Characterization of the Yeast Telomere Nucleoprotein Core

Rap1 BINDS INDEPENDENTLY TO EACH RECOGNITION SITE

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At the core of Saccharomyces cerevisiae telomeres is an array of tandem telomeric DNA repeats bound site-specifically by multiple Rap1 molecules. There, Rap1 orchestrates the binding of additional telomere-associated proteins and negatively regulates both telomere fusion and length homeostasis. Using electron microscopy, viscosity, and light scattering measurements, we show that purified Rap1 is a monomer in solution that adopts a ringlike or C shape with a central cavity. Rap1 could orchestrate telomere function by binding multiple telomere array sites through either cooperative or independent mechanisms. To determine the mechanism, we analyze the distribution of Rap1 monomers on defined telomeric DNA arrays. This analysis clearly indicates that Rap1 binds independently to each non-overlapping site in an array, regardless of the spacing between sites, the total number of sites, the affinity of the sites for Rap1, and over a large concentration range. Previous experiments have not clearly separated the effects of affinity from repeat spacing on telomere function. We clarify these results by testing in vitro the function of defined telomere arrays containing the same Rap1 binding site separated by spacings that were previously defined as low or high activity. We find that Rap1 binding affinity in vitro correlates with the ability of telomeric repeat arrays to regulate telomere length in vivo. We suggest that Rap1 binding to multiple sites in a telomere array does not, by itself, promote formation of a more energetically stabile complex.

Telomeres are specialized chromatin domains that control numerous DNA processes occurring at the termini of linear eukaryotic chromosomes. The telomere nucleoprotein complex regulates transcription of nearby genes, telomere duplication through semi-conservative DNA replication, de novo extension of telomere DNA by telomerase, telomere recombination, and chromosome stability. In the absence of proper telomere structure, these processes become deregulated, and cells normally halt the cell cycle; continued cell division can lead to telomere fusions and other aberrant DNA recombin-

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5 The abbreviations used are: DBD, DNA-binding domain; R/S, Rap1:DNA site ratio; NHEJ, non-homologous end-joining.
Telomere length-dependent control of telomerase action, NHEJ, transcriptional repression, and growth arrest is orchestrated by changes in the organization and/or number of Rap1 molecules and its protein partners bound to telomere DNA. Telomeres are proposed to fluctuate between open/extendible and closed/nonextendible states in a manner that is governed by telomere repeat length (15–18). The telomere length associated with changes between these states varies depending on the process. Repression of telomerase action in cis at a telomere is controlled by an incremental contribution of each Rap1 module (counting) when telomeres range from normal length to ~120 bp (19). This mechanism requires Rif1 and Rif2, effectors of Rap1 telomerase repression (13, 20, 21). In addition to this incremental repressive action of Rap1/Rif1/Rif2, a more abrupt increase in telomerase action at telomeres (and in recombinational lengthening mechanisms) occurs when telomeres are shorter than ~120 bp (17, 18). Although the minimum length required for NHEJ repression is unclear, telomere repeat lengths that direct binding of four Rap1 molecules are sufficient to protect a DNA end (and promote efficient extension of the repeat tract to normal length) (22, 23); telomere lengths of ~20–30 bp are subject to telomere fusion (24).

Despite extensive evidence for the fundamental roles Rap1 plays at telomeres, how Rap1 is organized along the length of telomere DNA to orchestrate changes between the open/extendible and closed/nonextendible telomere states is ill-defined. Here, we analyze Rap1 free in solution and when bound to arrays of telomeric recognition sites. Using a combination of electron microscopy, viscosity, and light scattering measurements, we provide a physical view of Rap1. In solution, Rap1 is a nonglobular monomer with a ringlike or C shape and a central cavity. In addition, we show conclusively that Rap1 monomers bind independently to each individual nonoverlapping recognition site within a telomeric DNA array, regardless of the occupancy of the other sites. Rap1 loading is rapid (our data and Ref. 3) and, once bound within the repeat array, Rap1 releases DNA much more slowly. Furthermore, Rap1 binds independently to each site regardless of 1) the number of array sites, 2) the recognition sequence, and 3) the spacing between adjacent sites (over the range of 15–31 bp). Finally, we dissect the effect of recognition site affinity and spacing on Rap1-controlled telomere length maintenance and show that the affinity of Rap1 for the repeat element, but not the spacing between, is critical for telomere “counting” in vivo. We discuss the implications of these results on the dynamic regulation of telomere structure.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—To make the His$_c$-Rap1 Escherichia coli expression construct pDL80 (pEH111143), the Rap1 gene was PCR-amplified from yeast genomic DNA, cloned into pET15b (Novagen), and verified by sequencing. To test Rap1 site spacing in vivo, yeast integration plasmids (pDL164–pDL170) containing different numbers of the 31-bp* sequence (see Table 1) were constructed by a reiterative cloning strategy. First, oligonucleotides oEH11256 and oEH11257 were annealed to create a single 31-bp* site, which was ligated to pE56 cut with BamHI + BglII. A clone, pDL164, containing one 31-bp* site in the correct orientation was identified by sequencing. Greater numbers of 31-bp* sites were then iteratively cloned into the BamHI site of pDL164, screened for correct orientation, and sequenced.

