Dual Functions of Single-stranded DNA-binding Protein in Helicase Loading at the Bacteriophage T4 DNA Replication Fork*

Received for publication, October 27, 2003, and in revised form, February 9, 2004
Published, JBC Papers in Press, February 9, 2004, DOI 10.1074/jbc.M311738200

Yujie Ma‡, Tongsheng Wang‡, Jana L. Villemain§, David P. Giedroc§, and Scott W. Morrical‡¶

From the ‡Department of Biochemistry, University of Vermont College of Medicine, Burlington, Vermont 05405 and §Department of Biochemistry and Biophysics, Texas A & M University, College Station, Texas 77843

Semi-conservative DNA synthesis reactions catalyzed by the bacteriophage T4 DNA polymerase holoenzyme are initiated by a strand displacement mechanism requiring gp32, the T4 single-stranded DNA (ssDNA)-binding protein, to sequester the displaced strand. After initiation, DNA helicase acquisition by the nascent replication fork leads to a dramatic increase in the rate and processivity of leading strand DNA synthesis. In vitro studies have established that either of two T4-encoded DNA helicases, gp41 or dda, is capable of stimulating strand displacement synthesis. The acquisition of either helicase by the nascent replication fork is modulated by other protein components of the fork including gp32 and, in the case of the gp41 helicase, its mediator/loading protein gp59. Here, we examine the relationship between gp32 and the gp41/gp59 and dda helicase systems, respectively, during T4 replication using altered forms of gp32 defective in either protein-protein or protein-ssDNA interactions. We show that optimal stimulation of DNA synthesis by gp41/gp59 helicase requires gp32-gp41 interactions and is strongly dependent on the stability of ssDNA binding by gp32. Fluorescence assays demonstrate that gp59 binds stoichiometrically to forked DNA molecules; however, gp59-forked DNA complexes are destabilized via protein-protein interactions with the C-terminal “A-domain” fragment of gp32. These previously published results suggest a model in which a mobile gp59-gp32 cluster bound to lagging strand ssDNA is the target for gp41 helicase assembly. In contrast, stimulation of DNA synthesis by dda helicase requires direct gp32-dda protein-protein interactions and is relatively unaffected by mutations in gp32 that destabilize its ssDNA binding activity. The latter data support a model in which protein-protein interactions with gp32 maintain dda in a proper active state for translocation at the replication fork. The relationship between dda and gp32 proteins in T4 replication appears similar to the relationship observed between the UL9 helicase and ICP8 ssDNA-binding protein in herpesvirus replication.

DNA helicase acquisition by a nascent DNA replication fork is essential for reconstituting rapid, processive DNA synthesis. This principle is illustrated dramatically by the bacteriophage T4 DNA replication system (1–2). T4 encodes two DNA helicases known to affect the movement and properties of DNA replication forks; the gene 41 protein (gp41) and the dda protein, respectively.

gp41 is the essential replicative helicase of the T4 phage. This hexameric enzyme translocates processively in a 5’ → 3’ direction on the displaced lagging strand of the dsDNA template, thus enhancing the rate and processivity of leading strand DNA synthesis catalyzed by the T4 DNA polymerase helozone (gp43, gp44/62, and gp45 proteins) (1–3). In addition, gp41 is an obligatory component of the T4 primosome (helicase/primase complex) and is, thus, essential for lagging strand DNA synthesis (4–5). A second T4-encoded DNA helicase, dda protein, stimulates leading strand DNA synthesis in vitro but is nonessential for phage DNA replication in vivo (6–8). Like gp41, dda unwinds dsDNA in a 5’ → 3’ direction and is presumed to translocate on the lagging strand; however, unlike gp41 the unwinding reaction proceeds by a distributive rather than processive translocation mechanism (6–7). dda is proposed to play specialized but nonessential roles in T4 replication including helping DNA replication forks bypass template-bound proteins such as RNA polymerase (9). In addition, a T4 dda deletion strain has a weak DNA delay phenotype, consistent with a role of dda protein in the initiation of DNA replication from T4 origins (10).

The effects of both gp41 and dda helicases on DNA replication have been studied using in vitro strand displacement DNA synthesis reactions in which replication is initiated from a nick in a dsDNA template. Strand displacement synthesis starting from a nick requires both DNA polymerase holoenzyme and gp32 (the T4 ssDNA-binding protein) and is stimulated by either T4 helicase (6–7, 11). gp32 facilitates a relatively slow strand displacement reaction that generates the ssDNA tail required for DNA helicase acquisition by the nascent replication fork. Helicase acquisition is clearly modulated by gp32 and other protein components of the nascent fork. In the case of dda, which interacts strongly and specifically with gp32, the latter protein is an essential cofactor for dda stimulation of strand displacement synthesis (6–7). In the case of gp41, which does not interact with gp32 directly, the cooperative binding of gp32 to the displaced strand inhibits gp41 loading, causing a lag time in the onset of rapid DNA replication (12). This lag time is eliminated by gp59, a mediator protein that loads gp41 onto gp32-saturated ssDNA molecules (12–16). gp59 also binds...
to specific DNA structures including forks and cruciforms and can recruit gp41 helicase to these structures (17–18). It is clear that the loading of both gp41 and dda helicases, enzymes that must bind to and translocate on ssDNA at the T4 replication fork, involves complex functional and/or physical relationships with gp32, a protein that rapidly saturates and sequesters replication fork ssDNA through tight and cooperative binding.

