Site-directed Mutations of T4 Helicase Loading Protein (gp59) Reveal Multiple Modes of DNA Polymerase Inhibition and the Mechanism of Unlocking by gp41 Helicase*

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The T4 helicase loading protein (gp59) interacts with a multitude of DNA replication proteins. In an effort to determine the functional consequences of these protein-protein interactions, point mutations were introduced into the gp59 protein. Mutations were chosen based on the available crystal structure and focused on hydrophobic residues with a high degree of solvent accessibility. Characterization of the mutant proteins revealed a single mutation, Y122A, which is defective in polymerase binding and has weakened affinity for the helicase. The interaction between single-stranded DNA-binding protein and Y122A is unaffected, as is the affinity of Y122A for DNA substrates. When standard concentrations of helicase are employed, Y122A is unable to productively load the helicase onto forked DNA substrates. As a result of the loss of polymerase binding, Y122A cannot inhibit the polymerase during nucleotide idling or prevent it from removing the primer strand of a D-loop. However, Y122A is capable of inhibiting strand displacement synthesis by polymerase. The retention of strand displacement inhibition by Y122A, even in the absence of a gp59-polymerase interaction, indicates that there are two modes of polymerase inhibition by gp59. Inhibition of the polymerase activity only requires gp59 to bind to the replication fork, whereas inhibition of the exonuclease activity requires an interaction between the polymerase and gp59. The inability of Y122A to interact with both the polymerase and the helicase suggests a mechanism for polymerase unlocking by the helicase based on a direct competition between the helicase and polymerase for an overlapping binding site on gp59.

The T4 replisome is considered to be a model for the more complex replication systems (1, 2). The eight protein replisome can be divided into three modules, the leading strand holoenzyme, the lagging strand holoenzyme, and the primosome (3). The leading and lagging strand holoenzymes are formed by the polymerase (gp43)8 and the polymerase clamp (gp45) with the aid of the clamp loader protein (gp44/62). Contained within gp43 polymerase are two distinct active sites. One is the polymerization site that catalyzes the 5’ to 3’ incorporation of deoxynucleotides (dNTPs) into the growing primer strand, and the other is the exonuclease site that is responsible for the removal of dNMPs in the 3’ to 5’ direction (4). gp45 is bound to the polymerase and encircles the DNA duplex, thereby increasing its processivity during DNA synthesis (5, 6). gp44/62 aids in the assembly of the polymerase-clamp complex by positioning the clamp at the 3’-OH of the primer-template junction and acting as a chaperone for the interaction between gp45 and gp43 (7, 8). The primosome is made up of the helicase (gp41), primase (gp61), single-stranded DNA-binding protein (gp32), and the helicase loading protein (gp59) (9). gp41 is a hexameric protein that travels along the ssDNA in a 5’ to 3’ manner using the energy of ATP hydrolysis to unwind duplex DNA (10, 11). gp61 is also hexameric and is responsible for synthesis of pentaribonucleotides on the lagging strand template that are used as primers for the lagging strand polymerase (12, 13). gp32 coats the ssDNA that is produced by the helicase and is thought to aid in the coupling of leading and lagging strand replication (14, 15). gp59 is responsible for the recruitment and proper loading of gp41 at the replication fork and appears to be involved in coordinating the assembly of the replisome (16–18).

The assembly of the T4 replisome at a replication fork has been the subject of extensive investigation. Functional and physical interactions have been described for all components of the replisome (2, 9, 19, 20). The polymerase physically interacts with gp32, which has important consequences for the properties of gp43, such as higher affinity for the primer-template junction and increased processivity during DNA synthesis (21). The polymerase is in turn attached to the clamp protein through a well described interaction between the C terminus of the polymerase and the subunit interfaces of the trimeric clamp protein (5, 6, 22). Based on functional evidence, an additional interaction between gp41 helicase and the polymerase has been proposed (23, 24).

gp59 is a central component of the primosome, making protein-protein contacts with all other primosomal proteins. Protein cross-linking experiments have detected molecular contacts between gp59, gp61, gp41, and gp32 proteins (25–27). Single molecule and ensemble fluorescence energy transfer experiments (FRET) have confirmed the cross-linking results for gp59-gp32 and gp59-gp41 interactions (9). Recently, we have characterized a protein-protein interaction between gp59 and the gp43 polymerase that inhibits the polymerase and exonuclease activities of gp43. Cross-linking and FRET studies indicate that a complex between these two proteins is formed at the replication fork prior to the initiation of DNA replication (9, 16, 27). With computational methods, a molecular model of the complex was generated that rationalizes the inhibition of the polymerase activities by gp59 where a protein segment of gp59 blocks partitioning of the DNA substrate between the polymerase and exonuclease sites of the enzyme (17). Unlocking of the polymerase is achieved through the loading of gp41 at the replication fork in the presence of ATP (28). In addition to the many protein-protein interactions involving gp59, it also binds to several types of DNA substrates, possessing the highest affinity for forked DNA.