**Rap1 Purification**—pDL80 was transformed into E. coli Rosetta pLysS cells (Novagen). Transformants were grown in LB + ampicillin + chloramphenicol in 2.8-liter baffled flasks at 15 °C. Rap1 expression was induced with 0.4 mM isopropyl β-d-thiogalactopyranoside. The cell pellet was resuspended in lysis buffer (50 mM sodium phosphate pH 8, 0.5 mM NaCl, 10 mM imidazole, 10% glycerol), frozen in liquid nitrogen, and stored at ~80 °C. Protease inhibitors were added during lysis: 10 μg/ml pepstatin (Sigma), 1 mM Pefabloc (Sigma), and Complete Protease Inhibitor Tablets without EDTA (Roche). The cells were lysed by sonication. His-tagged Rap1 was purified from the cleared lysate in three chromatography steps: 1) batch nickel-nitriilotriacetic acid resin (Qiagen), 2) gradient elution from a HiTrap heparin column in Hep buffer (50 mM Tris, pH 8, 10% glycerol, 1 mM EDTA, 1 mM DTT) over 0.1–1 mM NaCl, and 3) gradient elution from a HiTrap SP HP column in S buffer (50 mM HEPES, pH 7.6, 10% glycerol, 1 mM EDTA, 10% glycerol) over 50–350 mM NaCl. Rap1 ~80 °C storage buffer was 20 mM HEPES, pH 7.6, 50 mM NaCl, 1 mM DTT, 10% glycerol. Most experiments used His-tagged Rap1; however, the His tag was successfully removed from an aliquot of purified Rap1 upon treatment with thrombin. Removal of the His tag affected neither the gel filtration profile nor the Rap1 DNA array binding behavior (four sites, 22-bp spacing tested; data not shown).

**Molecular Weight and Size Determination**—Size exclusion chromatography was performed on a Superdex 200 10/300 GL column (GE Healthcare) in size exclusion chromatography buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM DTT, 5 mM MgCl$_2$); Rap1 was compared with globular protein standards (Bio-Rad). A Viscotek Tetra detector array was used to simultaneously measure UV, refractive index, right angle light scattering, and viscosity parameters in-line for the size exclusion chromatography eluted Rap1 peak. The Tetra detector array was calibrated in triplicate using lysozyme as a standard. Rh was calculated using Viscotek software.

**DNA Gel Shift**—DNA fragments were prepared by first digesting the appropriate plasmids (a gift of David Shore) (19, 25) with BamHI + BglII (to release the defined arrays) or EcoRI (to release the native telomere fragment). The small DNA fragments were then gel-purified (Qiagen). DNA fragments were radiolabeled using standard procedures. Unincorporated nucleotides were removed on a ProbeQuant G-50 microspin column (Amersham Biosciences).

Binding reactions (15 μl) for most experiments contained buffer A (20 mM HEPES, pH 7.9, 150 mM NaCl, 1 mM DTT, 10% glycerol, 5 mM MgCl$_2$), 0.3 mg/ml BSA (New England BioLab), 5–10 μg/ml dl/dc (Fluka/Biochimika), and the specified concentrations of Rap1 and DNA array fragment. The DNA fragment was a mix of unlabeled DNA and 32P-labeled DNA at 1/100th the concentration of the unlabeled DNA. DNA was diluted in buffer A plus 0.3 mg/ml BSA plus dl/dc; Rap1 was diluted appropriately in buffer A plus 0.3 mg/ml BSA. The reactions were initiated by adding 10-μl aliquots of the diluted DNA reaction mix to 5-μl aliquots of the different Rap1 dilutions. The reactions were incubated at 25°C for 30–60 min and were
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then loaded directly (without dye, glycerol added to 9.4%) onto a prechilled 1% agarose gel in 0.5\% TBE. The gel was run in 0.5\% TBE at 4°C at 10 V/cm for 5–6 h and transferred to DE81 paper (Whatman) by first pressing overnight and then drying under vacuum. Dried gels were quantified using Molecular Dynamics ImageQuant software upon exposure to a phosphor screen (GE Healthcare). Binding reactions were also performed in buffer B (20 mM HEPES, pH 7.6, 90 mM KCl, 1 mM DTT, 1% glycerol, 0.005% Nonidet P-40, 4 mM magnesium acetate). For these reactions, Rap1 and DNA were diluted in buffer B prior to mixing as described above.

The frequency ($P_j$) for obtaining each bound state DR$_i$ (where D is the DNA fragment and R is Rap1) given random assortment is a function of the relative concentrations of Rap1 and DNA, the number of Rap1 binding sites per array ($n$), and the number of ways of obtaining each DR$_i$. The relationship is shown in Equation 1, where $i$ is the number of bound Rap1 molecules/DNA molecule.

$$P_j = \left( \frac{[\text{Rap1}]}{n[DNA]} \right)^i \left( 1 - \frac{[\text{Rap1}]}{n[DNA]} \right)^{n-i} \cdot \frac{n!}{(n-i)!} \cdot \frac{n}{(n-i)!(n-i)!} \tag{1}$$

Equation 1 assumes that all Rap1 available in the reaction is bound to DNA. Therefore, binding was ensured by performing the reactions at DNA concentrations well above the $K_d$ for Rap1 binding to the final site in the array. The $K_d$ for binding the final site is equal to the binding affinity for a single site ($K_a$) multiplied by the number of sites ($n$), and the number of ways of binding the recognition sequence as a single site (via filter binding) multiplied by the number of sites within the array.

To compare the observed frequency of each DR$_i$ with that predicted from Equation 1, the frequency of each observed DR$_i$ for a given Rap1:DNA site ratio (R:S) was systematically compared with the frequency predicted for R:S values that varied from 0 to 1. The best fit distribution was obtained by minimizing the residual between each DR$_i$ of the observed and predicted distributions (minimize the sqrt(sum(residual$^2$))).

