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Research paper

Binding polarity of RPA to telomeric sequences and influence of G-quadruplex stability

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ABSTRACT

Replication protein A (RPA) is a single-stranded DNA binding protein that plays an essential role in telomere maintenance. RPA binds to and unfolds G-quadruplex (G4) structures formed in telomeric DNA, thus facilitating lagging strand DNA replication and telomerase activity. To investigate the effect of G4 stability on the interactions with human RPA (hRPA), we used a combination of biochemical and biophysical approaches. Our data revealed an inverse relationship between G4 stability and ability of hRPA to bind to telomeric DNA; notably small G4 ligands that enhance G4 stability strongly impaired G4 unfolding by hRPA. To gain more insight into the mechanism of binding and unfolding of telomeric G4 structures by RPA, we carried out photo-crosslinking experiments to elucidate the spatial arrangement of the RPA subunits along the DNA strands. Our results showed that RPA1 and RPA2 are arranged from 5′ to 3′ along the unfolded telomeric G4, as already described for unstructured single-stranded DNA, while no contact is possible with RPA3 on this short oligonucleotide. In addition, these data are compatible with a 5′ to 3′ directionality in G4 unfolding by hRPA.

1. Introduction

Replication protein A (RPA) is a highly conserved protein in eukaryotes [1,2] and is a key player in essential processes such as replication, recombination, and DNA repair [3]. RPA binds single-stranded DNA (ssDNA) with high affinity and no sequence specificity. RPA-coated ssDNA recruits ATR to stalled or broken replication forks through a direct interaction with its binding partner ATR-interacting protein (ATRIP) and activates the ATR checkpoint activation during S phase [4]. RPA is a heterotrimeric protein composed of 70-, 32-, and 14-kDa subunits that are commonly referred to as RPA1, RPA2, and RPA3, respectively. RPA carries six DNA-binding domains (DBD), four of them are located in RPA1 (DBD-A, DBD-B, DBD-C and DBD-F), one is located in RPA2 (DBD-D), and one belongs to RPA3 (DBD-E). It has been proposed that RPA binds unstructured ssDNA with four different binding modes covering 8–10 nt, 12–23 nt, 23–27 nt or 30-nt with a 5′ to 3′ polarity and implicating DBD-AB, DBD-ABC, DBD-ABCD or DBD-ABCDE, respectively [3,5].

The evidence for involvement of RPA in telomere maintenance stems from several studies showing that: (i) telomere shortening is induced by mutations in yeast and human RPA [6–8], (ii) telomerase is activated by RPA in yeast cells [9,10], and (iii) RPA is required during the processing of telomeres in ALT (alternative lengthening of telomeres) cells [11]. In addition, Cohen et al. showed that low concentrations of human RPA (hRPA) stimulate *in vitro* extension of G-rich DNA primers by the telomerase (whereas high concentrations are inhibitory) [12].

Although RPA binds ssDNA in a non-sequence specific manner, other ssDNA binding proteins containing OB-fold motifs, such as POT1-TPP1 and the CST complex, specifically recognize telomeric repeats [13,14]. Telomeres are composed of tandem repeats of the sequence TTAGGG and bear a 3′ ssDNA extension (G-overhang) that associates with POT1 and TPP1, two proteins of the shelterin complex [13]. The nucleoprotein structure at telomeres prevents

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the ends of chromosomes from being recognized as damaged DNA [15]. POT1 suppresses ATR activation at telomeres and antagonizes RPA binding to telomeric ssDNA [16,17]. Furthermore, the CST complex (CTC1-Stn1-Ten1), which is necessary for telomere replication [18,19], was recently shown to regulate telomerase activity through a competition with POT1-TPP1 for telomeric DNA [20].

G-rich telomeric strands can adopt non-canonical DNA conformations known as G-quadruplexes (G4) [21]. The guanine quartets in these four-stranded structures are stabilized by Hoogsteen hydrogen bonds and coordinated by central cations. The human telomeric sequence can form intramolecular G4 structures of different conformations [22]. Evidence is accumulating that G4 form in genomes [23–25]. Several helicases, including the Werner helicase (WRN), that are able to unwind G4 are essential for telomere stability and replication [26]. Both RPA and POT1 bind to and unfold telomeric G4 structures [27,28] and interact with WRN [29,30]. In the absence of WRN, the competition between POT1 and RPA results in either the uncoupling or the blockage of the replication fork at telomeres [17].

We previously reported that hRPA efficiently binds and unfolds the G4 structure adopted by an oligonucleotide of the telomeric sequence (htelo; see Table 1 for sequence) and proposed a sequential mode of binding of hRPA on telomeric G4 [27]. In this model, hRPA initially takes advantage of the natural G4 breathing that exposes ssDNA at its extremities. RPA then breaks Hoogsteen hydrogen bonds that stabilize the guanine quartets. Destabilization of a region of the G4 structure makes possible the binding of a second molecule of hRPA.

In this work, we used a combination of biochemical and biophysical approaches to gain insights into interaction between telomeric G4 and hRPA under conditions that modulate the stability of the G4 including in the presence of small G4 ligands. Our data revealed an inverse relationship between G4 stability and hRPA binding to telomeric DNA. Notably, unfolding of telomeric G4 by hRPA was strongly impaired by small G4-stabilizing ligands. In addition, we performed photo-crosslinking experiments to elucidate the positioning of the hRPA protein subunits along the unfolded G4. Our results showed that RPA1 and RPA2, but not RPA3, interact with the telomeric G4 and are arranged from 5′ toward 3′. A similar arrangement has been reported for the interaction with an unstructured ssDNA. Our data suggest a possible mechanism of binding and unfolding of telomeric G4 by hRPA.