The properties of gp32 (34 kDa, 301 amino acids) have been studied extensively (19–20). gp32 contains three modular domains, “A domain,” “B domain,” and “core.” The central core domain (amino acids 22–253) contains the ssDNA binding site consisting of an oligonucleotide/oligosaccharide binding (OB)-fold stabilized by a structural zinc atom (21). The acidic, C-terminal A domain (amino acids 254–301) is the site of protein-protein interactions with several T4 DNA replication and recombination proteins, including but not limited to gp59, dda, DNA polymerase (gp43), and primase (gp61) (14, 22–23). The N-terminal B domain (amino acids 1–21) contains residues essential for cooperative ssDNA binding and gp32-gp32 interactions (24). Several altered forms of gp32 have been purified and studied biochemically. The gp32-A fragment (amino acids 1–253) lacks the entire A domain and is deficient in protein-protein interactions with gp59, dda, gp43, etc. yet retains approximately wild-type levels of ssDNA binding affinity and cooperativity (14, 23, 25). The A-domain fragment (amino acids 213–301) lacks ssDNA binding ability but retains protein-protein interactions with the above-named replication proteins (14, 22, 26). The gp32-B fragment (amino acids 22–301) is devoid of cooperativity and gp32-gp32 interactions and exhibits severely reduced ssDNA binding affinity compared with wild type (24). Giedroc and Villemain (27, 28) also characterized a series of gp32 species with missense mutations at positions Lys8 and Arg4 that exhibit varying degrees of deficiency in ssDNA binding affinity and cooperativity. These mutant proteins as well as gp32-B retain protein-protein interactions with other replication factors by virtue of their intact A domains (26). We previously showed that the ability of different gp32 B domain mutants to support strand displacement DNA synthesis (in the absence of a helicase) correlates strongly with their hierarchy of relative ssDNA binding affinities, i.e., gp32 wild type > R4K > R4G > R4T > R4C > gp32-B (28).

The precise roles of gp32 protein-protein and protein-ssDNA interactions in helicase loading processes are poorly understood. To address these issues, we tested the abilities of gp41/ gp59 and dda to stimulate strand displacement DNA synthesis reactions in the presence of the various truncated and missense forms of gp32. Our findings demonstrate the importance of gp32-gp59 and gp32-dda protein-protein interactions in acquisition of the gp41 and dda helicases, respectively, by the T4 replication fork. Surprisingly, the data indicate that gp32 mutations which weaken gp32-ssDNA binding affect gp41 and dda helicase acquisition differentially; loading of gp41 at the replication fork by gp59 is strongly inhibited as gp32 species affinity for ssDNA decreases, whereas acquisition of the dda helicase is relatively unaffected by the same gp32 mutations. In addition, the results of fluorescence assays indicate that the A domain of gp32 attenuates gp59-fork DNA interactions. Together, the data suggest different models for the active forms of gp41 versus dda present at T4 replication forks and for the nucleoprotein intermediates that form during helicase assembly at replication forks.

MATERIALS AND METHODS

Reagents and Enzymes—Radiolabeled a-[32P]dATP and a-[32P]dCTP were purchased either from ICN or PerkinElmer Life Sciences. All nonradioactive ribonucleotides and deoxyribonucleotides were purchased from Amersham Biosciences. The bacteriophage fd gene 2 protein (gp2) was purified as described (30). All other chemicals, biochemicals, and commercial enzymes were purchased from Sigma unless specifically noted.

T4 DNA Replication and Recombination Proteins—Purification and storage conditions for T4 proteins including gp43 (DNA polymerase), gp44/62 and gp45 (DNA polymerase processivity factors), gp32 (ssDNA-binding protein; 34 kDa, 301 amino acids), gp41 and dda (DNA helicases), and gp59 (mediator/loading protein for gp41 helicase) were as previously described (12–13, 31–34). gp32 protein fragments gp32-A (amino acids 1–253), gp32-B (amino acids 22–301), and A domain (amino acids 213–301) were purified from overexpressing strains as described (14, 22, 24). gp32 point mutant species R4K, R4G, R4T, R4C, and K3A were also purified according to published procedures (27, 28). The concentrations of gp32 wild type and B-domain mutant species were determined by the absorbance at 280 nm using an extinction coefficient of ε280 = 4.13 × 10³ M⁻¹ cm⁻¹ determined from the amino acid sequence (35). The concentrations of other T4 protein stock solutions were determined by a spectrophotometric Bradford assay using bovine serum albumin and wt gp32 standards. All purified protein stocks were tested for contaminating endo- or exonuclease activities by incubating protein (at reaction concentrations) with circular plus linear ssDNA, supercoiled plus nicked circular dsDNA, or linear dsDNA samples as appropriate, then examining the DNA on agarose gels. All protein stocks used in these studies were nuclease-free according to these criteria.

Nucleic Acids—Oligonucleotides were purchased from Operon. Supercritical RFII DNA from bacteriophage M13mp19 was isolated as described (36). Nicked RFII DNA was generated by treating M13mp19 RFI DNA with the bacteriophage fd gene 2 protein as described (30). Circular single-stranded DNA from bacteriophage M13mp19 was isolated by extraction from purified phage particles (36–37). All DNA concentrations were determined by the absorbance at 260 nm using conversion factors of 50 µg/ml for dsDNA and 36 µg/ml for ssDNA and are expressed in units of µmol of nucleotide residues per µg.