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4 The abbreviations used are: gp, gene product; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; WT, wild type; OG, OR green; CPM, 7-diethylamino-3-(4-maleimidophenyl)-4-methylcoumarin; Y122A, Tyr122 → Ala gp59 mutant; FRET, fluorescence resonance energy transfer.
structures (29). A molecular model has been proposed for the interaction between gp59 and a forked DNA substrate (30). As predicted by this model, a site-directed mutation at residue Ile^{87} renders gp59 defective in regard to the binding of forked DNA (31).

In vivo, gp59 is indispensable for the loading of gp41 helicase onto a D-loop, which is formed through the action of UvsX/Y-catalyzed strand invasion of ssDNA into a homologous dsDNA template (32). T4 phage employs two strategies for the initiation of replication (33). At the early stages of replication, origin-dependent initiation dominates, using stably bound RNA transcripts called R-loops to serve as the primer and scaffold for replisome assembly (33). Subsequently, in the middle and late stages of the T4 infection, R-loop-dependent initiation ceases and the D-loop-dependent mode takes over (33).

Here we present the functional effects of site-directed mutation of several possible “hot spots” of protein-protein interactions involving the helicase loader protein gp59 (Fig. 1). Of the four mutants generated, a single mutation, Tyr^{122} to alanine (Y122A), has drastically altered the properties of the protein. The previously proposed gp59-gp43 interaction model places residue Tyr^{122} near the interface of these two proteins. Y122A could no longer load gp41 helicase onto DNA and failed to support coupled leading and lagging strand synthesis unless extremely high concentrations of gp41 are used. Moreover, FRET-based assays indicated that Y122A does not form a specific protein-protein contact with DNA polymerase. As a consequence of this deficiency, Y122A no longer inhibits the exounucleolytic activity of the polymerase but still retains the ability to inhibit the polymerization activity of the polymerase, revealing two distinct modes of polymerase inhibition by gp59. We propose a further refined model that features a competition between gp41 and gp43 for an overlapping binding site on gp59 that rationalizes the loss of both the helicase loading and polymerase inhibition activities upon mutation of Tyr^{122}.

**FIGURE 1. Location of site-directed mutations on the gp59 helicase loader protein.**

The backbone of gp59 protein is shown as a ribbon. The sites of mutation are indicated as labeled. The illustration was generated using the Protein Data Bank coordinates 1C1K using DeepView software.

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**MATERIALS AND METHODS**

[^{32}P]dCTP was purchased from PerkinElmer Life Sciences. Unlabeled ribonucleotides were purchased from Roche Applied Science. Both 7-diethylamino-3-(4’maleimidophenyl)-4-methylcoumarin (CPM) and Oregon Green 488 carboxylic acid, succinimidyl ester, 5-isomer (OG) were obtained from Molecular Probes (Eugene, OR). Bacteriophage T4 replication proteins gp41, gp61, gp43, gp44/62, gp45, and gp32 were purified as described (12, 24). Bacteriophage T4 recombination proteins were overexpressed in *Escherichia coli* using the pet28b (Stratagene) expression vectors and purified as described (34). The pGEM vector was from Promega.

Mutagenesis, Expression, and Purification of Wild-type and gp59 Mutants—Site-directed mutants were introduced into the gp59-intein fusion pet-IMPACT vector (12). Mutations were made in gp59 using the Quikchange mutagenesis method (Stratagene), and the entire gp59 open reading frame was sequenced using the dideoxy terminator method. The sequences of the mutagenic primers were as follows with the boldface underlined letters indicating the mutation site (reverse primers are the reverse complement of the forward): F111A, 5’-GGAGGCTTAAAGCAAATTAAAAGGTTCAAGT1TTGGTTTGAAGAGATATCCGC; Y217A, 5’-GAAACTCTGGAATCTTGGCAAGCTTGCCTTTTGCCAAAGGTTGAC; Y146A, 5’-ATCCAAAGGTCTTCAACGTTATTTTTAAAACCTTCTGCAACATG; and Y122A, 5’-GATATTGCAACATTATTGCTTTTAGTATAAAAAGTTGAAGTTTC.

Wild-type and gp59 mutants were transformed into BL21(DE3) cells and grown in 10 ml of Luria broth overnight at 37 °C. The overnight cultures were diluted 100-fold into 1 liter of LB and grown at 37 °C to an *A*_{600} of 0.8. The cultures were then allowed to cool to 18 °C, and protein expression was induced with 0.1 mM isopropyl-β-D-galactopyranoside. After 16 h of shaking, cells were collected by centrifugation at 6,000 × g and resuspended in 10 ml of chitin column binding buffer containing 1/5 of a protease inhibitor pellet (Roche Applied Science). Cells were lysed using sonication, and cell debris was pelleted at 25,000 × g. Cell-free extract was loaded onto 1 ml of chitin (New England Biolabs) columns and washed with 50 column volumes of chitin binding buffer. The chitin resin was resuspended in binding buffer plus 75 mM β-mercaptoethanol and incubated overnight at 4 °C to facilitate intein-mediated cleavage. Following overnight cleavage, full-length gp59 was eluted in binding buffer, dialyzed into storage buffer, and analyzed for purity using SDS-PAGE. Protein concentrations were determined by measuring the absorbance at 280 nm using an extinction coefficient of 37,800 M^{-1} cm^{-1}.

