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Detection of the strand exchange reaction using DNAzyme and *Thermotoga maritima* recombinase A

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ABSTRACT

We have designed multiple detection systems for the DNA strand exchange process. Thermostable *Thermotoga maritima* recombinase A (TmRecA), a core protein in homologous recombination, and DNAzyme, a catalytic DNA that can cleave a specific DNA sequence, are introduced in this work. In a colorimetric method, gold nanoparticles (AuNPs) modified with complementary DNAs (cDNAs) were assembled by annealing. Aggregated AuNPs were then separated irreversibly by TmRecA and DNAzyme, leading to a distinct color change in the particles from purple to red. For the case of fluorometric detection, fluorescein isothiocyanate (FITC)-labeled DNA as a fluorophore and black hole quencher 1 (BHQ1)-labeled DNA as a quencher were used; successful strand exchange was clearly detected by variations in fluorescence intensity. In addition, alterations in the impedance of a gold electrode with immobilized DNA were employed to monitor the regular exchange of DNA strands. All three methods provided sufficient evidence of efficient strand exchange reactions and have great potential for applications in the monitoring of recombination, discovery of new DNAzymes, detection of DNAzymes, and measurement of other protein activities.

Various factors such as ultraviolet (UV)[1] light exposure, ionizing radiation, chemical exposure, oxidative damages, and replication errors can cause DNA double-strand breaks (DSBs) [1–7]. This phenomenon leads to many events such as DNA repair, cell cycle arrest, and cell apoptosis [8–10]. If problems occur in the DNA repair system, including recombination, extrinsic DSBs can cause abnormalities in cells, leading to an increase in the occurrence of various diseases related to gene instability. In particular, cancer cells derived from extrinsic DSBs fail to control the cell cycle, which affects the surrounding normal cells and promotes tumor growth [11,12].

Because it is well known that an intrinsic DSB functions primarily as an intermediate of indispensable reactions such as meiotic recombination, mating-type switching, and V(D)J recombination in yeast, extrinsic DSBs must be repaired [13–15]. Among the diverse DNA repair systems, DNA recombination is essential for every living organism. DNA recombination can be divided into two mechanisms depending on the cell cycle: homologous recombination (HR) and nonhomologous end joining (NHEJ) recombination [16]. In particular, many investigations have focused on the HR system due to its generality. Because the correct operation of the HR system is crucial for preventing diseases, the detection of recombination is of valuable significance for research.

In HR, many proteins participate in the exchange of DNA strands [17,18]. A well-known example is *Escherichia coli* recombinase A (RecA). Although it requires the presence of other proteins for effective recombination, RecA is the core protein in DNA strand exchange [19]. In contrast to other homologs such as Rad51 and Dmc1, RecA catalyzes special functions such as three- or four-strand exchange, replication fork regression, coprotease function, and stimulation of the translesion DNA activity of DNA polymerase V [20,21]. Among several types of bacterial RecA, *Thermotoga maritima* RecA (TmRecA) has received a great deal of attention because of its high thermal stability [22]. This stability is a distinct advantage in experiments that require high temperatures.

DNAzyme is a catalytic nucleic acid that specifically cleaves the complementary strand [23–25] because it recognizes DNA containing a ribose nucleotide as a substrate in the presence of certain metal ions. DNAzyme has been used in applications such as metal ion sensors, nucleotide sensors, and therapeutic agents [26,27]. In addition, it can be used to detect recombination in an irreversible DNA strand exchange reaction. By employing DNAzyme in the

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[1] *Abbreviations used:* UV, ultraviolet; DSB, double-strand break; HR, homologous recombination; NHEJ, nonhomologous end joining; RecA, recombinase A; TmRecA, *Thermotoga maritima* recombinase A; AuNP, gold nanoparticle; ssDNA, single-stranded DNA; cDNA, complementary DNA; PCR, polymerase chain reaction; TEV, tobacco etch virus; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; dsDNA, double-stranded DNA; TLC, thin layer chromatography; AMP–PNP, adenosine 5′-(β,γ-imido) triphosphate tetralithium salt hydrate; RT, room temperature; UV–VIS, UV–visible; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; BHQ1, black hole quencher 1; PBS, phosphate-buffered saline.

