Cooperative Binding of Single-Stranded Telomeric DNA by the Pot1 Protein of *Schizosaccharomyces pombe*†

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ABSTRACT: The fission yeast Pot1 (*p*rotection *o*f *t*elomeres) protein is a single-stranded telomeric DNA-binding protein and is required to protect the ends of chromosomes. Its N-terminal DNA-binding domain, Pot1pN, shows sequence similarity to the first OB fold of the telomere-binding protein α subunit of *Oxytricha nova*. The minimal-length telomeric ssDNA required to bind Pot1pN was determined to consist of six nucleotides, GGTTAC, by gel filtration chromatography and filter-binding assay (K_D = 83 nM). Pot1pN is a monomer, and each monomer binds one hexanucleotide. Experiments with nucleotide substitutions demonstrated that the central four nucleotides are crucial for binding. The dependence of Pot1pN−ssDNA binding on salt concentration was consistent with a single ionic contact between the protein and the ssDNA phosphate backbone, such that at physiological salt condition 83% of the free energy of binding is nonelectrostatic. Subsequent binding experiments with longer ssDNAs indicated that Pot1pN binds to telomeric ssDNA with 3′ end preference and in a highly cooperative manner that mainly results from DNA-induced protein−protein interactions. Together, the binding properties of Pot1pN suggest that the protein anchors itself at the very 3′ end of a chromosome and then fills in very efficiently, coating the entire single-stranded overhang of the telomere.

The telomere is the DNA−protein complex found at the termini of all linear eukaryotic chromosomes, serving multiple functions in cells (*1*−*4*). The DNA component of telomeres usually consists of tandem repeats of a short 5−9 base pair sequence. Although the exact sequence of the telomeric DNA varies from species to species, clusters of three or more guanine residues are usually found on one strand. This G-rich strand is always oriented in the 5′ to 3′ direction toward the end of the chromosome (*2*, *3*, *5*).

Most of the telomeric DNA is double stranded, but the 3′ ends are single stranded in all organisms investigated to date, including ciliates (*6*), budding yeast (*7*), fission yeast (*8*), and human (*9*−*11*). These single-stranded extensions serve as substrates for the enzyme called telomerase, which elongates the 3′ ends using an internal RNA subunit as the template to direct the sequence added (*4*, *12*−*17*). By this means, telomerase ensures the complete replication of chromosome ends, which otherwise shorten with each cell division.

Telomere proteins that bind sequence-specifically to double-stranded telomeric DNA include budding yeast Rap1p (*18*), fission yeast Taz1p (*19*), and human TRF1 and TRF2 (*20*, *21*). Other factors are specific to single-stranded telomeric DNA, such as Cdc13p[1] from *Saccharomyces cerevisiae* (*22*) and telomere end binding proteins (TEBP) from *Oxytricha nova* and *Euplotes crassus* (*23*−*28*). Functions of telomere proteins include capping the ends of chromosomes, preventing nucleolytic degradation and thus confering stability (*29*, *30*), and the positive and negative regulation of telomerase (*31*−*34*).

Recently, a distant homologue of the α subunit of TEBP, Pot1p, was identified by taking advantage of the complete genome of fission yeast (*8*, *35*). Accordingly, Pot1p protein was shown to bind specifically to the single-stranded G-rich telomeric sequence of fission yeast in vitro. Moreover, deletion of the *pot1*$^+$ gene resulted in rapid loss of telomeric DNA as well as chromosome missegregation and chromosome circularization, diagnostic features of end-to-end chromosome fusion. Several Pot1 homologues, including human Pot1, have been found by means of BLAST searches against sequence databases (*8*). However, database searches have not revealed sequences corresponding to the β subunit of *Oxytricha* TEBP, which forms a ternary complex with the α subunit and ssDNA (*26*, *36*).

The sequence similarity between Pot1p and TEBPα is restricted to the OB (oligonucleotide/oligosaccharide binding) fold (*37*) at their N-termini, and this 20 kDa fragment of

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[1] Abbreviations: Pot1p or Pot1 protein, protection of telomeres protein; Pot1pN, N-terminal domain of *S. pombe* Pot1p consisting of amino acids 1−185; Cdc13p, an *S. cerevisiae* telomeric ssDNA-binding protein with structural and functional similarities to Pot1p; TEBP, telomere end-binding protein from the ciliated protozoan *Oxytricha*; ssDNA, single-stranded DNA; OB fold, oligonucleotide/oligosaccharide-binding motif found in diverse proteins; Tel, GGTTACA, which represents a common repeated sequence in *S. pombe* telomeric DNA; TelΔA, GGTTAC.

