Biochemistry and Biophysics Reports 35 (2023) 101518

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

A real-time fluorescent gp32 probe-based assay for monitoring single-stranded DNA-dependent DNA processing enzymes

Senthil K. Perumal

Department of Chemistry, 402 Wartik Laboratories, The Pennsylvania State University, University Park, PA, 16802, USA

ARTICLE INFO

Keywords:
SSB
Polymerase
Helicase
Protein displacement
Recombinase

ABSTRACT

Single-stranded DNA (ssDNA) generated during DNA replication, recombination and damage repair reactions is an important intermediate and ssDNA-binding proteins that binds these intermediates coordinate various DNA metabolic processes. Mechanistic details of these ssDNA-dependent processes can be explored by monitoring the generation and consumption of ssDNA in real time. In this work, a fluorescein-labeled gp32-based sensor was employed to continuously monitor various aspects of ssDNA-dependent DNA replication and recombination processes in real time. The gp32 protein probe displayed high sensitivity and specificity to a variety of ssDNA-dependent processes of T4 phage. Several applications of the probe are illustrated here: the solution dynamics of ssDNA-binding protein, protein-protein and protein-DNA interactions involving gp32 protein and its mode of interaction, ssDNA translocation and protein displacement activities of helicases, primer extension activity of DNA polymerase holoenzyme and nucleoprotein filament formation during DNA recombination. The assay has identified new protein-protein interactions of gp32 during T4 replication and recombination. The fluorescent probe described here can thus be used as a universal probe for monitoring in real time various ssDNA-dependent processes, which is based on a well-characterized and easy-to-express bacteriophage T4 gene 32 protein, gp32.

1. Introduction

Single-stranded DNA (ssDNA) is an important and ubiquitous intermediate generated during DNA metabolic reactions such as replication, recombination, and damage repair. When genomic DNA is unwound by DNA helicases, ssDNA generated is immediately coated by ssDNA binding proteins (SSB) to prevent degradation. SSBs are conserved across various species and examples include gp32 in T4 phage, gp2.5 in T7 phage, SSB in bacteria and Replication Protein A (RPA) in eukaryotes [1–4]. SSB-bound DNA nucleoprotein complexes serve as a template for various DNA replication and DNA damage repair processes [1,3,4]. The latter function is achieved through physical association of SSBs with DNA replication and damage repair proteins [2,5–8].

T4 phage gp32 protein is a prototypical member of the ssDNA binding protein [9,10]. T4 gp32 and T7 gp2.5 function as monomers unlike bacterial SSBs, archaeal SSBs and eukaryotic RPA, which exist as a homotetramer, homodimer and heterotrimer, respectively [1,3]. gp32 is composed of three domains: a core DNA binding domain (DBD), an N-terminal domain and a C-terminal domain [10]. The structure of the core DBD contains the oligosaccharide/oligonucleotide-binding (OB)-fold and a structural Zn^{2+} ion that is tightly bound to Cys77, Cys87 and Cys90(10). The DBD core also contains a solvent exposed reactive cysteine located at position 166. The N-terminal domain, also referred to as basic or "B-domain", is necessary for cooperative DNA binding of gp32 to generate gp32-DNA nucleoprotein complex [11]. The C-terminal domain contains an intrinsically disordered region predominantly composed of acidic residues, hence referred to as acidic or "A-domain". The C-terminal acidic tail is necessary for the functional and physical association of gp32 with replication and damage repair proteins [12,13].

gp32 is critical for coupling leading and lagging strand synthesis, homologous recombination, DNA damage repair, transcription and DNA packaging [9,14,15]. gp32 molecules bound to DNA are expelled by ATP-dependent unwinding activities of T4 gp41, Dda and UvsW helicases [16–19]. Parental DNA unwound by helicases is coated by gp32. The polymerase holoenzyme progressing forward during primer extension DNA synthesis displaces the DNA-bound gp32 proteins for efficient DNA synthesis [20]. During lagging strand DNA synthesis, gp32 recruits the primosome complex for Okazaki fragment synthesis [21]. gp32-coated DNA generated during recombination reaction are recognized by the recombinase UvsW●UvsY●ATP complex, and this complex displaces the gp32 molecules to form a nucleoprotein filament

E-mail address: senthil.kperumal@gmail.com.

https://doi.org/10.1016/j.bbrep.2023.101518
Received 23 March 2023; Received in revised form 17 July 2023; Accepted 19 July 2023

composed of DNA●UvsX molecules [16–18,22]. This nucleoprotein filament invades intact duplex DNA structures to initiate recombination-dependent DNA synthesis [23,24]. These proteins and reactions of the T4 phage recombination-dependent synthesis are also involved in the repair of DNA damages such as double strand breaks (DSB) and single-stranded nicks [23,25,26].

New methods to monitor various aspects of ssDNA-dependent DNA replication and recombination reactions in real time are of great interest and these methods will complement existing methods used for characterization of DNA/RNA unwinding reactions and polymerization [27–30]. Most of these assays are limited to monitoring resolution of DNA and RNA structures by helicases using FRET-based methods or monitoring NTP hydrolysis using coupled enzymatic methods [27–30]. Here, a fluorescein labeled gp32 protein was used as a probe to monitor in real time various ssDNA-dependent reactions. This assay will serve as a useful fluorescence method to detect reactions of single-stranded DNA and proteins, which is based on a fluorescein conjugate of the well-characterized and easy-to-express bacteriophage T4 gene 32 protein, gp32. An organic fluorescent dye was introduced at Cys166 and leveraged to study various ssDNA-dependent processes in a continuous assay format. This probe was used to investigate the kinetics of ssDNA translocation activity and protein displacement activities of T4 phage DNA helicases, kinetics of primer extension by the polymerase holoenzyme complex on gp32-coated ssDNA. This gp32 protein-based fluorescent probe was also explored for monitoring homologous recombination reaction and the formation of presynaptic nucleoprotein complex formation during the recombination reaction. Application of this probe in mechanistic studies of protein-protein interaction was exemplified by successfully investigating the physical interaction of UvsW helicase and replication protein gp41 with gp32 through the C-terminal acidic tail region of gp32.

2. Methods

2.1. Reagents

All oligonucleotides were purchased from IDT Technologies, Inc. ATP was purchased from Sigma-Aldrich. Single-stranded circular M13mp18 DNA was obtained using standardized procedures as previously described [31]. Primed M13mp18 DNA was obtained by mixing 100 nM *M13mp18* (7249 bp) ssDNA and 150 nM of 5′-[T] 20TGCGCCGCTACAGGGCGCGTAC-3′ primer in a 10 mM Tris (pH 7.8), 1 mM EDTA and 100 mM NaCl buffer, and heating the mixture in a water bath at 85 °C for 3 min and gradually cooled to room temperature. 5-Iodoacetamidofluorescein (5-IAF) was purchased from Thermo Scientific. All DNA-dependent reactions were carried out in a reaction buffer comprised of 20 mM Tris acetate (pH 7.8), 125 mM potassium acetate and 10 mM magnesium acetate [6,32,33].

