in the absence of ssDNA (Fig. 3A, middle left), proteolytic cleavage of the A-domain is inhibited, as evidenced by the remaining presence of full-length gp32 in the 5- and 10-min time points, and by the concomitant decrease in the rate of appearance of (gp32-A) and/or core fragments (compare with gp32-only reaction; Fig. 3A, left). gp59 does not appear to inhibit cleavage of the B-domain, since most of the gp32 molecules that are degraded go all the way to core fragment (Fig. 3A, middle left, 10-min time point). (However, it is difficult to determine the precise rate of B-domain cleavage, since the 5-min sample was overloaded.) When gp59 is added to (gp32-A) in the absence of ssDNA (Fig. 3B, middle left), there is no significant effect on the rate of core fragment appearance (compare with (gp32-A)-only reaction; Fig. 3B, left). These results demonstrate that gp59 does protect gp32 from proteolytic cleavage, and indicate that this protection occurs preferentially at the site adjacent to the A-domain of gp32.

gp59-dependent protection of gp32 from trypsinolysis is also evident in the presence of ssDNA (Fig. 3A). The addition of gp59 to gp32-ssDNA complexes (Fig. 3A, right) strongly retards the rate of gp32 to (gp32-A) conversion compared to the equivalent reaction in the absence of gp59 (Fig. 3A, middle right). Note that the formation of gp32 core fragment is completely suppressed in these reactions, indicating that all of the gp32 is bound cooperatively to ssDNA with its B-domain inaccessible to protease (Williams et al., 1994). Our results are therefore consistent with a mode of protease protection in which gp59 interacts with the A-domain of ssDNA-bound gp32, and thereby interferes with cleavage of the A-domain. In equivalent experiments with (gp32-A)-ssDNA complexes (Fig. 3B, right and middle right), the (gp32-A) is completely resistant to trypsinolysis over a 10-min time course, both in the presence and absence of gp59. These results demonstrate that (gp32-A) also interacts cooperatively with ssDNA in the reaction mixtures, resulting in B-domain protection consistent with previous studies (Williams et al., 1994), gp59 does not alter the accessibility of (gp32-A) to protease in any way that is detectable by this method. Presumably gp59 does not interact with ssDNA-bound (gp32-A) molecules. Taken together, the data in Fig. 3 argue persuasively for an interaction of gp59 with gp32-ssDNA complexes that involves a specific interaction between gp59 and the A-domain of ssDNA-bound gp32.

Interactions of gp59 with immobilized gp32-ssDNA Complexes—The ability of gp59 to interact with gp32-ssDNA complexes was further demonstrated by the experiments shown in Fig. 4 and Table II. Here, gp32-ssDNA complexes were immo-
Fig. 4. Simultaneous retention of gp32 and gp59 on ssDNA-cellulose. Affinity assays were carried out batch style as described under “Materials and Methods.” Quantitative protein recovery data is given in Table II. Lane 1-2, gp59 and gp32 markers, respectively. Lane 3, 10-μl sample of Reaction Buffer (RB) supernatant after addition of 0.75 mg of gp59 in 1 ml of RB to 100 μl of gp32-saturated ssDNA cellulose. Lane 4, 10-μl sample of high-salt wash, showing proteins recovered in the supernatant after resuspending the immobilized gp59-gp32-ssDNA in 1 ml of RB + 2.0 M NaCl buffer. See text for details.

The simplest interpretation of the data presented in Fig. 4 and Table II is that gp59 is capable of co-occupying ssDNA that is already saturated with gp32, forming a stoichiometric gp59-gp32-ssDNA complex. This interpretation is entirely consistent with results of protease protection experiments (above) and gp41 ATPase experiments (below). However, an alternative explanation of the data is also possible: that gp59 merely binds to portions of the ssDNA that are inaccessible to gp32 within the ssDNA-cellulose matrix. This trivial explanation is rendered unlikely by the following observations. 8) Saturating DNase I treatment of the ssDNA-cellulose batch used in these experiments releases 195 nmol of nucleotide residues per 100 μl of resin. The amount of gp32 retained by the same bed volume of ssDNA-cellulose is 1.0 mg (Table II), which is enough to bind 200 nmol of nucleotide residues (assuming a binding site size of n = 7), an amount that is virtually identical to the DNase I-accessible sites. Thus gp32 appears capable of saturating all of the protein-accessible ssDNA in the matrix. Realistically then, the only way to accommodate stoichiometric quantities of gp59 and gp32 simultaneously on the matrix is via the formation of some type of defined gp59-gp32-ssDNA complex.

