Published in final edited form as:

DNA Repair (Amst). 2015 January ; 25: 41–53. doi:10.1016/j.dnarep.2014.10.002.

Regulation of the Bacteriophage T4 Dda Helicase by Gp32 Single-stranded DNA-Binding Protein

Christian S. Jordan¹ and Scott W. Morrical^{1,*}

¹Department of Biochemistry, University of Vermont College of Medicine, Burlington, VT, United States of America, 05405

Abstract

Dda, one of three helicases encoded by bacteriophage T4, has been well-characterized biochemically but its biological role remains unclear. It is thought to be involved in origin dependent DNA replication, recombination-dependent replication, anti-recombination, and recombination repair. The Gp32 protein of bacteriophage T4 plays critical roles in DNA replication, recombination, and repair by coordinating protein components of the replication fork and by stabilizing ssDNA. Previous work demonstrated that stimulation of DNA synthesis by Dda helicase appears to require direct Gp32-Dda protein-protein interactions and that Gp32 and Dda form a tight complex in the absence of ssDNA. Here we characterize the effects of Gp32-Dda physical and functional interactions through changes in the duplex DNA unwinding and ATPase activities of Dda helicase in the presence of different variants of Gp32 and different DNA repair and replication intermediate structures. Results show that Gp32-Dda interactions can be enhancing or inhibitory, depending on the Gp32 domain seen by Dda. Protein-protein interactions with Gp32 stimulate the unwinding activity of Dda, an effect associated with increased turnover of ATP, suggesting a higher rate of ATPase-driven translocation. Dda-Gp32 interactions also promote the unwinding of DNA substrates at higher salt concentrations and in the presence of substrate-bound DNA polymerase. Conversely, the formation of Gp32 clusters on ssDNA can inhibit unwinding, suggesting that Gp32-ssDNA formation sterically regulates which portions of replication and recombination intermediates are accessible for processing by Dda helicase. The data suggest a mechanism of replication fork restart in which Gp32 promotes Dda activity in template switching while preventing premature fork progression.

Graphical abstract

*To whom correspondence should be addressed: Scott W. Morrical, Department of Biochemistry, University of Vermont College of Medicine, B407 Given Bldg., 89 Beaumont Avenue, Burlington, VT 05405. Tel.: 802-656-8260. Fax: 802-656-8229. ; Email: smorrlica@uvm.edu

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

helicase; single-stranded DNA binding protein; recombination; replication; repair

1. Introduction

The bacteriophage T4 replisome is a highly tractable system for studying the protein-protein and protein-DNA interactions that mediate DNA replication, recombination, and repair. DNA replication can be reconstituted *in vitro* using purified recombinant T4 proteins, with rate and fidelity comparable to *in vivo* reactions (Nossal, 1994). T4-encoded DNA helicases control the initiation and propagation of replication forks, and also play central roles in the coordination of DNA synthesis with recombination and repair activities (Kreuzer and Brister, 2010; Liu and Morrical, 2010; Maher et al., 2011).

T4 encodes three DNA helicases, Gp41, Dda, and UvsW, two of which (Gp41 and Dda) appear to participate directly in the initiation and/or propagation of DNA replication forks. Gp41 is the major replicative helicase in T4 (Nossal, 1994). A member of helicase Superfamily 4 (SF4), Gp41 is hexameric and highly processive. Gp41 is assembled onto replication forks by its loading protein, Gp59, and it interacts with Gp61 primase to reconstitute the T4 primosome during lagging strand DNA synthesis. It is required for T4 DNA replication in all *E. coli* host strains (Gauss et al., 1983). In contrast, the Dda helicase is non-essential for T4 DNA replication in most *E. coli* strains, but it is required for replication in an *E. coli optA⁻* strain (Gauss et al., 1983). T4 *dda⁻* strains exhibit a delay in the onset of replication, suggesting that this helicase is involved in the early, origin-dependent pathway of replication initiation, in a manner that is distinct from that of Gp41 helicase (Gauss et al., 1994). Furthermore, null mutations of Dda are synthetically lethal in a T4 *59⁻* background, indicating that Dda is required for T4 DNA replication when loading of the replicative Gp41 helicase is compromised (Gauss et al., 1994). These findings demonstrate that the Dda helicase performs significant replication functions during T4

infection in *E. coli* host cells, and that these functions are not wholly redundant with the replication functions of Gp41 helicase.

Dda, a member of Superfamily 1 (SF1), is a 49.9-kDa monomeric helicase, with 5'→3' polarity and low to moderate processivity (Nanduri et al., 2002; Raney and Benkovic, 1995). The biochemical properties of Dda are consistent with a helicase that promotes diverse transactions in DNA replication, recombination, and repair. Dda stimulates *in vitro* reactions including: strand displacement DNA synthesis catalyzed by T4 DNA polymerase (Jongeneel, Bedinger et al., 1984; Ma et al., 2004); replication fork progression past a template-bound RNA polymerase molecule (Bedinger et al., 1983); DNA branch migration promoted by T4 UvsX recombinase (Kodadek and Alberts, 1987); recombination-dependent DNA synthesis and amplification (Formosa and Alberts, 1986; Morrical and Alberts, 1990; Morrical et al., 1991); and error-free translesion DNA synthesis by template-switching (Bleuit et al., 2001; Kadyrov and Drake, 2004). Dda binds tightly and specifically to Gp32, the T4 ssDNA-binding protein, and to UvsX, the T4 recombinase (Formosa and Alberts, 1984; Hacker and Alberts, 1992; Hurley et al., 1993). Data suggest that the ability of Dda to stimulate strand displacement DNA synthesis depends on protein-protein interactions between Gp32 and Dda, and does not depend on Gp32-ssDNA interactions (Ma et al., 2004). These and other findings suggest a model in which Dda-Gp32 physical and functional interactions are critical in organizing, coordinating and regulating the helicase to promote strand displacement DNA synthesis at the T4 replication fork.

Gp32 is a highly cooperative ($\omega \approx 1000$) and nonspecific single-stranded DNA-binding protein (SSB) essential for T4 replication (Alberts, 1987; Nossal, 1994). It is a 34-kDa, 301-amino acid zinc metalloprotein with three functional domains that can be defined by partial proteolysis: the N-terminal basic (B) domain (residues 1–21), the core domain (residues 22–254), and the C-terminal acidic (A) domain (255–301) (Williams et al., 1979). The core domain contains the ssDNA-binding site (Spicer et al., 1979) and the A domain mediates critical interactions with heterologous replisome proteins such as Dda and Gp43 (Burke et al., 1980; Krassa et al., 1991). The B domain mediates self-association of Gp32 monomers in solution and cooperativity of ssDNA binding (Giedroc et al., 1990). Monomers of Gp32 associate on ssDNA in clusters, which is mediated by highly basic amino acids in the B domain (Casas-Finet et al., 1992; Churchill and Travers, 1991).

Gp32-B is a 30.8-kDa truncated version of Gp32 that lacks the B domain. Gp32-B is essentially devoid of self-association (dimerizes weakly at very high concentration, $K_a \approx 10 \text{ M}^{-1}$), exhibits no cooperativity ($\omega = 1$), and has approximately 10^4 -fold lower ssDNA-binding affinity compared with wild-type (Giedroc et al., 1990; Ma et al., 2004; Villemain et al., 2000; Xu et al., 2001). Gp32-B is therefore a useful reagent that separates the DNA-binding component from the protein-protein interaction functionality of Gp32, and thus allows direct and specific testing of the effects of Gp32-Dda protein-protein interactions on helicase activity.

Dda is among the proteins that interact most strongly with Gp32. However, the implications of this interaction have been difficult to characterize. Dda is quantitatively bound to Gp32 during chromatography over Gp32–agarose affinity columns and elutes at salt concentrations

ranging from 0.6 to 2.0 M, one of the highest of all major bound protein species (Formosa et al., 1983; Hurley et al., 1993; Jongeneel, Formosa et al., 1984). *In vitro* ATPase assays have established that Gp32-covered ssDNA does not stimulate Dda ATPase activity and that Gp32 acts as a competitive inhibitor of Dda by making ssDNA binding sites inaccessible to Dda. This suggests that *in vivo* the site of action of Dda may be on ssDNA not covered by Gp32 (Jongeneel, Bedinger et al., 1984). However, studies have also shown a positive interaction between Dda and Gp32 *in vitro*. Dda stimulates strand displacement DNA synthesis only in the presence of Gp32 (Jongeneel, Bedinger et al., 1984). This stimulation requires direct Gp32-Dda protein-protein interactions since it is relatively unaffected by mutations that destabilize the ssDNA-binding component of Gp32 (Ma et al., 2004). Even the extremely ssDNA-binding deficient truncation mutant, Gp32-B, facilitates the stimulation of strand displacement DNA synthesis by Dda, even though Gp32-B itself fails to stimulate DNA synthesis by T4 DNA polymerase holoenzyme (Ma et al., 2004; Villemain et al., 2000).

