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Regulation of the bacteriophage T4 Dda helicase by Gp32 single-stranded DNA–binding protein

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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.

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 [1]. 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 [2,3,4].

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 [1]. 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 *Escherichia coli* host strains [5]. 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 [5]. 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 [6]. 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 [6]. 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.

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.

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Dda, a member of Superfamily 1 (SF1), is a 49.9-kDa monomeric helicase, with $5' \rightarrow 3'$ polarity and low to moderate processivity [7,8]. 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 [9,10]; replication fork progression past a template-bound RNA polymerase molecule [11]; DNA branch migration promoted by T4 UvsX recombinase [12]; recombination-dependent DNA synthesis and amplification [13,14,15]; and error-free translesion DNA synthesis by template-switching [16,17]. Dda binds tightly and specifically to Gp32, the T4 ssDNA–binding protein, and to UvsX, the T4 recombinase [18,19,20]. 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 [10]. 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 ($\omega \geq 1000$) and nonspecific single-stranded DNA-binding protein (SSB) essential for T4 replication [1,21]. 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) [22]. The core domain contains the ssDNA-binding site [23] and the A domain mediates critical interactions with heterologous replisome proteins such as Dda and Gp43 [24,25]. The B domain mediates self-association of Gp32 monomers in solution and cooperativity of ssDNA binding [26]. Monomers of Gp32 associate on ssDNA in clusters, which is mediated by highly basic amino acids in the B domain [27,28].

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, $K_a \approx 10\,M^{-1}$), exhibits no cooperativity ($\omega = 1$), and has approximately 10^4-fold lower ssDNA-binding affinity compared with wild-type [10,26,29,30]. 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 [20,31,32]. *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 [9]. 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 [9]. This stimulation requires direct Gp32–Dda protein–protein interactions since it is relatively unaffected by mutations that destabilize the ssDNA-binding component of Gp32 [10]. 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 [10,29].

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 toward 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 [33]. 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 [34]. 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 [26,35]. Recombinant Gp32 protein was purified according to the protocol of [36], 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 [37]. All T4 protein stock solutions used in this study were nuclease-free according to published criteria [38]. 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