As a further test for gp59-gp32-ssDNA complex formation, we performed gel mobility shift assays (data not shown). M13mp19 ssDNA circles which were saturated with gp32 entered a 0.4% agarose gel, but had greatly reduced electrophoretic mobility with respect to naked ssDNA. In contrast, we observed that ssDNA circles which were saturated with gp59 did not enter a 0.4% agarose gel. Furthermore, when saturating, equimolar amounts of both gp32 and gp59 were added to ssDNA circles simultaneously, the DNA did not enter the gel. These results suggested that gp59 interacts with gp32-ssDNA complexes, consistent with results of the proteolysis and ssDNA-cellulose affinity experiments described above. However, the gel shift results were not very informative in and of themselves, since we were unable to observe a unique gel-shift band for gp59-gp32 ssDNA complexes, and therefore could not confirm by this method that both proteins were present on the ssDNA. The proteolysis and ssDNA-cellulose experiments were more informative in this regard. Yonesaki (1994) previously demonstrated that gp59 aggregates ssDNA oligonucleotides both in the presence and absence of gp32; our gel-shift results involving longer ssDNA molecules appear to be consistent with his observations.

gp59-A-Domain Contacts are Essential for Helicase Assembly onto gp32-ssDNA Complexes—To assess the functional role of gp59-A-domain contacts in the process of gp59-dependent assembly of the gp41 DNA helicase onto gp32-ssDNA complexes, we performed a series of experiments examining the ssDNA-stimulated ATPase activity of gp41 as a function of the gp32, (gp32-A), and gp59 proteins. Results are shown in Fig. 5. In the absence of either gp32 or (gp32-A), gp41 has a strong ssDNA-stimulated ATPase activity that is stimulated 20–25% by gp59 under the conditions of these experiments (see points on vertical axis, Fig. 5), a result consistent with previously published data (Morrical et al., 1994). In the absence of gp59, adding either native gp32 (open circles) or (gp32-A) (black circles) to the reactions causes a precipitous decrease in the observed ATPase activity of the helicase (Fig. 5). The residual ATPase activity observed at ssDNA-saturating concentrations of gp32 and (gp32-A) (saturation point denoted by vertical dashed line, assuming a binding site size of 7 nucleotide residues for both gp32 and (gp32-A); Newport et al. (1981), Kowalczykowski et al. (1981), Lonberg et al. (1981) is equal to the ssDNA-independent ATPase activity of the helicase (Fig. 5). The residual ATPase activity observed at ssDNA-saturating concentrations of gp32 and (gp32-A) (saturation point denoted by vertical dashed line, assuming a binding site size of 7 nucleotide residues for both gp32 and (gp32-A); Newport et al. (1981), Kowalczykowski et al. (1981), Lonberg et al. (1981) is equal to the ssDNA-independent ATPase activity of the helicase (Fig. 5). The residual ATPase activity observed at ssDNA-saturating concentrations of gp32 and (gp32-A) (saturation point denoted by vertical dashed line, assuming a binding site size of 7 nucleotide residues for both gp32 and (gp32-A); Newport et al. (1981), Kowalczykowski et al. (1981), Lonberg et al. (1981) is equal to the ssDNA-independent ATPase activity of the helicase (Fig. 5). The residual ATPase activity observed at ssDNA-saturating concentrations of gp32 and (gp32-A) (saturation point denoted by vertical dashed line, assuming a binding site size of 7 nucleotide residues for both gp32 and (gp32-A); Newport et al. (1981), Kowalczykowski et al. (1981), Lonberg et al. (1981) is equal to the ssDNA-independent ATPase activity of the helicase (Fig. 5). The residual ATPase activity observed at ssDNA-saturating concentrations of gp32 and (gp32-A) (saturation point denoted by vertical dashed line, assuming a binding site size of 7 nucleotide residues for both gp32 and (gp32-A); Newport et al. (1981), Kowalczykowski et al. (1981), Lonberg et al. (1981) is equal to the ssDNA-independent ATPase activity of the helicase (Fig. 5). The residual ATPase activity observed at ssDNA-saturating concentrations of gp32 and (gp32-A) (saturation point denoted by vertical dashed line, assume...
Protein contained approximately equimolar amounts of gp32 and gp59 as estimated from the SDS-PAGE gel shown in Fig. 4. In the saturation point for (gp32-A)-ssDNA complex formation only at (gp32-A) concentrations that are substantially below (gp32-A), however. Under these conditions, the addition of gp59 covered ssDNA molecules (Morrical et al., 1994). A different result is seen when the ssDNA in the reaction is bound by (gp32-A), however. Under these conditions, the addition of gp59 leads to significant stimulation of gp41's ATPase activity, but only at (gp32-A) concentrations that are substantially below the saturation point for (gp32-A)-ssDNA complex formation (Fig. 5, black squares). The stimulatory effect of gp59 is lost completely as the (gp32-A) concentration approaches the saturation point with respect to ssDNA binding sites (vertical dashed line). The residual ATPase activity seen at slightly subsaturating to saturating [(gp32-A)] is equal to the ssDNA-independent ATPase activity of gp41 (horizontal dashed line), which was determined independently in this experiment, and which is independent of both (gp32-A) and (gp32-A) (data not shown). These results demonstrate clearly that gp59 is unable to promote the assembly of the gp41 helicase enzyme onto ssDNA molecules that are covered with bound (gp32-A). Therefore, contacts between gp59 and the A-domain of gp32 appear to be essential for this “assembly factor” function of gp59.