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recombination process, the proper operation of RecA can be monitored.

Diverse methods, including colorimetry, electrochemical impedimetry, and fluorometry, are employed to trace the extent of reactions in many fields. A colorimetric method based on gold nanoparticles (AuNPs) offers an easy, rapid, and low-cost observation system [28–32]. Electrochemical impedance spectroscopy is also an attractive method because it is carried out in a label-free manner with direct detection; it is also more practical under various conditions compared with other methods [33–37]. In addition, fluorometry shows high sensitivity and simplicity [38,39]. All three detection techniques were used in this work. For colorimetry, AuNPs modified with a complementary single-stranded DNA (ssDNA) were hybridized. During the irreversible exchange catalyzed by TmRecA and DNAzyme, strand separation was accompanied by a color change from purple to red. Fluorometry was used to measure the distance between fluorophore-labeled DNAs and a quencher, which is increased by the irreversible strand exchange reaction and reflected in the change in fluorescence intensity. In addition, a characterized gold electrode with immobilized DNA is reacted with complementary DNA (cDNA) to anneal. The hybridized DNA strands are then irreversibly exchanged with DNAzyme, and the progress of the reaction is monitored by measuring impedance.

Materials and methods

Materials

Modified A-strand (5′-$(A)_{15}$GTGACTCACTAT(rA)GGAAGAGATG-3′), B-strand (5′-$(A)_{15}$CATCTCTTCCTATAGTGAGT-3′), C-strand (DNAzyme, 5′-CATCTCTTCTCCGAGGCGGTCGAAATAGTGAGT-3′), and D-strand (5′-CATCTCTTCTTATAGTGAGT-3′) were purchased from Bioneer (Korea) and used without further purification.

Cloning, overexpression, and purification of TmRecA protein

The open reading frame of *tmrecA* was amplified from the genomic DNA of the *T. maritima* MSB8 strain. The polymerase chain reaction (PCR) with *i*-pfu polymerase (iNtRON Biotechnology, Korea) was used for the amplification using gene-specific primers. Amplified genes were treated with restriction enzymes (*Bam*HI and *Xho*I) and then cloned into the pET-28aTEV vector (Novagen, USA) with the recognition site for the tobacco etch virus (TEV) protease. Cloned hexahistidine-tagged TmRecA was overexpressed from the *E. coli* BL21(DE3) strain (Novagen). Harvested cells were resuspended in 100 mL of lysis buffer (20 mM Tris–HCl [pH 8.0], 0.5 mM β-mercaptoethanol, and 5% [v/v] glycerol). Cells were lysed by adding 1 mg/ml lysozyme, 250 mM phenylmethylsulfonyl fluoride (PMSF), and 500 mM NaCl, followed by sonication. Lysis was followed by centrifugation at 4 °C for 60 min at 18,000 rpm, and the supernatant was applied to a 5-mL Hi-Trap Ni column (GE Healthcare, USA) preequilibrated with binding buffer (20 mM Tris–HCl [pH 8.0], 500 mM NaCl, 0.5 mM β-mercaptoethanol, and 5% [v/v] glycerol). The column was washed with 20% (v/v) elution buffer containing 60 mM imidazole, and the protein was eluted with 300 mM imidazole. The His-tag of TmRecA was removed by TEV digestion overnight at 4 °C. The protein was injected onto a desalting G-25 column preequilibrated with binding buffer. The protein was further purified with the Hi-Trap Ni column and applied to the desalting G-25 column preequilibrated with buffer A (20 mM Tris–HCl, 200 mM NaCl, 0.5 mM β-mercaptoethanol, and 5% [v/v] glycerol). The eluate was then injected into a MonoQ column (GE Healthcare) preequilibrated with buffer A and eluted with increasing concentrations of NaCl from 100 to 500 mM. For further purification, the protein was applied to a Superdex 200 HL column preequilibrated with buffer B (20 mM Tris–HCl, 200 mM KCl, 1 mM ethylenediaminetetraacetic acid [EDTA], 1 mM dithiothreitol [DTT], 5 mM $MgCl_2$, and 5% [v/v] glycerol). The purity of TmRecA was confirmed by denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and judged to be greater than 99% pure (see Fig. S1 in supplementary material).