DNA helicases typically assemble and translocate on ssDNA where they are likely to encounter bound SSBs, which may modulate helicase activity. In this paper we present our findings on how physical and functional interactions with Gp32 modulate the DNA binding, unwinding, and ATPase activities of Dda helicase. We report here the enhancement of Dda unwinding and ATPase activities by the non-cooperative Gp32-B on varied DNA substrates, and the inhibition of Dda unwinding activity towards certain DNA substrates by full-length Gp32. The results suggest that Gp32 plays both positive and negative roles in regulating Dda helicase activity, and that both effects may influence the outcome of complex DNA transactions such as replication fork restart.

2. Materials and Methods

2.1. Reagents and resins

Buffers and solutions were prepared with purified, deionized water. Analytic grade chemicals and reagents were purchased from Sigma unless otherwise indicated. 1× TBE buffer contained 100 mM Tris, 90 mM boric acid, 1 mM EDTA. ssDNA-cellulose resin was prepared as described (Alberts and Herrick, 1971). DEAE and hydroxyapatite resins were purchased from BioRad. ATP was purchased from USB. γ -[^{32}P]-labeled ATP was purchased from Perkin Elmer. 6× protein-loading dye was purchased from Promega. Concentrations of chemical reagents, proteins, and nucleic acids given in the text are final concentrations.

2.2. Nucleic acids and polyamide nucleic acids

All DNA concentrations are expressed in moles per liter of oligonucleotide molecules. Polyacrylamide gel electrophoresis- and high-performance liquid chromatography-purified mixed sequence oligonucleotides were purchased from IDT. The concentration of each oligonucleotide was calculated using absorbance measurement at 260 nm and extinction coefficients provided by the manufacturer. Certain oligonucleotides were 5'-[^{32}P]-labeled using γ -[^{32}P]-labeled ATP and T4 polynucleotide kinase (NEB) according to manufacturer's instructions. The kinase was heat-inactivated at 95 °C for 5 min following the labeling reaction. Polyamide nucleic acid (PNA) was prepared by BioSynthesis, concentrations were calculated using absorbance measurement at 260 nm and extinction coefficients provided by

the manufacturer, and was heated to 65 °C for 15 min prior to use. PNA is not a substrate for Dda helicase (Tackett et al., 2002). The sequences of all oligonucleotides used are listed in Table 1.

2.3. Overexpression and purification of bacteriophage T4 proteins

Recombinant Dda and Gp32-B proteins were overexpressed and purified from *Escherichia coli* as described (Morris et al., 2001; Giedroc et al., 1990). Recombinant Gp32 protein was purified according to the protocol of Ishmael et al. (2001), with the following modifications: the *E. Coli* NCZYM medium was replaced with LB; a Mono Q (GE Healthcare) chromatography step was added after the chitin chromatography step; and the Gp32 storage buffer was modified to be 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.1 mM EDTA, 1 mM β -mercaptoethanol, and 10% glycerol. Recombinant Gp43-D219A, an exonuclease-deficient Gp43 polymerase mutant, was a gift from Dr. Linda Reha-Krantz (University of Alberta). Concentrations of protein stock solutions were calculated from extinction coefficients at 280 nm: 41,306 M⁻¹ cm⁻¹ for Gp32 and Gp32-B, 59,010 M⁻¹ cm⁻¹ for Dda, and 136,030 M⁻¹ cm⁻¹ for Gp43-D219A, all calculated based on the amino acid sequence (Gill and von Hippel, 1989). All T4 protein stock solutions used in this study were nuclease-free according to published criteria (Morrical et al., 1994). All proteins were determined to be 95% pure by SDS-PAGE analysis.

2.4. DNA electrophoretic mobility shift assays

DNA electrophoretic mobility assays were carried out to compare the protein-ssDNA complexes formed by Gp32, Gp32-B, and Dda alone and in combination. Reactions contained 175 nM (10.5 μ M nucleotides) of 5'-[³²P]-labeled oligo F (mixed-sequence 60-mer) at 25 °C in 20 mM Tris-acetate (pH 7.8), 90 mM potassium acetate, 10 mM magnesium acetate in a total reaction volume of 20 μ l. Reactions were initiated by the addition of Gp32, Gp32-B, and/or Dda to a final concentration of 1.5 μ M to ensure saturation of the ssDNA by Gp32, which has a binding site size of 7 nucleotide residues per protomer (Kowalczykowski, et al., 1986). Reactions were incubated for ten minutes at 25 °C and prepared for electrophoresis by the addition of non-denaturing gel loading buffer (0.1% bromophenol blue, 0.1% xylene cyanol in 6% glycerol). Protein-DNA complexes were separated from unbound ssDNA by electrophoresis in a 6% non-denaturing polyacrylamide gel in 1 $\times$ TBE buffer. Gels were stained with Coomassie Blue to visualize protein; and subsequently ssDNA was visualized by phosphorimaging using a BioRad Molecular Imager FX.

2.5. Protein electrophoretic mobility shift assays

Electrophoretic mobility shift assays were carried out to compare the effects of ssDNA (oligo D) and PNA oligonucleotides of complementary sequences and identical lengths (27 bases) on the electrophoretic mobility of Gp32 and Gp32-B proteins on a 6% non-denaturing polyacrylamide gel. 175 nM (10.5 μ M nucleotides) PNA or oligo D and 1.5 μ M Gp32 or Gp32-B were incubated at 25 °C in 20 mM Tris-acetate (pH 7.8), 90 mM potassium acetate, 10 mM magnesium acetate. After ten minutes, non-denaturing gel loading buffer (0.1% bromophenol blue, 0.1% xylene cyanol in 6% glycerol) was added, samples were loaded on

a non-denaturing 6% polyacrylamide gel in 1× TBE buffer, electrophoresed at low voltage (45 V), and proteins were visualized by staining with Coomassie Blue.

2.6. Construction of helicase substrates for unwinding assays

Construction of helicase substrates was based on a previously published protocol (Jones et al., 2004). Oligonucleotide compositions of the several different helicase substrates are shown in Table 1 (substrate structures are shown schematically in Fig. 4). Tailed duplex substrate was prepared by addition of 1.2 equivalents of unlabeled oligo A to the 5'-[³²P]-labeled oligo B in 10 mM Tris-HCl (pH 8.0) and 200 mM NaCl, heating to 95 °C for 5 min, and slowly cooling to room temperature over a period of three hours. Gapped duplex substrates were prepared under the same conditions, mixing one equivalent of oligo D with 1.2 equivalents of a 5'-[³²P]-labeled version of either oligo C, K, L, M, or N (for the 6-, 4-, 2-, 1-, or 0-nucleotide gap lengths, respectively).

Pseudofork substrate was made by annealing 5'-[³²P]-labeled oligo E with 1.5 equivalents of unlabeled oligo F in 10 mM Tris-HCl (pH 8.0) and 200 mM NaCl and heating to 95 °C for 5 minutes, followed by 60 min at 68 °C. The heated DNA was allowed to slowly cool to room temperature over a period of three hours and then purified by centrifugation through a Probe Quant G-50 spin column (Amersham Biosciences).

Fork and inverted fork mimic substrates were made by annealing 5'-[³²P]-labeled oligo E with unlabeled oligo F and either oligo G (for the inverted fork mimic) or H (for the fork mimic) at a ratio of 1:1.5:10 E:F:(G or H) under the same conditions used to anneal the pseudofork DNA substrate. Fork and inverted fork substrates were purified by electroelution from 6% polyacrylamide gels in 1× TBE buffer using an electroelution device (Owl Scientific). Following electroelution, substrates were concentrated using Microcon YM10 centrifuge filter devices (Millipore).

Occluded and inverted occluded fork mimics substrates were made by annealing 5'-[³²P]-labeled oligo E with unlabeled oligos F, H, and I (for the occluded fork mimic) or oligos F, G, and J (for the inverted occluded fork mimic) at a ratio of 1:1.5:10:10 (E:F:H:I or E:F:G:J) under the same conditions used to anneal the pseudofork DNA substrate. Occluded and inverted occluded fork mimics were purified by the same procedure used for fork and inverted fork mimics.

2.7. DNA unwinding assays

DNA unwinding assays were performed as described (Nanduri et al., 2001). 1 nM Dda was first incubated with 100 nM DNA substrates for two minutes, then with 100 nM Gp43-D219A, if present, for two minutes, and finally with indicated concentrations of Gp32 or Gp32-B for two minutes at 25 °C in reaction buffer containing 25 mM HEPES (pH 7.5), 10 mM potassium acetate, 0.1 mM EDTA, 2 mM β-mercaptoethanol, 100 µg/ml BSA, 10 U/ml pyruvate kinase, and 4 mM phosphoenolpyruvate, and the reaction was initiated by the simultaneous addition of 4.6 mM ATP, 5 mM magnesium acetate, and 300 nM PNA. Aliquots were withdrawn at various times and added to an equal volume of 200 mM EDTA to quench the reaction. Non-denaturing gel loading buffer (0.1% bromophenol blue, 0.1% xylene cyanol in 6% glycerol) was added to each sample and each sample was analyzed on

native polyacrylamide gels ranging from 4% to 20%, depending on product size, visualized by exposure on a PhosphorImaging K screen, quantified in a BioRad Molecular Imaging FX, and analyzed by densitometry using BioRad Quantity One software. The fraction of unwound product was determined by calculating the ratio of single-stranded product to the total density per lane, as described (Amaratunga and Lohman, 1993).