2.2. Proteins

T4 phage ssDNA binding protein gp32 and gp32-A mutant lacking the C-terminal acidic tail were cloned, expressed, and purified as previously described [6,17,22,34,35]. T4 recombinase proteins UvsX and UvsY, helicases gp41 and UvsW, replication proteins gp43 polymerases, gp59 helicase loader, gp45 processivity clamp and gp44/62 clamp loader proteins were obtained as previously reported [36–42]. T4 Dda protein was a generous gift from Dr. Kevin D. Raney, University of Arkansas for Medical Sciences.

Fluorescein labeled gp32 and gp32A proteins were prepared as follows: the concentrated pure protein (gp32 or gp32-A) was buffer exchanged into a buffer containing 20 mM HEPES (pH 7.40), 150 mM NaCl and 10% (w/v) glycerol. A 10-fold excess of 5-acetamidofluorescein in DMF was mixed with gp32 or gp32A solution and incubated overnight at 4 °C. The reaction was stopped using freshly prepared 100 mM β-mercaptoethanol and dialyzed against a buffer containing 20 mM HEPES (pH 7.50), 100 M NaCl, 10 mM β-mercaptoethanol and 10% (w/v) glycerol. The labeling efficiency of gp32-F and gp32A-F were calculated from the absorbances at 492 and 280 nm. The labeled protein was concentrated, and flash frozen in liquid N2 and stored at −80 °C. The protein was determined to contain approximately 1 mol of fluorescein dye per mole of gp32 or gp32-A protein.

2.3. Steady-state fluorescence measurements

Steady state fluorescence measurements were carried out on a FluoroMax 4 spectrofluorometer from HORIBA at 25 °C. Fluorescence emission spectra for gp32 proteins carrying a fluorescein probe were obtained by exciting fluorescein at 480 nm and the emission between 490 and 600 nm was recorded. Corrections for the fluorescence intensity from dilution and inner filter effects were made by using the formula $F = F_{obs}$ antilog $(A_{ex}/2)$, where A_{ex} is the absorbance at the excitation wavelength of 480 nm.

2.4. Stopped-flow kinetic measurements

Kinetics of gp32-F and gp32-A-F binding to DNA and dissociation were measured on an Applied Photophysics stopped-flow apparatus equipped with a fluorescence detector. For the experiments involving gp32 proteins binding to DNA loading, the reaction was initiated by mixing the ssM13 DNA (200 nM) with gp32-A-F (1 μM), and the kinetics was monitored by signal change ($\lambda_{em} > 515$ nm) upon excitation at 480 nm. For the kinetics of gp32-F dissociation from DNA, the reaction was initiated by mixing a preorganized complex of gp32-F●DNA with the appropriate enzyme or enzyme complex, and the kinetics being performed as previously stated.

3. Results

3.1. Generation and characterization of fluorescein-labeled gp32 protein

gp32 protein was selectively labeled at the solvent exposed reactive Cys166 (Fig. 1A) by fluorescein using 5-Iodoacetamidofluorescein involving thiol chemistry. Both the wild-type protein as well as the mutant gp32-A protein that lacks the C-terminal acidic tail were labeled to obtain fluorescein-labeled gp32 (gp32-F) and gp32-A (gp32-A-F) proteins with a 1:1 stoichiometry of dye to protein ratio. Ligation of the thiol reactive organic dye to Cys166 is very selective with one label per protein without altering the zinc bound thiols, this is agreement with reported labeling selectivity of the only accessible cysteine of gp32 protein [17,43]. Cys166 labeling of gp32 does not alter the activity of the protein [17,22,34,35,44]. These fluorescein-labeled ssDNA-binding protein gp32 were employed to understand the interaction of T4 replication proteins with gp32 protein [6]. This probe could be exploited further to uncover the mechanistic details of various ssDNA-dependent processes because much of the DNA replication and recombination reactions involve ssDNA as an intermediate.

Labeled gp32 protein was validated for its robustness as a probe by measuring both the equilibrium binding as well as the kinetics of gp32 protein binding to ssDNA. These studies were carried out by mixing ssDNA and fluorescein-labeled gp32 proteins and monitoring the signal change of fluorescein-labeled gp32 bound to DNA. Both gp32-F and gp32-A-F displayed a three-fold change in the signal of fluorescein-labeled gp32 upon binding ssDNA (Fig. 1B and C). The binding of gp32-F to ssDNA involved a single-exponential kinetics with an observed rate constant of $0.1946 \pm 0.0052\ s^{-1}$ suggesting a simple bimolecular association (Fig. 1D). However, the behavior of gp32-A mutant binding to DNA exhibited double-exponential kinetics with observed rate constants of $0.548 \pm 0.010\ s^{-1}$ and $0.034 \pm 0.007\ s^{-1}$. This result suggests that the binding involves complex steps due to the absence of the acidic tail (Fig. 1E). gp32 binding to DNA is rapid and diffusion limited as observed in the case of *Escherichia coli* SSB protein [45,46].

