Direct visualization of RecQ helicase–DNA interaction with fluorescence microscopy and atomic force microscopy

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Abstract

RecQ helicase–DNA interactions were directly visualized with fluorescence microscopy. DNA–RecQ complexes formed in binding and unwinding reaction were stretched onto the hydrophobic surface by molecular combing method. The complexes can be observed with fluorescence microscope because the DNA molecules were labeled with dye molecules of YOYO-1. The DNA binding and unwinding activity of RecQ helicase leads to reduced lengths of the observed DNA molecules. More direct observations with atomic force microscopy were also made. It was seen that RecQ is mainly monomeric both in solution and after binding to DNA.

Keywords: RecQ helicase; DNA molecule; Molecular combing; Fluorescence microscopy; Atomic force microscopy

1. Introduction

DNA helicases are motor proteins responsible for separating the individual strands of double-stranded DNA (dsDNA) to provide single-stranded DNA (ssDNA) for key cellular processes such as DNA repair, DNA recombination, and replication [1,2]. DNA helicases use the chemical energy derived from nucleoside 5'-triphosphate binding or hydrolysis to mechanically disrupt the hydrogen bonds between the two strands in dsDNA and to translocate along DNA for processive unwinding [3].

The RecQ helicase family, which is widespread in diverse organisms from Escherichia coli to humans, has received a great deal of attention since the discovery that several human diseases, such as Bloom’s, Werner’s and Rothmund–Thompson syndromes, are linked with these enzymes [4–6]. Analyses of these and related diseases, at the cellular and molecular levels, led to the conclusion that RecQ helicase plays a conserved role in the preservation of genomic integrity [7–9]. E. coli RecQ helicase (610aa, molecular weight 68920 Da) displays a 3'-5' polarity in DNA unwinding, and can unwind diverse DNA substrates including DNA with blunt ends, with 5' or 3' overhangs, nicked or forked DNA, and three or four-way junctions as well as G4 DNA [10,11].

Study of the DNA binding and unwinding activities of helicases is important for understanding the mechanism of interaction between helicases and their DNA substrate. The DNA unwinding activity can be investigated by a variety of techniques. Gel electrophoresis was a standard assay for measuring helicase activity [10,12]. Some continuous fluorometric assays were carried out and the process of DNA unwinding was monitored by measuring the decrease in fluorescence as the dye ligands were displaced from the duplex molecule [13–15]. Recently we have taken advantage of the substantial change in fluorescence polarization anisotropy to simultaneously monitor DNA binding and unwinding of E. Coli RecQ helicase [16]. We have also used this anisotropy method to determine the thermodynamic parameters for the interaction of RecQ with DNA [17]. Magnetic tweezer was used to monitor real-time changes in extension of a single, stretched, nicked dsDNA as it was unwound by single UvrD molecules [18].
In the present study, we use fluorescence microscopy and atomic force microscopy (AFM) to investigate the DNA binding and unwinding activities of RecQ. For observation with fluorescence microscopy, we labeled lambda DNA with fluorescent dye YOYO-1 after reaction, then stretched the DNA–RecQ complexes on PMMA hydrophobic surface. With AFM, we show that RecQ is mainly monomeric both in solution and after binding to DNA.

2. Materials and methods

2.1. Hydrophobic surface preparation

Quartz glass plates (21×42 mm², 1 mm thickness) were immersed in hot NaOH (0.5 M) for about 10 min, and then rinsed thoroughly with high-purity water (Millipore S.A., France). Glass surfaces were rendered hydrophobic by coating the surfaces with PMMA as described [19,20]. Briefly, a droplet (0.2 ml) of PMMA (560F, Japan) in chloroform [10% wt/wt] was dripped down onto the center of a cleaned glass, which was mounted horizontally on a spin-coating machine using a double-sided tape, and spread by spin-coating at 6000 rpm for 1 min. After spin coating, the PMMA film was formed evenly on the whole glass surface. To further improve the film uniformity, the glass was then baked at 145 °C for ~30 min and then stored at room temperature in a dust-free environment.

2.2. Reagents and buffers

Lambda DNA was purchased from Sino-American Biotechnology Company (China). DTT, EDTA, ATP, NaCl, Tris, and MgCl₂ were purchased from Sigma Company (USA). RecQ was expressed and purified as described [16]. All buffers were made with 18.2 MΩ water purified through the Milli-Q Water Purification System (Millipore Corporation, France).