Time course experiments were performed as described above with the following changes. Excess (1 $\mu$l containing 10× or 15×) unlabeled telomeric DNA array fragment (competitor) was added to 15–20 $\mu$l reaction mixtures containing the DNA and Rap1 at the indicated times. For loading experiments, the zero time point was generated by first mixing the competitor and labeled DNA prior to adding Rap1. Also, for loading experiments, each time point was placed on ice after competitor addition, glycerol was added immediately as above, and the sample was loaded onto a running agarose gel within two minutes as described above. For release experiments, Rap1 and DNA mixtures were incubated at 25°C for 30 min prior to adding competitor. Incubation at 25°C continued, and time point aliquots were removed and placed on ice. The samples were analyzed by gel electrophoresis as above.

**DNA Filter Binding**—DNA fragments containing a single recognition sequence were made by annealing the following complementary oligonucleotides: oEHBl6094/16095 (23 bp consensus), oEHBl6096/16097 (17 bp), oEHBl6102/16103 (27 bp), oEHBl6104/16105 (31 bp), oEHBl11256/11257 (31 bp), and oEHBl11260/11261 (random, low affinity Rap1 binding site). Annealing reactions contained 0.1 M NaCl. DNA fragments containing two recognition sequences were purified from plasmids as above. Binding reactions were as above, with 500–$\mu$l DNA mixtures added to 250–$\mu$l Rap1 mixtures containing the appropriate dilution. Nitrocellulose membranes (Schleicher & Schuell) were prewetted in water and then equilibrated in buffer A prior to assembling in a dot blot apparatus (Minifold I, Schleicher & Schuell). Each binding reaction was applied as a separate dot under vacuum. Each dot was washed two times with 500 $\mu$l of buffer A.

**In Vivo Counting**—This method was described previously (21). Briefly, the distal tip of chromosome VIII, including the native telomere, was replaced by a construct containing the URA3 gene, an array of Rap1 binding sites of differing numbers ($n = 0–16$) with regular site spacing (27 bp or 31 bp) and a distal 81-bp TG$_{L+3}$ telomeric seed. The following integrating plasmids were digested with EcoRI and Sall and transformed into EHB11114: pE56, pE192, pE198, pE202, pE207, pE205, pE219, pE208, pE243, and pDL164–pDL170. Multiple Ura+ transformants were selected and streaked twice on SD-Ura plates and once on YPD plates. Genomic DNA was prepared, and telomere Southern blots were probed for URA3.

**Electron Microscopy**—Rap1 was diluted to 100–500 $\mu$m in buffer A, applied to carbon-coated grids, and negatively stained with 2% uranyl acetate. The samples were imaged at 86,000× magnification with an FEI Tecnai T12 transmission electron microscope operated at 120 kV. The magnification was calibrated from negatively stained catalase crystals. The images were examined with the EMAN software suite (55).

**RESULTS**

Rap1 Is a Monomer with a Ringlike Conformation—To investigate the biochemical and biophysical properties of Rap1 bound to an array of telomere DNA binding sites, we first characterized the oligomeric state and size dimensions of purified recombinant Rap1 free in solution. Absolute values for these parameters can be obtained by measuring the hydrodynamic and light scattering properties of a known, or determined, concentration of purified Rap1 (Fig. 1A; see also “Experimental Procedures”). These properties were simultaneously measured in-line for Rap1 eluted from a size exclusion column. Light scattering data showed that Rap1 is a homogeneous monomer in solution with a molecular mass of 92.6 ± 9.8 kDa (Fig. 1B), in accordance with the predicted monomeric molecular mass of 92.5 kDa. In addition, the intrinsic viscosity [$\eta$] of Rap1 (20 ml/g) was large. This value of [$\eta$] for Rap1 is larger than that observed for most globular proteins (3–4 ml/g, regardless of molecular weight) but is smaller than might be predicted if Rap1 were a random coil (65 ml/g, see Fig. 1 references).
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Two lines of evidence indicated that this value reflects the hydrated volume, shape, and charge of active Rap1. First, the elution profile of purified Rap1 on size exclusion chromatography showed a single monodispersed peak (Fig. 1C), with less than 2% of the protein in the peak aggregated. Second, DNA binding studies that determine the percentage of active protein demonstrated that greater than 90% of the Rap1 preparation bound telomere DNA (data not shown). Together, these data suggest that active Rap1 has a nonglobular or elongated shape.

Another measurement of the size of a protein molecule is its hydrodynamic radius. Although the intrinsic viscosity of a protein in solution depends on the hydrated volume, shape, and charge of that protein in solution, an estimate for the hydrodynamic radius ($R_h$) can be calculated from [η] and molecular weight by assuming a model for shape. Thus, the hydrodynamic radius for monomeric Rap1 was determined by using the standard hard sphere Einstein model (see “Experimental Procedures”). As expected given the large [η], the $R_h$ value for Rap1 (6.6 nm) was also large when compared with other globular proteins of a similar molecular weight (Fig. 1B). This $R_h$ value is consistent with the elution behavior of Rap1 on size exclusion chromatography (Fig. 1C). Comparison of the elution time for Rap1 with those for a set of globular protein molecular weight standards showed that Rap1 eluted at a position equivalent to an ~360-400-kDa globular protein, predicted to have a hydrodynamic radius close to 6 nm.

