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Alternative arrangements of telomeric recognition sites regulate the binding mode of the DNA-binding domain of yeast Rap1

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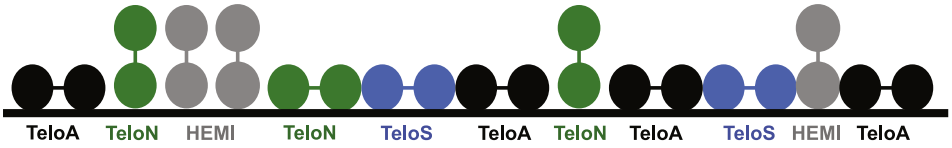
HIGHLIGHTS

- Yeast telomeres contain a heterogeneous distribution of possible Rap1 binding sites.
- Rap1 binds to all these different arrangements of sites using different binding modes.
- Rap1 binds to an isolated half-site using only one of its two Myb-like domains.
- At telomeres Rap1 forms a heterogeneous population of bound-states.

GRAPHICAL ABSTRACT



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ABSTRACT

The function of yeast Rap1 as an activator in transcription, a repressor at silencer elements, and as a major component of the shelterin-like complex at telomeres requires the known high-affinity and specific interaction of the DNA-binding domain (DBD) with its recognition sequences. In addition to a high-affinity one-to-one complex with its DNA recognition site, $Rap1^{DBD}$ also forms lower affinity complexes with higher stoichiometries on DNA. We proposed that this originates from the ability of $Rap1^{DBD}$ to access at least two DNA-binding modes. In this work, we show that $Rap1^{DBD}$ binds in multiple binding modes to recognition sequences that contain different spacer lengths between the hemi-sites. We also provide evidence that in the singly-ligated complex $Rap1^{DBD}$ binds quite differently to these sequences. $Rap1^{DBD}$ also binds to a single half-site but does so using the alternative DNA-binding mode where only a single Myb-like domain interacts with DNA. We found that all arrangements of Rap1 sites tested are represented within the telomeric sequence and our data suggest that at telomeres Rap1 might form a nucleoprotein complex with a heterogeneous distribution of bound states.

1. Introduction

The Rap1 protein from budding yeast *Saccharomyces cerevisiae* is an important regulator of transcription and genomic integrity. Rap1 is responsible for activating transcription of ribosomal protein genes and silencing transcription at HM silent mating-type loci [1,2]. A highly abundant protein [3], Rap1 is also found in large numbers at telomeres where it is involved in establishing architectural nucleoprotein complexes [4], silencing transcription at telomeres [5,6], and acts as a negative regulator of telomere length [2,7,8]. As a consequence of its wide genomic activities, the *RAP1* gene is essential [9]. Loss of function mutations in the Rap1 protein leads to improper telomere elongation [2], telomere fusion [10], and failure to silence target genes and telomeres [5,11].

Rap1 is a DNA-binding protein that recruits other protein factors to carry out its diverse genomic functions. The DNA-binding domain (DBD) of Rap1 contains two Myb-type motifs and is centrally positioned within the 827 amino acid sequence [12]. Crystal structures of $Rap1^{DBD}$ bound to DNA show that the two Myb-type motifs of the DBD are bound

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with the two half-sites of a Rap1 recognition sequence in a one-to-one complex [12–14]. Most of the interactions with other protein factors occur with the Rap1 C-terminal domain (RCT) [15,16]. Proteins that interact directly with the RCT include members of the silent information regulators (SIR) Sir3 and Sir4, forming a complex with Rap1 on DNA during gene silencing [6]. The RCT domain also binds the Rap1 interacting factors Rif1 and Rif2, forming the core scaffold of the shelterin-like complex at telomeres [15]. The Rap1–Rif complexes are recruited to the repetitive T(G_{1-3}) arrays of telomeric DNA forming a "cap" at the chromosome ends together with other protein complexes like Ku70–Ku80 and Cdc13–Stn1–Ten1 [17–19]. This nucleoprotein complex shelters the chromosome ends from being recognized as double-strand breaks, suppressing the DNA damage response pathway [20].

Rap1 plays an important role in regulating telomere length homeostasis. It has been proposed that Rap1 is a central component of a counting mechanism where the cell monitors and responds to the number of Rap1 molecules (or Rap1–Rif2) present at the telomere as a way of regulating access to telomerase [21,22]. The recent crystal structure of a $Rap1^{RCT}$–Rif2 complex suggests that Rif2 may in fact bind two Rap1 molecules from two different binding surfaces [23].

Crystal structures of $Rap1^{DBD}$ bound to DNA have added to the current available model where the two Myb-type motifs of the DBD bind simultaneously and with high affinity with the two half-sites of a Rap1 recognition sequence [12–14]. However, previous work from Del Vescovo et al. showed that Rap1 at a minimum is capable of binding a single hemi-site [24]. Recently we provided evidence that while the DBD indeed binds its recognition sequence in a high-affinity singly-ligated complex, it can also access higher stoichiometry complexes on both telomeric and non-telomeric dsDNA substrates [25]. We proposed that the ability of $Rap1^{DBD}$ to achieve stoichiometries on DNA higher than the expected one-to-one is due to its ability to switch between at least two DNA-binding modes. In one mode, observed in the crystal structures, both Myb-like domains bind with high affinity to both hemi-sites in the recognition sequence; in the alternative mode only one Myb-like domain interacts with lower affinity with DNA. In this work, we extended our study to other recognition sequences that contain different spacer lengths between the hemi-sites or a single half-site. All of the sequences tested are potential Rap1 recognition sites found in the heterogenous yeast telomeres [26–28]. The ability of $Rap1^{DBD}$ to bind to a single half-site within a short dsDNA substrate provides direct evidence that a single myb-like domain is sufficient for interaction and that the alternative binding mode can be populated. We propose that the ability of Rap1 to bind different arrangements of sites at telomeres and its ability to access different binding modes could lead to the formation of a heterogeneous nucleoprotein complex.

