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ORIGINAL PAPER

Direct Observation Method of Individual Single-Stranded DNA Molecules Using Fluorescent Replication Protein A

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Abstract Direct observation studies of single molecules have revealed molecular behaviors usually hidden in the ensemble and time-averaging of bulk experiments. Direct single DNA molecule analysis of DNA metabolism reactions such as DNA replication, repair, and recombination is necessary to fully understand these essential processes. Intercalation of fluorescent dyes such as YOYO-1 and SYTOX Orange has been the standard method for observing single molecules of double-stranded DNA (dsDNA), but effective fluorescent dyes for observing single molecules of single-stranded DNA (ssDNA) have not been found. To facilitate direct single-molecule observations of DNA metabolism reactions, it is necessary to establish methods for discriminating ssDNA and dsDNA. To observe ssDNA directly, we prepared a fusion protein consisting of the 70 kDa DNA-binding domain of replication protein A and enhanced yellow fluorescent protein (RPA-YFP). This fusion protein had ssDNA-binding activity. In our experiments, dsDNA was stained by SYTOX Orange and ssDNA by RPA-YFP, and we succeeded in staining ssDNA and dsDNA by using RPA-YFP and SYTOX Orange simultaneously.

Keywords Direct single-molecule observation · λDNA · Single-stranded DNA · Replication protein A (RPA) · DNA-protein interaction

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Introduction

Analysis of the interactions between DNA and proteins has been an important component of studies investigating DNA metabolism reactions such as DNA replication, repair, and recombination [1–5]. Fluorogenic single-stranded DNA (ssDNA)-binding proteins, for example, have been used to study helicase activity [6]. Modification of ssDNA-binding proteins with fluorescent dye contributed to investigations of the mechanism of interaction between ssDNA and proteins [7] and to studies of the mechanical effects of ssDNA-binding proteins on bound DNA molecules [8]. However, these studies did not involve direct microscopic observation of the individual molecules. While the use of ssDNA-binding proteins has helped to elucidate the nature of DNA-protein interactions during DNA metabolism reactions, a complete understanding of this behavior remains elusive due to limitations of *in vitro* multimolecular measurements. Previous studies have described the average behavior of many molecules; thus, the properties of each molecule could not be clarified, and as a result the role of many enzymes remains largely unknown. Thus, direct single-molecule observation is a potentially useful technology for elucidating the interactions between DNA and proteins during DNA metabolism reactions [9–11].

Direct single-molecule observation involving fluorescent techniques has been successfully applied to the study of DNA-protein interactions [12]. In particular, fluorescent observation enables a better understanding of the relevant phenomena because DNA-protein interactions can be directly detected. For example, fluorescent labeling has been used to directly observe the sliding motion of RNA polymerases on double-stranded DNA (dsDNA) [10, 13], dsDNA hydrolysis by RecBCD helicase [14], and the influence of DNA form or tension on exonuclease III activity [15–17]. To observe DNA reproduction, restoration, rearrangements, and transcription, a fluorogenic reagent that can label dsDNA, ssDNA, and protein is needed. However, in contrast to dsDNA and protein, no fluorescent reagent for labeling ssDNA is currently available [15–17]. Therefore, we devised a direct observation method for rendering ssDNA visible using a fluorogenic ssDNA-binding protein.

The fragility of ssDNA relative to dsDNA poses an inherent problem for direct observation. It is difficult to control the formation of ssDNA in solution, and effective intercalating fluorescent dyes, such as YOYO-1, cannot be used for single molecule analysis of ssDNA [18]. These factors have limited development of imaging technologies for direct observation of a single ssDNA molecule. In this study, we focused on replication protein A (RPA), an ssDNA binding-protein involved in DNA metabolism [19]. This major eukaryotic single-stranded-specific DNA-binding protein was first identified as an essential factor for Simian Virus 40 DNA replication *in vitro* [19]. The activities of RPA were determined by its DNA-binding properties, as well as by its ability to interact with a variety of proteins, such as DNA polymerases, DNA damage recognition proteins, recombination factors, and transcriptional activators [20]. RPA is composed of three subunits, termed RPA70, RPA32, and RPA14, with molecular weights of 70 kDa, 32 kDa, and 14 kDa, respectively. The major ssDNA-binding function is associated with the two central DNA-binding domains of the RPA70 subunit [21]. We postulated that it would be possible to stabilize and directly observe ssDNA using fluorescently labeled RPA, and that this technology could be used for direct single-molecule observation of DNA metabolism reactions, such as DNA replication, repair, and recombination.