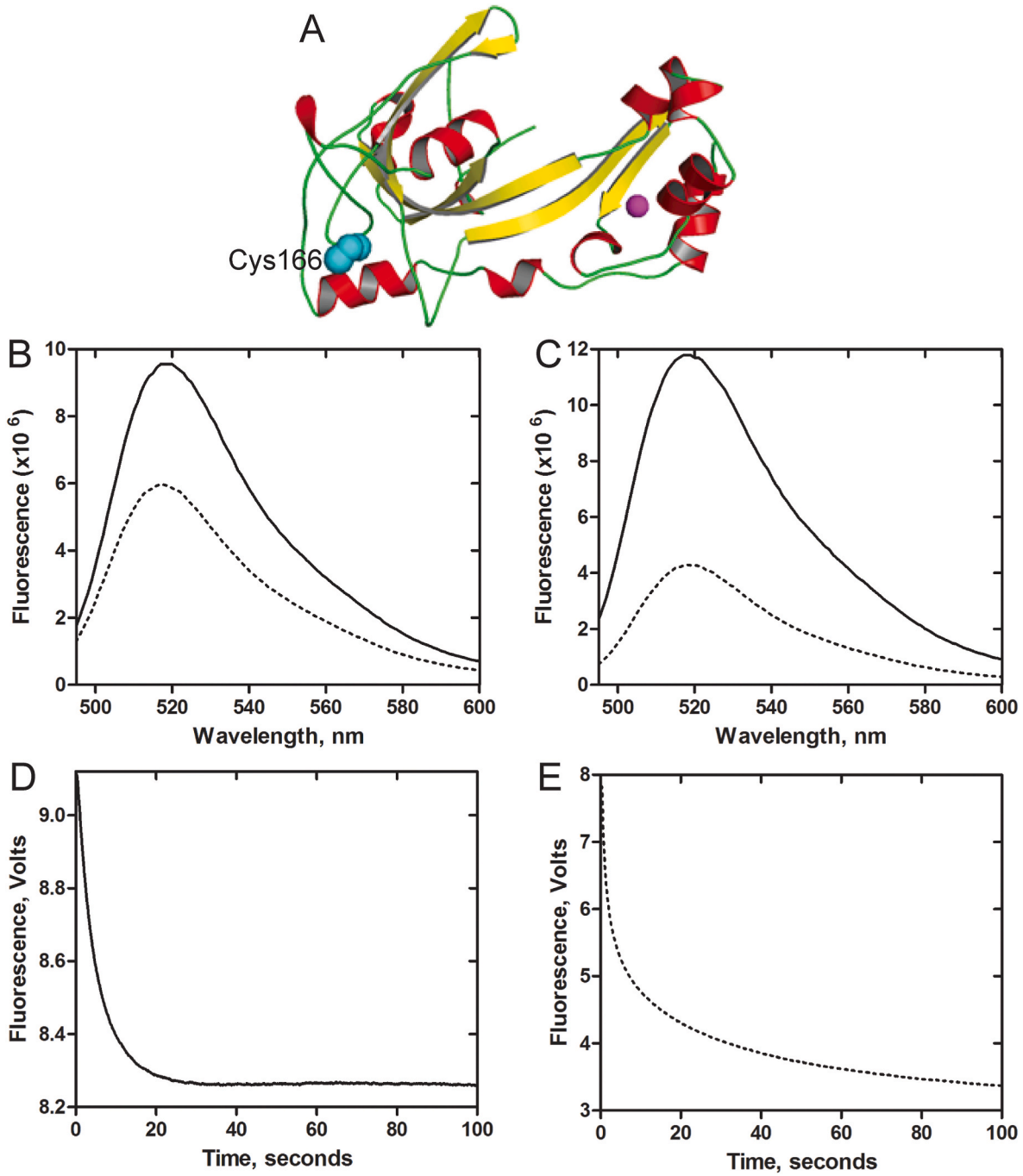

Fig. 1. Fluorescence change associated with binding of the fluorescein-labeled ssDNA binding protein gp32 to DNA (PDB code: 1GPC). (A) Structure of gp32 highlighting the location of Cys166 (spherical representation of side chain shown). Zn atom shown as sphere. Fluorescence emission spectra (solid line) of gp32-F (0.5 μM) (C) and gp32-A-F (0.5 μM) (D) and change in the emission upon binding ssM13 DNA (10 μM nt) (broken line). Stopped-flow kinetic time trace for the binding of labeled gp32-F (0.5 μM) (E) and gp32-A-F (0.5 μM) (F) proteins binding to ssM13 DNA substrate (10 μM nt). Upon excitation at 480 nm, the kinetics were monitored by following the fluorescence signal change ($\lambda_{em} > 515$ nm).

3.2. Application of the probe to study the dynamics of gp32●DNA interaction

gp32 protein binds to ssDNA with very high affinity and also displays high cooperativity (ω = 1000) [47], but their exchange on DNA is not clear. Spontaneous dissociation of gp32 protein in solution was investigated by mixing a preformed complex against 10-fold excess of a competing unlabeled gp32 trap protein. This excess protein would prevent the rebinding of fluorescein-labeled protein to DNA. Exchange kinetics of gp32 on DNA involved a double-exponential behavior with observed concentration-independent rate constants of 0.997 ± 0.011 s^{-1} and 0.159 ± 0.060 s^{-1} (Fig. 2A). The exchange of gp32-A also exhibited a similar kinetic behavior in solution; however, the exchange rates were much slower with observed rate constants of 0.132 ± 0.02 s^{-1} and 0.015 ± 0.003 s^{-1} (Fig. 2B) *ca.*10-times slower ($t_{1/2}$ = 10–40 s) than the wild-type protein, suggesting that the wild-type protein exchanges much faster in solution than the gp32-A mutant protein.

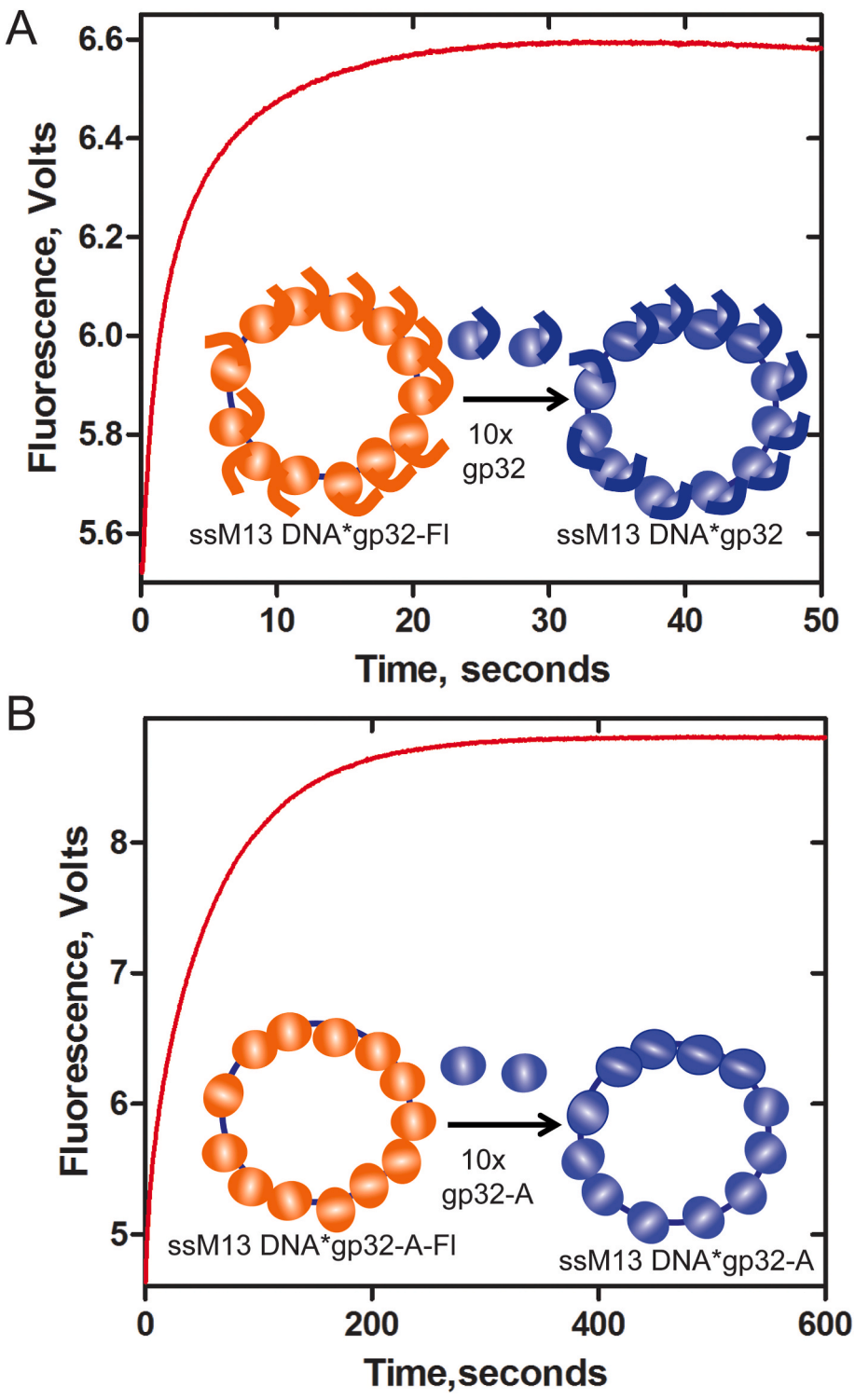

Fig. 2. Dynamics of DNA-bound gp32 proteins in solution. Spherical representation of the wild-type gp32 and the gp32-A mutant are differentiated by the presence of the C-terminal acidic tail (wild-type protein). (A) Stopped-flow kinetic time trace of dissociation of gp32-F (0.5 μM) from DNA (10 μM nt) into solution monitored in the presence of 10-fold excess of unlabeled gp32 (5 μM) trap protein (schematic representation shown in inset). The reaction was initiated by mixing the trap protein against the complex of gp32-F●DNA, and the kinetics were monitored by following the signal above 515 nm. (B) Kinetic time trace of gp32-A-F (0.5 μM) dissociating from DNA (10 μM nt) in the presence of 10-fold excess of unlabeled gp32-A (5 μM) trap protein (Inset shows a schematic representation of the process).