2.8. ATPase assay

ATPase assays were based on published spectrophotometric-coupled assays (Morrical et al., 1994). For all ATPase assays a Varian Cary Bio 50 UV-visible spectrophotometer was used. The buffer used for ATPase reactions comprised 25 mM Tris-acetate, pH 7.5, 150 mM potassium acetate, and 10 mM magnesium acetate. Components were incubated in a cuvette in a total volume of 150 μ l at 25 °C: Either 11 or 22 nM Dda, plus variable concentrations of DNA oligonucleotides and Gp32-B, depending on the experiment. Each reaction was initiated by the addition of 4.5 mM ATP. Absorbance was monitored at 340 nm over a time course of 10 minutes following ATP addition. The rate of ATP hydrolysis was calculated by converting NADH absorbance to NADH concentration using an extinction coefficient of NADH at 340 nm of 6,200 M⁻¹ cm⁻¹ and plotting NADH concentration versus time. Rates of ATP hydrolysis were determined by calculating the slope at the linear portion of each absorbance scan. The decrease in concentration of NADH (μ moles L⁻¹ s⁻¹) is equivalent to the concentration of ADP released (μ moles L⁻¹ s⁻¹) by Dda, and thus the rate of ATP hydrolysis.

2.9. Statistical analyses

Error bars represent the standard error of the mean. Statistical analyses were performed using GraphPad Prism software. Significance was assessed at the p = 0.05 level.

3. Results

3.1. Dda forms similar tripartite complexes with ssDNA and either Gp32 or Gp32-B

Native gel electrophoretic mobility shift assays (Fig. 1) demonstrate that Dda forms complexes with either Gp32 or Gp32-B, both in the presence and absence of 5'-[³²P]-labeled ssDNA. Experiments were performed in the absence of ATP to prevent Dda translocation on and release of ssDNA. As shown in Fig. 1A, Gp32 alone (Fig. 1A, lane 1) migrates as a diffuse band with relatively low apparent molecular weight. The smearing of this band is likely due to the formation of small mixed oligomers of Gp32 under native conditions (Carroll et al., 1975). The mobility of the Gp32 band appears to be shifted upon addition of Dda (Fig. 1A, lane 2), while Dda alone migrates as a diffuse band at relatively high apparent molecular weight (Fig. 1A, lane 9). This result is consistent with Gp32-Dda interactions occurring in the absence of ssDNA. On the native gel, ssDNA and Gp32 co-migrate as a uniform band with a relatively high apparent molecular weight (Fig. 1A, lane 3) that is quantitatively supershifted upon addition of Dda (Fig. 1A, lane 4). The ssDNA that is present in the Gp32-ssDNA complex is also quantitatively supershifted upon addition of Dda (Fig. 1B, lanes 3–4). In the absence of Gp32, the addition of ssDNA causes Dda to migrate as a more uniform band with slightly higher apparent molecular weight than is seen with Dda alone (Fig. 1A, lanes 9–10). The band contains ssDNA as shown in Fig. 1B, lane 10.

The electrophoretic mobility of the Dda-ssDNA complex (Fig. 1A–B, lane 10) is distinct from that of the Dda-Gp32-ssDNA complex (Fig. 1A–B, lane 4). Together, the native gel data in Fig. 1 indicate that Dda, Gp32, and ssDNA form a stable tripartite complex in the absence of ATP.

On the native gel, Gp32-B alone (Fig. 1A, lane 5) migrates at a uniform apparent molecular weight due to its monomeric (non-cooperative) nature and appears to undergo a mobility shift in the presence of Dda (Fig. 1A, lane 6). A small fraction of Gp32-B migrates at a slightly higher apparent molecular weight in the presence of ssDNA (Fig. 1A, lane 7), consistent with the very weak and non-cooperative interactions of Gp32-B with ssDNA (Giedroc et al., 1990). Both Gp32-B (Fig. 1A, lane 8) and ssDNA (Fig. 1B, lane 8) bands are supershifted by the addition of Dda. The electrophoretic mobility of ssDNA in the Dda/Gp32-B/ssDNA mixture is distinct from that seen in the Dda-ssDNA complex (Fig. 1B, lanes 8 and 10), and similar to that seen in the Dda-Gp32-ssDNA complex (Fig. 1B, lane 4). These observations indicate that Dda, Gp32-B, and ssDNA form a tripartite complex similar to the Dda-Gp32-ssDNA complex in the absence of ATP.

3.2. Gp32 and Gp32-B do not bind polyamide nucleic acid (PNA)

Gp32 and Gp32-B could interfere with helicase assays by binding to the ssDNA trap that is used to prevent re-annealing of the unwound products. To guard against this possibility we employed a polyamide nucleic acid (PNA) trap that can anneal via base pairing to the ssDNA product but lacks a negatively charged sugar-phosphate backbone and therefore does not bind to Gp32 or Gp32-B. To confirm the lack of binding, we performed the EMSA experiment shown in Fig. 2. We compared the effects of a PNA molecule 27 bases in length, and of a ssDNA oligonucleotide of the same length and complementary sequence, on the electrophoretic mobility of Gp32 and Gp32-B in a 6% non-denaturing polyacrylamide gel. The addition of ssDNA caused a significant decrease in the electrophoretic mobility of Gp32 (Fig. 2, lanes 1–4), whereas PNA had no effect on the mobility of Gp32 (Fig. 2, lanes 1–3). The electrophoretic mobility of Gp32-B was not affected by either PNA or ssDNA (Fig. 2, lanes 5–8). These data indicate that Gp32 and Gp32-B do not bind PNA, a conclusion that is supported by fluorescence data (unpublished results). The data in Fig. 2, in addition to published data showing Dda does not bind PNA (Nanduri et al., 2001), make PNA an ideal trap for helicase DNA unwinding assays.

3.3. Effect of Gp32 and Gp32-B on Dda DNA unwinding activity

DNA unwinding assays, an example of which is depicted schematically in Figure 3A, were conducted using heteroduplex DNA substrates of different structures and varying ssDNA lengths. 5'-[³²P]-labeled substrates were designed so that unwinding catalyzed by Dda helicase and trapping by PNA would generate a labeled product that can be separated from the substrate by non-denaturing PAGE, allowing the reaction to be quantified by phosphorimaging. Figure 3A illustrates this principle for a substrate that mimics a replication fork. The data in Figure 3B shows a time course for the unwinding of the fork mimic by Dda helicase. Quantitative analysis of this data is presented in Figure 4G, below.

Experiments in Figure 4A–G were designed to investigate the possibility that Gp32 and Gp32-B have differential effects on Dda DNA unwinding activity in the context of different DNA structures, which are shown schematically in each panel of Figure 4. The structures model a number of different intermediates that Dda helicase might encounter during DNA replication/recombination/repair. The substrates tested included a tailed duplex (Fig. 4A), gapped duplex (Fig. 4B), pseudo-fork (Fig. 4C), inverted fork mimic (Fig. 4D), fork mimic (Fig. 4E), occluded fork mimic (Fig. 4F), and inverted occluded fork mimic (Fig. 4G). ssDNA gaps, when present, were 6 nucleotides in length in all experiments in Figure 4. Reactions with each substrate contained a constant amount of Dda, and, when present, a constant amount of either Gp32 or Gp32-B. For all substrates, rates of unwinding were negligible in the presence of Gp32 alone or Gp32-B alone (data not shown). The forked substrates examined in Figure 4C–F are stable in the presence of Gp32 (Jones et al., 2004; Branagan et al., 2012, 2014), due to the fact that Gp32 is kinetically blocked from melting native duplex DNA (Jensen et al., 1976). With all substrates, the observed rates of Dda-catalyzed DNA unwinding decreased in the presence of Gp32, and, in all substrates except the simple 12:24 tailed duplex (Fig. 4A), the rates of Dda-catalyzed unwinding increased in the presence of Gp32-B (Fig. 4B–G). Dda helicase unwinds the 12:24 tailed duplex substrate very efficiently and the rate of unwinding is not increased by Gp32-B at any concentration tested (data not shown).