Material and Methods

Materials

Enzymes and DNA, including ExTaq DNA polymerase, *Kpn* I, *Nco* I, λDNA, M13mp18 ssDNA, and 100x Halt Protease Inhibitor Cocktail (EDTA-Free solution), were purchased from TAKARA (Shiga, Japan). SYTOX Orange dye (excitation 547 nm/emission 570 nm) used for staining dsDNA was purchased from Invitrogen (Carlsbad, CA, USA). Chemically synthesized primers were obtained from JBioS (Saitama, Japan). Ni-NTA Superflow was obtained from QIAGEN (Dusseldorf, Germany), while Heparin Sepharose, Q-Sepharose Fast Flow, and S-Sepharose Fast Flow were obtained from GE Healthcare UK Ltd. (Buckinghamshire, England). Bradford reagent and molecular weight markers for SDS-PAGE were purchased from Bio-Rad (Hercules, CA, USA), and the S-Tag AP Western Blot Kit was purchased from Novagen (Darmstadt, Germany). The enhanced yellow fluorescent protein (eYFP) fusion protein expression system of the pET32 modified vector (pET32-eYFP) was a kind gift from Prof. Hiroshi Ueda (Department of Chemistry and Biotechnology, University of Tokyo) [22]. All other reagents were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Wako Chemicals (Osaka, Japan).

Construction of the eYFP-mouse RPA70 (aa 191–438) Vector

The DNA-binding domain nucleotide sequence of mouse RPA70 was amplified by PCR with the following primers: RPA70-DBD-F: 5′-CGG GGT ACC CCG CAG CCA AAG TGG TGC CCA T-3′, and RPA70-DBD-R: 5′-CAT GCC ATG GCA TGT AAG GCT TGT CCT TCT GAG TCA AA-3′. The pET32-eYFP vector containing the ssDNA-binding domain nucleotide sequence of mouse RPA70 was digested with *Kpn* I and *Nco* I. This fusion protein contained several tags for purification, detection, and solubilization. The N-terminal domain of the fusion protein has Trx-, His-, and S-Tags, while the C-terminal domain has an eYFP and a His-Tag.

The recombinant protein comprising the ssDNA-binding domain of RPA and eYFP (RPA-YFP) was expressed and purified as follows. The vector construct was transformed into Rosetta (DE3) cells (Novagen) by heat shock, and one colony was used to inoculate 5 ml of Luria Bertoni (LB) medium containing 1% glucose, 100 μg/ml ampicillin, and 34 μg/ml chloramphenicol. The culture was grown overnight at 30°C and then 3 ml was used to inoculate 300 ml of 1% glucose in LB medium. After 1 h of growth at 30°C, 0.5 mM IPTG was added, and after an additional 2 h of growth, the culture was centrifuged at 15,000×*g* for 10 min at 4°C. The pellet was resuspended in TG buffer (50 mM Tris–HCl (pH 8.0), 10% glycerol, 0.01% Triton X-100, and 1x Halt Protease Inhibitor Cocktail). The resuspended cells were sonicated 10 times for 30 s each, then centrifuged for 20 min at 30,000×*g* at 4°C. The supernatant was then mixed with 10 ml of washed Ni-NTA Fast Flow beads. The column was washed with TG buffer containing 1 M NaCl,

and proteins bound to the resin were eluted with TG buffer containing 500 mM imidazol. The eluted recombinant RPA-YFP (fraction I) was further purified using Q-Sepharose Fast Flow and Heparin Sepharose.

Fraction I (with ssDNA-binding activity) was diluted with 10 volumes of TG buffer and loaded onto a Q-Sepharose Fast Flow column that had been pre-equilibrated with TG buffer containing 5 mM 2-mercaptoethanol. After the column was washed, the ssDNA-binding activity was fractionated again using a linear gradient of 0–1 M NaCl in TG buffer containing 5 mM 2-mercaptoethanol. The ssDNA-binding activity first appeared at around 0.3 M NaCl. These fractions from the Q-Sepharose Fast Flow column were collected, dialyzed, and then loaded onto a Heparin Sepharose Fast Flow column. The column was pre-equilibrated and washed in TG buffer containing 5 mM 2-mercaptoethanol, and elution was performed with a linear gradient of NaCl (0–1 M) in the TG buffer containing 5 mM 2-mercaptoethanol. The active protein, found in only one fraction eluting at approximately 0.4 M NaCl, was collected, dialyzed and concentrated using TG buffer containing 0.01% Triton X-100, 1 mM EDTA, 250 mM sucrose, and 50% glycerol solution, and stored at −80°C.