Activity measurements of TmRecA

The activity of TmRecA was measured by two methods. Qualitative analysis involved measuring the amount of labeled ADP/ATP. The reaction mixture consisted of 3 μM TmRecA, reaction buffer (25 mM Tris–OAc [pH 7.5], 10 mM $Mg(OAc)_2$, and 3 mM [^{32}P] ATP), and 10 μM 20-mer ssDNA or double-stranded DNA (dsDNA) (Bioneer). After incubation at 50 °C for 20 min, thin layer chromatography (TLC) imaged using a bioimaging analyzer (FLA-2000, Fujifilm, Japan) was employed to identify the amount of ADP and ATP (see Fig. S2 in supplementary material).

Quantitative analysis was performed using Malachite Green. Reactions contained 25 mM Tris–OAc (pH 7.5), 10 mM $Mg(OAc)_2$, 3 mM ATP, and 3 μM TmRecA and were carried out at either 37 or 50 °C. The concentration of ssDNA and dsDNA used in the reactions was 10 μM. In addition, 3 mM nonhydrolyzable ATP [adenosine 5′-(β,γ-imido) triphosphate tetralithium salt hydrate, AMP–PNP, Sigma, USA] was used for the control experiment. The amount of phosphate released by ATP hydrolysis was monitored using a Malachite Green Phosphate Assay Kit (cat. no. POMG-25H).

Preparation, modification, and hybridization of AuNPs

AuNPs (~13 nm in diameter) were prepared by a citrate reduction of $HAuCl_4$. Prior to use, all glassware was cleaned using a 3:1 mixture of HCl/HNO_3 and rinsed with deionized water. An aqueous $HAuCl_4$ solution (final concentration of 0.40 mM) was refluxed while stirring, and then trisodium citrate (final concentration of 1.52 mM) was added rapidly. The solution was refluxed again for 1 min and cooled to room temperature (RT) under gentle stirring. The concentration of AuNPs was determined by UV–visible (UV–VIS) spectroscopy (Libra S22, Biochrom, UK) at 520 nm. Strands A and B were immobilized on the surface of the AuNPs. By salt aging with NaCl, a 100-fold molar excess of each DNA was introduced onto the nanoparticles. Several centrifugations were carried out to remove any remaining free DNA and to exchange the buffer with the annealing buffer (30 mM tetramethylammonium chloride, 0.001% [w/w] *N*-lauryl sarcosine, 500 μM Tris–HCl [pH 8.0], and 40 μM EDTA). A- and B-strand-modified nanoparticles were incubated together in a 1:1 ratio for 10 min, heated to 90 °C, and then cooled to 20 °C for 6 h to anneal the A- and B-strands.

Colorimetric detection

The hybridized AuNPs were resuspended in a reaction buffer (600 μM tetramethylammonium chloride, 0.00002% [w/w] *N*-lauryl sarcosine, 10 μM Tris–HCl [pH 8.0], and 800 nM EDTA). For the detection of the strand exchange reaction, aggregated AuNPs were incubated with 4 μM TmRecA, 5 μM C-strand (DNAzyme), and 50 μM Pb^{2+}. In addition, three control reactions with only DNAzyme, only TmRecA, or the addition of the 5 μM D-strand (a sequence complementary to the A-strand without DNAzyme activity) were carried out. All reactions were conducted at 50 °C for 6 h. Nonrelated proteins such as bovine serum albumin (BSA) and lysozyme were used at 4 μM concentrations to act as negative controls; these reactions were carried out at 37 °C for 10 h to preserve protein activity. The change in color was confirmed in photographic images and was measured quantitatively by UV–VIS spectroscopy.