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RPA-1 from *Leishmania amazonensis* (LaRPA-1) structurally differs from other eukaryote RPA-1 and interacts with telomeric DNA via its N-terminal OB-fold domain

R.S. Pavani [a,1,2], C. Fernandes [b,2], A.M. Perez [c], E.J.R. Vasconcelos [d], J.L. Siqueira-Neto [e], M.R. Fontes [b], M.I.N. Cano [a,*]

[a] Departamento de Genética, Instituto de Biociências, Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP), Botucatu, SP 18618-970, Brazil
[b] Departamento de Física e Biofísica, Instituto de Biociências, Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP), Botucatu, SP 18618-970, Brazil
[c] Laboratório Nacional de Biociências – LNBio, Centro Nacional de Pesquisa em Energia e Materiais – CNPEM, Campinas, SP 13083-970, Brazil
[d] Seattle Biomedical Research Institute, Seattle, WA, USA
[e] Skaggs School of Pharmacy and Pharmaceutical Sciences, 9500 Gilman Dr, University of California, San Diego, La Jolla, CA 92093, USA

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ABSTRACT

Replication protein A-1 (RPA-1) is a single-stranded DNA-binding protein involved in DNA metabolism. We previously demonstrated the interaction between LaRPA-1 and telomeric DNA. Here, we expressed and purified truncated mutants of LaRPA-1 and used circular dichroism measurements and molecular dynamics simulations to demonstrate that the tertiary structure of LaRPA-1 differs from human and yeast RPA-1. LaRPA-1 interacts with telomeric ssDNA via its N-terminal OB-fold domain, whereas RPA from higher eukaryotes show different binding modes to ssDNA. Our results show that LaRPA-1 is evolutionary distinct from other RPA-1 proteins and can potentially be used for targeting trypanosomatid telomeres.

1. Introduction

Leishmaniasis is an emerging human disease, which is caused by a trypanosomatid parasite belonging to the *Leishmania* genus. This disease is endemic in many countries, and there are no efficient methods for its control or eradication [1]. Currently, telomeres have been considered good targets for the development of new therapies due to their role in the maintenance of genome stability [2]. Telomeres are the protective DNA–protein complexes found at the ends of eukaryotic chromosomes [3]. Telomeric proteins have been found in many organisms, such as vertebrates, yeasts and ciliates [4–7]. In trypanosomatids, in spite of the presence of LaRbp38, only the orthologs of TRF and Rap-1 reported in other eukaryotes were found to interact with parasite double-stranded telomeres [8–11], and very few typical 3′ G-overhang binding proteins have been reported in these protozoa [12–14]. Some of them, including LaRPA-1 [13,14], were isolated in *Leishmania amazonensis* telomerase-positive nuclear extracts using affinity chromatography on the telomeric G-rich strand DNA. Recently, a trimeric RPA-like complex, which binds specifically to telomeric DNA, was described as a component of the telomerase holoenzyme of the ciliated protozoa *Tetrahymena thermophila* [15]. However, in *Leishmania* and in contrast to expectations, affinity purified LaRPA-1 was not co-purified with the other two subunits of the canonical RPA heterotrimeric complex [13].

In budding yeast and higher eukaryotes, RF-A (replication factor A) or RPA respectively, is the main single-stranded DNA-binding protein that plays multiple roles in DNA processing pathways, and it is composed of the subunits RPA-1 (also named RPA70 or Rfa1), RPA-2 (also named RPA32 or Rfa2) and RPA-3 (also named RPA14 or Rfa3) [16,17]. RPA-1 and RPA-2 interact with

Abbreviations: CD, circular dichroism; DBD, DNA binding domain; OBF domain, oligonucleotide–oligosaccharide fold domain; Rfa1, replication factor 1; RPA-1, replication protein A subunit 1; RPA70, replication protein A subunit 70 kDa; ssDNA, single-stranded DNA

* Corresponding author. Fax: +55 14 3815 3131.
E-mail address: micano@ibb.unesp.br (M.I.N. Cano).
[1] Address: Laboratório Especial de Toxinologia Aplicada, Instituto Butantan, São Paulo, Brazil.
[2] These authors contributed equally to this work.