Gel Mobility Shift Assay of the RPA-YFP and DNA Complexes

For DNA-binding assays, single-stranded M13mp18 DNA (280 ng) was incubated for 2 h on ice in 300 pmol of RPA-YFP in 20 μl of 30 mM HEPES-NaOH (pH 8.0), 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% Tween 20, and 5 mM 2-mercaptoethanol. The resulting protein-DNA complexes were detected with ethidium bromide (EtBr) after agarose gel (0.8%) electrophoresis in TBE buffer.

Preparation of Amino Silane-Treated Coverslips

Amino silane-treated coverslips were prepared as reported by Lee and Swaisgood, with some modifications [23]. Coverslips (18 mm×26 mm; Matsunami) were cleaned by soaking in 1% Contaminon alkaline detergent (Wako) overnight at room temperature and were then washed, first with acetone and then milli-Q water. The coverslips were soaked in 30% hydrogen peroxide for at least 3 h and then washed with deionized water and blown dry with air. After the glass surface was cleaned, coverslips were soaked in 10% 3-aminopropyltriethoxysilane (Shin-Etsu Chemicals; Tokyo, Japan) for 2 h at room temperature, washed with milli-Q water, and then baked overnight at 100°C after blowing off the water with dry air. Next, DNA and the RPA-YFP complex solution was combed onto the modified coverslips using the moving droplet method [24].

Visualization of Single-Stranded λDNA (ssλDNA) for Direct Observation of Single DNA Molecules

We prepared two types of substrate DNA. The first was ssλDNA produced by heat denaturation (95°C, 10 min). The second substrate DNA consisted of half dsDNA and half ssDNA, and was prepared using an elongation reaction as follows. The elongation reaction mixture consisted of 1X rTaq buffer, 100 μM dNTPs, 1 μg λDNA, synthetic oligonucleotide (25 mer; 5′-CGTAACCTGTCGGATCACCGGAAAG-3′) and 0.5 units ExTaq DNA polymerase. The reaction (final volume of 100 μl) was carried out as follows: 95°C for 5 min, 55°C for 1 min, 72°C for 15 min, and then retention at 4°C. The reaction was stopped by the addition of 0.5 M EDTA (final concentration of 20 mM).

For the ssDNA visualization reaction mixture (final 100 μl), 680 pmol of RPA-YFP and 300 ng of ssDNA were added to 25 mM HEPES-NaOH (pH 8.0), 1 mM EDTA, 100 mM NaCl, 0.1% Tween 20, 5 mM 2-mercaptoethanol, and 10% glycerol. The ds/ssDNA visualization reaction mixture (final 100 μl) was made by adding 290 pmol of RPA-YFP and 200 ng of ds/ssDNA to 25 mM HEPES-NaOH (pH8.0), 1 mM EDTA, 100 mM NaCl, 0.1% Tween 20, 5 mM 2-mercaptoethanol, and 10% glycerol. After 2 h incubation on ice, SYTOX Orange (base pair:dye=75:1) was added to a final concentration of 200 nM and the mixture was incubated for 30 min on ice.

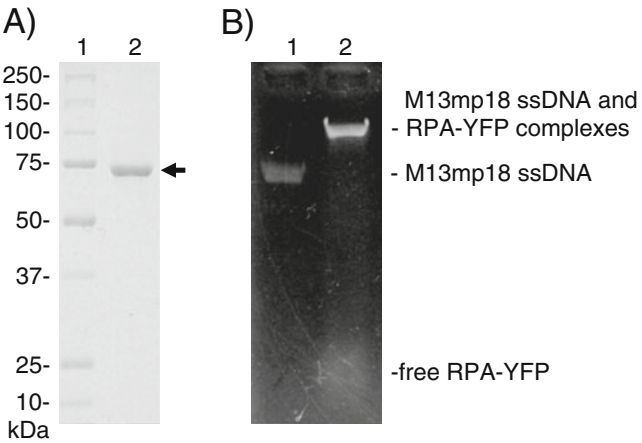

Fig. 1 **a** Purified fusion protein of YFP and the ssDNA-binding domain of replication protein A 70 kDa (RPA-YFP). The molecular weight of RPA-YFP was about 70 kDa. Purified RPA-YFP was electrophoresed on a 10% SDS-PAGE gel and visualized by staining with Coomassie Brilliant Blue R-250. Lane 1: Molecular weight marker. Lane 2: purified RPA-YFP. **b** Gel mobility shift assay of RPA-YFP using M13mp18 ssDNA. M13mp18 ssDNA with/without RPA-YFP complexes was analyzed using 0.8% (w/v) agarose gel electrophoresis and visualized with ethidium bromide staining. Lane 1: M13mp18 ssDNA without RPA-YFP. Lane 2: M13mp18 ssDNA with RPA-YFP