**DNA Constructs**—Single-stranded M13 phage DNA (ssM13) was purified from infected XL1-Blue cells using the QiagenTM Qiaprep Spin miniprep kit according to the manufacturer’s instructions. Briefly, ssM13 DNA (0.5 mg/ml) was incubated with 2 μl chloroacetaldehyde in 20 mM potassium phosphate (pH 5.5) for 8 h. The reaction was then dialyzed against 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA at 4 °C for 16 h and transferred to a 55 °C oven for an additional 8 h to allow for maturation of the derivatized bases. The integrity of the modified DNA (eM13) was confirmed by Tris acetate-EDTA (TAE)-agarose electrophoresis. The absorbance maximum and minimum were identical to published values (36), and the concentration of the eM13 was calculated using an extinction coefficient of 16,268 M^{-1} cm^{-1}.

The 3540-bp nicked substrate used for rolling circle replication (pGEM_nick) was made by specifically nicking a modified pGEM vector at position 1353 with the enzyme N.BbvC I (New England Biolabs).
The nicking site was introduced through Quickchange™ mutagenesis of a pGEM vector containing a 530-bp insertion of phage T4 DNA corresponding to positions 114,754–115,284 of the T4 genome. The sequence of the forward mutagenic primer (reverse primer is the reverse complement of the forward) was 5'-GCT TAA CTA GTG AGG CAC CTA CGT TCT GTC TAT TCC GGT, where the boldface underlined letter indicates the mutation site, and ^ indicates the nicking position. The nicking reaction was carried for 2 h at 37 °C in buffer 4 (New England Biolabs) containing 10 μg of the pGEM_nick plasmid and 20 units of N.BbvC1B. Following digestion, the reaction was heated to 70 °C for 20 min to completely denature the nicking enzyme. The substrate was used without further treatment. The efficiency of the nicking reaction was monitored with 1% TAE-agarose electrophoresis. As a control, pGEM vector without the nick site was treated in an identical fashion, and no change in mobility was observed using TAE electrophoresis.

The sequences of the oligonucleotide substrates used in the unwinding assays were as follows: fork lead, 5'-CAT GCA GGA CAG TCG GAT CGC AGT CAG ATT TAC TCT GTG ATC TAG TAC GTA TCC AG; fork lag, 5'-TAA GGT ATT CAA GAT ACC TCG TAC TCT GTA CTG ACT GCG ATC CGA CTG TCC TGC ATG ATG; and trap, 5'-CTG ACT GCC ATC CGA CTG TCC TGC ATG. The unwinding fork was made by mixing fork lead and fork lag DNA in equal molar amounts.

The 80-base oligonucleotide (ss80mer) used for the D-loop experiments is homologous to the coding strand of phage M13 between positions 6240 and 6320. PCR was used to amplify a 1500-bp region of M13 DNA, 50 nM trap ssDNA, 400 nM gp32, 5 mM ATP, 150 nM gp41, and 150 nM gp59 (monomorphic concentrations). The reaction was carried out at 37 °C, and various aliquots were removed at the time points indicated and quenched with an equal volume of 250 mM EDTA, 0.2% SDS, and loading buffer (50% glycerol, 1 μg/ml bromophenol blue, 1 μg/ml xylene cyanol FF). Reaction products were separated by 10% PAGE in TBE buffer and analyzed using a PhosphorImager.

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**Helicase Unwinding Assay**—The helicase unwinding assays were performed in the standard replication buffer (25 mM Tris acetate (pH 7.8), 125 mM KOAc, and 10 mM Mg(OAc)₂) with 10 nM unwinding fork DNA, 50 nM trap ssDNA, 400 nM gp32, 5 mM ATP, 150 nM gp41, and 150 nM gp59 (monomorphic concentrations). The reaction was carried out at 37 °C, and various aliquots were removed at the time points indicated and quenched with an equal volume of 250 mM EDTA, 0.2% SDS, and loading buffer (50% glycerol, 1 μg/ml bromophenol blue, 1 μg/ml xylene cyanol FF). Reaction products were separated by 10% PAGE in TBE buffer and analyzed using a PhosphorImager.

**Labeling gp43 and gp59 with Fluorophores**—Labeling of WT and mutant gp59s (F111A, Y122A, Y146A, and Y217A) with CPM was carried out essentially as described previously (17). Briefly, after dialyzing against the labeling buffer (20 mM Tris-HCl (pH 7.3), 150 mM NaCl, 10% glycerol), WT and gp59 mutants were labeled with a 4-fold excess of CPM dye, and the labeling reactions were allowed to proceed in dark for 4 h at 4 °C. Excess dye was removed by buffer exchange with a Centricron (Millipore) until no free dye was detectable in the filtrate. The protein concentration and the amount of dye were measured by the absorbance at 280 and 384 nm, respectively. The labeling efficiency ([dye]/[protein]) was generally between 60 and 70%. The labeled proteins were frozen in aliquots and stored at −70 °C. The procedure for labeling the N terminus of gp43 with Oregon Green 488 maleimide (OG) is as described previously (17).