3.3. Application of the gp32 probe to study protein-protein interactions

Since gp32 plays a central role in various processes involving ssDNA●protein complexes, gp32-F could be used to study protein-proteins interactions. Binding of a protein partner could change the fluorescence of gp32-F, and this change could be utilized to monitor the binding and the kinetics of protein interactions involving gp32. When binding of UvsW helicase to gp32-F in the absence of DNA was carried out, the fluorescence of gp32-F was significantly quenched (Fig. 3A) suggesting that these two proteins interact in solution. When the kinetics of the binding between UvsW and gp32 were measured by rapid mixing of gp32-F and UvsW, a large time-dependent reduction in the fluorescence of gp32-F was observed (Fig. 3B, green). The trace displayed a biphasic kinetic behavior with a total amplitude change of 0.8. This drop in fluorescence signal corresponded to the association of gp32-F molecules with UvsW helicase in solution forming a stable gp32-F●UvsW complex.

The helicase-loader protein gp59 forms a complex with the gp32 protein on DNA [8,48–51]. It is possible for gp59 to interact with gp32 protein in the absence of DNA. When this interaction was evaluated by

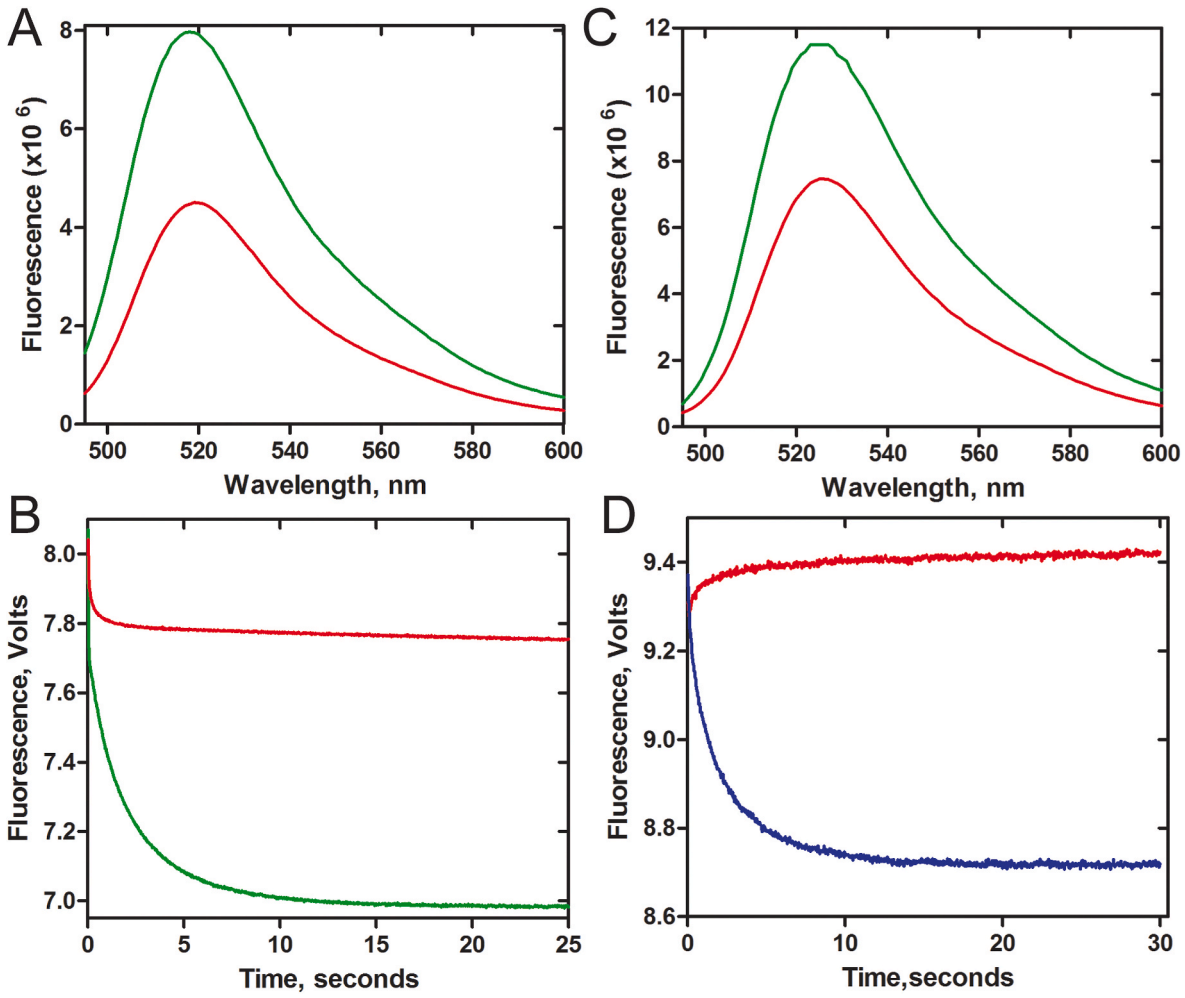

Fig. 3. Analyses of protein-protein interactions. (A) Fluorescence emission spectra of gp32-F (0.5 μM) in the absence (green) and presence (red) of UvsW helicase (0.5 μM). (B) Stopped-flow kinetic trace for the binding of gp32-F (0.5 μM) (green) and gp32-A-F (0.5 μM) (red) to UvsW helicase (0.5 μM) in solution. The kinetics were monitored by following the fluorescence signal change ($\lambda_{em} > 515$ nm) upon excitation at 480 nm. (C) Fluorescence emission spectrum of gp32-F (0.5 μM) in solution (green). The emission spectrum of gp32-F in the presence of 0.5 μM gp59 helicase-loader complex (red). (D) Stopped-flow kinetic trace for the association of gp32-F (0.5 μM) (red) and gp32-A-F (0.5 μM) (blue) proteins to 0.5 μM gp59 helicase loader. The kinetics were monitored as in (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

adding gp59 helicase loader to gp32-F in solution, a significant decrease in the fluorescence of gp32-F from the addition of gp59 was observed (Fig. 3C). The kinetics of association of gp59 and gp32 proteins using gp32-F was initiated by mixing gp59 protein against gp32-F. The kinetic trace displayed a significant time-dependent decrease in the fluorescence of gp32-F (Fig. 3D, blue) with a single-exponential binding with an amplitude of 0.514 and an observed rate constant of 0.42 ± 10 s^{-1}. This drop in signal could arise from local conformational changes associated with the interaction of gp32 and gp59 proteins in solution. These observations disclosed a new interaction of gp59 helicase loader protein and gp32 in solution.