To more completely understand the relationship between the hydrodynamic radius and molecular weight of Rap1, we used electron microscopy to measure the dimensions of Rap1 monomers. Examination of the surface-deposited and negatively stained monomers revealed that Rap1 adopts a circular, ringlike conformation in which the density in the center of the ring is much reduced (Fig. 2). As expected, no such structures were observed in control samples that lacked Rap1 (data not shown). Measurement of 255 monomer images established a diameter for Rap1 of 120 ± 13.5 Å, a size that correlates well with the hydrodynamic radius calculated from the intrinsic viscosity of 66 Å.

Rap1 Independently Binds Individual Recognition Sites in a DNA Array in Vitro—To gain insight into how Rap1 molecules bind multiple adjacent recognition sequences in an array of telomere repeat DNA and thereby organize the telomere core complex, we asked whether multiple Rap1 molecules bind independently or nonrandomly to individual recognition sites within a defined DNA array of Rap1 binding sites. Because of the normally nonprocessive action of yeast telomerase, repeat elements (the Rap1 recognition sites) in native S. cerevisiae telomes are somewhat heterogeneous in sequence, irregularly spaced, and may even overlap. This irregular array complicates the assessment of binding independence. Therefore, we first examined this question by using defined arrays of equally spaced Rap1 recognition sites, which have previously been shown to mimic the action of telomeres in vivo by their capacity to both control telomere length maintenance when placed proximal to a short stretch of native telomere sequence and to prevent NHEJ (19, 25–27). Each synthetic telomere array contained a known number of identically oriented Rap1 binding sites, each separated by a defined spacing between adjacent sites. Using radiolabeled DNA fragments containing a particular defined telomere array, we then analyzed the distribution of Rap1 bound among the DNA molecules. Complexes containing different numbers of Rap1 molecules bound per DNA fragment (i.e. different bound states, DR) were separated by agarose gel electrophoresis. In these experiments, the concentration of Rap1 varied but was always kept below or equal to the concentration of DNA recognition sites. Under these limiting Rap1 conditions, all of the recognition sites compete for Rap1 binding. Assessment of independence then comes from a comparison of the observed distribution of Rap1 among the DNA molecules to the distribution predicted by random assortment.

Analysis of the distribution of Rap1 bound to DNA fragments containing four recognition sequences is first presented in Fig. 3A. In vivo, placement of a defined array of four Rap1 binding sites or an 80-bp stretch of telomere repeat sequence (which also contains four Rap1 binding sites) adjacent to an induced double-strand break is sufficient to prevent NHEJ repair of the double-strand break end, to recruit other telomere binding proteins, and to allow efficient extension by telomerase to create a normal length telomere (28–32). Mobility shift

![FIGURE 2. Rap1 adopts a ringlike shape in solution.](https://example.com/figure2)

Transmission electron microscopy images of Rap1 are shown. Left, field view. Right, representative images at 86,000× magnification.
Although Rap1 did not exhibit obvious cooperative binding to sites within the four-site array, quantitative analysis was applied to assess whether Rap1 indeed bound individual sites within this array independently. We compared the observed distribution of bound states (DR), for various input ratios of Rap1 to DNA sites (R:S), to the distribution predicted by multisite random assortment (see “Experimental Procedures” for details and equations). Although some amount of radiolabeled DNA migrated diffusely in the gel shift experiments, most migrated as discreet bands (greater than 90%, data not shown), allowing quantification. To eliminate uncertainty in knowing precisely the concentrations of Rap1 and DNA in each reaction, the amount of each DR, species was normalized to the total radiolabeled DNA in each reaction and compared with the frequency of each DR, species predicted by random assortment. The distribution that best fit the observed data was determined by minimizing the difference between each DR, in the predicted and observed distributions (Fig. 3, C and D; see also “Experimental Procedures”). The similarity between the best fit and observed distributions can be visualized by plotting the pair-wise values for each DR, from the best fit and observed distributions. Analysis of these pair-wise values for each DR, from seven separate experiments encompassing 31 observed distribution sets that range in R:S from 0.15 to 0.97 shows close clustering of the pair-wise DR, values about the axis of perfect correlation (Fig. 3E). Thus, the observed distribution of each bound state for a given R:S is highly similar to the frequency predicted for each bound state based on random assortment. Under the limiting Rap1 condition employed in these experiments, cooperative Rap1 binding behavior is expected to increase the frequency of both the higher bound and the unbound states while decreasing the frequency of the lower bound states as compared with random assortment. Such cooperative behavior would lead to a diffusion of the pair-wise DR, % bound values away from the axis of perfect correlation, which we do not observe. The tight correlation between predicted and observed distributions allowed the R:S in each reaction to be back-calculated. As expected, the back-calculated values were similar to the estimated R:S for each reaction and increased linearly with increasing Rap1 concentration (data not shown). Together, these data indicate that Rap1 monomers bind independently to each site within a four-site array.

In native S. cerevisiae telomeres, potential Rap1 binding sites are irregularly spaced and vary somewhat in the consensus recognition sequence. We therefore also investigated whether the sequence of the recognition site or the spacing between adjacent sites affected the array binding behavior of Rap1. To test whether Rap1 binds independently within DNA arrays regardless of site spacing, the distribution of Rap1 on DNA fragments carrying four-site arrays with 17-, 22-, 27-, 31-bp, or an alternating 15/20-bp spacing was investigated. In each case, independent binding by Rap1 was observed upon both qualitative inspection of the gel shift patterns and upon quantitative assessment as described above (Fig. 4 and data not shown). These arrays with different spacings also differed somewhat in sequence at the 5′ and 3′ sides of the recognition consensus (Table 1). We measured the affinity of Rap1 for the single DNA binding sites present in each of the differently spaced arrays.