**Steady-state FRET Experiments**—Steady-state FRET experiments were carried out on an ISA FluoroMax-2 spectrophotometer at 25 °C. gp59-CPM (600 nM) was mixed with gp43-OG (400 nM) in the presence of a forked 32/64/73-mer DNA substrate (100 nM) constructed as described previously (17). An excitation wavelength of 390 nm was used. Donor (gp59-CPM) quenching and acceptor (gp43-OG) sensitization due to FRET were observed over a wavelength range between 430 and 600 nm. Slit widths were adjusted between 1 and 3 nm to keep the spectrum on scale. FRET signals between gp43 and WT/mutant gp59s were measured.

Three reactions were performed for each gp43/gp59 pair. In the first reaction, CPM-labeled gp59 was mixed with OG-labeled gp43 to measure the energy transfer between the donor and the acceptor. In the second reaction, unlabeled gp59 was mixed with OG-labeled gp43 to measure the background signal of acceptor sensitization under the experimental conditions. This spectrum was then subtracted from the one obtained in the first reaction to correct the FRET signal. In the third reaction, CPM-labeled gp59 was mixed with unlabeled gp43 to measure the amount of donor quenching by gp53 and was subtracted from the first reaction.
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mined by using Equation 1, with the fluorescence signal being replaced with counts/min.

Rolling Circle Replication—The rolling circle replication reactions were performed in the standard replication buffer containing 5 nM pGEM nick, 200 nM gp43, 200 nM 44/62, 200 nM gp45, 400 nM gp41, 400 nM gp61, 100 nM gp59 (WT or mutant as indicated), 4 μM gp32, 100 μM each of CTP, GTP, and UTP, 2 mM ATP, 100 μM dNTPs, and 10 μCi of [α-32P]dCTP, in a reaction volume of 45 μL. The reactions were quenched at the time points indicated with an equal volume of 50 mM EDTA. Titration of WT-gp59 and Y122A was performed in a total volume of 10 μL, and the reaction was quenched after 10 min. IC50 values were determined by using Equation 1, with the fluorescence signal being replaced with counts/min. In experiments where the concentration of gp41 was varied, a total reaction volume of 5 μL was used, and reactions were quenched after 7 min. The DNA products were analyzed through 0.8% alkaline-agarose gel electrophoresis (30 mM NaOH and 5 mM EDTA) for 48 h.

D-loop Initiated Replication—For the polymerase initiation reactions, the 5'-32P-labeled ss80mer (10 nM) was preincubated with 1.5 μM UvsX and 0.25 μM UvsY for 10 min at 37 °C prior to mixing with a 3-fold molar excess of dsM13 (30 nM) in the presence of 2 mM ATP, 50 μM dNTPs, 10 μM creatine phosphate, and 5 units/ml of creatine kinase followed by the immediate addition of the replisomal proteins at the following concentrations: 200 nM gp43 exo-, gp44/62, and gp45; 400 nM gp32; and 100 nM wild-type or Y122A gp59 mutant. Aliquots were removed at the indicated time points, quenched with 500 mM EDTA, and separated on 0.8% alkaline-agarose gels for 24 h. For the exonuclease inhibition reactions, the 5'-32P-labeled ss80mer (10 nM) was preincubated with 1.5 μM UvsX and 0.25 μM UvsY for 10 min at 37 °C prior to mixing with a 3-fold molar excess of supercoiled dsM13 (30 nM) in the presence of 2 mM ATP, 50 μM dNTPs, 10 μM creatine phosphate, and 5 units/ml of creatine kinase. D-loop formation was allowed to occur for 20 min before the addition of the replisomal proteins (same as above except for replacement of gp43 exo- with WT-gp43). Aliquots were removed at the indicated time points, quenched with 500 mM EDTA, and separated on 6% denaturing PAGE for 2.5 h.

RESULTS

DNA Binding Experiments—Because we are mainly concerned with gp59 protein-protein interactions that occur while bound to DNA, it is essential that the mutant proteins have the same DNA binding properties as the wild-type enzyme. eM13 DNA has been extensively used to characterize the binding properties of DNA-binding proteins, including T4 phage replication proteins such as gp59 and UvsX (37, 38). The binding of a protein to etheno-modified DNA is known to produce a 2.2–2.5-fold increase in fluorescence intensity when monitored at 405 nM (35). Under conditions of low salt ionic strength, WT and all four gp59 mutants bind to eM13 and cause a 2.2–2.5-fold increase in fluorescence (Table 1). All mutants display a similar resistance to NaCl as compared with the wild-type protein (IC50 values for NaCl vary by <1.5-fold), indicating that the mutants are not altered in their affinity for ssDNA. There is a moderate amount of cooperativity in the dissociation of WT-gp59 and mutant gp59s from eDNA by NaCl (Hill coefficients from 1.4 to 2.1). Ionic strength-dependent changes in cooperativity of gp59 binding to eDNA have been observed previously and are thought to reflect a transition between two different binding modes of gp59 on ssDNA (36).

![Figure 2](https://example.com/figure2.png)

**FIGURE 2. Effect of gp59 mutants on helicase activity unwinding assay.** Fraction remaining duplex was calculated by dividing the amount of radioactivity in the duplex band by the total amount of radioactivity contained in the duplex and ssDNA bands. The time courses shown are in the presence of WT-gp59 (open circles), F111A (open squares), Y122A (filled diamonds), Y146A (filled squares), Y217A (filled circles), or in the absence of gp59 protein (filled triangles).