The C-terminal acidic tail of gp32 is essential for the interaction of gp32 with its various protein partners [6,39]. This role was investigated using gp32-A-F mutant protein that lacks the C-terminal acidic tail. Kinetics of gp32-A-F mutant protein binding to UvsW (Fig. 3B, red) showed that, unlike the wild-type gp32-F protein, the interaction of the mutant protein is weak as evident by the reduced amplitude of the signal change (0.24 vs 0.84 for gp32-F). A similar observation was made with the gp59 and gp32-A-F pair (Fig. 3D, red) displaying a small increase in signal which could arise from the conformational change associated with the interaction of gp32A and gp59 proteins that increases the fluorescence signal of fluorescein on gp32A protein. The interaction of gp59 with gp32 protein is different completely different when the C-terminal tail was not present. These observations suggest that the C-terminal acidic tail of gp32 is necessary for the interaction of gp32 with UvsW and gp59 proteins in solution.

Next the requirement of the C-terminal tail for interaction of gp32 with its protein partners on DNA was tested using UvsW and gp41 helicases. The kinetics of gp32 interaction with UvsW●ATP on DNA exhibited a signal change with an amplitude of 1.1 and this was associated with the displacement of gp32 protein from DNA by UvsW in an ATP-dependent manner (Fig. 4A, blue). When gp32-A-F mutant protein was used, UvsW had significantly reduced protein displacement activity with an amplitude of 0.2 (Fig. 4A, red). These observations implied that the C-terminal tail of gp32 plays a critical role for its interaction with UvsW helicase on DNA. When the gp41●gp59●ATP complex was mixed with gp32-F●DNA, the ssDNA-binding proteins were displaced from DNA as evident by an increase in the fluorescence of the displaced gp32-F molecules (Fig. 4B, blue). When gp32A-F was used in the assay a significant loss of protein displacement activity was observed from the reduced fluorescence quenching (Fig. 4B, red). The reduction in fluorescence signal could arise from the mode of interaction of gp59 and gp32A on DNA that could reduce the signal of fluorescein on gp32A. These data confirmed that the C-terminal tail is important for the interaction of gp41 helicase as well as UvsW on DNA with gp32 protein.

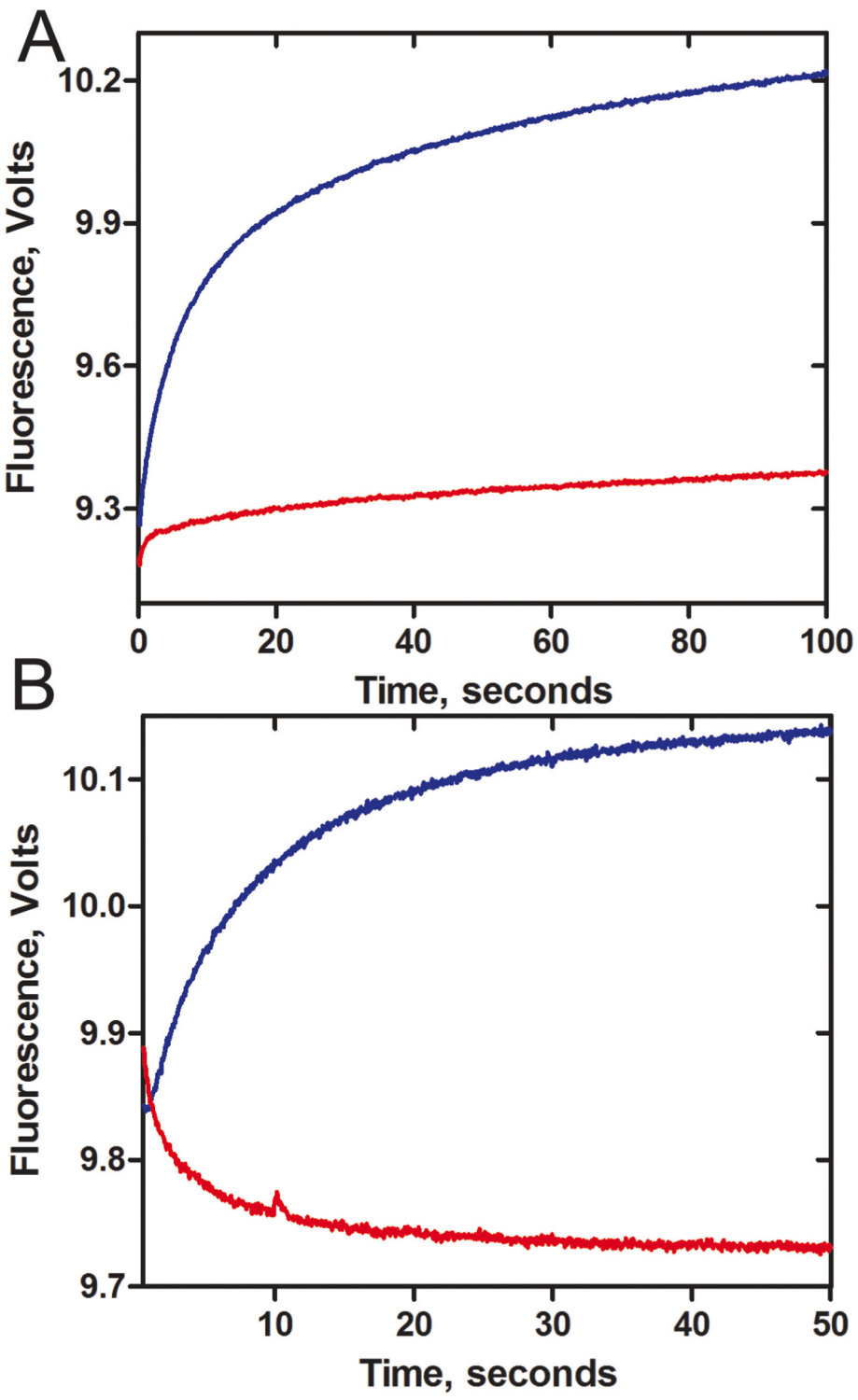

Fig. 4. Protein-protein interactions of gp32 using the fluorescent probe on DNA. (A) Stopped-flow kinetic trace for the displacement of gp32-F from DNA (blue) and gp32-A-F from DNA (red) catalyzed by UvsW helicase. Here, DNA●gp32 complex derived from ssM13 DNA (10 μM nt) and gp32 (0.5 μM) was rapidly mixed with UvsW helicase (0.5 μM), and the kinetics were monitored by following the fluorescence signal change ($\lambda_{em} > 515$ nm) upon excitation at 480 nm. (B) Kinetic trace for the displacement of gp32-F from DNA (blue) and gp32-A-F from DNA (red) catalyzed by gp41 helicase. Here, DNA●gp32 complex was rapidly mixed with gp41●gp59●ATP mixture derived from 40 nM gp41 helicase, 180 nM gp59 and 2 mM ATP, and the kinetics were monitored as in (A). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. Utilization of the probe to monitor ssDNA translocation and protein displacement by DNA helicases

When UvsW translocates on ssDNA, it displaces gp32 molecules bound to DNA(6). This translocation coupled to protein displacement activity was investigated by using a preorganized gp32-F●DNA complex and then initiating the reaction by addition of UvsW and ATP in a stopped-flow mixing chamber. The ATP-dependent translocation led to the displacement of gp32-F from DNA in a biphasic manner (Fig. 5A). When the concentrations of UvsW were increased, the amplitude and rate of the fast phase of the biphasic traces increased as a function of helicase concentration. However, the slow phase was concentration independent with an observed rate constant of 0.160 ± 0.005 s^{-1}. At 63 nM, 126 nM and 189 nM UvsW concentrations, the amplitude changes were 0.339, 0.497 and 0.615 with a rate of 2.69 ± 0.45 s^{-1}, 4.70 ± 0.56 s^{-1} and 6.36 ± 0.95 s^{-1}, respectively. The observed burst phase was attributed to a single event of helicase-mediated protein displacement whereas the slow phase describes dissociation of UvsW involving rate-limiting rebinding of UvsW and the displaced gp32 proteins and the associated steady-state protein displacement by the helicase.