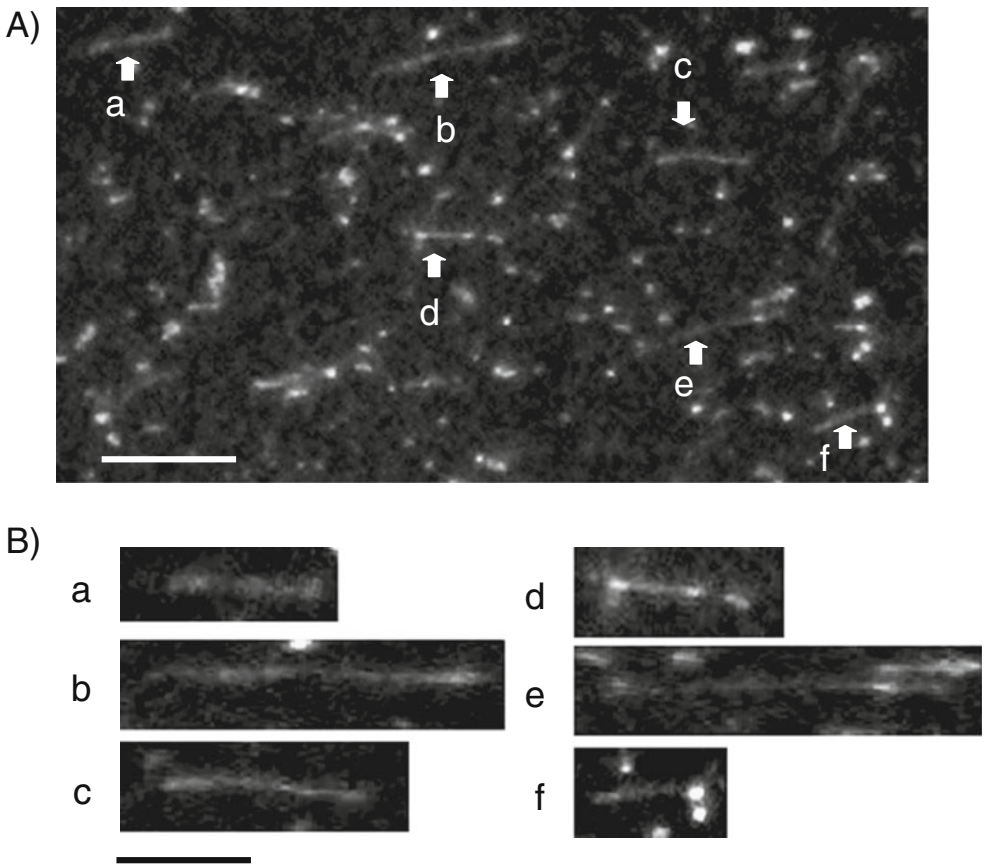

Fig. 2 Visualization of ssλDNA using RPA-YFP. **a** Direct observation of RPA-YFP fluorescence. White bar: 10 μm. **b** Higher magnification showing a linear signal. Black bar: 5 μm

The RPA-YFP-ssDNA or RPA-YFP-ds/ssDNA complexes were then combed onto coverslips using the moving droplet method [24].

Optical System for Visualization of DNA Molecules

DNA-protein complexes on coverslips were observed with a fluorescence microscope (Nikon ECLIPSE TE2000-U; Tokyo, Japan) equipped with a 100×1.3 numerical aperture (NA) oil immersion objective lens, and images were captured with a digital camera (Nikon DIGITAL CAMERA D80). DNA molecule lengths were determined by comparison with the appropriate scale on the captured images.

Other Methods

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 10% gels using the standard Laemmli method. After electrophoresis, gels were stained with Coomassie Brilliant Blue (CBB) R-250 (Wako) or EzStain Silver kit (ATTO). The protein concentration was determined according to the method of Bradford using a protein assay kit (Bio-Rad) with bovine serum albumin (BSA) as the standard.