### Experimental Procedures

The similarity between the best fit and observed distributions can be visualized by plotting the pair-wise values for each DR, from the best fit and observed distributions. Analysis of these pair-wise values for each DR, from seven separate experiments encompassing 31 observed distribution sets that range in R:S from 0.15 to 0.97 shows close clustering of the pair-wise DR, values about the axis of perfect correlation (Fig. 3E). Thus, the observed distribution of each bound state for a given R:S is highly similar to the frequency predicted for each bound state based on random assortment.

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using a filter binding assay and found that Rap1 bound two of these recognition sequences with significantly reduced affinity (Table 1). Despite the difference in Rap1 DNA binding affinity by a factor of ~500, Rap1 bound each site independently in four-site arrays containing either the lower or higher affinity recognition sequences (DNA fragment and Rap1 concentrations were adjusted to compensate for the lower binding affinity). Therefore, our data indicate that Rap1 binds independently to individual sites within a four-site array regardless of the spacing between sites (as long as the sites do not overlap; data not shown).

Telomere function in vivo is directly linked to the length of telomere repeat DNA sequence and the consequent number of bound Rap1 molecules. The processive action of telomerase at telomeres increases substantially at telomere repeat lengths of 100–120 bp and shorter, whereas NHEJ at telomeres is increased when telomeres are, in general, shorter than 80 bp (18, 24, 29). Although a change in Rap1 binding behavior from independent to more cooperative seems unlikely for arrays that are shorter than the four sites (68–108 bp) used above, it is possible that Rap1 may not bind each site independently when arrays contain greater numbers of binding sites. To determine whether the number of sites within an array affected the DNA binding behavior of Rap1, arrays containing various numbers of sites were tested. Rap1 bound independently to individual sites within defined arrays of 2, 3, 4, 6, and 16 sites (Fig. 5 and data not shown). A native 273-bp telomere sequence with 14 predicted Rap1 binding sites was also tested. Qualitative inspection of the distribution of Rap1 bound states revealed that, as observed above for the short, defined arrays and as reported previously (4), Rap1 bound individual sites within this native telomere array in a noncooperative manner (Fig. 6). Thus, Rap1 binds independently to the multiple sites in an array regardless of the total number of array sites.

We tested three other experimental variables for possible effects on Rap1 array binding. First, the concentration of Rap1 (and DNA) was tested over a range of 10–500 nM DNA fragment and 4–2000 nM Rap1. Second, an alternate set of buffer conditions was tested. Studies of the cooperative binding properties of single-strand binding (Ssb) protein, for example, indicate that the binding behavior of single-strand binding protein can be greatly altered by the salt and divalent metal ion concent-

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**TABLE 1**

Rap1 binding affinities for the recognition sequences and spacings used in this study

| Spacing | Sequence | $K_d$ |
|---------|----------|-------|
| Consensus | 23 bp | 5'-CACACCAACCCACCCACACAC-3' | 20±2 μM |
| Competent for Telomere Length Maintenance | 22 bp | 5'-GATCCTAAGCTCCGATCC-3' | 78±12 μM |
| 2x27 bp | 5'-GATCCTAAGCTCCGATCC-3' | 44±4 μM |
| 27 bp | 5'-GATCCTAAGCTCCGATCC-3' | 41±10 μM |
| 31 bp | 5'-GATCCTAAGCTCCGATCC-3' | 44±8 μM |
| 17 bp | 5'-GATCCTAAGCTCCGATCC-3' | 40±5 μM |
| 2x17 bp | 5'-GATCCTAAGCTCCGATCC-3' | 25±5 μM |

* Binding affinities were measured for a single binding site unless otherwise indicated. A $K_d$ of 13 μM is reported for the rpg-1 sequence, containing the Rap1 recognition site: 5'-CACACCATACATT-3' (60).

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**FIGURE 4.** Rap1 binds independently to each site within a four-site array regardless of the spacing between recognition sites. Representative gel shift data are shown for Rap1 binding to DNA fragments containing four-site arrays with the indicated spacing between sites. The dotted lines indicate data from three separate gels. The black triangles represent the set of expected R:S values used in each experiment: 0.054, 0.1075, 0.215, 0.43, and 0.86. For the 17- and 22-bp spacings, the [DNA] is 500 nM; for the alternating 15-bp/20-bp and the 27- and 31-bp spacings, the [DNA] is 100 nM. Experiments for each spacing were performed at least three times with similar results.

**FIGURE 5.** Rap1 binds independently to sites within arrays of two, three, and six binding sites. A, Rap1 binding data for DNA fragments containing two recognition sites with either the 17- or the 22-bp sequence and spacing between sites. [DNA] is 200 nM; expected R:S is 0.054, 0.108, 0.215, 0.43, and 0.86 (range indicated by black triangles). Top panel, gel shift; bottom panel, graph of correlation between observed and best fit predicted values for frequency of each bound state at a given R:S. Dotted line, axis of perfect correlation. Multiple separate experiments are plotted. For 17 bp (open circles), there were three experiments with 11 observed distribution sets, $R^2 = 0.9917$; for 22 bp (closed diamonds), there were seven experiments with 29 observed distribution sets, $R^2 = 0.998$. B, same as A, but six recognition sites. [DNA] is 500 nM. For 17 bp, there were four experiments with 12 observed distribution sets, $R^2 = 0.9923$; for 22 bp, there were six experiments with 21 observed distribution sets, $R^2 = 0.9913$. C, same as A, but six recognition sites. [DNA] is 100 nM. For 17 bp, there were five experiments with 19 observed distribution sets, $R^2 = 0.9712$; for 22 bp, there were five experiments with 19 observed distribution sets, $R^2 = 0.9915$. 