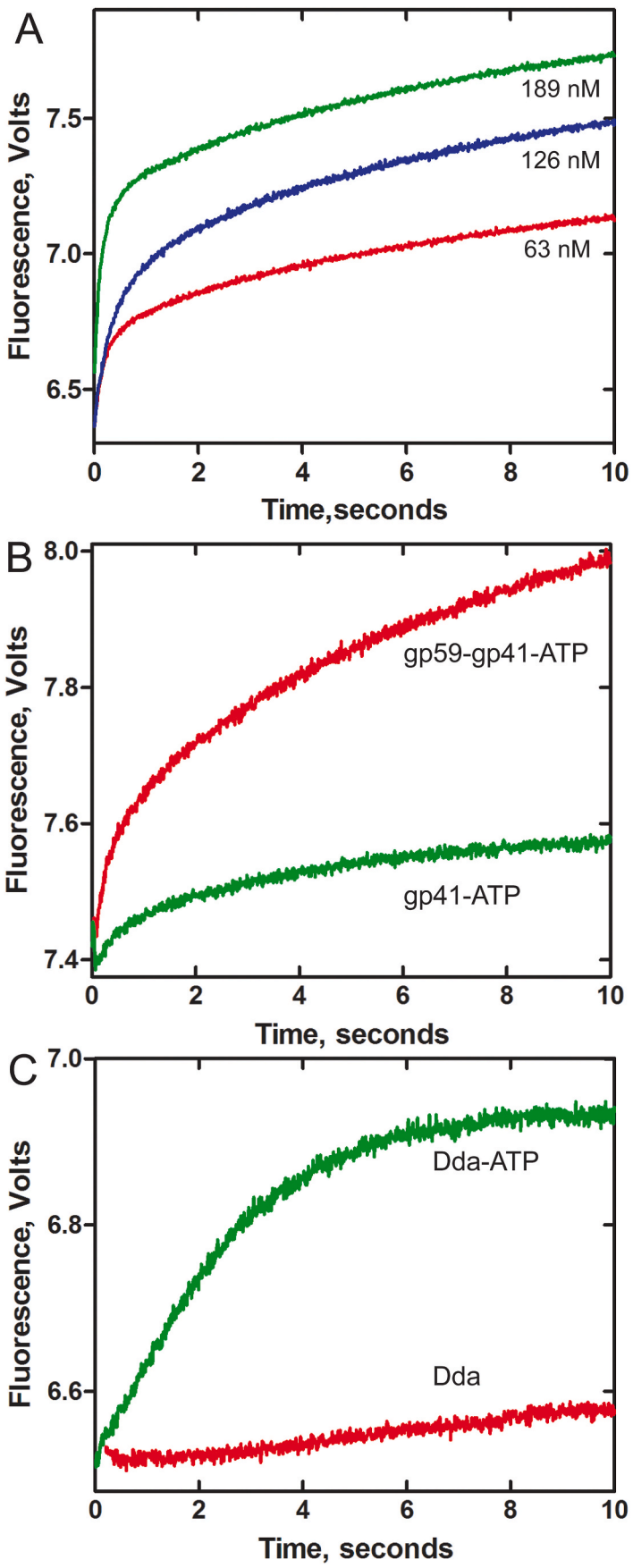

Fig. 5. Protein displacement activity of phage T4 helicases. (A) Stopped-flow kinetic traces for the displacement of gp32-F (0.5 μM) bound to ssM13 DNA (10 μM nt) substrate by varying concentrations of UvsW helicase. The translocation reaction was initiated by mixing a pre-organized complex of gp32-F and ssM13 DNA against a mixture containing UvsW and ATP (2 mM). The kinetics were monitored by following the fluorescence signal change (λ_{em} > 515 nm) upon excitation at 480 nm. (B) Stopped-flow kinetic trace of gp41 helicase translocation and displacement of gp32-F on DNA (red). Kinetic trace for gp41 translocation and protein displacement in the presence of the helicase-loader complex gp59 (green). A preincubated complex of gp32-F (0.5 μM) and ssM13 DNA (10 μM nt) was mixed against a mixture containing ATP (2 mM) and 100 nM gp41 (red) in the presence of 300 nM gp59 (green). The kinetics were monitored as in (A). (C) Time trace for ssDNA translocation and gp32 displacement by Dda helicase in the absence (red) and presence (green) of ATP. Here, a mixture of gp32-F and DNA was rapidly mixed with Dda helicase (40 nM) and ATP (1 mM). The kinetics were carried out as in (A). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

gp41 helicase is the replicative helicase in the T4 phage and is involved in unwinding the parental strand of DNA during replication [37]. gp41 helicase-mediated translocation and gp32 protein displacement activities on DNA were initiated by the addition of gp41●ATP to a pre-formed gp32-F●DNA complex. The signal change from the protein displacement due to the translocating helicase exhibited biphasic kinetics. The kinetics displayed a burst phase with a rate constant k_1 of 2.05 ± 0.82 s^{-1}, followed by a steady-state slow phase with a rate constant of 0.145 ± 0.023 s^{-1}, arising from steady-state displacement of gp32-F (Fig. 5B). When ATP is excluded from the reaction, no change in the signal was observed (data not shown). Efficiency of spontaneous assembly of gp41 onto DNA is low, but the efficiency of this process on DNA is greatly enhanced by gp59 protein, this is evident from the six-fold increased amplitude change of 0.693 for gp32 displacement by gp41●gp59 complex (cf. 0.192 in the absence of gp59) involving a three-fold increase in the rate with gp59 helicase loader.

T4 Dda helicase is involved in replication initiation and interacts with recombination proteins [52,53]. Dda helicase-mediated DNA translocation and protein displacement reactions were initiated by mixing gp32-F●DNA with Dda●ATP and the signal change from gp32-F protein displaced from DNA was monitored (Fig. 5C). A significant change in the signal with a rate of 0.39 ± 0.07 s^{-1} (amplitude change: 0.452) implies that similar to gp41 and UvsW, Dda also translocated on DNA and displaced gp32 molecules. In the absence of ATP, no change in the signal was observed confirming that the observed signal is associated with ATP-dependent ssDNA translocation of Dda helicase.