Unwinding Assays—A simple helicase unwinding assay (39) was employed to measure the stimulatory effect of wild-type and gp59 mutants on gp41 helicase activity (Fig. 2). Cross-linking and FRET studies have shown that this effect is mediated through a direct contact between the two proteins at the replication fork (26). As shown, under conditions of our assay, gp41 helicase itself has an extremely slow on-rate with only 10% of duplex unwound in 1 min. Inclusion of WT-gp59 increases the binding rate of gp41 by ~6-fold (60% of duplex unwound in 1 min). F111A and Y217A increase the unwinding rate by an extent similar to that of the WT-gp59. Y146A stimulates unwinding to a lesser extent (30% unwound in 1 min), whereas Y122A does not increase the unwinding rate as compared with gp41 alone.

Rolling Circle Replication—It has been suggested that gp43 is involved in the loading of gp41 at the replication fork (23). If so, it may be possible that the helicase loading defect in Y122A could be overcome by the inclusion of gp43 in the reaction. To test this possibility, we employed a nicked 3.6-kb circular plasmid as a substrate for rolling circle replication (Fig. 3). Upon treatment of the pGEM_nick plasmid with the N.BbvC IB enzyme a single 3′ end is created at position 1353, which serves as a primer for the holoenzyme polymerase. Following the incorporation of 30–50 nucleotides by the polymerase, a sufficient amount of ssDNA is produced, which allows the loading of the helicase onto the lagging strand (40). In this assay, nucleotide incorporation by the polymerase is used as an indicator of productive helicase loading. As shown in Fig. 3, the efficient loading of gp41 helicase onto the replication fork is achieved when gp32 and WT-gp59 are included in the reaction mixture. Consistent with the helicase unwinding assay, F111A and Y217A stimulate rolling circle replication to an extent equal to the wild-type protein. Y146A only partially stim-

**TABLE 1**

| -Fold enhancement* | NaCl IC50* | Hill coefficient |
|--------------------|-----------|-----------------|
| WT                 | 2.44 ± 0.03 | 89 ± 3          | 1.9 ± 0.1 |
| F111A              | 2.53 ± 0.02 | 94 ± 3          | 1.4 ± 0.1 |
| Y122A              | 2.35 ± 0.02 | 61 ± 2          | 1.6 ± 0.1 |
| Y146A              | 2.21 ± 0.03 | 58 ± 3          | 1.8 ± 0.1 |
| Y217A              | 2.22 ± 0.06 | 54 ± 5          | 2.1 ± 0.3 |

* - Fold enhancement is defined as the ratio of the fluorescence of the etheno-DNA in the presence of a saturating amount of gp59 protein to the fluorescence of the etheno-DNA in the absence of protein.
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FIGURE 3. Rolling circle replication in the presence of WT and mutant gp59 proteins. A schematic illustrating the initiation of rolling circle replication using the pGEM_nick substrate. Holoenzyme polymerase performs slow strand displacement synthesis in the presence of gp32, producing ssDNA that is used as a scaffold for the loading of gp41. B, reactions 1– 6 show the DNA synthesis products at 1–3 min in the presence of WT-gp59, no gp59, F111A, Y122A, Y146A, and Y217A, respectively.

FIGURE 4. Concentration dependence of gp41 on rolling circle replication in the presence of WT-gp59 or Y122A. A plot of the DNA synthesis products produced from rolling circle replication carried out on the pGEM_nick substrate in the presence of all eight replisomal proteins (see "Materials and Methods") for WT-gp59 (squares), Y122A (triangles), and in the absence of gp59 (diamonds), respectively.

gp41 required to observe helicase-dependent synthesis is increased by 3–4-fold.

Steady-state FRET Experiments—Steady-state FRET was used to characterize the ability of the mutant gp59s to interact with gp32 and gp43. As shown in Fig. 5A, the interaction of WT-gp59 with gp32 results in the expected amount of FRET between the labeled gp59 and gp32. All four labeled gp59 mutants display a FRET signal with gp32 (Table 2), indicating the ability to interact with gp32 has not been affected by the mutations. The cause of the increased FRET signal displayed by F111A is unclear. Because all function assays carried out on F111A suggest that it is identical to the wild-type enzyme, it may be due to a change in the environment of the CPM fluorophore and not its interaction with gp32. Using a similar assay, F111A, Y146A, and Y217A retain their interaction with gp43 (Table 3). However, using the FRET assay as a measure, Y122A is unable to form a close association with gp43 (Table 3 and Fig. 5B).