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**Figure 6. Noncooperative binding of Rap1 to recognition sites within a long stretch of native telomeric repeat DNA.** Gel shift of Rap1 binding a DNA fragment containing 273 bp of native telomeric sequence. [DNA] is 10 nM. [Rap1] from left to right is 15, 30, 60, 75, 90, 105, and 120 nM.

Despite this slower release rate, the rate constant for attaining equilibrium under these conditions is dominated by the extremely rapid association of Rap1 with the specific recognition sites in the array (61). Together these data indicate that the independent binding observed for Rap1 to each recognition site within a telomeric DNA array reflects both the loading and release of Rap1, at least under buffer condition 1. Under the buffer condition that slows the rate of Rap1 release, this independence largely reflects the loading of Rap1 onto the array where Rap1 remains stably bound for an extended period of time.

**Telomere Length Regulation in Vivo Is Sequence-specific but Is Independent of Site Spacing**—The experiments above indicated that Rap1 binds individual recognition sites within a DNA array independently, regardless of spacing, array length, and likely independent of sequence. Previous experiments in *vivo* that investigated effects of Rap1 recognition site sequence and spacing on telomere length regulation have yielded somewhat conflicting results. Most experiments yield results similar to our in *vitro* Rap1 array binding studies and show that control of telomerase-mediated telomere extension by the number of Rap1 binding sites (counting) occurs in a manner independent of spacing between these sites, for spacings ranging between 13 and 38 bp (25, 27). However, Rap1 binding sites spaced 17 or 31 bp apart were not well counted (25). In the latter study, although all of the functional spacings carried the same sequence, the spacings that functioned poorly or not at all also differed in sequence at the edges of the consensus recognition site. The authors note that the sequence of the 17-bp spacing site did not bind Rap1 *in vitro*, whereas the 31-bp sequence/site showed high affinity Rap1 binding (25), leading them to conclude that either the unique spacing of the 31-bp site or a feature of this sequence that was independent of Rap1 binding failed to allow these 31-bp spaced sites to be counted. These sequences and spacings are in fact the same ones used in our

![Buffer A and Buffer B](image_url)
study, and during the course of our in vitro array binding experiments, we examined the affinity of Rap1 for these sequences. In contrast to Grossi et al. (25), we discovered that both of the nonfunctional sites in vivo also bound Rap1 with reduced affinity in vitro (Table 1). We therefore re-examined whether arrays carrying the previous 31-bp spaced sites functioned poorly in vivo because of differences in sequence or spacing.

To test directly whether Rap1 binding sites spaced 31 bp apart can be efficiently counted in vivo, we converted the recognition sequence in the 31-bp spacing arrays from the original low affinity sequence to the same high affinity sequence found in the 22- and 27-bp spacings (called 31 bp*). Filter binding experiments with a single site confirmed that Rap1 bound the 31-bp* sequence with an affinity comparable with that of the 27-bp sequence (Table 1). DNA arrays containing different numbers of the 31-bp* site were then placed next to a single telomere in vivo (Fig. 8A), and the length of the adjacent native TG1–3 sequence was measured by telomere-specific Southern blot. Analysis of the average length of this telomere revealed that as the number of sites in each array increased, the length of the native TG1–3 sequence decreased equivalently, regardless of whether the sites were separated by 27 bp or 31 bp* (Fig. 8, B–D). The previous study shows that, in contrast, arrays containing the original 31-bp construct were not counted as well as arrays containing the high affinity 27-bp spacing construct (25). Together, these data indicate that spacing between adjacent Rap1 binding sites not only has no effect on independent binding by Rap1 to arrays of recognition sequences in vitro but also has little effect on Rap1 controlled telomerase-mediated telomere extension and length maintenance in vivo. Furthermore, these results suggest that the sequence near the edges of each Rap1 binding site within the array can have effects on counting in vivo.

DISCUSSION

Rap1 Is a Ringlike Monomer—How the different domains of Rap1 are organized to perform its DNA binding and DNA regulatory functions remains unsolved. We began to address these questions by using electron microscopy to image Rap1 and by analyzing the hydrodynamic and light scattering properties of Rap1 in solution. These studies reveal that Rap1 is a monomer in solution and that each monomer forms a ringlike or C-shaped structure with a radius of ∼6–6.6 nm (the measured EM and hydrodynamic radii, respectively). The crystal structure of the Rap1 DBD bound to a cognate DNA site shows DNA completely encircled by the interactions of the two linked homeodomains and the C-terminal tail of the DBD. It is tempting to speculate that this DBD is found at the internal surface of the ringlike shape of full-length Rap1 observed here via EM. In light of the ringlike shape of the Rap1 protein (and, indeed, the DBD that completely encircles DNA), how Rap1 binds DNA remains an interesting question. Rap1 is capable of binding recognition sites embedded within a circular plasmid (35) and within promoters in vivo. Thus, if the DBD resides within the interior of the Rap1 C or ringlike shape observed here, a conformational change in Rap1 may be required to bind internal sites on duplex DNA; Rap1 binding to a telomere end could employ either a threading mechanism or a similar conformational change. Resolution of these possibilities awaits kinetic analysis of Rap1 binding to recognition sites within a linear versus a circular DNA context.