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single-stranded DNA via four OB (oligonucleotide/oligosaccharide-binding)-fold domains named DNA binding domains A-D in order of decreasing affinity for ssDNA (single-stranded DNA); DBD A-C are in RPA-1 and DBD D is in RPA-2 [18–21]. Furthermore, in contrast to LaRPA-1, the RPAs from higher eukaryotes bind to ssDNA through at least three different modes depending on the length of the ssDNA and the number of ssDNA-binding domains involved [18].

In this manuscript, we have expressed and purified three truncated mutants of LaRPA-1 to better understand its telomere interactions. Here, we confirmed that LaRPA-1 interacts with G-rich single-stranded telomeric DNA using only its N-terminal OBF1 domain. We also created in silico structural models to show relevant structural differences in the LaRPA-1 tertiary structure compared with that of human and yeast RPA-1/Rfa1 and to explain the LaRPA-1:telomeric DNA interaction at the molecular level.

2. Materials and methods

2.1. Bacterial strain and plasmid vector

The pET-28a+ vector was used to clone the truncated mutants: LaRPA-1$^{\Delta C\text{-term}}$ containing OBF1 and OBF2, both located at the N-terminal of LaRPA-1; LaRPA-1$^{\Delta OB1,2}$ containing OBF3 located at the C-terminal of LaRPA-1 and LaRPA-1$^{\Delta OB1}$, which lacks the canonical OBF1 domain. The cloning strategy was based on PCR and was designed to generate recombinant proteins with an N-terminal 6×His-tag. The constructs were used to transform the *Escherichia coli* BL21-CodonPlus (DE3)-RP cells (Stratagene).

2.2. Protein expression, purification and refolding

To produce the recombinant proteins, *E. coli* was transformed using each recombinant plasmid. For expression assays, the transformants were then cultivated in LB medium supplemented with 50 μg of kanamycin/ml and 50 μg of chloramphenicol/ml at 37 °C for 2 h. Protein expression was induced using 1 mM isopropyl thio-β-D-galactopyranoside (IPTG) at 37 °C for an additional 3 h. Cells were harvested at 4 °C, and pellets were suspended in lysis buffer (50 mM Tris–HCl pH 8.0, 50 mM NaCl, 10 mM EDTA pH 8.0 and 1X protease inhibitor cocktail), disrupted by sonication and incubated with 5U DNAse I, followed centrifugation at 4 °C. The final pellet was suspended in 50 mM glycine pH 8.0 containing 7 M urea, in the presence of 1X protease inhibitor cocktail (Sigma) and the suspension was loaded in a HiTrapChelating (GE Healthcare) column previously equilibrated with starting buffer (20 mM Tris HCl pH 7.0, 0.5 M NaCl, 7 M urea, 71 μl/L β-mercaptoethanol). Proteins were eluted using a linear gradient of 25–500 mM imidazole in elution buffer (20 mM Tris HCl pH 7.0, 0.5 M NaCl, 7 M urea and 500 mM imidazole) and an FPLC (AKTAprimeplus). Fractions containing the recombinant proteins were separated in 12% SDS–PAGE, pooled and renatured by dialysis in 10 volumes of renaturing buffer (20 mM Tris HCl pH 7.0 and 20 mM NaCl) at 4 °C. Heparin (50 μg/ml) was added to each protein suspension before dialysis to prevent precipitation (see Lira et al. [9] for details). The protein concentration was determined spectrophotometrically in a Jasco V530 series spectrophotometer (Jasco, Japan) using the extinction coefficient of the denatured protein. The purity of the proteins was confirmed by measuring A280 nm/A260 nm and using SDS–PAGE.