The results obtained in Fig. 5 raised several interesting questions. Primarily, can gp59 promote helicase assembly onto ssDNA that is covered with a mixture of (gp32-A) and gp32? If so, is the ssDNA-minimum density of ssDNA-bound gp32 molecules in the complex that is required for gp59-promoted helicase assembly? To address these questions, we performed the gp41 ATPase experiments described in Fig. 6, wherein the mole fraction of (gp32-A) in a mixture of gp32 and (gp32-A) was varied. The total concentration of gp32 species [(gp32)_{total} + (gp32-A)_{total}] was constant, and was in 2-fold excess with respect to ssDNA binding sites, gp59 was present in all reactions. In Fig. 6, the black squares denote ATPase activities observed at various mole fractions of (gp32-A), where the mole fraction is expressed as a function of the total (ssDNA-bound + unbound) concentration of both gp32 species. The horizontal dashed line in Fig. 6 represents the ssDNA-independent ATPase activity of gp41, which was equal to the rate seen in all reactions if gp59 was omitted (data not shown). The ssDNA-stimulated ATPase activity of gp41 decreases steadily with increasing (gp32-A) mole fraction (Fig. 6, black squares), and becomes indistinguishable from ssDNA-independent ATPase activity at (gp32-A) mole fractions of 0.84 and higher.

The observation (Fig. 6) that gp59 stimulates helicase ATPase activity at relatively high mole fractions of (gp32-A) strongly suggests that gp59 is capable of promoting helicase assembly onto ssDNA molecules that are partially covered with (gp32-A), and partially with native gp32. To estimate the minimum density of ssDNA-bound gp32 required for gp59-promoted helicase assembly, we calculated the mole fraction of ssDNA-bound (gp32-A) as a function of the total concentration of ssDNA-bound (gp32-A) and gp32. The data were re-plotted experiment. This result is consistent with previously published observations that gp59 promotes gp41 assembly onto gp32-covered ssDNA molecules (Morrical et al., 1994). A different result is seen when the ssDNA in the reaction is bound by (gp32-A), however. Under these conditions, the addition of gp59 leads to significant stimulation of gp41’s ATPase activity, but only at (gp32-A) concentrations that are substantially below the saturation point for (gp32-A)-ssDNA complex formation (Fig. 5, black squares). The stimulatory effect of gp59 is lost completely as the (gp32-A) concentration approaches the saturation point with respect to ssDNA binding sites (vertical dashed line). The residual ATPase activity seen at slightly subsaturating to saturating [(gp32-A)] is equal to the ssDNA-independent ATPase activity of gp41 (horizontal dashed line), which was determined independently in this experiment, and which is independent of both (gp32-A) and (gp32-A) (data not shown). These results demonstrate clearly that gp59 is unable to promote the assembly of the gp41 helicase enzyme onto ssDNA molecules that are covered with bound (gp32-A). Therefore, contacts between gp59 and the A-domain of gp32 appear to be essential for this “assembly factor” function of gp59.