Protection of telomere DNA involves binding of multiple sites within a DNA array by site-specific DNA binding proteins across many species. Both Schizosaccharomyces pombe and vertebrates use proteins containing homeodomains to bind the double-stranded region of telomere DNA and regulate telomere function (36, 37). These proteins, Trf1 and Trf2 in vertebrates and Taz1 in S. pombe, are considered the functional analogs of scRap1 with respect to DNA binding. In contrast to
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the two homeodomains present in each scRap1 molecule, each molecule of Trf1, Trf2, and Taz1 contains only one. Trf1, which controls telomere extension (38), forms a dimer in vitro and thus binds human telomere repeats, effectively recreating the two homeodomains observed in scRap1. Likewise, hTrf2, which controls recombination processes at the telomere (39), forms homodimers in vitro capable of binding DNA (40). Although these proteins use two homeodomains to bind duplex telomere DNA, the DBDs of these homodimers may not completely encircle DNA-like scRap1. Crystal structures of the DBDs from Trf1 and Trf2 show two homeodomains bound to opposite sides of duplex telomere DNA, presumably driven by the spacing of the binding sites on DNA (41). In both Trf1 and Trf2, the homeodomain lies at the extreme C terminus of the protein, and neither protein carries an appreciable C-terminal tail. In S. pombe Taz1, a short amino acid stretch lies between the C terminus of the proteins and the homeodomain; whether this region is required for high affinity DNA binding is unclear. Interestingly, EM studies suggest that Taz1 may also form a ringlike shape when binding DNA (42). Although hTrf1 and hTrf2, in isolation, may not completely encircle DNA like scRap1, it is possible that these proteins may cooperate with other telomere-associated proteins to similarly encircle telomere DNA. Vertebrates and S. pombe also carry a homolog of scRap1, which contains a single homeodomain as well as the BRCT and RCT regions. Although isolated hRap1 does not bind DNA, hRap1 interacts with hTrf2 to inhibit NHEJ in vitro, and the hRap1-hTrf2 complex binds DNA (43, 44). Whether homeodomains from hTrf2 and hRap1 can cooperate to bind duplex DNA and whether hRap1 can affect the binding affinity of hTrf2 remain interesting questions.

Fundamental Properties of the Rap1-Telomere DNA Core Complex—Telomeres of the yeast S. cerevisiae possess, as a fundamental core, multiple Rap1 molecules bound to multiple recognition sites within the TG-rich telomere repeat tract. Using a quantitative biochemical method, we demonstrate that Rap1 contributes to telomere structure by independently binding individual recognition sites within an array of nonoverlapping sites. Moreover, we show that this independent binding occurs regardless of the spacing between adjacent sites, the total number of recognition elements, and the binding affinity of Rap1 for the particular sequence of the individual sites. Furthermore, we establish that these binding studies reflect the equilibrium binding state of Rap1 to an array of sites. Previous results show that Rap1 binds DNA fragments carrying a single recognition site as a monomer (14) and that Rap1 binds multiple sites within a repeat array without obvious cooperativity (3, 4). However, these experiments did not determine whether binding conditions were performed at equilibrium or whether Rap1 loaded onto or released multiple sites in a telomere array in a manner that was truly independent of the Rap1 occupancy of the neighboring site (4). Our analysis extends these early data to show not only that Rap1 exists as a monomer in solution but also that Rap1 monomers load onto and release (buffer condition 1) each site within an array of specific repeat binding elements without regard to the occupancy of the other sites within the array. In addition, we confirm that Rap1 binds DNA fragments containing telomere repeat elements rapidly (3) and demonstrate that Rap1 releases each recognition element of telomere DNA slowly (loading in under 2 min under these conditions; release $t_{1/2} = \sim 30$ min or greater, depending on the buffer conditions). As noted previously for different reaction conditions, we find that the speed of Rap1 release is dependent on the buffer conditions (35). Thus, under these slow release conditions, Rap1 loads onto multiple sites in an array independent of the occupancy of the other sites and then remains bound to that site for a relatively long time. Although the relationship of these in vitro conditions to those in vivo is unclear, the long time frame of Rap1 release suggests that a mechanism may be required in vivo to remove Rap1 from telomeres (and other high affinity sites) before replication can proceed.

Whether analogs of scRap1 like Trf1, Trf2, and Taz1 also bind independently to multiple sites in a telomere array remains to be demonstrated. Trf1 binds multiple sites in a telomere array without a large degree of cooperativity (45), although whether these results reflect equilibrium binding is unclear. The binding characteristics of hTrf2 dimers to multiple sites within a telomere array remains to be determined. In addition, other proteins in the human shelterin complex (e.g. hRap1, Tin2, Tpp1, and Pot1) can form complexes with the Trf proteins (36, 46, 47) and may modulate DNA binding. Similar considerations hold in S. cerevisiae, where the binding behavior of Rap1 could be modulated, for example, by proteins such as Rif1, Rif2, and Sirs.