Inhibition of gp43 Polymerase by WT-gp59 and Y122A—In order to further characterize the interaction between gp59 and gp43, the ability of WT-gp59 and Y122A to inhibit the idling reaction of the polymerase was explored (Fig. 6A). The template strand of Bio25/75 is made up of only A, T, G, except for 3 Cs located at positions 50–52. Upon the omission of dGTP, the wild-type enzyme undergoes repeated cycles of incorporation of dCTP and excision of dCMP at the base pair prior to the stall site. The Kd of the polymerase holoenzyme that is stalled by nucleotide omission is 0.005 s⁻¹ (see Ref. 41; data not shown). At the time of gp59 addition to the reaction (15 s after mixing of holoenzyme with DNA), 100% of the holoenzyme has assembled and replicated up to the stall site, and because of the extremely slow dissociation of the polymerase, it remains bound to the DNA template for the duration of the assay (1 min). As shown, wild-type gp59 potently inhibits the WT-gp43 idling reaction (IC⁵₀ = 0.45 ± 0.06 μM, n = 1.3 ± 0.2); however, Y122A has no effect over the concentration range tested.

D-loops are formed by the invasion of single-stranded DNA into homologous dsDNA templates and serve as the primer for the polymerase (32). gp59 is absolutely essential for loading gp41 helicase at D-loop and is thought to play a role in preventing the bubble migration synthesis that occurs in the presence of Dda helicase (42). We tested the ability of WT-gp59 and the Y122A to inhibit the polymerization activity of gp43 at a D-loop structure (Fig. 6B). D-loop formation was catalyzed through a combination of the T4 recombination proteins UvsX and UvsY. A linear dsDNA template was used, eliminating the requirement for topoisomerase activity. In the absence of gp59 and gp41, a significant amount of replication occurs, which is produced by a strand displace-
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**FIGURE 5.** Fluorescent spectra demonstrating the presence or absence of FRET between WT-gp59 and Y122A with gp32 and gp43. A, fluorescent spectrum demonstrating FRET between gp32 and WT-gp59. The fluorescence spectrum is shown of a solution containing 0.6 μM N-terminally labeled Oregon Green 488 gp32, 0.6 μM gp59 labeled with thiol reactive CPM, and 100 nM forked 32/64/73-mer DNA substrate. B, fluorescent spectrum showing the amount of FRET between gp43 and WT-gp59 or Y122A. The fluorescence spectrum is shown of a solution containing 100 nM forked 32/64/73-mer DNA substrate with either 0.6 μM N-terminally labeled Oregon Green 488 gp43 (light gray), 0.6 μM N-terminally labeled Oregon Green 488 gp43 and 0.6 μM WT-gp59 labeled with thiol reactive CPM (black), and 0.6 μM N-terminally labeled Oregon Green 488 gp43 and 0.6 μM Y122A labeled with thiol reactive CPM (dark gray), respectively.

**TABLE 2**

Fluorescence resonance energy transfer between Oregon Green-labeled gp32 and CPM-labeled gp59 proteins

| gp59         | Normalized FRET* |
|--------------|------------------|
| WT           | 0.43             |
| F111A        | 0.44             |
| Y122A        | 0.36             |
| Y146A        | 0.41             |
| Y217A        | 0.41             |
| None*        | 0.33             |

* Normalized FRET is ratio of the fluorescence intensity at 465 nm to the fluorescence intensity at 515 nm.

* Normalized FRET in the absence of gp59-CPM.

The exonucleolytic activity of wild-type gp43 polymerase can remove D-loops (Fig. 6C). In this case, the homologous dsDNA template was supercoiled dsM13, which stabilizes the D-loop after strand invasion. The 20-min incubation prior to the addition of holoenzyme ensured that the strand invasion reaction was complete. Wild-type gp43 holoenzyme alone cannot perform strand displacement synthesis when initiating from a D-loop; instead the D-loop is removed by repeated cycles of nucleotide excision and fork regression. In this situation, presumably, the polymerization activity of gp43 is overcome by its exonuclease activity, resulting in the removal of a single nucleotide, which is the first half of the polymerase idling reaction. Next, rather than the incorporation of a nucleotide, the DNA strands rearrange causing the polymerase to remain at its current position. The following nucleotide excision by gp43 results in another round of fork regression. Upon repeated cycles of this process, the net result is the removal of the D-loop. Inclusion of WT-gp59 in the reaction mixture increases the lifetime of the D-loop by inhibiting the exonuclease activity of the polymerase (Fig. 6C, reaction 2). The Y122A mutant, however, does not bring the same amount of stability to the D-loop (Fig. 6C, reaction 3).

**DISCUSSION**

Following the first few rounds of origin-initiated replication, the majority of DNA replication in T4 phage is initiated through strand invasion of ssDNA into homologous dsDNA templates catalyzed through the combined action of UvsX, UvsY, and gp32 (43). The result of this strand invasion is a DNA structure termed a D-loop, which serves a dual role as a primer for DNA synthesis and a scaffold for the assembly of the replisome. This strand invasion mechanism is also thought to play a major role in recombination-mediated DNA repair pathways such as double strand break repair and single-stranded lesion bypass (44). In *vivo*, the helicase loader protein gp59 is absolutely required for initiation of replication at D-loops. This is supported by *in vitro* studies showing that gp59 abrogates bubble migration synthesis and promotes the semi-conservative type of replication that is the hallmark of normal DNA synthesis (40). These results suggest a crucial role of gp59 during replisome assembly.