Strand Displacement DNA Synthesis Reactions—Strand displacement DNA synthesis reaction mixtures contained the following components (final concentrations): 20 mM Tris acid, pH 7.4, 80 mM potassium acetate, 10 mM magnesium acetate, 100 µM/ml bovine serum albumin, 0.5 mM dithiotreitol, 10 µg/ml creatine phosphokinase, 10 µM creatine phosphate, 2 mM each ATP and GTP, 100 µM each dATP, dCTP, dGTP, and dTP, 10 µC [32P]dATP or [32P]dCTP (specific activity = 3000 Ci/mmol), and 10 µM (nucleotides) M13mp19 RFII DNA plus T4 proteins gp43 (4 µg/ml), gp44/62 (24 µg/ml), and gp45 (8 µg/ml). Reactions were carried out in the presence or absence of gp41 (18 µg/ml when present), gp59 (5 µg/ml when present), dda (5 µg/ml when present), and the various gp32 species (each 1.45 µg/ml when present). Exact halflife and gp32 compositions for each reaction are given in the figure legends. Constant salt conditions were maintained between reactions through the addition of appropriate amounts of gp32, gp59, gp41, and/or dda storage buffers.

All reactions were carried out at 37 °C. All reaction components except DNA, dNTPs, and a-[32P]dATP or d-[32P]dCTP were preincubated for 3 min at 37 °C, then the reaction was initiated by the addition of the missing components. Reactions were allowed to proceed for up to 10 min at 37 °C depending on the experiment. DNA synthesis was monitored both quantitatively via trichloroacetic acid precipitation and qualitatively via alkaline agarose gel electrophoresis. Time courses of DNA synthesis reactions were obtained as follows. From an initial reaction mixture of 50 µl, 10 µl aliquots were removed at the indicated times, spotted onto Whatman GF/A glass filters; these were soaked for 15 min in 250 ml of cold 5% trichloroacetic acid, 10% saturated sodium pyrophosphate solution at 4 °C, then washed successively with 4 × 250 ml of cold 1 m HCl and 2 × 250 ml of cold 95% ethanol, dried under a heat lamp, and transferred to a scintillation counter where the extent of incorporation of label was quantitated. One-dimensional gel point assays and electrophoretic analyses, reactions in 25 µl (final volume) were carried out for 10 min at 37 °C and then stopped by the addition of 28 µl EDTA (final concentration) and transferred to an ice-water bath. A portion of each sample was removed for quantitative analysis by trichloroacetic acid precipitation as described above. The remaining gel was processed for alkaline agarose gel electrophoresis by adding 10% sucrose, 30 mM NaOH, and 0.04% bromocresol green (final concentrations). Samples were then loaded onto a horizontal 0.8% alkaline agarose gel that was prepared and run as described (38). After electrophoresis, gels were neutralized by soaking for 30 min in TBE (89

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2 Y. Ma and S. Morrical, unpublished data.
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RESULTS

gp32 Truncations Differentially Affect Helicase Stimulation of Strand Displacement DNA Synthesis—We compared the effects of no gp32, full-length gp32, gp32-A, gp32-B, and gp32 A domain fragment on strand displacement DNA synthesis reactions catalyzed by the T4 DNA polymerase holoenzyme (gp43, gp44/62, and gp45 proteins) in the presence and absence of the gp41/gp59 and dda helicases (Fig. 1). Fig. 1A shows time courses of strand displacement synthesis in the absence of gp41, gp59, and dda. Here, significant amounts of DNA synthesis are observed only with full-length gp32 and with the gp32-A truncation mutant. The rate of DNA synthesis is ~2-fold higher for full-length gp32 than for gp32-A (Fig. 1A), suggesting that gp32-polymerase interactions are required for optimal strand displacement DNA synthesis in the absence of a DNA helicase. This observation is consistent with previously published data (23). Note that reactions with the gp32-B and A-domain fragments are indistinguishable from the reaction with no gp32; all are essentially at background (Fig. 1A). Clearly the low affinity, distributive binding of gp32-B to ssDNA is insufficient to support strand displacement synthesis under these conditions. Likewise, the failure of the A domain fragment to support the reaction demonstrates that gp32-polymerase interactions alone are insufficient for strand displacement synthesis.

The time courses shown in Fig. 1B represent reactions performed in the presence of the dda helicase; conditions were otherwise identical to Fig. 1A. Under these conditions, the dda helicase affords a 2–3-fold stimulation of the rate of strand displacement synthesis in the presence of full-length gp32 relative to the equivalent reaction in the absence of dda (Fig. 1, A and B). dda does not stimulate strand displacement synthesis in reactions lacking gp32 (Fig. 1, A and B), consistent with previous reports (6, 7). dda also fails to stimulate reactions containing gp32-A (Fig. 1, A and B), suggesting that dda-gp32 protein-protein interactions are required for dda stimulation of strand displacement synthesis. This conclusion is supported by the observation that gp32-B supports moderate levels of strand displacement synthesis in the presence of dda protein (Fig. 1B), whereas gp32-B does not support the reaction in the absence of dda (see Fig. 1A). The activity of gp32-B is less than that of full-length gp32 but substantially higher than with gp32-A, which does not respond to dda (Fig. 1B). These results suggest that dda-gp32 protein-protein interactions play a central role in the stimulatory activity of the dda helicase toward the strand displacement DNA synthesis reaction and that the ssDNA binding affinity and cooperativity of the gp32 species are relatively unimportant in this process. Intriguingly, the addition of the A-domain fragment of gp32 to reactions containing dda causes a small but reproducible increase in strand displacement synthesis above background levels (Fig. 1B and data not shown), suggesting that dda-A domain interactions may be sufficient to drive a small number of helicase molecules into an active conformation (see “Discussion”).