2.3. Helicase assays

The standard reaction buffer consisted of 25 mM Tris–HCl (pH 7.4), 40 mM NaCl, 2 mM MgCl₂ and 0.1 mM DTT. The reaction solution (50 µl volume) contained standard reaction buffer, lambda DNA (0.13 nM), and RecQ helicase. The concentration of RecQ helicase was 20–200 times that of lambda DNA. The binding and unwinding reaction was performed at 25 °C. Lambda DNA and RecQ helicase were incubated together for about 10 min, and the unwinding reaction was carried out by quickly adding ATP (1 mM) into the reaction solution. The unwinding reaction was stopped by adding EDTA (10 mM) 10 min later.

2.4. DNA labeling

After binding and unwinding reaction, the RecQ-bound DNA was stained with fluorescent dye, oxazole yellow dimer (YOYO-1, Molecular Probes, USA) at a ratio of ten base pairs per dye molecule (bp/dye = 10) by mixing DNA-RecQ complex sample with a specific volume of freshly prepared 0.1 μM dye solution (10 mM Tris/1 mM EDTA buffer, pH 8.0). The mixed solution was incubated for approximately 30 min in dark, and then diluted to 6.5 μM in a 25 mM Tris–HCl buffer (pH 7.4).

2.5. Molecular combing

A droplet (~1.2 µl) of the stained DNA solution was deposited onto a PMMA surface. After ~5 min, the air-water interface started to recede due to significant drying of the droplet, and DNA molecules originally bound to the surface with one extremity were extended and immobilized on the dried surface, whereas unbound molecules were swept away by the moving interface. As a result, a significant number of fixed DNA molecules were fully elongated and aligned radically.

2.6. DNA imaging by fluorescence microscopy and data analysis

Fluorescently stained DNA molecules were observed using an inverted optical microscopy (IX-70; Olympus, Japan) by epifluorescence with a 20× objective. The images were captured with a cooled CCD camera (CoolSNAP-HQ, 1392×1040 pixels, 12-bit digitization; Roper Scientific, Inc.). YOYO-1 has an excitation maximum at 491 nm and an emission maximum at 509 nm. A 100 W mercury lamp was used in combination with a U-MWB excitation cube (BP450-480, DM500, BA515). The CCD acquisition time we used was 3 s. A MetaMorph software (Universal Imaging Co.) was used for the system control, data acquisition and data processing.

2.7. Deposition of DNA–RecQ complexes on mica and AFM observation

After binding and unwinding reaction, the sample (5 µl) was deposited onto a fresh mica surface. After 10 min, the surface was rinsed slowly with high-purity water and then dried with compressed nitrogen. A Multimode AFM with a NanoScope IIIa controller (Digital Instruments) was used to take images in air with the ‘tapping mode’. The scan rate was 1–2 Hz with a scan range of 1–4 µm. Images were displayed without modification except for flattening.

3. Results

3.1. The binding affinity of RecQ for DNA is high

In the binding experiments, ATP was not added into reaction solution. To investigate the binding affinity of RecQ for DNA, we kept the DNA concentration at a constant (0.13 nM), and the RecQ concentration was 20–200 times that of DNA. We found that, compared with combed DNA, the combed DNA–RecQ complexes are shorter and denser. With increasing RecQ concentration, the lengths of combed DNA–RecQ complexes decreased and their density increased correspondingly (Fig. 1). These indicate that RecQ helicase
made DNA condensed, and enhanced DNA’s binding to hydrophobic surface. From these results we concluded that RecQ bound to DNA easily, and once bound together, the complexes were stable.

To obtain reliable results for the effect of RecQ helicase on the length of combed DNA, the lengths of combed DNA and DNA–RecQ complexes were measured, and the results are given in Fig. 2. It can be seen that the position of the highest column was \( \sim 28 \) \( \mu \)m in the case of combed DNA, and \( \sim 15 \) \( \mu \)m in the case of combed DNA–RecQ complexes. The mean lengths are \( \sim 24.7 \) and \( \sim 18.2 \) \( \mu \)m, respectively.