Based on recent studies, gp59 appears to be a central player in the T4 replisome, making contacts with components of the leading strand holoenzyme, primosome, and the DNA substrate (9, 17, 29). For the most part, the functional consequences of these interactions are speculative. We have characterized a series of mutations with the goal of elucidating the effects of the loss of one or more of these protein-protein interactions. Guided by the crystal structure, we chose conserved hydrophobic residues that have a high degree of solvent exposure. Residues with these properties are thought to be hot spots of protein-protein contacts (45). Site-directed mutations were introduced separately into each of the four positions chosen: F111A, Y122A, Y146A, and Y217A. Each mutant was tested for its helicase loading activity, ssDNA binding, gp32 ssDNA-binding protein interaction, polymerase interac-

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**TABLE 3**

Fluorescence resonance energy transfer between Oregon Green-labeled gp43 and CPM-labeled gp59 proteins

| gp59         | Normalized FRET* |
|--------------|------------------|
| WT           | 0.43             |
| F111A        | 0.44             |
| Y122A        | 0.36             |
| Y146A        | 0.41             |
| Y217A        | 0.41             |
| None*        | 0.33             |

* Normalized FRET is ratio of the fluorescence intensity at 465 nm to the fluorescence intensity at 515 nm.

* Normalized FRET in the absence of gp59-CPM.

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gp59 Helicase Loader Protein-Protein Interactions

Two mutants, F111A and Y217A, showed no altered functionality in the assays used and were not subjected to a more extensive characterization. Also, recently Jones et al. (31) demonstrated that mutation of these two residues did not affect the affinity of gp59 for forked DNA substrates. Similarly, Y146A displayed only minor changes in helicase loading activity and was fully active in all other assays. For these reasons, the majority of this Discussion will focus on the properties of the Tyr122 → Ala gp59 mutant (Y122A). When standard conditions are employed, Y122A is completely defective in helicase loading activity. This deficiency cannot be attributed to a lack of DNA binding capability because Y122A binds eM13 DNA as well as the WT-gp59 and displays a similar resistance to increased NaCl concentrations. It is noteworthy that much less DNA synthesis is observed in a reaction containing gp41 and Y122A as compared with a reaction with gp41 helicase alone. This is likely due to the inhibition of strand displacement activity that is observed in reactions containing WT-gp59 and strongly suggests that Y122A is able to bind to the replication fork, but cannot serve as a scaffold for the productive loading of gp41 helicase. Additionally, when gp41 concentrations were elevated, Y122A supported a moderate amount of helicase-dependent DNA synthesis consistent with the interaction between Y122A and gp41 having been altered and not the interaction between gp59 and the DNA substrate.

We have employed two different FRET-based assays to characterize interactions between gp59 and other components of the T4 replisome (9, 17). These assays were used to test the mutants with respect to gp32 and gp43 interactions. All mutants interacted with gp32 normally, both on and off DNA. This result is consistent with the structural model for gp59-gp32 interaction based on cross-linking data (25). The residues chosen for mutation lie on the opposite face of gp59 from where gp32 interacts. With the exception of Y122A, the mutants displayed similar levels of energy transfer with gp43 as compared with the wild-type protein. Y122A showed very little FRET signal with labeled gp43, demonstrating that the interaction between these two proteins has been disrupted by the introduction of the mutation at position 122.

The disruption of the gp43-Y122A interaction was unexpected, because Y122A retains the ability to inhibit the strand displacement activity of gp43 using the nicked circular substrate. It is likely that strand displacement inhibition only requires that gp59 be capable of binding to the forked DNA, indicating that the mode of inhibition is steric to impede the forward movement of the polymerase holoenzyme. What then is the purpose of a direct protein-protein interaction between gp59 and gp43? To address this question we tested the ability of Y122A to inhibit a holoenzyme polymerase that is idling because of nucleotide omission. The order of addition was controlled to ensure that all substrate was saturated by the holoenzyme before addition of WT-gp59 or Y122A. Under this condition, gp59 would only inhibit a polymerase activity through a direct protein-protein interaction and not through any other mechanism such as competition for the primer-template junction or sequestering of gp43 from the DNA substrate. As expected, WT-gp59 potently inhibits idling of the polymerase holoenzyme; however, Y122A has no effect at concentrations up to 1.2 μM.

We further tested the inhibition of polymerase activity by Y122A at a D-loop, which is the in vivo substrate for gp59 helicase loading. The in vitro system for studying D-loop-dependent initiation of replication enabled us to study the effect of WT-gp59 and Y122A on the exonuclease activity of gp43(exo−) and the polymerization activity of gp43(exo−) using the similar substrates. The results mirror that of the experiments using other templates. WT-gp59 inhibits both the exonuclease and polymerase activities of gp43 at the D-loop. Although the Y122A mutant inhibits the polymerization activity of the holoenzyme, it is unable to prevent the removal of the D-loop by the exonuclease activity. This finding, in conjunction with the result from the idling experiment, strongly suggests that a direct interaction between gp59 and gp43 is necessary to inhibit the exonuclease activity of gp43. However, binding of gp59 to the replication fork alone will result in the inhibition of the strand displacement activity of the holoenzyme.