The reactions in Fig. 1C were performed in the presence of the gp41 helicase; otherwise, conditions were identical to Fig. 1A. In reactions containing either no gp32, gp32-A, or full-length gp32, the results obtained with gp41 as helicase were extremely similar to the corresponding data obtained with dda as helicase (Fig. 1, B and C) (the concentration of gp41 used was chosen empirically so as to approximately match the levels of DNA synthesis seen with dda). One dramatic difference is apparent. The gp32-B species fails to support strand displacement synthesis in the presence of gp41 (Fig. 1C), whereas this truncated form of gp32 is active in the dda-stimulated reaction (Fig. 1B). These results appear to support the notion that significant gp32-dependent strand displacement synthesis must occur before gp41 helicase can load at the nascent replication fork (1–2, 4–5). The A-domain fragment of gp32 also fails to support the reaction in the presence of gp41 (Fig. 1C). Note the rather concave appearance of the reaction time course with full-length gp32, an effect due to the slow loading of gp41 helicase in the absence of its loading factor, gp59 (12). Note also that little gp41 stimulation of the gp32-A-containing reaction is apparent, consistent with previously published results (23).

The reactions represented in Fig. 1D contain both gp41 and its loading factor, gp59. For the reactions with full-length gp32,
the combination of gp59 + gp41 yields a marked enhancement in the rate of strand displacement synthesis over that seen with gp41 alone (Fig. 1, C and D). Note that the presence of gp59 causes the reaction time course to be nearly linear, in contrast with the more concave time course in Fig. 1C. These data are consistent with published reports that gp59 reduces the lag time in helicase acquisition by the replication fork (12).

Two other observations are pertinent here. First, the combination of gp41 plus gp59 slightly stimulates the reactions with gp32-B, A domain, or no gp32 compared with the equivalent reactions with gp41 alone (Fig. 1, C and D). This observation is consistent with observations that gp59 can facilitate a low level of gp41 helicase loading at nicks in the absence of gp32 (12).3 Second, the gp41 plus gp59 combination significantly inhibits the reaction with gp32-A compared with reactions lacking gp59 (Fig. 1, C and D). The inhibition may be due to the formation of gp59-gp32-A-ssDNA structures that interfere with initiation and/or movement of replication forks (39).

Control reactions shown in Fig. 1E demonstrate that in the absence of gp41, gp59 has a general inhibitory effect on strand displacement synthesis with all gp32 species including full-length gp32 (compare with Fig. 1A). This inhibition is consistent with previously published data (12, 40–41) and may result from the formation of nonproductive gp59-DNA structures that impede DNA synthesis (17–18, 39).

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activity in reactions containing the R4G, R4T, K3A, and R4Q gp32s compared with equivalent reactions lacking gp59. DNA synthesis activities in the presence of R4Q and K3A are enhanced 1.5–2-fold, measured relative to wt gp32 control, in the presence of gp41/gp59 versus gp41 alone (Fig. 2B). The largest improvement is seen with the very weak ssDNA binding mutants R4T and R4G, which in combination with gp41 helicase alone promoted DNA synthesis levels barely above background (Fig. 2A). Here, the addition of gp59 plus gp41 to reactions with R4T or R4G gp32 yields DNA synthesis levels 8–10% of that seen with wt gp32 under the same conditions (Fig. 2B), corresponding to a 4–5-fold increase in absolute activity for these mutants (data not shown). Nevertheless, even in the presence of gp59 reactions rates are an order of magnitude lower with R4T and R4G than with wt gp32. The inhibition of DNA synthesis by gp41 plus gp59 in the presence of gp32-A, first noted in Fig. 1D, is also evident in Fig. 2B. It is interesting to note that even the R4T and R4G gp32 species, which bind weakly to ssDNA but retain protein-protein interactions with gp59, support stronger strand displacement synthesis reactions in the presence of gp41 plus gp59 than does gp32-A, which binds strongly to ssDNA but lacks interactions with gp59 (Fig. 2B).

When the dda helicase replaces gp41 (+gp59) in strand displacement DNA synthesis reactions, the landscape of gp32 mutational effects on the reaction is altered dramatically. The data in Fig. 2C show that all of the gp32 B domain missense mutants support a reaction 50–70% as efficient as wt gp32, including the two weakest ssDNA binding mutants R4T and R4G. Even the gp32-B truncation mutant, similar to background in reactions with gp41 (+gp59), in the presence of dda helicase supports DNA synthesis to a level ~35% of that seen with wt gp32 (Fig. 2C). This despite the fact that gp32-B binds very weakly and non-cooperatively to ssDNA and cannot saturate the lattice under our reaction conditions (29). The common thread between these six gp32 mutant species (R4K, R4Q, K3A, R4T, R4G, gp32-B) is that despite their drastic differences in ssDNA binding affinity, all retain protein-protein interactions with the dda helicase. These six mutants also contain an intact core (ssDNA binding) domain; however, as indicated in Fig. 1B the core domain of gp32, although powerfully stimulatory, may not be absolutely essential for dda function in DNA synthesis reactions (see “Discussion”).