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as a function of the ssDNA-bound mole fraction of (gp32-A), and are shown in Fig. 6 (open circles). The calculated data indicate that ATPase activity becomes indistinguishable from ssDNA-independent ATPase activity only at ssDNA-bound (gp32-A) mole fractions of 0.91 or higher. This would correspond to 9% of ssDNA-independent ATPase activity of gp41, determined independently. Data points represent reactions containing 5 μg/ml gp41, 5 μg/ml gp59, 10 μM M13mp19 ssDNA, 2.5 mM ATP, and variable concentrations of gp32 and (gp32-A). The combined concentration of (gp32-A) and gp32 was held constant at 2.90 μM in each reaction mixture. The concentrations of other reaction components are described under "Materials and Methods." Black squares (■) represent ATPase velocities plotted versus the total mole fraction of (gp32-A), e.g., [(gp32-A)]total, [(gp32)-ssDNA]/[(gp32-A)-ssDNA]. Open circles (○) represent ATPase velocities plotted versus the ssDNA-bound mole fraction of (gp32-A), e.g., [(gp32-A)-ssDNA]/[(gp32)-ssDNA]. The ssDNA-bound mole fractions were calculated assuming a 3-fold higher association constant for (gp32-A)-ssDNA than for gp32-ssDNA, and assuming equal cooperativity and binding site size parameters for (gp32-A) and gp32.

FIG. 6. ssDNA-stimulated ATPase activity of gp41 as a function of (gp32-A)/gp32 ratio. Velocities of ATP hydrolysis (determined by coupled assay) are plotted versus the mole fraction of (gp32-A) in a mixture of gp32 and (gp32-A). The horizontal dashed line represents the ssDNA-independent ATPase activity of gp41, determined independently. Data points represent reactions containing 5 μg/ml gp41, 5 μg/ml gp59, 10 μM M13mp19 ssDNA, 2.5 mM ATP, and variable concentrations of gp32 and (gp32-A). The combined concentration of (gp32-A) and gp32 was held constant at 2.90 μM in each reaction mixture. The concentrations of other reaction components are described under "Materials and Methods." Black squares (■) represent ATPase velocities plotted versus the total mole fraction of (gp32-A), e.g., [(gp32-A)]total, [(gp32)-ssDNA]/[(gp32-A)-ssDNA]. Open circles (○) represent ATPase velocities plotted versus the ssDNA-bound mole fraction of (gp32-A), e.g., [(gp32-A)-ssDNA]/[(gp32)-ssDNA]. The ssDNA-bound mole fractions were calculated assuming a 3-fold higher association constant for (gp32-A)-ssDNA than for gp32-ssDNA, and assuming equal cooperativity and binding site size parameters for (gp32-A) and gp32.

**DISCUSSION**

Our studies of gp59-gp32 protein-protein interactions have led us to the following major conclusions: 1) gp59 binds to gp32 predominantly via contacts with the acidic, C-terminal A-domain of gp32; 2) gp59 binds to both free and ssDNA-bound gp32 molecules via gp59-A-domain contacts; 3) gp59 interacts with gp32-ssDNA to form gp59-gp32-ssDNA complexes that may contain stoichiometric amounts of both proteins; and 4) the interactions of gp59 molecules with the A-domains of ssDNA-bound gp32 molecules are essential for gp59-dependent helicase-ssDNA assembly reactions. The evidence for, and implications of, these conclusions are summarized below.

Our primary conclusion is that gp59-gp32 protein-protein interactions involve specific contacts between gp59 and the C-terminal A-domain of gp32. This conclusion is based on direct affinity measurements as well as on indirect biochemical assays of gp59 and gp32 structure and function. The affinity evidence is summarized in Table I, and results of specific affinity chromatography experiments are documented in Figs. 1 and 2. The data demonstrate that removal of the A-domain from gp32 virtually abolishes its ability to interact with gp59 that has been immobilized via covalent linkage to agarose beads (Fig. 1). Under identical experimental conditions, both native gp32 and the isolated A-domain of gp32 retain the ability to bind to gp59-agarose (Fig. 1). In the reciprocal experiment, gp59 binds very strongly to an affinity column containing immobilized gp32 A-domain (Fig. 2). Taken together, our affinity chromatography data argue convincingly for specific gp59-gp32 interactions occurring predominantly via the A-domain of gp32. This argument is further buttressed by partial trypsinolysis data, which show that proteolytic removal of the A-domain from native gp32 is inhibited strongly and specifically in the presence of gp59 (Fig. 3). A model for gp59-gp32 protein-protein interactions is presented in Fig. 7A.