DNA unwinding assays were performed with substrates designed to mimic features of DNA replication/recombination/repair intermediates. Reactions promoted by Dda alone, in the absence of Gp32 or Gp32-B, help to define DNA structural features that are optimal for its helicase activity (Fig. 4A–G). The data show that Dda alone unwinds a wide variety of partially single-stranded substrates including pseudoforks and fork mimics. As predicted by the 5'-to-3' polarity of the helicase, no unwinding products were observed with the occluded fork mimic (Fig. 4F). The highest rates of unwinding by Dda alone were observed with substrates containing 5' ssDNA extensions of 12–30 nucleotides. This includes, in descending order, tailed duplex DNA (Fig. 4A), fork mimic (Fig. 4E), and pseudo-fork substrates (Fig. 4C). Substrates containing 6-nucleotide ssDNA gaps, including gapped duplex (Fig. 4B), inverted fork mimic (Fig. 4D), and inverted occluded fork mimic (Fig. 4G), were also unwound by Dda alone, but at reduced rates compared to substrates with longer ssDNA extensions. Dda was not able to unwind the occluded fork mimic, which lacks lagging strand ssDNA (Figure 4F). These observations suggest that to unwind a fork-shaped substrate, Dda requires lagging strand ssDNA or its equivalent. Dda tolerates a fully duplex leading strand arm of a fork substrate (Fig. 4G), but does not tolerate a fully duplex lagging strand arm (Fig. 4F). The implications of Dda's substrate preferences for its DNA repair functions are described in the Discussion.

The enhancement of DNA unwinding in the presence of Gp32-B was specific to the DNA substrate structure. The effect was most pronounced in the inverted occluded fork (Fig. 4G), the inverted fork mimic (Fig. 4D), the gapped duplex (Fig. 4B), the pseudo-fork (Fig. 4C), and the fork mimic (Fig. 4E) structures, in descending order. The enhancement effect of Gp32-B was not observed with the tailed duplex substrate, a strong substrate for Dda alone (Fig. 4A). The occluded fork mimic (Fig. 4F) was not unwound significantly in the presence or absence of Gp32-B. These results are consistent with the notion that Gp32-B can help

Dda load onto lagging strand ssDNA to promote replication fork progression (Ma et al., 2004).

Full-length Gp32 severely inhibits Dda-catalyzed unwinding of the inverted fork mimic (Fig. 4D). This is in marked contrast with the inverted occluded fork mimic (Fig. 4G), which is similar in structure and was only slightly inhibited by Gp32. This can be explained by the structural difference between the substrates and suggests that Gp32 bound to an extended patch of leading strand ssDNA can prevent Dda from binding to the ssDNA gap on the lagging strand. The implications of this finding are explored in the Discussion. The unwinding of other DNA substrates by Dda was weakly inhibited by full-length Gp32 (Fig. 4A–C,E), except for the occluded fork mimic, which was not unwound significantly under any conditions (Fig. 4F).

3.4. Gp32-B enhances the DNA unwinding activity of Dda on substrates containing short ssDNA gaps

A series of DNA unwinding assays was conducted using a set of partial duplex DNA substrates that varied only in the size of the ssDNA gap present, ranging from a gap size of zero (single-strand nick) to a six-base gap, to test whether Gp32 or Gp32-B can promote unwinding of substrates that Dda helicase alone does not unwind efficiently (Figure 5A). Consistent with published data (Byrd and Raney, 2005), it was determined that Dda alone unwinds a 6-base gapped substrate at a moderate rate (Fig. 5B), but its unwinding activity decreases dramatically as the gap length is shortened to 4, 2, or 1 bases (Figs. 5C–E). No unwinding activity is detectable on the nicked substrate (gap length 0) (Fig. 5F). Even though Dda does not efficiently unwind substrates with gaps of fewer than six bases, the rates are enhanced by the presence of Gp32-B in all cases except the nicked duplex, which did not undergo any detectable unwinding (Fig. 5B–F). In contrast, Gp32 slightly inhibits unwinding at all gap lengths tested (Fig. 5B–E), and did not facilitate unwinding of the nicked substrate (Fig. 5F).

3.5. Gp32-B promotes the DNA unwinding activity of Dda helicase at elevated NaCl concentrations

Consistent with published data (Jongeneel, Formosa, et al., 1984), the DNA unwinding activity of Dda helicase is inhibited by the addition of 75–150 mM NaCl (Fig. 6B–D). Addition of Gp32-B, but not Gp32, raises Dda DNA unwinding activity under all salt conditions tested (Fig. 6B–D), using the six-base gapped substrate (Fig. 6A). The initial rate of DNA unwinding by Dda is dramatically higher in the presence of Gp32-B than in its absence, even as the initial rate decreases with increasing salt concentration. The final extent of unwinding, however, appears to be independent of salt concentration in the presence of Gp32-B (Figure 6B–D). Control experiments were performed under the same conditions with Gp32 and Gp32-B in the absence of Dda and no DNA unwinding was detected (data not shown).

3.6. Gp32-B promotes Dda-catalyzed unwinding of a gapped duplex bound by DNA polymerase

Gp43-D219A, an exonuclease-deficient T4 DNA polymerase mutant, was incubated with the six-base gapped duplex substrate in the absence of dNTPs to create conditions simulating a stalled DNA polymerase (Fig. 7A). Under these conditions, addition of Gp43-D219A inhibits Dda unwinding activity in the absence of Gp32-B (Fig. 7B). Addition of Gp32-B, but not of Gp32, stimulates Dda strand displacement activity to levels similar to those seen with Dda + Gp32-B in the absence of polymerase (Figs. 4B and 7B). Gp32 is unable to promote unwinding of the polymerase-bound DNA substrate by Dda, since the time courses of reactions containing Dda and Gp43-D219A are indistinguishable in the presence/absence of Gp32 (Fig. 7B).

3.7. Gp32-B stimulates the ssDNA-dependent ATPase activity of Dda helicase

Previous studies established that Gp32 inhibits the ssDNA-dependent ATPase activity of Dda helicase (Jongeneel, Formosa et al., 1984). Here, the effects of Gp32-B on the kinetics of Dda ATPase activity were explored. With ssDNA as the variable ligand, and at saturating ATP, the addition of a four-fold excess of Gp32-B with respect to Dda concentration caused an increase in ATPase activity at all ssDNA concentrations examined (a 1000-fold range) (Fig. 8). Similarly, with ATP as the variable substrate, and at saturating ssDNA, a four-fold excess of Gp32-B increased Dda ATPase activity at all ATP concentrations examined (Fig. 9A). As reported in Table 2, the stimulation of Dda ATPase activity by Gp32-B was due to an approximately 2-fold increase in the k_{cat} of the reaction in the presence of a four-fold excess of Gp32-B. Gp32-B also lowered the K_m for ATP by approximately 2-fold, but it appeared to have no effect on the apparent affinity of Dda for ssDNA, since the activation constant $K_{\text{act}}(\text{ssDNA})$ was unchanged (Table 2). Therefore Gp32-B increases the catalytic efficiency of Dda-catalyzed ATP hydrolysis, as reflected by the 4-fold increase in $k_{\text{cat}} / K_m(\text{ATP})$ values (Table 2). The data shown in Figures 8 and 9 (performed using homopolymer (dT)₆₀) were identical to those obtained in experiments using mixed-sequence 20-mer, mixed-sequence 60-mer, and homopolymer (dT)₂₀, ruling out effects resulting from secondary structure and oligonucleotide length (data not shown).

The ATPase activity of 11 nM Dda increases linearly as the Gp32-B concentration increases from zero to 110 nM (a ten-fold excess) (Fig. 9B), suggesting that the concentrations of Gp32-B used were below the K_d for its interactions with Dda-ssDNA. Alternatively, the stimulation of Dda by Gp32-B has a more complex dependence on protein concentration, as we explore in the Discussion.

4. Discussion

Dda helicase unwinds a variety of DNA substrates containing accessible amounts of single-stranded DNA. The data show that Dda most efficiently unwinds substrates containing relatively long 5' ssDNA extensions of 12–30 nucleotide residues, such as the tailed duplex, pseudo-fork, and fork mimic substrates visited in Figure 4A,C and E. Dda also unwinds substrates containing ssDNA gaps. A 6-nucleotide gap in an otherwise duplex substrate is sufficient to allow unwinding by Dda, however the rate of unwinding decreases dramatically

as the gap length decreases, and Dda fails to unwind a nicked substrate (Fig. 5). The requirement of a 6-nucleotide or greater ssDNA gap for efficient unwinding activity is consistent with the binding site size of monomeric Dda on ssDNA, which was previously determined to be 6 nucleotides (Byrd and Raney, 2004). The substrate specificity of Dda, combined with its 5' → 3' polarity, is consistent with a helicase that can translocate on lagging strand ssDNA to stimulate strand displacement DNA synthesis by the leading strand polymerase. The resistance of the occluded fork mimic substrate, which has a fully duplex lagging strand arm, to unwinding by Dda (Fig. 4F) further emphasizes the requirement for lagging strand ssDNA as an assembly site for the enzyme at the replication fork. Meanwhile, the ability of Dda to initiate unwinding from a single-stranded gap in otherwise duplex DNA suggests a mechanism for promoting the restart of stalled replication forks (see Fig. 11, below).