2. Materials and methods

2.1. Reagents and buffers

All chemicals used were reagent grade. All solutions were prepared with distilled and deionized Milli-Q water (18 MΩ at 25 °C). All oligonucleotides were purchased from Integrated DNA Technology (IDT, Coralville, IA). The oligonucleotides used for binding experiments were all HPLC purified, suspended in TE buffer (10 mM Tris–HCl, pH 8.3 and 0.1 mM EDTA) and the concentration was determined spectrophotometrically using the extinction coefficients provided. The sequence composition of the "top" strand of the oligonucleotides used is shown in Table 1 and the position of the FAM or Cy3 fluorescent labels is indicated in the text. All annealed duplex dsDNAs were prepared by mixing equimolar concentrations of each oligonucleotide strand in 20 mM HEPES (pH 7.4), 50 mM NaCl, 10% *v*/*v* glycerol, 2 mM $MgCl_2$ and incubated in a pre-heated 95 °C water bath, followed by slow cooling to room temperature.

Table 1
Sequence of the "top" strands of the dsDNA substrate used. The half-site of the Rap1 recognition sequence at telomeres is in bold.

	L_{bp}	S_{bp}	h_{bp}	5′-3′ "top" strands
TeloA	21	3	4	CCGC**ACACC**CAC**ACACC**AGTG
$TeloA_{h3}$	19	3	3	CGC**ACACC**CAC**ACACC**ATG
$TeloS_{h5}$	21	1	5	CGCGC**ACACCC**A**CACC**AGTGG
TeloS	19	1	4	CCGC**ACACCC**A**CACC**AGTG
$TeloS_{h2}$	15	1	2	CC**ACACCC**A**CACC**AG
$TeloS_{h1}$	13	1	1	C**ACACCC**A**CACC**G
TeloN	18	0	4	CCGC**ACACCACACC**AGTG
$TeloN_{h5,6}$	21	0	5,6	CGCGC**ACACCACACC**AGTAGG
HEMI	13	–	4	CCGC**ACACC**AGTG
HEMI-L	13	–	1,7	C**ACACC**AGTCGCG
HEMI-R	13	–	7,1	CAGTCGC**ACACC**G

2.2. Purification of Rap1 constructs

The DNA-binding domain of Rap1 comprising residues 358–601 [14] was cloned and overexpressed in *Escherichia coli* Rosetta2(DE3)pLysS (EMD Chemicals, Novagen, Gibbstown, NJ). The $Rap1^{DBD}$ was purified with a two-column purification protocol as described [25]. Briefly, following cell lysis the clarified supernatant was incubated with 0.3% *v*/*v* polyethyleneimine, recovered in the supernatant and incubated overnight with Glutathione Sepharose 4 Fast Flow GST-affinity resin. After cleavage of the GST-tag the $Rap1^{DBD}$ was directly loaded on a Poros 50 HE Heparin and eluted at 600 mM NaCl. Purified $Rap1^{DBD}$ was dialyzed against Storage Buffer (20 mM HEPES (pH 7.4), 400 mM NaCl, 40% *v*/*v* glycerol, 1 mM DTT, and 0.5 mM EDTA) and then stored at −80 °C. Before the experiments, $Rap1^{DBD}$ was dialyzed against Buffer HN_{50} (20 mM HEPES pH 7.4, 50 mM NaCl, 2 mM $MgCl_2$, 10% *v*/*v* glycerol) and the concentration was determined using an extinction coefficient of 24,870 M^{-1} cm^{-1} [29,30].

2.3. Analysis of telomeric sequences

We analyzed a series of telomeric sequences that range in size from 34 nt to 363 nt in total length. These include: Tel270 and Telo80 [26]; wt1–7 [28]; M34310–M34313 (GenBank, [27]); TEL01L-TR, TEL01R, TEL03L, TEL03R, TEL04L, TEL06R, TEL08L-TR, TEL08R, TEL09L, TEL10L, TEL10R, TEL11L, TEL11R, TEL12L, TEL13L, TEL13R and TEL14R (SGD, strain S288C). In order to identify all possible unique arrangements of Rap1 binding sites, we applied a few simple rules. First, we assumed that Rap1 sites are occupied from the telomeric end and moved inward. Second, the order of binding follows a simple affinity rule based on the spacing between half-sites, where 3 bp > 2 bp > 1 bp > 0 bp > half-site. Finally, no overlap between identical sites was allowed when assuming that only the site proximal to the telomeric end is occupied.

2.4. Analytical ultracentrifugation

All sedimentation experiments were collected on an Optima XL-A analytical ultracentrifuge using a An60Ti rotor (Beckman Coulter, Brea, CA). Sedimentation velocity experiments with 2 μM Cy3-labeled DNA were performed using Epon charcoal-filled double-sector centerpieces at 55,000 rpm with 0.03 cm spacing and recording scans every 8 min at 545 nm. Sedimentation equilibrium experiments were performed using Epon charcoal-filled six-sector centerpieces at the appropriate rpm with 0.001 cm spacing, scanned every 4 h, averaged from 10 replicates and recorded at 545 nm. Sedimentation velocity and equilibrium data were processed and analyzed with SedFit/SedPhat (Peter Schuck). The apparent molecular weights of the complexes were determined as described [25].