2. Materials and methods

2.1. Materials

BSA was from Roche, $\gamma[^{32}P]$ATP (3000 Ci/mmol) was from PerkinElmer, and T4 polynucleotide kinase (PNK) was from NEW England BioLabs. The sequences of the oligonucleotides used here are listed in Table 1. DNA concentrations were determined by UV spectroscopy using the extinction coefficients provided by the manufacturer. Oligonucleotides were from Eurogentec.

Table 1
Sequences of oligonucleotides.

Name	Sequence
htelo	GGGTTAGGGTTAGGGTTAGGG
htelo-mut	GGCTTACGGTTAGCGTTACGG
T21	TTTTTTTTTTTTTTTTTTTT
htelo-S_0	s^4TGGGTTAGGGTTAGGGTTAGGG
htelo-S_{10}	GGGTTAGGGs^4TTAGGGTTAGGG
htelo-S_{22}	GGGTTAGGGTTAGGGTTAGGGs^4TT
F-htelo-T	Fluorescein-GGGTTAGGGTTAGGGTTAGGG-Tamra

Recombinant hRPA was expressed in the *Escherichia coli* BL21 (DE3) strain transformed with the plasmid pET_{11a}hRPA that permits the co-expression of RPA1, RPA2, and RPA3. hRPA was purified over Affi-Gel Blue, Hydroxyapatite (Biorad), and Q-Sepharose chromatography columns as described previously [31]. Mouse anti-RPA1 monoclonal antibody was from Millipore. Mouse anti-RPA2 monoclonal antibody was from Novus Biologicals.

Telomestatin was a generous gift from Kazuo Shin-ya (University of Tokyo, Japan). The pyridine dicarboxamide derivatives 360A and 360A-Br were synthesized by Patrick Mailliet in our laboratory and provided by Marie-Paule Telaude-Fichou (Institut Curie, Orsay, France), respectively. Telomestatin, 360A, and 360A-Br were dissolved at 10 mM and diluted in 100% DMSO solution before use.

2.2. Electrophoretic mobility shift assay

Oligonucleotides were labelled with $\gamma[^{32}P]$ATP using T4 polynucleotide kinase. Unincorporated $\gamma[^{32}P]$ATP was removed using a Biospin 6 column (Bio-Rad) equilibrated in TE buffer. For all electrophoretic mobility shift assays (EMSA), hRPA was diluted and pre-incubated (10 min at 4 °C) in buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM DTT, 10% glycerol, 0.2 mg/mL BSA, and 0.1 mM EDTA. Radiolabeled oligonucleotide (2 nM) in reaction buffer [50 mM HEPES, pH 7.9, 0.1 mg/mL BSA, and 100 mM KCl (or NaCl or LiCl when indicated) and 2% glycerol] was incubated with various amounts of protein for 15 min at 20 °C (10 μL). Longer incubation times (up to 1 h) did not affect the patterns or intensities of the bands, indicating that the systems had reached thermodynamic equilibrium within 15 min. Samples were then loaded on a native 1% agarose in 0.5× TBE buffer containing or not the corresponding cations (10 mM). Electrophoresis was performed for 90 min at 5 V/cm at room temperature. For experiments studying the effect of the temperature, electrophoresis was performed at the temperature of hRPA-DNA incubation. After electrophoresis, the gel was dried and exposed to a phosphorimager screen. After being exposed for at least 10 h, the screen was scanned with the Phosphorimager TYPHOON instrument (Molecular Dynamics). The samples in the gel were quantified using ImageQuant version 5.1. Each experiment was reproduced at least twice. When shown, error bars correspond to the standard deviation calculated from at least two independent experiments. When G4 ligands were used, radiolabeled oligonucleotide (2 nM) was pre-incubated with G4 ligands (0.5 μM) for 10 min at room temperature in the standard reaction buffer before adding hRPA.

2.3. Energy transfer measurements

Fluorescence spectra of 100 nM the 5′-fluorescein and 3′-Tamra labelled htelo oligonucleotide (F-htelo-T) were taken at 20 °C in a buffer containing 50 mM KCl, 2 mM $MgCl_2$, and 5 mM lithium cacodylate, pH 7.2. The total volume was 50 μL. F-htelo-T was pre-incubated (10 min at 20 °C) with G4 ligand (0.5 μM). Protein (0.5 μL) was directly added to the F-htelo-T solution or the F-htelo-T G4 ligand mixture. The spectra were collected after 2 min of incubation between 490 and 660 nm while exciting at 470 nm. Longer incubation times (up to 15 min) did not affect the spectrum, indicating that the systems reached thermodynamic equilibrium within 2 min. The ratio P was calculated as $P = ID/(ID + IA)$, where ID and IA are the emission intensities of the donor fluorescein at 518 nm and of the acceptor Tamra at 586 nm, respectively.

2.4. Spectroscopic studies

UV-melting profiles were acquired on an Uvikon XL spectrometer (Secoman). Absorbance of oligonucleotides was recorded at