Previous studies demonstrated that full-length Gp32 inhibits the ssDNA-dependent ATPase activity of Dda by competing for binding sites on the ssDNA (Jongeneel, Formosa et al., 1984). Nevertheless Dda requires Gp32 or Gp32-B in order to stimulate strand displacement DNA synthesis by the T4 DNA polymerase holoenzyme *in vitro* (Jongeneel, Bedinger et al., 1984; Ma et al., 2004). Therefore Gp32 promotes Dda activity within the context of a replication fork. Results of this study demonstrate that Gp32 is a regulator of Dda helicase activity. Gp32 negatively regulates the unwinding rate of Dda, as shown by its weak inhibition of reactions with several substrates. Gp32 also controls the substrate specificity of Dda as shown by its strong inhibition of reactions with an inverted fork substrate (Fig. 4D). The N-terminal truncation of Gp32 to generate Gp32-B reveals a cryptic helicase stimulatory activity of Gp32, however, one that appears to depend solely on Gp32-Dda protein-protein interactions. It seems probable that at the wild-type T4 replication fork *in vivo* or *in vitro*, this cryptic activity may be revealed in the context of a moving polymerase, or through direct interactions of Gp32 with other replisome components including polymerase.

With one key exception, the unwinding of a variety of DNA substrates by Dda helicase is weakly inhibited by Gp32 but strongly stimulated by Gp32-B. The exception is the inverted fork mimic shown in Figure 4D. Unwinding of this substrate by Dda is severely inhibited by full-length Gp32, but strongly stimulated by Gp32-B. A possible mechanism to explain the different effects of Gp32 and Gp32-B on the unwinding of fork and inverted fork substrates by Dda is shown in Figure 10. According to this model, Gp32 cluster formation on ssDNA and, importantly, the orientation of such clusters, regulate the unwinding activity of Dda. Gp32-B does not form clusters on ssDNA, but instead stimulates Dda through direct protein-protein interactions. This leads to high rates of unwinding of both fork and inverted fork substrates (Fig. 10). On the other hand, Gp32 forms clusters on exposed ssDNA in both substrates, but the clusters are in opposite orientations with respect to their common duplex arm. Gp32-ssDNA clusters are asymmetric and therefore differ at their 5' and 3' ends (illustrated by the round and pointed ends, respectively, of the Gp32 protomers as drawn in Fig. 10). The model suggests that Dda only interacts productively with the 3' end of a Gp32-ssDNA cluster. With the fork substrate, this interaction would place Dda on the lagging strand ssDNA and in the correct orientation to unwind the duplex arm via 5' → 3' translocation. This model is consistent with the idea put forward by Nossal and co-workers

that Gp32 clusters preferentially assemble in the 5' to 3' direction on replication fork ssDNA (Jones et al., 2004). The 5' → 3' polarity of Gp32 cluster assembly would ensure that ssDNA generated by strand displacement DNA synthesis is available for Dda assembly before Gp32 can fully saturate it. Gp32 is proposed to slide freely along ssDNA (Shamoo et al., 1995; Lohman and Kowalczykowski, 1981). Therefore the Gp32 cluster could be dragged along by Dda and thus act as a brake on the helicase, slowing down its net rate of movement (Fig. 10), which would explain the observation that Gp32 weakly inhibits Dda unwinding activities on fork substrates (Fig. 4C–E,G). In contrast, Gp32-ssDNA cluster formation on the inverted fork substrate places the cluster on leading strand ssDNA with its 5', not 3', end oriented towards the duplex arm (Fig. 10). If recruited to this cluster, 5' → 3' translocation by Dda would be in the wrong direction to unwind the duplex arm. Dda alone is capable of unwinding the inverted fork, and is greatly stimulated by Gp32-B (Fig. 4D). Therefore, the severe inhibition of inverted fork unwinding by Gp32 indicates that the 5' end of the Gp32-ssDNA cluster specifically interferes with the unwinding of this structure by Dda helicase (Fig. 10).

The structure of the inverted fork substrate resembles a replication fork in which the leading strand polymerase has stalled at DNA damage, leaving an extended region of ssDNA on the leading strand, while Okazaki fragment synthesis has continued on the lagging strand (Fig. 11). Thus the inverted fork structure resembles a DNA intermediate that is proposed to occur during the process of recombination repair (Kadyrov and Drake, 2004). Our findings suggest a possible model for how Gp32 might direct the activities of Dda helicase in order to help bring about replication fork restart during recombination repair (Fig. 11). When the leading strand polymerase stalls at DNA damage, leading and lagging strand synthesis are uncoupled. The uncoupling allows synthesis of an overlapping Okazaki fragment by the lagging strand synthesis machinery, while allowing an ssDNA gap to open up in front of the stalled leading strand polymerase. A Gp32 cluster would assemble on this gap, which data suggest would regulate Dda activity in the following ways: First, as described above (Figs. 4D and 10), the 5' end of the Gp32 cluster blocks the stimulation of fork progression by Dda. Second, interactions with the 3' end of the Gp32 cluster could recruit Dda to remove the stalled polymerase and unwind the 3' end of the daughter strand from its template, as suggested by results in Figure 7. Dda is capable of translocation past common DNA lesions such as abasic sites (Eoff et al., 2005), and it is conceivable that interactions with Gp32 could further promote this activity. The unwinding of the stalled leading strand is a prerequisite for template switching promoted by UvsX recombinase in the presence of Dda (Bleuit et al., 2001; Kadyrov and Drake, 2004). As shown by Drake and colleagues, successive UvsX- and Dda-dependent template switching events are necessary to regress a lesion-stalled replication fork and subsequently to promote error-free translesion DNA synthesis (Kadyrov and Drake, 2004). The results of this study suggest that helicase trafficking by Gp32 could play a role in preventing premature fork progression until the required template switching events have been completed.

The increased rates of unwinding of various DNA substrates observed for Dda in the presence of Gp32-B could be the result of an increase in translocation velocity, an increase in processivity, or both. Gp32-B increases the catalytic efficiency of Dda for ssDNA-dependent ATP hydrolysis (Table 2). This suggests an increase in the efficiency of Dda as a

helicase, which means a higher probability of a productive step coupled to each ATP hydrolytic event. This effect in itself could result in a higher observed rate of unwinding. An SSB functioning as a processivity factor for a DNA helicase has been described in at least two different systems (Boehmer, 1998; Shereda, Bernstein, and Keck, 2007). Processivity in Dda is enhanced when multiple copies of Dda interact with the same region of ssDNA, giving rise to the notion of a “cooperative inchworm” mechanism for Dda helicase activity (Byrd and Raney, 2005; Eoff and Raney, 2010; Spurling et al., 2006). We propose as a working hypothesis that Gp32-B (or cryptically, Gp32) promotes the assembly of oligomeric Dda species on ssDNA as it is exposed through the unwinding activity of the initial monomeric helicase, leading to the observed rate acceleration via an enhanced cooperative inchworm mechanism. Detailed pre-steady-state and steady-state kinetics measurements will be needed to address this hypothesis, in future studies.

Understanding the mechanism of DNA helicase activity and its regulation by other DNA binding proteins is essential for progress in the fields of genome stability, oncology, and drug development. Since DNA helicases are molecular motors that translocate on ssDNA, it stands to reason that ssDNA-binding proteins play important roles in regulating helicase activity. Results of this study indicate that in the T4 system, the Gp32 SSB can have a dual regulatory function with respect to Dda helicase activity: it can interfere with Dda loading onto certain DNA structures, and thereby regulate the manner in which different substrates may be recognized and processed by Dda. This effect may be important for the mechanism of recombination repair (Fig. 11). In addition, within the context of the replication fork, Gp32 may stimulate the translocation rate and/or processivity of Dda to enhance unwinding. These findings indicate that SSBs, far from being passive players in the disruption of duplex DNA by helicases, are more likely to be active players in coordinating helicase activities with other events at the replication fork or in repair and recombination complexes. In addition to T4, the significance of SSB-helicase interactions has been established in other biologic systems, including *E. coli* and HSV-1 (Boehmer, 1998; Cadman and McGlynn, 2004). It seems certain that regulation of DNA helicase enzymes by SSBs will be an important general feature of DNA metabolism in many if not all organisms.

Acknowledgments

The authors thank Dr. Stephen J. Benkovic for providing the overexpression vector for Gp32 and Jennifer Tomczak for technical support. This work was supported by National Institutes of Health research grant no. R01GM48847 to SWM. CSJ was supported by NIH training grant no. T32ES07122. The sponsors had no involvement in study design, the collection, analysis and interpretation of data, in the writing of the report, or in the decision to submit the article for publication.