The truncated forms of gp32 used in this study, (gp32-A) (amino acids 1–253) and A-domain (amino acids 213–301), share a common, overlapping sequence of 40 amino acids (residues 213–253). The affinity chromatography results described above suggest that these 40 residues do not play a major role in gp59-gp32 protein-protein interactions, and that most of the gp59-ssDNA binding energy involves contacts between gp59 and portions of the C-terminal 49-amino acid sequence of gp32. This segment carries a large negative charge (−13 over the final 42 residues), and is believed to function as a hinged domain that swings out upon gp32-ssDNA binding to allow interactions with other T4 proteins (Williams and Konigsberg, 1978; Kowalczykowski et al., 1981). The results of our studies add gp59 to a growing list of T4 proteins that are either known or inferred to interact with gp32 via contacts with its acidic C-terminal region. This list includes several T4-encoded DNA replication and recombination proteins, most notably gp43 (DNA polymerase), gp61 (primase), dda (DNA helicase), uvsX (strand transferase), and uvsY (recombination accessory protein) (Hurley et al., 1993; Jiang et al., 1993).

Our results also lead us to conclude that gp59 interacts with ssDNA-bound gp32 molecules, and that once again this interaction occurs via gp59-A-domain contacts. Previous studies of gp59-dependent helicase and primosome activation have inferred that direct contacts between gp59 and ssDNA-bound gp32 molecules are important for gp59 function (Barry and Alberts, 1994a; Morrical et al., 1994; Salinas and Kodadek, 1995; see Introduction). In this study we have provided both direct and indirect biochemical evidence for the interaction of gp59 with gp32-ssDNA. Partial trypsinolysis reveals that gp59
specifically protects the A-domain-proximal cleavage site of gp32 both in the presence and absence of ssDNA (Fig. 3). The "+ssDNA" trypsinolysis experiments were conducted at saturating [ssDNA] with respect to gp32, and at salt concentrations that give rise to tight, stoichiometric binding of gp32 to ssDNA. The gp32 in these experiments, in addition to exhibiting gp59-dependent protection of A-domain cleavage, also exhibited ssDNA-dependent protection of B-domain cleavage that is characteristic of gp32-ssDNA complexes (Williams and Konigsberg, 1978). Therefore there is little doubt that the proteolysis protection pattern observed in the presence of gp59 and ssDNA (Fig. 3) is a result of specific gp59 interactions with the A-domains of ssDNA-bound gp32 molecules. The ability of gp59 to interact with gp32-ssDNA complexes is further demonstrated by the results of ssDNA-cellulose affinity experiments (Fig. 4, Table II), in which gp59 was observed to interact stoichiometrically with immobilized gp32-ssDNA complexes. An intriguing feature of this data is that gp59 appears to bind to gp32-ssDNA complexes without displacing gp32 from the ssDNA (Fig. 4). Presumably contacts with gp32's A-domain, which is believed to be oriented away from the ssDNA-binding site in the core-domain (and therefore relatively distant from the ssDNA itself) (Williams et al., 1994), allows gp59 to localize with the gp32-ssDNA complex without disrupting gp32-ssDNA interactions.

However, it is possible that gp59-gp32 interactions alter the ssDNA-binding properties of gp32 without disrupting gp32-ssDNA interactions. Results of the gp41 ATPase experiments presented in this study provide a compelling case for the functional importance of gp59-gp32 protein-protein interactions. Once again the data point out the central role of the gp32 A-domain in making functional as well as physical contacts with gp59, and the importance of gp59 interactions with ssDNA-bound gp32 molecules is highlighted. The data in Figs. 5 and 6 indicate that ssDNA molecules which are saturated with (gp32-A) are completely refractory to helicase assembly, even in the presence of excess gp59. In contrast, gp59 readily assembles gp41 onto gp32-saturated ssDNA molecules (Fig. 5; Morrical et al., 1994). In the presence of (gp32-A), gp59 only promotes helicase-ssDNA assembly when the (gp32-A) concentration is substantially subsaturating with respect to the ssDNA (Fig. 5), suggesting that gp59 is able to promote helicase binding to regions of ssDNA that are free of (gp32-A). This result appears to be consistent with observations that gp59 markedly stimulates the loading of gp41 onto short linear ssDNA molecules (Barry and Alberts, 1994a). gp59 is also capable of promoting helicase assembly when sufficient numbers of native gp32 molecules are incorporated into a (gp32-A)-ssDNA complex (Fig. 6), indicating that gp59 specifically recognizes the A-domain of gp32 as a target site for helicase-ssDNA assembly.