The failure of the Y122A mutant to interact with the polymerase is consistent with the proposed interaction model for these two proteins (17). This model was generated through the use of an online docking program, Cluspro, using the Protein Data Bank files for gp59 and gp43.
Several preliminary models were generated, and the most favorable model was chosen on the basis of cross-linking and FRET data. On the other hand, the mutations in this paper were chosen on the basis of hydrophobicity and solvent exposure and not the interaction model. Tyr122 of gp59 is located directly between the two regions (helix H7 and loop H6-H7) that were predicted to interact with gp43. On the basis of this central location, the role of Tyr122 may be to stabilize helix H13 and the loop H6-H7 in their interactions with gp43. Indeed, helix H7, which contains Tyr122, makes contacts with both helix H6 and H13. Because the Y122A mutant is defective in polymerase binding and Tyr122 is positioned very close to the predicted interface between gp43 and gp59, it represents an independent confirmation of the interaction model.

The fact that binding of gp59 to the replication fork is the only requirement for inhibition of the polymerase activity of gp43, yet both proteins have co-evolved to maintain the gp59-gp43 interaction, suggests that the inhibition of the exonuclease activity of the polymerase is of critical importance. gp59 inhibition of D-loop removal by the polymerase may be required for efficient origin-independent initiation of replication. In the absence of gp59, loading of gp41 onto fork structures is relatively slow (on the order of minutes). This allows enough time for the highly active exonuclease activity of gp43 to remove either a portion of or the entire invading strand of the D-loop. The invading strand of the D-loop used in this study was 80 bp and could be completely degraded within 1 min. Based on recombination frequencies, the invading strand in vivo can vary between 30 and several hundred base pairs (46). It seems quite plausible that the invading strand of smaller D-loops could be completely removed prior to gp41 loading unless gp59 binds to the D-loop and locks the polymerase in place until the helicase can load.

It is quite surprising that the mutation of a single residue on gp59 eliminates interactions with both the polymerase and the helicase. This result could be rationalized if the mutation altered the overall structure of the protein. However, this seems unlikely because several other properties such as DNA binding and the interaction with gp32 have not been affected by the mutation. It is plausible that the polymerase and the helicase share overlapping binding sites on gp59. If this is the case, how then does gp59 break its interaction with the polymerase to interact with gp41? We propose two possible models for polymerase unlocking and helicase loading that are based on a direct competition between gp41 and gp43 for binding gp59. In the first model (Fig. 7A), a hexameric gp59 is proposed to be the functional form. This is based on the cross-linking studies indicating that gp59 has the ability to form higher order structures on DNA or in the presence of gp41 and gp32. Moreover, gp59 stimulates the unwinding activity of the hexameric gp41 maximally at a 1:1 gp59:gp41 ratio. In this scenario, a single subunit of a hexameric gp59 is involved in a protein-protein interaction with the polymerase. This type of arrangement leaves the other five subunits exposed and

FIGURE 7. Two possible models for unlocking of gp43 and loading of gp41. The various protein components are colored as follows: clamp protein (black), polymerase (gray), gp59 (red), and gp41 (blue). The sites of interaction between gp43 and gp59 are colored green and yellow, respectively. The site of interaction between gp41 and gp59 are colored fuchsia and yellow, respectively. Details of model A and B can be found in the “Discussion.”
available for helicase binding. The binding of helicase subunits to the gp59 monomers not involved in the polymerase interaction induces a conformation change throughout the gp59 hexamer, which breaks the interaction with the polymerase and exposes the last gp59 monomer to gp41. Loading of the final gp41 monomer triggers ATP hydrolysis and causes gp59 to dissociate from the fork, which initiates helicase-dependent DNA synthesis.

The second model is similar in many respects to the first; however, it can accommodate either a gp59 monomer or hexamer bound to the replication fork (Fig. 7B). In this model, a portion of the gp41-binding site on gp59 remains exposed even while gp59 is bound to gp43. The binding of gp41 to the ternary complex of DNA-gp59-gp43 causes a conformational change in gp59 that releases gp43 and creates a larger binding interface, which includes residue 122. Following the conformational change by gp59, gp41 repositions itself and achieves the final state that is competent for ATP hydrolysis, gp59 dissociation, and DNA unwinding. In this scenario, the gp41 can still bind to Y122A at the replication fork but its active, final form is not achieved because of the absence of the Tyr122 interaction.

The role of gp32 was not explicitly mentioned in either of the above models. However, it is clear that the ssDNA is coated with gp32 prior to loading (30) (Fig. 2). Based on these and other results, Morrical and co-workers (14) have proposed that the complex of gp59-gp32-ssDNA forms a unique topological structure referred to as a helicase loading complex. The hexameric conformational change mechanism (Fig. 7A) is best suited for accommodating the proposed helicase loading complex structure. Recently, Nossal and co-workers (49) have confirmed the structural change by gp59, gp41 repositions itself and achieves the final state that is required for both the loading of gp41 in the presence of ATP (28). However, electron microscopy experiments have shown that in some cases gp59 is retained as a complex of gp32-gp59 in the lagging strand loop in some replicating molecules (48). Clearly, further investigation into the mechanism of polymerase unlocking and helicase loading is required, and the two mechanisms presented here will serve as good working models for additional experimentation.

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