Abbreviations

NA	nucleic acid
PNA	polyamide nucleic acid
dsDNA	double-stranded deoxyribonucleic acid
ssDNA	single-stranded deoxyribonucleic acid

SSB	single-strand deoxyribonucleic acid binding protein
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
EMSA	electrophoretic mobility shift assay

References

- Alberts BM. Prokaryotic DNA replication mechanisms. *Philos Trans R Soc Lond B Biol Sci.* 1987; 317(1187):395–420. [PubMed: 2894677]
- Alberts BM, Herrick G. DNA-cellulose chromatography. *Methods in Enzymology.* 1971; 21:198–217.
- Amaratunga M, Lohman TM. Escherichia coli rep helicase unwinds DNA by an active mechanism. *Biochemistry.* 1993; 32(27):6815–6820. [PubMed: 8392863]
- Bedinger P, Hochstrasser M, Jongeneel CV, Alberts BM. Properties of the T4 bacteriophage DNA replication apparatus: the T4 dda DNA helicase is required to pass a bound RNA polymerase molecule. *Cell.* 1983; 34(1):115–123. [PubMed: 6136341]
- Bleuit JS, Xu H, Ma Y, Wang T, Liu J, Morrical SW. Mediator proteins orchestrate enzyme-ssDNA assembly during T4 recombination-dependent DNA replication and repair. *Proc Natl Acad Sci U S A.* 2001; 98(15):8298–8305. [PubMed: 11459967]
- Boehmer PE. The herpes simplex virus type-1 single-strand DNA-binding protein, ICP8, increases the processivity of the UL9 protein DNA helicase. *J Biol Chem.* 1998; 273(5):2676–2683. [PubMed: 9446572]
- Branagan AM, Klein JA, Jordan CS, Morrical SW. Control of helicase loading in the coupled DNA replication and recombination systems of bacteriophage T4. *J Biol Chem.* 2014; 289(5):3040–3054. [PubMed: 24338568]
- Branagan AM, Maher RL, Morrical SW. Assembly and dynamics of Gp59-Gp32-single-stranded DNA (ssDNA), a DNA helicase loading complex required for recombination-dependent replication in bacteriophage T4. *J Biol Chem.* 2012; 287(23):19070–19081. [PubMed: 22500043]
- Burke RL, Alberts BM, Hosoda J. Proteolytic removal of the COOH terminus of the T4 gene 32 helix-destabilizing protein alters the T4 in vitro replication complex. *J Biol Chem.* 1980; 255(23):11484–11493. [PubMed: 6254971]
- Byrd AK, Raney KD. Protein displacement by an assembly of helicase molecules aligned along single-stranded DNA. *Nat Struct Mol Biol.* 2004; 11(6):531–538. [PubMed: 15146172]
- Byrd AK, Raney KD. Increasing the length of the single-stranded overhang enhances unwinding of duplex DNA by bacteriophage T4 Dda helicase. *Biochemistry.* 2005; 44(39):12990–12997. [PubMed: 16185067]
- Cadman CJ, McGlynn P. PriA helicase and SSB interact physically and functionally. *Nucleic Acids Res.* 2004; 32(21):6378–6387. [PubMed: 15576682]
- Carroll RB, Neet K, Goldthwait DA. Studies of the self-association of bacteriophage T4 gene 32 protein by equilibrium sedimentation. *J Mol Biol.* 1975; 91(3):275–291. [PubMed: 241852]
- Casas-Finet JR, Fischer KR, Karpel RL. Structural basis for the nucleic acid binding cooperativity of bacteriophage T4 gene 32 protein: the (Lys/Arg)³(Ser/Thr)² (LAST) motif. *Proc Natl Acad Sci U S A.* 1992; 89(3):1050–1054. [PubMed: 1736285]
- Churchill ME, Travers AA. Protein motifs that recognize structural features of DNA. *Trends Biochem Sci.* 1991; 16(3):92–97. [PubMed: 1647556]
- Eoff RL, Raney KD. Kinetic mechanism for DNA unwinding by multiple molecules of Dda helicase aligned on DNA. *Biochemistry.* 2010; 49(21):4543–4553. [PubMed: 20408588]
- Eoff RL, Spurling TL, Raney KD. Chemically modified DNA substrates implicate the importance of electrostatic interactions for DNA unwinding by Dda helicase. *Biochemistry.* 2005; 44(2):666–674. [PubMed: 15641792]

- Formosa T, Alberts BM. The use of affinity chromatography to study proteins involved in bacteriophage T4 genetic recombination. *Cold Spring Harb Symp Quant Biol.* 1984; 49:363–370. [PubMed: 6335687]
- Formosa T, Alberts BM. DNA synthesis dependent on genetic recombination: characterization of a reaction catalyzed by purified bacteriophage T4 proteins. *Cell.* 1986; 47(5):793–806. [PubMed: 3022939]
- Formosa T, Burke RL, Alberts BM. Affinity purification of bacteriophage T4 proteins essential for DNA replication and genetic recombination. *Proc Natl Acad Sci U S A.* 1983; 80(9):2442–2446. [PubMed: 6302682]
- Gauss P, Doherty DH, Gold L. Bacterial and phage mutations that reveal helix-unwinding activities required for bacteriophage T4 DNA replication. *Proc Natl Acad Sci U S A.* 1983; 80(6):1669–1673. [PubMed: 6300866]
- Gauss P, Park K, Spencer TE, Hacker KJ. DNA helicase requirements for DNA replication during bacteriophage T4 infection. *J Bacteriol.* 1994; 176(6):1667–72. [PubMed: 8132462]
- Giedroc DP, Khan R, Barnhart K. Overexpression, purification, and characterization of recombinant T4 gene 32 protein 22-301 (g32P-B). *J Biol Chem.* 1990; 265(20):11444–11455. [PubMed: 2195020]
- Gill SC, von Hippel PH. Calculation of protein extinction coefficients from amino acid sequence data. *Anal Biochem.* 1989; 182(2):319–326. [PubMed: 2610349]
- Hacker KJ, Alberts BM. Overexpression, purification, sequence analysis, and characterization of the T4 bacteriophage dda DNA helicase. *J Biol Chem.* 1992; 267(29):20674–20681. [PubMed: 1328208]
- Hurley JM, Chervitz SA, Jarvis TC, Singer BS, Gold L. Assembly of the bacteriophage T4 replication machine requires the acidic carboxy terminus of gene 32 protein. *J Mol Biol.* 1993; 229(2):398–418. [PubMed: 8429554]
- Ishmael FT, Alley SC, Benkovic SJ. Identification and mapping of protein-protein interactions between gp32 and gp59 by cross-linking. *J Biol Chem.* 2001; 276(27):25236–25242. [PubMed: 11309384]
- Jensen DE, Kelly RC, von Hippel PH. DNA “melting” proteins. II. Effects of bacteriophage T4 gene 32-protein binding on the conformation and stability of nucleic acid structures. *J Biol Chem.* 1976; 251(22):7215–7228. [PubMed: 791945]
- Jones CE, Mueser TC, Nossal NG. Bacteriophage T4 32 protein is required for helicase-dependent leading strand synthesis when the helicase is loaded by the T4 59 helicase-loading protein. *J Biol Chem.* 2004; 279(13):12067–12075. [PubMed: 14729909]
- Jongeneel CV, Bedinger P, Alberts BM. Effects of the bacteriophage T4 dda protein on DNA synthesis catalyzed by purified T4 replication proteins. *J Biol Chem.* 1984; 259(20):12933–12938. [PubMed: 6092352]
- Jongeneel CV, Formosa T, Alberts BM. Purification and characterization of the bacteriophage T4 dda protein. A DNA helicase that associates with the viral helix-destabilizing protein. *J Biol Chem.* 1984; 259(20):12925–12932. [PubMed: 6092351]
- Kadyrov FA, Drake JW. UvsX recombinase and Dda helicase rescue stalled bacteriophage T4 DNA replication forks in vitro. *J Biol Chem.* 2004; 279(34):35735–35740. [PubMed: 15194689]
- Kodadek T, Alberts BM. Stimulation of protein-directed strand exchange by a DNA helicase. *Nature.* 1987; 326(6110):312–314. [PubMed: 2950327]
- Kowalczykowski SC, Paul LS, Lonberg N, Newport JW, McSwiggen JA, von Hippel PH. Cooperative and noncooperative binding of protein ligands to nucleic acid lattices: experimental approaches to the determination of thermodynamic parameters. *Biochemistry.* 1986; 25(6):1226–1240. [PubMed: 3486003]
- Krassa KB, Green LS, Gold L. Protein-protein interactions with the acidic COOH terminus of the single-stranded DNA-binding protein of the bacteriophage T4. *Proc Natl Acad Sci U S A.* 1991; 88(9):4010–4014. [PubMed: 2023949]
- Kreuzer KN, Brister JR. Initiation of bacteriophage T4 DNA replication and replication fork dynamics: a review in the Virology Journal series on bacteriophage T4 and its relatives. *Virology J.* 2010; 7:358. [PubMed: 21129203]