The model presented in Fig. 7B outlines the importance of gp59-A-domain contacts in the formation of gp59-gp32-ssDNA complexes, and the central role that these complexes play in helicase-ssDNA assembly. We propose that gp59 directs the assembly of the gp41 helicase onto gp32-ssDNA complexes by binding to ssDNA-bound gp32 and forming a localized target for helicase binding (Fig. 7B). As proposed elsewhere (Morrical et al., 1994), gp59 may act as a molecular adapter and recruit gp41 subunits onto the protein-ssDNA matrix. The A-domain of gp32 appears to be an essential recognition site for assembly of the gp59-gp32-ssDNA intermediate; therefore gp59 cannot localize on (gp32-A)-ssDNA complexes, and cannot promote helicase assembly within regions of ssDNA that are bound by (gp32-A) (Fig. 7B). This principle may explain the observation that both gp59 and native gp32 are required to activate the gp41 helicase in the presence of T4 recombination proteins uvsX and uvsY (Salinas and Kodadek, 1995). Presumably, gp59-gp32 interactions serve to target helicase assembly to regions of gp32-ssDNA (and away from uvsX-uvsY-ssDNA presynaptic filaments). This strategy makes sense in light of

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10 Y. J. Ma and S. W. Morrical, unpublished data.
the mechanism of recombination-initiated DNA synthesis in bacteriophage T4 (Kreuzer and Morrical, 1994), wherein gp41 must be assembled on the lagging strand template in order to reconstitute a functional replication fork. Invading primer strands would be covered with uvsX/uvsY and therefore refractory to gp59/gp41, whereas the lagging strand template would be rapidly coated with gp32 as it is produced during strand displacement DNA synthesis, effectively reconstituting (via gp32-A domains) target sites for gp59-directed helicase assembly.

The fact that greater than ~100 molecules of gp32 must be incorporated into a (gp32A)-ssDNA complex on average before gp59-dependent helicase-ssDNA assembly is detected (Fig. 6 and related discussion) suggests a potentially interesting feature of gp59 function: that gp59 may require a patch of contiguously bound gp32 molecules to reconstitute a helicase assembly site. This possibility arises from a statistical consideration of the arrangement of native gp32 and (gp32A) molecules within a combined gp32-(gp32A)-ssDNA complex. For a gp32-(gp32A)-ssDNA complex containing 0.09 mole fraction of gp32-ssDNA (the highest mole fraction of gp32-ssDNA at which no ssDNA-stimulated helicase activity is detected; Fig. 6), the probability of any one site on the ssDNA being occupied by gp32 as opposed to (gp32A) is 0.09; the probability of 2 contiguous gp32 molecules in the complex is 0.0081; that of 3 contiguous gp32s is 0.000729, etc. (assuming equal site size and cooperativity parameters, and random incorporation). This would give, on average, ~90 gp32 monomers per M13mp19 ssDNA circle, ~8 gp32 dimers, ~0.8 gp32 trimers, etc. If a single native gp32 molecule surrounded by (gp32A) is a sufficient target site, then gp59 should readily assemble helicase onto gp32-(gp32A)-ssDNA complexes containing on average 90 gp32s per ssDNA circle. Since this is not the case, it appears that gp59 must inhibit the helicase activity (Salinas and Kodadek, 1995). They suggested that this residual activity may be due to the ability of gp59 to interact partially with (gp32A).

Although we have detected some very weak interactions between gp59 and (gp32A) (see above), our ATPase data argue against this interpretation. We suggest that a more likely possibility is the following. The generation of branched DNA structures present in the recombination in vitro system may create some limited opportunities for gp59 to promote helicase-ssDNA assembly by a mechanism that does not strictly depend on interactions between gp59 and gp32’s A-domain.

The enzymes of bacteriophage T4 DNA metabolism encounter single-stranded DNA as it exists within a complex of tightly and cooperatively bound gp32. The assembly of enzymes such as the gp41 helicase onto gp32-ssDNA complexes must be coordinated both spatially and temporally in order to guarantee the proper assembly of the T4 DNA replication fork, as well as the integration of DNA synthesis with elements of the phase DNA recombination and repair machineries. It is evident that gp59 has evolved to play a highly specialized role in assembling gp41 onto gp32-covered ssDNA, and that specific protein-protein interactions between gp59 and both gp32 and gp41 are extremely important in this assembly process. Through further biochemical and molecular dissection, we hope to gain a detailed understanding of the nucleoprotein structures formed by gp59 and its ligands, and of their roles in T4 DNA synthesis, recombination, and repair.

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