**Fig. 2.** Effects of gp32 missense and truncation mutant proteins on strand displacement DNA synthesis activity of T4 DNA polymerase holoenzyme in the presence of gp41, gp41/gp59, or dda helicase systems. A, reactions containing 18 µg/ml gp41 and 5 µg/ml gp59. B, reactions containing 18 µg/ml gp41 and 5 µg/ml gp59. C, reactions containing 5 µg/ml dda. In each panel the gp32 species present in a given reaction is indicated below the datum. The concentration of gp32 species was 1.45 µM in each reaction. All other reaction components, concentrations and conditions were as described under “Materials and Methods.” Reactions were run for 10 min and then stopped by trichloroacetic acid precipitation and analyzed for ^32P incorporation as described under “Materials and Methods.” The data in each panel are normalized with respect to the activity observed with wild-type gp32 (~100%).
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Fig. 3. Effects of gp32 missense and truncation mutant proteins on product distribution in strand displacement DNA synthesis reactions containing gp41 (A), gp41/gp59 (B), or dda helicase systems (C). Samples from reactions represented in Fig. 2 were run on an alkaline agarose electrophoretic gel, and the products were visualized by autoradiography as described under “Materials and Methods.” The gp32 species present (1.45 μM) is indicated above each lane.

although this species promotes a healthy (>50% of wt) strand displacement synthesis reaction in the absence of a helicase (Fig. 1A), replication forks so generated appear to be incapable of recruiting gp41 helicase. This observation is consistent with previously published data (23). Fifth, although DNA synthesis levels observed with the R4Q and K3A mutants are only ~20% of those seen with wt gp32 (Fig. 2A), the data in Fig. 3A argue that gp41 helicase acquisition by replication forks may actually be more efficient with these “intermediate” mutants than with the wt and R4K gp32 species under these reaction conditions. This conclusion derives from the fact that a greater proportion of the incorporated label appears in the helicase plus band in the R4Q and K3A reactions (Fig. 3A). A possible explanation for this interesting effect will be presented in a later section.

Product distributions in reactions containing both gp41 and gp59 (Fig. 3B; same reactions as in Fig. 2B) were markedly different from those seen with gp41 alone. Here, none of the reactions displayed the type of bimodal product distribution observed with several of the gp32 species in Fig. 3A. Instead, for reactions in which significant replication occurred, the vast majority of replication products appeared as unresolved, high molecular weight strands, and therefore, appear to be of the helicase plus variety (Fig. 3B). This result suggests that gp59 efficiently delivers gp41 helicase to any nascent fork that has initiated gp32-dependent strand displacement synthesis regardless of which gp32 missense mutant is employed. Nevertheless, there still is a wide range of activities between the different missense mutants. Wild-type and R4K gp32s promoted the strongest reactions; R4Q and K3A mutants promoted strong reactions too, but band intensities were noticeably less intense than with the previous pair, whereas reactions with R4T and R4G gp32s were much weaker still (Fig. 3B). DNA synthesis products observed with the gp32-B and A-domain species appear to be indistinguishable from the reaction with no gp32 (Fig. 3B), indicating that these mutants provide no advantage to strand displacement synthesis, even in the presence of gp59 plus gp41. However, the “background” in these reactions does appear to be higher than that in the equivalent reactions in Fig. 3A, consistent with the slight stimulatory effects of gp59 plus gp41 noted in Fig. 1D. Furthermore, the faint products seen in reactions with no gp32, gp32-B, or A domain appear to concentrate in the area of the unresolved helicase plus band (Fig. 3B), suggesting that the low levels of DNA synthesis here are due to a very small number of forks acquiring helicase in a gp59-dependent manner even in the absence of functional gp32 (12). The gp32-A reaction is unique in Fig. 3B in that its products show a hint of a bimodal distribution. Although overall synthesis levels are depressed with this mutant compared with Fig. 3A (and consistent with data in Figs. 1–2), the product distribution includes a weak but significant band in the helicase plus region as well as a smear of smaller products (Fig. 3B). Thus, paradoxically, although gp59 inhibits overall strand displacement synthesis supported by gp32-A, the data suggest that it simultaneously allows an inefficient gp41 helicase-loading reaction at nascent forks generated in the presence of gp32-A. This idea is supported by earlier data suggesting that gp59 can load gp41 onto ssDNA in the presence of sub-saturating concentrations of gp32-A (14). Once assembled, the movement of helicase plus forks would be impeded by unproductive gp59-gp32-A-ssDNA structures, which may explain the smear pattern in this reaction (Fig. 3B).

In the presence of the dda helicase, strand displacement synthesis reactions with all gp32 species except gp32-A and A-domain generate products that are remarkably similar both in distribution and intensity (Fig. 3C). Even the gp32-B mutant supports the formation of replication products that are indistinguishable in size from those formed with wild-type gp32, except for their slightly lower abundance. These data combine with those in Figs. 1–2 to demonstrate that dda is relatively indifferent to the affinity/cooperativity of the gp32 species present even to the point of promoting an efficient strand displacement synthesis reaction in concert with gp32-B. Note that the dda helicase plus forks in Fig. 3C have broader product distributions than their correspondents in the gp41 and gp59 plus gp41 sets (Fig. 3A and B) due to the lower processivity of dda versus gp41 helicases (6, 7).