The telomere nucleoprotein complex controls telomerase-mediated telomere extension using a mechanism that depends on the number of telomere repeats. Previous experiments that either tethered multiple Rap1 molecules through a heterologous DNA-binding domain to telomeres or replaced portions of native telomere DNA with defined arrays of Rap1 binding sites showed that the number of bound Rap1 molecules incrementally regulates telomerase-mediated telomere extension through the Rap1 associating factors Rif1 and Rif2 (19, 21). In addition, the frequency of telomere extension (telomerase activity at the telomere) observed in vivo for telomeres over the length range of $\sim 120–350$ bp (18) also gradually increases as telomere length decreases. Telomeres are postulated to fluctuate between extendible and nonextendible states. One possible mechanism to explain this conversion could be an increased stability of the telomere nucleoprotein complex as a function of the number of bound Rap1 molecules. Our data indicate that, at the root of formation of the telomere nucleoprotein complex, Rap1 alone binds independently to multiple sites in telomere repeat DNA based solely on the affinity of Rap1 for each of the individual sites.

Unlike the incremental inverse relationship between telomere length and Rap1-controlled telomerase extension frequency observed in vivo, telomeres also exhibit more abrupt length-dependent changes in protecting the DNA ends from NHEJ, increasing the effective processivity of telomerase at short telomere ends, and in cell cycle arrest. A model for this length-dependent control postulates that a “closed telomere state” exists in which a certain minimum number of Rap1 molecules are bound to telomere DNA. If Rap1 binding to telomere repeat elements in vivo is also independent of adjacent site occupancy as observed here and is therefore governed by affinity, then one
possible model for this minimum Rap1 requirement is that an interaction between bound Rap1 molecules and a second telomere-associated protein is altered when the number of Rap1 molecules falls below a certain threshold. Alternatively, Rap1 binding to DNA could exhibit length-dependent cooperativity in vivo that is not observed here in vitro. The telomere repeat length at which telomeres convert from “closed” to “open” is different for the different actions specified above: effective telomerase processivity is increased at a repeat length of 100–125 (six or seven Rap1 binding sites), whereas Rap1-mediated inhibition of NHEJ may require two to four Rap1 binding sites (24, 26, 48). Because of the different lengths at which these different “conversions” occur, we favor a model for telomere structure in which Rap1 binds independently to each repeat element in a manner governed by affinity and that the interaction of secondary proteins with Rap1 changes as different numbers of Rap1 molecules are telomere-bound.

Sequence Dependence of Telomere Length Regulation in Vivo—Telomere sequence in S. cerevisiae varies because of the normally nonprocessive action of telomerase, although repeat elements conform to a consensus high affinity binding site for Rap1. We have discovered that certain deviations from the consensus Rap1 binding sequence at the outside edge affect both the high affinity DNA binding of Rap1 in vitro and telomerase-mediated telomere extension in vivo. Thus, we establish that two Rap1 recognition sequences observed previously to function poorly as part of a 17- or 31-bp spaced telomere array (25) also bind Rap1 with reduced affinity relative to that observed for the telomere consensus (2000× and 22× reduced, respectively). These poorly functioning Rap1 recognition sequences contain a G within the C-rich strand at either the 5′ or 3′ end of the consensus region. Studies that characterize the Rap1 consensus sequence based on both telomere and promoter regions indicate that a G is found less than 4% of the time at this 5′ position and less than 2% of the time at this 3′ position (50, 51). When we converted the low affinity Rap1 sequence in the previously reported 31-bp arrays to the higher affinity Rap1 sequence (10× change in affinity), the new 31-bp arrays (31 bp*) functioned in vivo as part of the telomere in a manner equivalent to the high affinity arrays. Previously, it was suggested that one possible explanation for the inability of arrays containing the low affinity 31-bp sequence to function as part of a telomere was due to a particular deficiency with this spacing in forming the proper telomere structure. We show that this is unlikely to be the case and that the low affinity sequence itself is deficient in telomere function.

Several models could explain the functional difference between the low and high affinity Rap1 binding sites in telomere arrays. One model is that Rap1 does not occupy the lower affinity sites in vivo, and therefore these recognition sites do not contribute to the telomere nucleoprotein complex. The abundance of Rap1 (nuclear concentration, ~1 μM) (52) and the ability of Rap1 to occupy lower affinity promoter recognition sequences in vivo (51) would seem to argue against this model. However, Rap1 binding to these promoter sites may be influenced by other co-repressor and co-activator proteins. In support of the hypothesis that the affinity of Rap1 for each telomere repeat element influences telomere repeat function, a small increase in Rap1 gene expression results in shorter telomere repeat length (53). If shortened telomeres are a consequence of increased occupancy of telomeres by Rap1, then perhaps not all possible repeat elements within telomeres are normally bound.

Recent chromatin immunoprecipitation data also suggest that an array of low affinity Rap1 recognition sites is not bound by Rap1 in vitro when placed next to an induced double-strand break (26). A second model is that these low affinity sites fail to promote a sequence-dependent conformational change in Rap1 that alters Rap1 interaction with itself or other proteins. Our in vitro data showed no evidence for cooperativity between Rap1 molecules bound to either low or high affinity recognition site arrays. Crystal structures of the isolated DBD of Rap1 bound to three different recognition sequences show that DNA binding can be accommodated by small changes in organization of amino acid side chains in the binding pocket (54). Thus, this model seems unlikely unless other regions of Rap1 interact with the outside edges of the Rap1 binding site. A third model is that the poorly functioning repeat sequences fail to bind another unidentified telomere sequence-specific DNA binding factor, such as Rf1 or Rf2. Resolution among these models awaits further biochemical characterization of the relevant telomere proteins.

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