2.3. Western blotting

The recombinant proteins (0.5 μg each) and proteins obtained from *L. amazonensis* nuclear extract [22] were separated using 10% SDS–PAGE and subjected to Western blot analysis with anti-LaRPA-1 according to Siqueira-Neto et al. [14]; the blots were developed with an alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Bio-Rad) using an Amplified Alkaline Phosphatase Immun-Blot Assay Kit (Bio-Rad) according to the manufacturer's instructions (Millipore).

2.4. Circular dichroism spectroscopy

CD measurements were obtained over the spectral ranges of 195–260 nm (for LaRPA-1$^{\Delta OBF1}$), 197–260 nm (for LaRPA-1$^{\Delta Cter}$) and 200–260 nm (for LaRPA-1$^{\Delta OBF1,2}$) using a JASCO J-815 spectropolarimeter (JASCO Spectroscopic Co., Ltd., Japan) equipped with a Peltier thermo-controller. The experiments were performed at 293 K using an optical path length of 0.5 nm, a scanning speed of 100 nm/min, a response time of 1 s, a bandwidth of 2 nm and a data pitch of 0.5 nm. Twenty spectra were acquired, averaged and corrected for the buffer solution (baseline) in the presence and absence of Tel1 and then normalized to the residual molar ellipticity [θ]. The CD spectra of both buffer and Tel1 gave negligible signals on the concentrations tested. All LaRPA-1 mutants were analyzed in buffer containing 20 mM Tris HCl and 20 mM NaCl, and the effect of telomeric DNA was evaluated by the addition of 150 pmol of Tel1 $(TTAGGG)_3$ to the protein samples. Deconvolution of the CD spectra was performed using the Dichroweb online server [23] with the CDSSTR algorithm and reference set 4 [24].

2.5. LaRPA-1 and yeast Rfa1 modeling and molecular dynamics (MD) simulations

Extensive linkers with several very flexible residues connect OBF2 and OBF3 in LaRPA-1 and DBD-B and DBD-C in the yeast Rfa1. In both cases, the linkers do not show homology with any protein DNA-binding structure; therefore, we created separated models for the LaRPA-1$^{\Delta Cter}$ and LaRPA-1$^{\Delta OBF1,2}$ truncated mutants and for Rfa1$^{DBD\text{-}A/DBD\text{-}B}$ and Rfa1$^{DBD\text{-}C}$ (GenBank Acc. number P22336). Chain A (PDB ID 1JMC) of human RPA70 (hRPA70) was selected as the best template for initial in silico models of LaRPA-1$^{\Delta Cter}$ (Score 380.51; *E*-value: 3e-57; Identity: 40%) and Rfa1$^{DBD\text{-}A/DBD\text{-}B}$ (Score: 373.7; *E*-value: 5.4e-60; Identity: 42%). Chain C (PDB ID 1L1O) of human RPA70 (hRPA70) was selected as the best template for initial in silico models of, LaRPA-1$^{\Delta OBF1,2}$ (Score 309.71; *E*-value: 3.4e-48; Identity: 30%) and Rfa1$^{DBD\text{-}C}$ (Score: 328.71; *E*-value: 2.2e-53; Identity: 36%). The best templates were chosen according to data obtained from the profile-based threading method program HHPred [25]. Initial in silico models were generated using the program MODELLER v.9.10 [26] and the selected templates without a DNA molecule, in the case of the 1JMC-based model. Subsequently, these initial models were subjected to molecular dynamics (MD) simulations executed by GROMACS (Groningen Machine for Chemical Simulation) v.4.5.3 [27] in the presence of explicit water molecules. The protonation states of charged groups were set according to pH 7.0. Counter ions were added to neutralize the system, and the GROMOS 96 53a6 force field [28] was chosen to perform the MD simulations. First, 200 ps of MD simulation with position restraints applied to the protein (PRMD) was executed to relax the system gently. Then, 40 ns of unrestrained MD simulations were performed to evaluate the stability of the structures.

3. Results and discussion

The search for proteins with homology to the crystallographic data of RPA-1 from *L. amazonensis* (LaRPA-1) using the HHPred server [25] resulted in sequences containing the DNA-binding