The A Domain of gp32 Attenuates gp59-Fork DNA Interactions—Previously we demonstrated that gp59 and gp32 form a unique beaded complex on ssDNA in which the interactions of both proteins with ssDNA appear to be attenuated (39). Formation of the beaded complex requires gp59-gp32 protein-protein interactions mediated via the A domain of gp32. Meanwhile, gel mobility shift data indicated that gp59 binds preferentially to branched DNA structures including forks and cruciforms (17, 18), raising the obvious question of whether the A domain of gp32 also attenuates structure-specific DNA binding activities of gp59. To address this issue we devised a fluorescence assay for gp59-fork DNA interactions, allowing us to study complex formation continuously in solution and in the presence or absence of gp32 A domain. The fluorescent base analog 2-aminopurine (2AP) was placed either in an ssDNA arm of a fork DNA molecule (dDNA1) or in the duplex arm of an otherwise identical fork DNA molecule (dDNA2) as described under “Materials and Methods.”

gp59 binding to 2AP-labeled fork DNA molecules causes a
The forward titration of fDNA with gp59 saturates at a ratio of 1:1 gp59:A domain (Fig. 4), indicating weaker binding to this region of the fork DNA. Indeed, binding monitored via the 2AP-duplex signal should be considered a lower limit since it is not clear that all molecules of gp59 bound per fork DNA molecule, but this has been demonstrated under "Materials and Methods." Titrations and fluorescence measurements were performed as described under "Materials and Methods." F − F<sub>0</sub> denotes the fluorescence signal with background subtracted (background as the fluorescence of free 2AP-labeled DNA). All fluorescence signals were corrected for the effects of dilution, inner filter effects, and intrinsic protein fluorescence as described under "Materials and Methods." Filled diamonds denote data from a titration of fDNA1, containing a single 2AP label in one of the ssDNA arms of the fork DNA. Open circles denote data from a titration of fDNA2, containing a single 2AP label in the duplex arm of fork DNA. Detailed descriptions of fDNA1 and fDNA2, reaction conditions, components, and concentrations are described under "Materials and Methods." SS, single strand; DS, double strand.

The degree of 2AP fluorescence enhancement observed with the fDNA2 molecule at saturation is only ~50% that observed with fDNA1, consistent with the duplex environment of the label in the former. The possibility that 2AP fluorescence enhancement in fDNA2 occurs via gp59-induced unwinding of the duplex region is eliminated by the results of gel mobility shift experiments, which show that fork DNA molecules fDNA1, fDNA2, and their unlabeled equivalents remain base-paired even when saturated with gp59 (data not shown; Refs. 17 and 18). Together, the data in Fig. 4 suggest that gp59-fork DNA interactions may initiate via high affinity interactions with the ssDNA arms and then propagate via lower affinity interactions into the duplex region.

Titrations of gp59-fork DNA complexes with gp32 A domain indicate that A domain destabilizes the complex (Fig. 5), consistent with the idea that gp41 loading only occurs after sufficient gp32-dependent strand displacement DNA synthesis by T4 DNA polymerase holoenzyme requires the tight and cooperative binding of gp32 to the displaced ssDNA (29). Mutations in the B domain of gp32 that decrease the stability of gp32-ssDNA cooperative clusters also dramatically decrease the rate and processivity of strand displacement DNA synthesis (29). The results of this study demonstrate that overall, the same gp32 B domain mutations also compromise the acquisition of gp41 helicase by nascent replication forks, consistent with the idea that gp41 loading only occurs after sufficient gp32-dependent strand displacement synthesis has occurred (1–2, 4–5). The helicase-loading protein gp59 strongly enhances gp41 helicase acquisition by replication forks in the presence of wild-type gp32 and also promotes a low level of gp41 helicase acquisition by partial replisomes formed in the absence of gp32 (Ref. 12 and this study). Nevertheless, gp59 provides at best a modest improvement in gp41 helicase acquisition by replisomes containing B domain mutants of gp32. The data indicate that efficient gp59-promoted helicase loading requires stable cluster formation by gp32 on lagging strand ssDNA.

Previous studies examined the binding of B domain mutant gp32s to the model single-stranded homopolymer, poly(A), at equilibrium as well as the kinetics of binolecular association and salt-induced dissociation of these gp32s from poly(A) under the same solution conditions (24, 27, 28, 43). These studies reveal that R4K, R4Q, R4T, and K3A gp32s bind with high cooperativity and show relatively small diminutions (~2–80-fold) in binding affinity to poly(A) at equilibrium (Table 1); in

Fig. 4. Forward titrations of 2-aminopurine-labeled fDNA molecules with gp59 protein, monitored via the enhancement of 2AP fluorescence. Titrations and fluorescence measurements were performed as described under "Materials and Methods." F − F<sub>0</sub> denotes fluorescence signal with background subtracted (background as the fluorescence of free 2AP-labeled DNA). All fluorescence signals were corrected for the effects of dilution, inner filter effects, and intrinsic protein fluorescence as described under "Materials and Methods." Filled diamonds denote data from a titration of fDNA1, containing a single 2AP label in one of the ssDNA arms of the fork DNA. Open circles denote data from a titration of fDNA2, containing a single 2AP label in the duplex arm of fork DNA. Detailed descriptions of fDNA1 and fDNA2, reaction conditions, components, and concentrations are described under "Materials and Methods." SS, single strand; DS, double strand.