3.5. Application of gp32 probe in assaying DNA polymerization by T4 polymerase holoenzyme

T4 DNA polymerase holoenzyme composed of a complex of gp43 polymerase and gp45 sliding clamp carries out processive DNA synthesis [54]. Due the discontinuous nature of Okazaki fragment synthesis, gp32 proteins are more predominant on the lagging strand and the polymerase holoenzyme encounters gp32 protein more often during Okazaki fragment synthesis. gp32-F probe was explored to monitor polymerase holoenzyme catalyzed DNA synthesis by following the displacement of gp32 from DNA during primer extension synthesis. This was tested by using polymerase holoenzyme mediated DNA synthesis on a gp32 coated primed-DNA substrate. The reaction was initiated by the addition of gp43 polymerase, gp45 clamp, gp44/62 clamp loader, dNTPs, and ATP to a preassembled complex of primed-M13 DNA●gp32-F. The polymerase holoenzyme assembled at the primer-template DNA junction extends the primer using the dNTPs. The primer extension caused the displacement of gp32-F molecules from the templating DNA thus reporting on the kinetics of DNA polymerization reaction (Fig. 6A). The kinetics displayed a biphasic behavior with a rate constant k_1 of 0.049 ± 0.009 s^{-1} (amplitude: 0.161) and a slow second phase with a k_2 of 0.0063 ± 0.0002 s^{-1} (amplitude: 0.450). Under limiting holoenzyme conditions, the fast phase corresponded to burst single binding kinetics involving processive synthesis by the holoenzyme complex. The slow phase corresponded to steady-state primer extension from the rebinding of holoenzyme to the excess substrate in solution. When the dNTPs required for primer extension were excluded from the reaction, gp32 protein displacement by the polymerase holoenzyme was not observed. This established that the observed signal in the reaction was associated with nucleotide incorporation during primer extension by the polymerase holoenzyme complex.

3.6. Probing presynaptic nucleoprotein complex formation in recombination by utilizing the gp32 probe

T4 phage encodes an array of proteins to carry out homologous recombination reaction. This includes gp59 protein, gp46/47 (Mre11/Rad50) complex, UvsX recombinase, UvsX-loader complex UvsY, UvsW helicase and gp32 protein [23,25,26,55]. The first step in the initiation

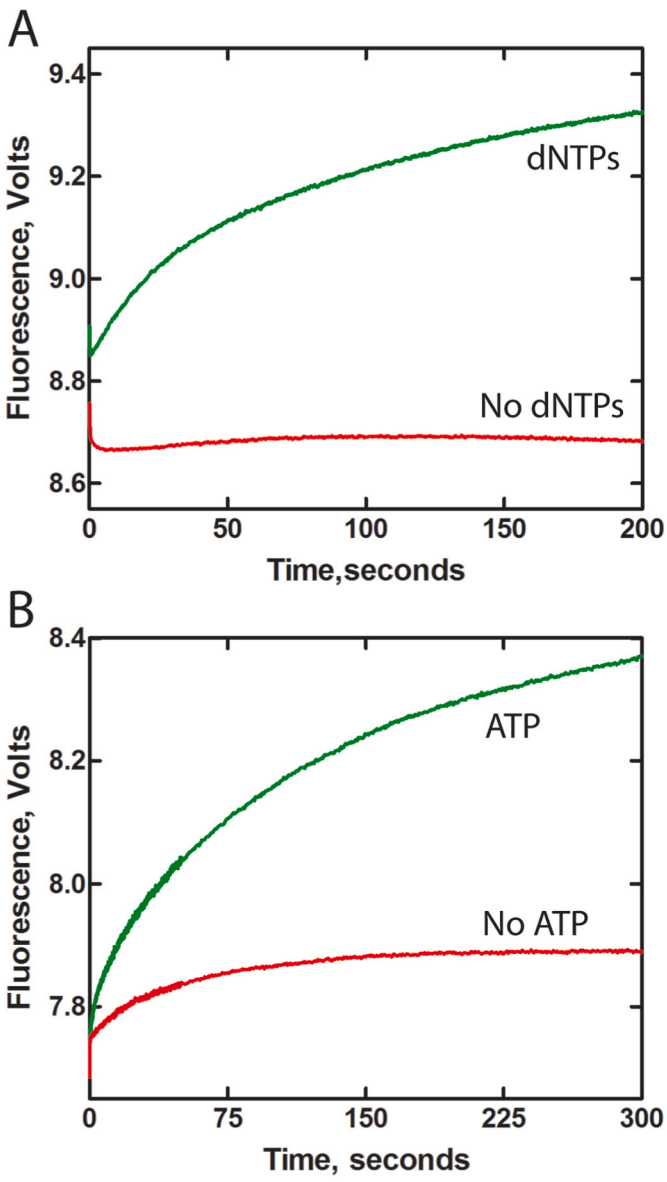

Fig. 6. Real-time analysis of DNA polymerization and recombination initiation reactions using fluorescently labeled gp32 probe. (A) Stopped-flow kinetic trace for the holoenzyme catalyzed primer extension on a primed-ssM13 DNA substrate (10 μM nt) coated with gp32-F (0.5 μM). The reaction was initiated by mixing a preincubated complex of gp32-F and ssM13 DNA with mixture containing gp43 (30 nM), gp44/62 (100 nM), gp45 (100 nM), ATP (2 mM) in the absence (red) and presence of dNTPs (800 μM, green). The kinetics were monitored by following the fluorescence signal change (λ_{em} > 515 nm) upon excitation at 480 nm. (B) Stopped-flow kinetic trace for the formation of the UvsX-DNA-UvsY-ATP nucleoprotein filament formation. A preincubated complex of gp32-F (0.5 μM) and ssM13 DNA (10 μM nt) was rapidly mixed against a mixture containing UvsX (0.5 μM), UvsY (0.5 μM) in the absence (red) and presence of 2 mM ATP (green). The kinetics were monitored as in (A). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

of DNA recombination and RDR reactions is the generation of ssDNA by the gp46/47 nuclease complex [23,26]. In the T4 system, UvsX●ATP●UvsY system recognizes gp32-coated ssDNA and initiates the formation of the UvsX-bound nucleoprotein complex. This complex formation is essential for the homologous recombination reaction. UvsX is the recombinase protein that is loaded onto the gp32-coated DNA by the UvsX-loader protein, UvsY, in the presence of ATP. When the DNA●UvsX●ATP●UvsY complex assembles on DNA, the gp32 protein

is displaced. The kinetics of pre-synaptic recombination nucleoprotein complex formation was evaluated in real time by monitoring gp32 displacement using the gp32-F probe. This was investigated by mixing the recombinase complex to gp32-coated DNA. The kinetics displayed a single-exponential behavior with a rate constant of 0.022 ± 0.004 s^{-1} and an amplitude change of 0.56 (Fig. 6B, black solid line). Requirement of ATP on the kinetics of gp32-F displacement by UvsX●UvsY complex was tested by excluding the nucleotide in the reaction (Fig. 6B, broken line), and a significantly reduced signal was observed (amplitude change of 0.12) when compared to the ATP mediated. This observation confirmed that the observed signal arises from the ATP-dependent formation of the presynaptic filament following the displacement of gp32-F molecules from DNA.