- Liu J, Morrical SW. Assembly and dynamics of the bacteriophage T4 homologous recombination machinery. *Virol J.* 2010; 7:357. [PubMed: 21129202]
- Lohman TM, Kowalczykowski SC. Kinetics and mechanism of the association of the bacteriophage T4 gene 32 (helix destabilizing) protein with single-stranded nucleic acids. Evidence for protein translocation. *J Mol Biol.* 1981; 152(1):67–109. [PubMed: 6279865]
- Ma Y, Wang T, Villemain JL, Giedroc DP, Morrical SW. Dual functions of single-stranded DNA-binding protein in helicase loading at the bacteriophage T4 DNA replication fork. *J Biol Chem.* 2004; 279(18):19035–19045. [PubMed: 14871889]
- Maher RL, Branagan AM, Morrical SW. Coordination of DNA replication and recombination activities in the maintenance of genome stability. *J Cell Biochem.* 2011; 112(10):2672–2682. [PubMed: 21647941]
- Morrical SW, Alberts BM. The UvsY protein of bacteriophage T4 modulates recombination-dependent DNA synthesis in vitro. *J Biol Chem.* 1990; 265(25):15096–15103. [PubMed: 2144282]
- Morrical SW, Hempstead K, Morrical MD. The gene 59 protein of bacteriophage T4 modulates the intrinsic and single-stranded DNA-stimulated ATPase activities of gene 41 protein, the T4 replicative DNA helicase. *J Biol Chem.* 1994; 269(52):33069–33081. [PubMed: 7806535]
- Morrical SW, Wong ML, Alberts BM. Amplification of snap-back DNA synthesis reactions by the uvsX recombinase of bacteriophage T4. *J Biol Chem.* 1991; 266(21):14031–14038. [PubMed: 1649833]
- Morris PD, Tackett AJ, Babb K, Nanduri B, Chick C, Scott J, Raney KD. Evidence for a functional monomeric form of the bacteriophage T4 Dda helicase. Dda does not form stable oligomeric structures. *J Biol Chem.* 2001; 276(23):19691–19698. [PubMed: 11278788]
- Nanduri B, Byrd AK, Eoff RL, Tackett AJ, Raney KD. Pre-steady-state DNA unwinding by bacteriophage T4 Dda helicase reveals a monomeric molecular motor. *Proc Natl Acad Sci U S A.* 2002; 99(23):14722–14727. [PubMed: 12411580]
- Nanduri B, Eoff RL, Tackett AJ, Raney KD. Measurement of steady-state kinetic parameters for DNA unwinding by the bacteriophage T4 Dda helicase: use of peptide nucleic acids to trap single-stranded DNA products of helicase reactions. *Nucleic Acids Res.* 2001; 29(13):2829–2835. [PubMed: 11433029]
- Nossal NG. The Bacteriophage T4 DNA Replication Fork. In: Karam, JD., editor. *Molecular Biology of Bacteriophage T4*. Washington, D.C: American Society for Microbiology; 1994. p. 43–53.
- Raney KD, Benkovic SJ. Bacteriophage T4 Dda helicase translocates in a unidirectional fashion on single-stranded DNA. *J Biol Chem.* 1995; 270(38):22236–22242. [PubMed: 7673202]
- Shamoo Y, Friedman AM, Parsons MR, Konigsberg WH, Steitz TA. Crystal structure of a replication fork single-stranded DNA binding protein (T4 gp32) complexed to DNA. *Nature.* 1995; 376(6538):362–366. Erratum in: *Nature*, 376(6541):616. [PubMed: 7630406]
- Shereda RD, Bernstein DA, Keck JL. A central role for SSB in Escherichia coli RecQ DNA helicase function. *J Biol Chem.* 2007; 282(26):19247–19258. [PubMed: 17483090]
- Spicer EK, Williams KR, Konigsberg WH. T4 gene 32 protein trypsin-generated fragments. Fluorescence measurement of DNA-binding parameters. *J Biol Chem.* 1979; 254(14):6433–6436. [PubMed: 221499]
- Spurling TL, Eoff RL, Raney KD. Dda helicase unwinds a DNA-PNA chimeric substrate: evidence for an inchworm mechanism. *Bioorg Med Chem Lett.* 2006; 16(7):1816–1820. [PubMed: 16439125]
- Tackett AJ, Corey DR, Raney KD. Non-Watson-Crick interactions between PNA and DNA inhibit the ATPase activity of bacteriophage T4 Dda helicase. *Nucleic Acids Res.* 2002; 30(4):950–957. [PubMed: 11842106]
- Villemain JL, Ma Y, Giedroc DP, Morrical SW. Mutations in the N-terminal cooperativity domain of gene 32 protein alter properties of the T4 DNA replication and recombination systems. *J Biol Chem.* 2000; 275(40):31496–31504. [PubMed: 10906124]
- Williams KR, Sillerud LO, Schafer DE, Konigsberg WH. DNA binding properties of the T4 DNA helix-destabilizing protein. A calorimetric study. *J Biol Chem.* 1979; 254(14):6426–6432. [PubMed: 221498]

Xu H, Wang Y, Bleuit JS, Morrical SW. Helicase assembly protein Gp59 of bacteriophage T4: fluorescence anisotropy and sedimentation studies of complexes formed with derivatives of Gp32, the phage ssDNA binding protein. *Biochemistry*. 2001; 40(25):7651–7661. [PubMed: 11412119]

Highlights

- Stimulation of DNA synthesis by Dda helicase requires Gp32 ssDNA-binding protein.
- Gp32-Dda protein-protein interactions stimulate Dda helicase and ATPase activities.
- Gp32 strongly inhibits unwinding of inverted replication fork structures by Dda.
- Gp32 controls where Dda may assemble on DNA replication and repair intermediates.
- Gp32 regulation of Dda may be important for the mechanism of replication restart.

Figure 1. Complex formation between Dda and Gp32 or Gp32-B in the presence and absence of ssDNA

Electrophoretic mobility shift assays were performed as described in Materials and Methods. The ssDNA used was 5'-[³²P]-labeled oligo F (mixed sequence 60-mer), which was present at a concentration of 175 nM (10.5 μM nucleotides). The concentration of each protein, when present, was 1.5 μM. **A)** Result of EMSA by 6% non-denaturing polyacrylamide gel electrophoresis and Coomassie blue protein staining. **B)** Result of same EMSA gel visualizing 5'-[³²P]-labeled ssDNA by autoradiography. DNA binding by protein(s) is observed as a decrease in the electrophoretic mobility of the DNA molecule.

Figure 2. Gp32 and Gp32-B do not bind to PNA

Electrophoretic shift assays (EMSAs) were performed as described in Materials and Methods. Reactions contained either 0 (–) or 175 nM (+) ssDNA (oligo D), or 0 (–), 175 (+), or 350 nM (++) PNA as indicated above the gel. Reactions in lanes 1–4 contained 1.5 μ M Gp32; those in lanes 5–8 contained 1.5 μ M Gp32-B. Following 6% non-denaturing polyacrylamide gel electrophoresis, the gel was stained with Coomassie Blue to visualize protein. ssDNA or PNA binding is observed as a decrease in the electrophoretic mobility of the protein.

Figure 3. Schematic representation of DNA unwinding assay

A) Helicase unwinding of DNA substrate in which one oligonucleotide component, depicted in red, is 5' radio labeled with [^{32}P], depicted by an asterisk. The PNA trap anneals to the exposed, complementary ssDNA in the labeled product, preventing reaction reversal caused by reannealing of labeled and unlabeled products. DNA unwinding assays were based on a previously published protocol (Nanduri et al., 2001) and performed as described in Materials and Methods. **B)** Intact and unwound DNA substrates are resolved by non-denaturing PAGE, visualized by autoradiography, and quantified by densitometry. The fraction of unwound product is determined by calculating the ratio of product density to the total density per lane, as described (Amaratunga and Lohman, 1993).

Figure 4. Dda unwinding activity of various DNA substrates in the presence and absence of Gp32 or Gp32-B

Helicase unwinding of DNA substrates in which one strand, depicted in red, contains a 5'-[³²P] radiolabel, depicted by an asterisk. DNA unwinding assays were performed as described in Materials and Methods. For each reaction, 125 nM DNA substrate was added to reaction buffer lacking ATP and magnesium, followed sequentially by 1 nM Dda, and when present 100 nM Gp32 or Gp32-B. Reactions were initiated by the simultaneous addition of 4.6 mM ATP, 5 mM magnesium acetate, and 300 nM PNA, then timed aliquots were removed and quenched with EDTA. Panels A–G depict results of DNA unwinding reactions using A) tailed duplex, B) gapped duplex, C) pseudo-fork, D) inverted fork mimic, E) fork

mimic, **F**) occluded fork mimic, and **G**) inverted occluded fork mimic. In each panel, reaction time courses in *blue* are for Dda alone, those in *green* are for Dda + Gp32-B, and those in *red* are for Dda + Gp32.

Figure 5. Dda unwinding of gapped duplex substrates as a function of ssDNA gap length
 ssDNA gap length was varied in a partial duplex substrate as shown in the reaction schematic in panel A. Reaction conditions were as described in Materials and Methods. Unwinding reactions contained final concentrations of 125 nM DNA substrate, 1 nM Dda, and either 0 or 100 nM of Gp32 or Gp32-B. Otherwise identical substrates contained ssDNA gap lengths of (B) 6 nucleotides, (C) 4 nucleotides, (D) 2 nucleotides, (E) 1 nucleotide, and (F) 0 nucleotides (nick). In panels B–F, reaction time courses in *blue* are for Dda alone, those in *green* are for Dda + Gp32-B, and those in *red* are for Dda + Gp32.