Fig. 5. Titrations of preformed gp59-2AP-fDNA complexes with the A domain fragment of gp32. F − F<sub>0</sub> denotes fluorescence signal with background subtracted (background as the fluorescence of free 2AP-labeled DNA). All fluorescence signals were corrected for the effects of dilution, inner filter effects, and intrinsic protein fluorescence as described under "Materials and Methods." Filled diamonds denote data from a titration of A domain fragment into preformed gp59-fDNA1 complex, containing a single 2AP label in one of the ssDNA arms of the fork DNA. Open circles denote data from a titration of A domain into preformed gp59-fDNA2 complex, containing a single 2AP label in the duplex arm of fork DNA. The concentrations of gp59 and DNA were 2 and 12 μM (nucleotides), respectively, in each experiment. All other reaction conditions, components and concentrations were as described under "Materials and Methods." SS, single strand; DS, double strand.
stable clusters on ssDNA and in combination with gp59 allows high levels of gp41 helicase acquisition by replication forks. In contrast, the K3A and R4Q mutants form saturated but kinetically labile (higher $k_e$) clusters on ssDNA (Table I) and correspondingly facilitate relatively low levels of gp59-dependent helicase acquisition. By the same logic, the severe defects of R4T, R4G, and gp32-B mutants in gp59-dependent helicase acquisition may be attributed to their drastically reduced kinetic lifetimes on ssDNA.

**Properties of gp59-Fork DNA Complexes and Their Modulation by gp32**—Quantitative analysis of gp59-fork DNA interactions by means of a 2-aminopurine fluorescence enhancement assay demonstrates that gp59 binds to fDNA and ssDNA molecules with the same apparent binding site size (Fig. 4; Ref. 42). A single fDNA molecule is bound and saturated by multiple molecules of gp59, which appear to occupy both ss- and dsDNA arms of the fork DNA (Fig. 4). gp59 affinity for fDNA and other branched DNA species exceeds its affinity for unbranched ssDNA or dsDNA (17–18); therefore, it seems likely that a fork may serve as a nucleation point for the assembly of larger gp59-fork DNA complexes. The initial localization of gp59 at a DNA fork structure could serve to orient and/or control the phasing of the larger complex.

Destabilization of gp59-fDNA by the A domain of gp32 (Fig. 5) suggests that gp32-induced destabilization of gp59-fDNA interactions is a global phenomenon. Similar destabilization of gp59-ssDNA interactions by gp32 A domain has been documented (39). A variety of evidence indicates that gp32 and gp59 continue to co-occupy DNA even while mutually weakening each other’s observed DNA binding affinities (13–14, 18, 26, 39). One possible outcome of this destabilization is an enhanced mobility of sequence-nonspecific gp59-gp32-fDNA complexes via sliding; that is, the lateral translocation of a complex along DNA without complete release of the DNA. gp59 and gp32 may form structures on DNA (e.g. the beaded complexes observed on ssDNA alone or the condensed structures seen at replication forks; Refs. 39 and 45) that are strongly linked to the DNA in a topological sense yet are designed to slide. Sliding could allow lagging strand ssDNA to spool through the complex as the ssDNA is generated during strand displacement synthesis (see Fig. 6). A similar mechanism has been proposed by Griffith, Nossal, and co-workers (45).

**A ‘Slide and Lock’ Model for gp59/gp32-mediated Loading of gp41 Helicase at DNA Replication Forks**—Our working model of gp41 helicase loading is shown in Fig. 6 and invokes the following features based on data presented in this study and elsewhere (13–14, 17–18, 26, 39, 45–47). In step 1 a nascent strand displacement replication fork incorporates a critical cluster of gp59 and gp32 molecules co-occupying the lagging strand ssDNA. gp59 affinity for branched DNA may help to nucleate cooperative binding and to properly orient the cluster adjacent to the core replisome. In step 2 the gp59/gp32 cluster remodels itself into a condensed particle topologically bound to the DNA, which we will call the helicase loading complex (HLC). The model supposes a specific stoichiometry for the HLC, which is six molecules each of gp59 and gp32, around which ssDNA is spoiled. The precise stoichiometry of the HLC is unknown; however, there is evidence that gp59 can hexameterize under certain conditions (47) and that each gp59 molecule interacts with one gp32 molecule (26). In step 3 the HLC acts as a spool around which the ssDNA thread slides. Sliding effectively mobilizes the HLC, allowing it to remain associated with the core replisome as it moves along the template. In step 4 the HLC recruits gp41 and locks it into the replisome by triggering the assembly of the ring-hexamer form of this helicase onto the lagging
strand ssDNA. gp59 may promote this reaction by inducing a high affinity nucleoside triphosphate binding conformation of gp41 helicase, the conformation necessary for gp41 hexamerization, as reported previously (13). Helicase recruitment may be accompanied by release of gp32 from the HLC, although this remains to be tested experimentally. In step 5 gp41 in turn recruits gp61 primase, reconstituting the T4 primosome and allowing lagging strand DNA synthesis to commence. gp59 may remain bound to gp41 through this step and through subsequent cycles of the replisome; however, this remains to be tested. Other testable features of this model such as the ability of the HLC to slide and translocate with the core replisome (step 3) are currently under investigation.