4. Discussion

DNA replication, recombination, and damage repair processes are mediated by several protein-protein interactions that carefully orchestrate the assembly of the functional replisome and recombinase complexes on DNA. It is important to study the details of these protein-protein interactions to understand the assembly of various DNA-dependent processes. Such studies necessitate a versatile probe that could be used to study various protein-protein and protein-DNA interactions. SSBs play critical roles in DNA replication, recombination, and damage repair reactions. These proteins maintain the integrity of genomic DNA by protecting the ssDNA from nucleases and recruit proteins essential for various DNA-dependent reactions. A fluorescein-labeled gp32 based real-time fluorescence assay described here would complement other existing radiometric and FRET methods to study these various ssDNA-dependent processes such as DNA polymerization, unwinding of duplex DNA by helicases, and initiation of recombination reactions.

gp32 probe was effectively used to characterize the dynamics of gp32 proteins on DNA. Studies showed that bacterial SSB proteins slide on DNA [45,56,57], however, because DNA wraps around the bacterial SSB tetramer, it is stably bound with restricted spontaneous dissociation in solution. Monomeric gp32 molecules, on the other hand, bind DNA by tucking the DNA into the binding site, and this DNA-gp32 complex is thought to be a stable complex. The observations here suggest that, albeit their high affinity for long ssDNA with a K_D of 100 nM, gp32 dissociates readily from the DNA ($t_{1/2} = 1–4$ s). The observations suggest that gp32 is dynamic on DNA and exchanges readily in solution. We noticed that the gp32-A mutant protein has reduced spontaneous dissociation from DNA in solution. The C-terminal tail of T7 ssDNA-binding protein gp2.5 and *E. coli* SSB mimics the electrostatics of DNA backbone and regulates the binding of the protein to DNA [46, 58–60]. However, when the acidic tail is removed, these proteins maintain a strong interaction with DNA due to the absence of competing electrostatics. gp32 displays a similar behavior due to the lack of competition from the auto-inhibitory C-terminal acidic tail and hence has a reduced rate of dissociation from DNA and forms a more stable complex on DNA than the wild-type protein. Collectively these observations suggest that gp32 protein is dynamic when bound to DNA and the stability of the protein on DNA is enhanced without the C-terminal acidic tail.

Several direct protein-protein interactions of gp32 within the T4 replisome have been established [6,12,13]. The change in the fluorescence of gp32-F protein upon binding DNA and other protein partners was exploited in identifying protein-protein interactions such as UvsW●gp32 and gp59●gp32. The gp32-F probe also identified a previously unknown interaction of gp32 and gp59 proteins in solution. This illustrates the effectiveness of the gp32-F probe in identifying unique protein-proteins interactions involving gp32. The gp32-A mutant probe was used to study gp32 mediated protein-proteins interactions on DNA such as UvsW and gp41 through the C-terminal acidic tail of gp32. These observations demonstrate that the fluorescein-labeled gp32 could serve as excellent probe for exploring protein-protein interactions.

Helicases catalyze the conversion of duplex DNA into ssDNA using ATP hydrolysis energy. T4 phage encodes there helicases namely gp41, UvsW, and Dda belonging to SF1, SF2, and SF4 family of helicases, respectively. UvsW plays an important role in DNA recombination and RDR reactions [24,61,62] by catalyzing replication fork remodeling through strand reannealing and unwinding activities [6,38,63]. gp41 helicase has ATP-dependent unwinding and ssDNA translocation activities[20,21,22,] [37,64]. Dda helicase possesses unwinding and ssDNA translocation activities with a biased 5′-to-3′ directionality [65–68]. Here fluorescein-labeled gp32 was used as an effective probe for monitoring the DNA unwinding-associated activities of DNA helicases such as ATP-dependent ssDNA translocation and protein displacement. This fluorescent probe complements gel mobility and radiometric assays used to study protein displacement activity of Dda helicase [35,68,69]. This assay provided a fast and easy real time method for monitoring ssDNA translocation coupled protein displacement activity of helicase and mapping out the requirement of the C-terminal acidic tail for the physical interaction of these helicases with gp32 both on DNA and in solution. ATP-dependent translocation of these helicases on ssDNA involved the enzymes latching on to the C-terminal tail of gp32 to displace these proteins from the DNA. These observations demonstrate that the fluorescein-labeled gp32 could serve as excellent probe for monitoring translocation activity of helicase.

DNA-templated primer extension reactions by the polymerase were characterized by gp32-F displacement reactions. When the polymerase holoenzyme encounters DNA-bound gp32 proteins in its path, it displaces the proteins coupled to the rate of primer extension and hence this could be used to monitor the kinetics of primer extension by the polymerase holoenzyme on DNA. The measurements of DNA primer extension by polymerase holoenzyme suggest that the gp32 probe could be exploited for detailed analyses of nucleotide incorporation rates, processivity of the holoenzyme complex from the number of gp32 molecules, and the change in the rate of gp32 displaced from the dissociation and reassembly of the polymerase holoenzyme complex on DNA. This assay also presents an easy and potentially novel method for measuring polymerase reaction in real time upon establishing the details of the molecular events of primer extension.

One of the first steps of DNA recombination and RDR is the generation of gp32-coated DNA. The gp32-coated nucleoprotein filament is recognized and bound by the recombinase complex, which displaces the bound gp32 protein. The formation of the ATP-dependent presynaptic recombination filament was monitored in real time from the displacement of gp32-F probe from DNA. The gp32 probe can be explored further in detail to understand the various molecular events of recombinant reactions in T4 phage. This method allows for the characterization of recombination reaction kinetics in the T4 system and could also be extended to other systems.

A highly sensitive fluorescent gp32 probe has been developed and explored beyond DNA binding assessment of gp32. This probe is easy to generate, implement and monitor several processes that involve ssDNA in DNA replication, recombination, and damage repair reactions. gp32 has the following advantages: it is easy to express and purify with very high yields; it is a monomeric protein; it can be labeled site-specifically without altering the functions of the protein; binding of gp32 to DNA is rapid and diffusion-limited; and the gp32-F probes are highly sensitive. When bound to DNA, the fluorescence of gp32-F and gp32-A-F are significantly quenched that was exploited to use these proteins are effective probes. We demonstrated that the Cys166-fluorescein labeled gp32 is an excellent probe that can be universally used to monitor several ssDNA-dependent reactions. This probe has broad applicability to study DNA metabolic reactions that involve ssDNA intermediate. Further, this probe complements other radiometric, gel-based, and single-molecule methods to survey ssDNA-dependent processes by reporting several protein-protein and protein-DNA interactions in real time. Fluorescent probes like gp32-F could be developed for other

systems to study these various DNA-dependent reactions. This approach could be expanded to similar DNA binding proteins. These proteins should be amenable to labeling with an organic dye through a naturally occurring solvent exposed Cys or a Cys introduced through site-directed mutagenesis. Such modification should retain the activity of the protein of interest.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

This work was supported by National Institutes of Health Grant GM013306 (to Stephen J. Benkovic). I would like to thank Dr. Stephen J. Benkovic for generously supporting this work, and for comments on the manuscript. I would like to thank Dr. Kevin D. Raney at University of Arkansas for Medical Sciences for providing us with T4 Dda helicase.

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