Figure 6. Gp32-B stimulates the DNA unwinding activity of Dda helicase at elevated salt concentrations

(A) Schematic representation of the DNA unwinding reaction employing a partial duplex substrate with a 6-nucleotide ssDNA gap. The DNA unwinding activity of Dda was measured in the absence and presence of Gp32-B or Gp32 under (B) low salt conditions (10 mM potassium acetate), (C) intermediate salt conditions (75 mM sodium chloride plus 10 mM potassium acetate), and (D) high salt conditions (150 mM sodium chloride plus 10 mM potassium acetate). In panels B–D, reaction time courses in *blue* are for Dda alone, those in *green* are for Dda + Gp32-B, and those in *red* are for Dda + Gp32. Reaction conditions were identical to those in Figure 5 except for the added NaCl in panels C and D. The gapped duplex substrate was identical to that used in reactions in Figures 4B and 5B.

Figure 7. Inhibition of Dda helicase activity by primer/template-bound DNA polymerase is relieved by Gp32-B

(A) Schematic of the reaction. The exonuclease-deficient T4 DNA polymerase Gp43-D219A is incubated with the 6-base gapped duplex substrate in the absence of dNTPs before the unwinding reaction is initiated by the addition of Dda helicase and Mg-ATP. (B) Time courses of unwinding reactions catalyzed by Dda alone (*blue*), in the presence of Gp43-D219A (*orange*), in the presence of Gp43-D219A and Gp32-B (*green*), and in the presence of Gp43-D219A and Gp32 (*red*). The substrate and reaction conditions were identical to those in Figure 5B except for the addition of Gp43-D219A, when present.

Figure 8. ATPase activity of Dda helicase as a function of ssDNA concentration, in the presence and absence of Gp32-B

Steady-state reaction velocities were determined by measuring ADP release in a continuous coupled assay at saturating ATP concentration (4.5 mM) and at the indicated concentrations of Dda, Gp32-B, and homopolymer (dT)₆₀ ssDNA. All other reaction conditions were as described in Materials and Methods.

Figure 9. ssDNA-dependent ATPase activity of Dda helicase as a function of ATP and Gp32-B concentrations. Assays were carried out as described in Materials and Methods. (A) Kinetics of Dda-catalyzed, ssDNA-dependent ATP hydrolysis in the presence and absence of a four-fold excess of Gp32-B (44 nM) over Dda (11 nM). (dT)₆₀ ssDNA concentration was saturating (25 μM) and ATP concentration was varied. (B) Effect of Gp32-B concentration on Dda ATPase activity at saturating concentrations of both (dT)₆₀ (50 μM) and ATP (4.5 mM). Dda concentration was 11 nM.

Figure 10. Gp32 and Gp32-B differentially regulate Dda activity on forks and inverted forks Dda unwinding of the **A)** fork and **C)** inverted fork substrates is greatly stimulated by monomeric Gp32-B. **B)** Gp32 weakly inhibits Dda unwinding activity on fork substrates, where the cluster forms on leading strand ssDNA with its 3' end oriented towards the duplex arm. We propose the Gp32-ssDNA cluster is dragged by Dda, acting as a brake on Dda net rate of movement. **D)** In contrast, Gp32 severely inhibits inverted fork unwinding by Dda. Here, Gp32 -ssDNA cluster formation takes place on leading strand ssDNA, with the 5' end of the cluster oriented towards the duplex arm and the 3' end away from the duplex arm. In our proposed model, the 3' end of the Gp32-ssDNA cluster recruits Dda and loads the helicase in the opposite direction than is necessary for duplex arm unwinding while the 5' end of the cluster specifically interferes with Dda unwinding of the duplex.

Legend

DNA	Enzymes
Daughter lagging strand	Gp43 polymerase
Daughter leading strand	Dda helicase
Damaged leading strand template (e.g., abasic site, oxidized base)	Gp32 SSB

Figure 11. Proposed model for Gp32 SSB regulation of Dda helicase in replication fork restart during recombination repair

A) When leading strand polymerase (red figure) stalls at DNA damage (depicted by a bold X), leading and lagging strand syntheses are uncoupled. The lagging strand synthesis machinery can synthesize an Okazaki fragment (green line) overlapping the damaged site, while allowing an ssDNA gap to open up in front of the stalled leading strand polymerase, which is covered by Gp32 (orange figures). **B)** The Gp32 cluster blocks Dda (blue figure) stimulation of fork progression by preventing its loading onto the lagging strand and promotes Dda unwinding of the 3' end of the template daughter strand. **C)** UvsX recombinase and Dda catalyze template switching (Bleuit et al., 2001; Kadyrov and Drake, 2004) to regress the lesion-stalled replication fork and **D)** allow subsequent error-free

translesion DNA synthesis (Kadyrov and Drake, 2004). This model describes a mechanism by which Gp32 regulation of Da helicase can prevent premature fork progression and allow replication fork restart and error-free translesion DNA synthesis during recombination repair.

Table 1

Upper Panel: Oligonucleotide compositions of the various DNA substrates used in unwinding reactions. The structures of relevant substrates are shown schematically in each figure. *Lower Panel:* Lengths and sequences of oligonucleotides used to assemble DNA substrates for unwinding reactions. The PNA oligo was used as a trap in unwinding reactions.

Substrate	Oligonucleotides
Tailed duplex	A + B
Gapped duplex (6-nt gap)	C + D
Gapped duplex (4-nt gap)	D + K
Gapped duplex (2-nt gap)	D + L
Gapped duplex (1-nt gap)	D + M
Nicked duplex	D + N
Pseudofork	E + F
Inverted fork	E + F + G
Fork	E + F + H
Occluded fork	E + F + H + I
Inverted occluded fork	E+F+G+J

Oligonucleotide	Sequence (5' to 3' for DNA; N to C for PNA)	Length
A	GCGACATCAGCG	12
B	TTTTTTTTTTTCGCTGATGTCGC	24
C	TGAGCGCGCATCTGAAGCATCAGCTTTG CTGATGCTTCAGATGCGCGCTCACTGCG CATTTGCGATAGTCGAGATTCTATGGCA	84
D	TGCCATAGAATCTCGACTATCGCAAAT	27
E	TAACGTATTCAAGATACCTCGTACTCTGTAC AGGTTGCGATCCGACTGTCCTGCAT	56
F	GATCATGCAGGACAGTCGGATCGCAACC TGATTACTGTGTCATATAGTACGTGATTACAG	60
G	GTACGAGGTATCTTGAATACGTTA	24
H	CTGAATCACGTACTATATGACACA	24
I	TACAGAGTACGAGGTATCTTGAATACGTTA	30
J	CTGAATCACGTACTATATGACACAGTAAAT	30
K	AGTGAGCGCGCATCTGAAGCATCAGCTT TGCTGATGCTTCAGATGCGCGCTCACTG CGCATTTGCGATAGTCGAGATTCTATGGCA	86
L	GCAGTGAGCGCGCATCTGAAGCATCAGC TTTGCTGATGCTTCAGATGCGCGCTCACT GCGCATTTGCGATAGTCGAGATTCTATGGCA	88
M	CGCAGTGAGCGCGCATCTGAAGCATCAG CTTGCTGATGCTTCAGATGCGCGCTCA CTGCGCATTTGCGATAGTCGAGATTCTATGGCA	89
N	GCGCAGTGAGCGCGCATCTGAAGCATCAGC TTTGCTGATGCTTCAGATGCGCGCTCACTGC CATTTGCGATAGTCGAGATTCTATGGCA	90
PNA	ATTGCGATAGTCGAGATTCTATGGCA	27

Table 2

Kinetic parameters of the ssDNA-dependent ATPase activities of Dda helicase in the absence and presence of a four-fold molar excess of Gp32-B.

	Dda	Dda + Gp32-B
$V_{\max}$ (x 10^{-6} M s $^{-1}$) ^a	0.9 ± 0.1	1.6 ± 0.1
k_{cat} (s $^{-1}$) ^a	80 ± 10	150 ± 10
$K_{\text{m (ATP)}}$ (x 10^{-6} M) ^a	200 ± 80	90 ± 30
$k_{\text{cat}} / K_{\text{m (ATP)}}$ (x 10^{-6} M $^{-1}$ s $^{-1}$) ^a	0.4 ± 0.2	1.7 ± 0.6
$K_{\text{act (ssDNA)}}$ (x 10^{-9} M) ^b	30 ± 10	30 ± 0

^aParameters derived from reactions performed with ATP as the variable substrate and with saturating ssDNA.

^bParameters derived from reactions performed with ssDNA as the variable ligand and with saturating ATP. $K_{\text{act (ssDNA)}}$ is the activation constant for ssDNA, the units of which are given in micromoles of oligonucleotide molecules per liter.