To investigate the DNA binding affinity of mutant RecQ (K55A), in which a lysine residue at amino acid position 55 (AAG) was substituted by an alanine (GCG) residue [16], mutant RecQ of different concentrations were examined under the same experimental condition as that for RecQ. The results indicate that mutant RecQ could also enhance DNA binding to hydrophobic surface. With increasing mutant RecQ concentration, the lengths of combed DNA-mutant RecQ complexes decreased and their density increased correspondingly (Fig. 3). Thus, mutant RecQ binds easily to DNA too, which is consistent with previous observation by fluorescence polarization anisotropy [16].

3.2. RecQ helicase is mainly monomeric in solution as well as after binding to DNA

To investigate the oligomeric state of RecQ molecules when they are in reaction buffer alone and when they bind to DNA, we resort to AFM. We found that at low concentration (1 nM), RecQ molecules are monomeric in reaction buffer solution. They appear as small balls with diameters of \( \sim 10 \) nm (Fig. 4(a)). Even at concentration of 50 nM most RecQ molecules are monomeric, only a small part of the molecules aggregate on the mica surface (Fig. 4(b)).
When RecQ and DNA are incubated together in unwinding reaction buffer but without ATP, we found that RecQ molecules bind to DNA randomly. Some are as separated monomers while others bind to DNA collectively (Fig. 4(c)). This excludes the possibility that RecQ molecules unwind DNA as dimers or hexamers. That is, RecQ should be a monomeric helicase.

3.3. Unwinding of lambda DNA by RecQ

To determine whether RecQ helicase could unwind lambda DNA (48 kbp), we performed unwinding experiments. Lambda DNA and RecQ helicase were incubated together in reaction solution for 10 min, then the unwinding reaction was started by adding ATP (1 mM). After 10 min, EDTA was added to stop the unwinding reaction. Control experiments were performed under the same conditions except that no ATP was added.

We have observed that the lengths of the combed DNA–RecQ complexes became much shorter after unwinding reaction (Fig. 5). These DNA–RecQ complexes appear as short fragments on the PMMA surface. In our experiment, we took advantage of the fluorescence dye molecules of YOYO-1 to observe DNA unwinding. YOYO-1 molecules bind strongly to dsDNA and the fluorescence quantum yields of the bound dyes are very high [21]. Thus, what we can observe are dsDNA that are left unwound. The average fluorescence intensity of the DNA molecules was measured. It was 194.6 without ATP and 173.7 with 1 mM ATP. This means that the molecules observed in the latter case are indeed dsDNA rather than ssDNA.

The lengths of combed DNA–RecQ complexes were measured and given in Fig. 6. The position of the highest column is at ~15 μm in the case of no ATP. In the case of 1 mM ATP, the position of the highest column is at ~5 μm. The mean lengths are ~17.5 and ~6.4 μm, respectively.
Thus with fluorescence microscopy, we can observe the effect of DNA unwinding by RecQ. It can be seen that the unwinding effect was significant: in 10 min, almost all lambda DNA molecules in the reaction solution were unwound. In fact, we have also used AFM to observe more directly the unwinding effect. We can see clearly that a large part of the dsDNA is unwound to ssDNA (Fig. 7).

4. Conclusion

In this report, RecQ helicase binding to and unwinding of lambda DNA were observed directly with fluorescence microscopy and AFM. Many methods have been used to study the activity of RecQ helicase, such as electrophoresis and fluorescence based assays. But in most of these experiments, one usually uses shorter DNA molecules with lengths of 10–50 bp and these short DNA molecules could be unwound by RecQ helicase obviously. To our knowledge, the study of RecQ helicase on lambda DNA (48 kbp) has not been previously reported.

Molecular combing combined with fluorescence microscopy has been used for the first time to provide the direct evidence for DNA binding and unwinding by a helicase. In the previous studies, single molecule techniques such as magnetic trapping and laser trapping have been used to study DNA-helicase interaction, but such approaches involved extensive manipulations and were not well suited to high-throughput analysis. In our experiments, the stretching of DNA–RecQ complexes relied on passive evaporation effect, thus large number of the complexes could be combed onto the same surface and analyzed at the same time.

Previously we have studied the conformational and functional properties of the E. Coli RecQ helicase using a wide array of biochemical and biophysical techniques. We deduced that RecQ helicase is monomeric in solution and also functions as a monomer in DNA unwinding [22]. This conclusion was further verified by direct AFM observation in the present study.

In summary, fluorescence microscopy and AFM techniques are demonstrated to be useful for studying the DNA binding and unwinding behaviors of RecQ helicase. These methods were simple and effective. They could be used for the study of other helicases.

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