FIG. 6. Slide and lock model for gp59/gp32-mediated loading of gp41 helicase at DNA replication forks. A mobile helicase loading complex comprised of gp59 and gp32 proteins co-localized on ssDNA recruits gp41 helicase to the lagging strand of the replication fork. See “Discussion” for details.
dda-gp32 Protein-Protein Interactions Are Critical for dda-driven Strand Displacement DNA Synthesis—Early in vitro studies of dda helicase activity in the T4 replication system revealed a close relationship between dda and gp32; dda was shown to stimulate strand displacement DNA synthesis by DNA polymerase holoenzyme in the presence of low concentrations of gp32 but not in its absence (6–7). dda interacts tightly with gp32 via contacts with the A domain of the latter (22). Our studies demonstrate the central importance of this protein-protein interaction in facilitating dda-driven strand displacement DNA synthesis reactions. Furthermore and most surprisingly, our data show that dda/gp32 function in strand displacement synthesis is insensitive to mutations affecting the DNA binding affinity and cooperativity of gp32 (Figs. 1–3). The data confirm that significant gp32-mediated strand displacement synthesis is not a prerequisite for dda helicase loading at replication forks. Instead, dda-gp32 protein-protein interactions are of paramount importance in loading and activating the helicase.

The effects of the gp32 A-domain fragment on dda-driven replication are intriguing; apparently this fragment can allow a very low level of dda-driven strand displacement synthesis even though the A domain is devoid of ssDNA binding activity due to the absence of the core domain (Fig. 1). Nevertheless reactions with A domain are seriously deficient compared even to other gp32 mutants such as gp32-B, indicating that the gp32 core domain does play a major role in promoting dda replication functions. Two possibilities (not mutually exclusive) for the role of core domain are the following. 1) dda/gp32 stimulation of strand displacement DNA synthesis strongly requires at least some residual ssDNA binding activity on the part of the gp32 species employed; such residual activity would have to reside in gp32 core domain. 2) In addition to contacting residues in the gp32 A domain, dda may also require contacts within the core domain of gp32 in order for the full replication functionality of dda/gp32 to be expressed.

A “Mixed Oligomer” Model for dda/gp32-driven Strand Displacement DNA Synthesis—For activity in strand displacement DNA synthesis, dda requires protein-protein interactions with gp32 but does not require stable protein-ssDNA interactions from its gp32 partner. The simplest explanation is that gp32-dda interactions induce an active form of the helicase. dda alone does not form stable quaternary structures in solution (48), and there is strong evidence that monomeric dda can unwind model DNA substrates (49). One possibility is that an oligomeric form of dda is required for strand displacement synthesis reactions and that gp32 binding triggers this oligomerization. Such a model is shown schematically in Fig. 7, in which a mixed oligomer of dda and gp32 subunits is portrayed as the actual translocating species at a replication fork. A second possibility (not shown) is that dda continues to function as a monomer but in association with one or more subunits of gp32 serving as cofactors to alter the helicase properties of the dda molecule. Distinguishing between these and other possibilities will require further knowledge of the stoichiometry and structure of dda-gp32 complexes, which are under investigation.

The Functional Relationship of dda and gp32 Resembles That of a Herpesvirus Helicase/ ssDNA-binding Protein System—The dependence of dda helicase replication functions on protein-protein interactions with gp32 appears to parallel the relationship between the UL9 helicase and ICP8 ssDNA-binding protein of herpes simplex virus 1. UL9 and ICP8 interact specifically, resulting in a dramatic stimulation of UL9 functions including its DNA helicase and DNA-dependent ATPase activities (50). The stimulatory effects of ICP8 are apparently conferred solely via protein-protein interactions with the UL9 helicase, since stimulation is observed even after blockage of the ssDNA binding site of ICP8 by covalent modification (50). Thus, protein-protein interactions between ICP8 and UL9 confer new properties upon the helicase, which is the case for gp32-dda interactions. The UL9-ICP8 complex has been implicated in the unwinding of HSV-1 origins of replication (51, 52), suggesting a mechanism of action similar to the one we have proposed for dda-gp32 complex translocation at T4 replication forks (Fig. 7).

In summary, the gp32 ssDNA-binding protein plays important dual roles in DNA helicase acquisition by T4 replication forks initiating strand displacement DNA synthesis. Lacking direct interactions with gp41, gp32 must work indirectly to promote acquisition of this processive helicase. It does so in two ways; first, by promoting the initial strand displacement reaction to generate a lattice for helicase recruitment and, second, with gp59, forming a helicase loading complex on this lattice, which efficiently targets gp41 assembly onto the lagging strand of the replisome. gp32 plays an altogether different role in recruitment of the non-processive dda helicase. Here, gp32 and dda interact directly, and the interaction confers new properties upon the helicase that allow it to initiate strand displacement synthesis de novo in concert with DNA polymerase holoenzyme. Further studies of gp32 functions in gp41 and dda helicase transactions will continue to provide important new insights into the dynamics of ssDNA-binding protein functions in DNA replication systems.

Acknowledgments—We thank Drs. Nancy Nossal and Deborah Hinton for providing gp41- and gp59-overproducing strains as well as Drs. J. Michael Hurley and Larry Gold for providing an overproducer of the A-domain fragment of gp32.

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Dual Functions of Single-stranded DNA-binding Protein in Helicase Loading at the Bacteriophage T4 DNA Replication Fork
Yujie Ma, Tongsheng Wang, Jana L. Villemain, David P. Giedroc and Scott W. Morrical

doi: 10.1074/jbc.M311738200 originally published online February 9, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M311738200

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