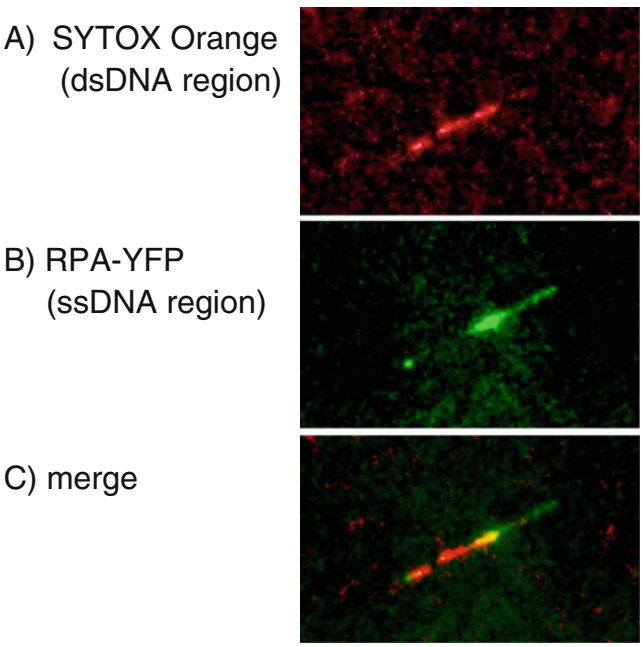

Fig. 3 Staining and direct observation of dsDNA and ssDNA. **a** The dsDNA region was stained with SYTOX Orange, producing a red signal. **b** The ssDNA region was stained with RPA-YFP, producing a yellow-green signal. **c** Merged image of a) and b). *White bar*: 10 μm

Results and Discussion

Purification and Gel Mobility Shift Assay

The RPA-YFP was isolated from bacterial extracts using the His-Tag system. The purified protein was subjected to 10% SDS-PAGE and the gel was stained with CBB (Fig. 1a). The gel indicated that purified RPA-YFP was about 70 kDa, which agreed with the molecular weight deduced from the amino acid sequence. The purified RPA-YFP was then used for the gel mobility shift assay. Figure 1b shows that the M13mp18 ssDNA with RPA-YFP band was "shifted", there being a distinct migratory difference between bands with (Lane 2) and without (Lane 1) RPA-YFP. The results indicated that RPA-YFP has ssDNA-binding activity.

Visualization of ssDNA Molecules

Heat-denatured ssλDNA molecules were visualized using RPA-YFP (Fig. 2a), which involved reacting the ssλDNA molecules with RPA-YFP and combing by the moving droplet method. No fluorescent substances were added except RPA-YFP; therefore, all the fluorescence signals were due to emission from RPA-YFP. Two types of fluorescent signal were observed: linear and spot. The ssλDNA molecules binding a large amount of RPA-YFP molecules were observed as linear fluorescent signals after the combing operation. In the spot signals, RPA-YFP was bound locally or in small amounts to the ssλDNA molecules. Unbound RPA-YFP formed the background signal, and no bright spots were observed (data not shown). Figure 2b shows the linear RPA-YFP bound ss-λDNA molecules at a greater magnification to illustrate that the use of RPA-YFP permits the visualization of ssDNA.

Staining and Direct Observation of ssDNA and dsDNA within the Same Molecule

As shown in Fig. 2, ssDNA molecules can be visualized using RPA-YFP. For analysis of DNA metabolism reactions using single-molecule observation, the ability to discriminate single- and double-stranded regions of DNA is crucial; therefore, we attempted to apply this method to enable such discrimination. λDNA molecules containing both ssDNA and dsDNA regions were prepared and stained with SYTOX Orange (Ex 547 nm/Em 570 nm) for dsDNA and RPA-YFP (Ex 513 nm/Em 527 nm) for ssDNA. Figure 3a shows that the dsDNA region of λDNA stains red with SYTOX Orange, while Fig. 3b shows that ssDNA stains yellow-green with RPA-YFP. Figure 3c is a merged image of Figs. 3a and b. Owing to the intercalating dye and its strong excitation light, some cleavages were observed in the double-stranded region of the DNA. This phenomenon is quite often observed when intercalating fluorescent dyes (YOYO-1 and SYTOX Orange etc.) are used for visualization of DNA [25]. On the other hand, the ssDNA visualized with RPA-YFP was quite stable, and no cleavage was observed during the 5 min of observation. Even the photocleavage was successfully suppressed; however, photobleaching precluded observation for a prolonged time. Furthermore, it should be noted that it is still difficult to observe quite short single stranded regions of DNA. This is because the strength of the fluorescence signal is proportional to the length of the single-stranded region. Despite these limitations, this method will promote analysis of DNA metabolic reactions at the single molecule level in the future, because it permits not only visualization of single-stranded regions of DNA, but also enables discrimination of single- and double-stranded regions.

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