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Schizosaccharomyces pombe Pot1p, Pot1pN, has been shown to bind single-stranded telomeric DNA (*8*). There is a tradition of studying the interactions of DNA-binding domains of proteins with their DNA sites, such as in the case of transcription factors and repressors, and with Pot1p the ease of expression of the N-terminal domain has provided a great practical advantage. To begin to understand how Pot1p interacts with telomeres, we have analyzed the DNA-binding properties of its N-terminal domain. The results of both gel filtration chromatography and filter-binding assays reveal that a six base long single-stranded DNA (GGTTAC) defines the minimum binding site for Pot1pN and that this sequence is bound with high specificity. We also demonstrate that multiple Pot1pN proteins can bind along long telomeric ssDNA with high cooperativity with a 3′end preference.

EXPERIMENTAL PROCEDURES

Oligonucleotide Preparation. Telomeric ssDNAs were made on an Applied Biosystems 380B DNA synthesizer and purified by polyacrylamide gel electrophoresis.

Purification of S. pombe Pot1pN Expressed in Escherichia coli. SpPot1N was expressed in *E. coli* BL21(DE3) cells harboring plasmid pET11a (Novagen). Cells were grown in 2 L baffled flasks containing 1 L of PTY medium to which ampicillin was added to 100 μg/mL. The cells were grown at 37 °C in a shaker incubator to an optical density of OD_{600} ~0.6 and were then cooled to room temperature and grown to an OD_{600} of ~1.0. Production of the protein was induced by addition of isopropyl thiogalactoside (IPTG) to 0.2 mM, and the cells were grown for an additional 5−6 h at room temperature. The cells were harvested by centrifugation and stored at −20 °C. Expression of the Pot1pN protein was confirmed by western blotting with anti-6×His antibody (Invitrogen). Approximately 20 g of cells (wet weight) were resuspended in 50 mL of lysis buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM 2-mercaptoethanol, 5 mM benzamidine, and 1 mM PMSF) and incubated on ice for 40 min. Then DNase I and 15 mM $MgCl_2$ were added to the cells, which sat on ice for another 20 min. The cells were lysed by sonication, and cell debris was removed by centrifugation. The supernatant containing His-Pot1pN protein was mixed with 5 mL of Ni-NTA−agarose beads and rocked at 4 °C for 4−5 h before elution with 50 mM imidazole. Then 3C protease was added to remove the N-terminal 6×His tag. Pot1pN protein was further purified by gel filtration chromatography (Superdex 75 from Pharmacia). Protein at this stage was >99% pure and was concentrated to 22 mg/mL by Centricon 10 (Millipore) and stored at −80 °C.

Gel Filtration Chromatography. Protein dilutions were made up fresh for each experiment. Typically, a gel filtration sample included 5 μL of 1 mM protein and 10 μL of telomeric ssDNA at a molar ratio of 1:1.2 and 100 μL of binding buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1 mM DTT). Reaction mixtures were allowed to equilibrate for 1 h at 4 °C before injection onto a Superdex 75 gel filtration column. The column was preequilibrated in the binding buffer and run at 4 °C. The entire process was monitored at two absorption wavelengths, 260 and 280 nm. The elution volume and ratio of A_{260}/A_{280} were evaluated using the software provided by Pharmacia.

Filter-Binding Assay. Filter-binding experiments were performed in a 96-well dot blot apparatus essentially as described (*38, 39*). All oligonucleotides used in filter-binding experiments were radiolabeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. Radiolabeled oligonucleotides were separated from free [γ-^{32}P]ATP on denaturing polyacrylamide gels. Trace amount (<0.05 nM) labeled oligonucleotides were incubated with Pot1pN in a 50 μL total volume of binding buffer (20 mM Tris-HCl, pH 7.5, 1 mM DTT, and 15−450 mM NaCl as indicated) for 1 h at room temperature (~23 °C). The mixtures were then filtered through a membrane sandwich containing a top layer of protein nitrocellulose membrane (Millipore), a middle of layer of Hi-bond positively charged nylon membrane (Amersham), and a bottom layer of filter paper (Whatman). Before sample application, the filter was pretreated with 200 μL of the binding buffer. After application of the samples, the filters were washed twice with 100 μL of binding buffer. A flow rate of ~20 μL/s was used to give optimal data. Then the membranes were separated and dried at room temperature for 30 min and quantified using a PhosphorImager (Molecular Dynamics). The data were analyzed with GraphPad Prism software.

Ionic Strength Dependence of Pot1pN−TelΔA Complex Formation. The salt dependence of the binding of Pot1pN to TelΔA was determined by conducting titration experiments in filter-binding buffer with different sodium chloride concentrations. The effect of the salt concentration on complex formation has been described by the relation

$$-\partial \ln [K_D]/\partial \ln [Na^+] = m'\Psi \quad (1)$$

where m' is the number of ion pairs formed and Ψ is the fraction of counterion bound per phosphate group, which is assumed to be 0.71 (*40*). Assuming that m' is equivalent to N, the number of ion pairs, the nonelectrostatic contribution to the standard free energy of binding (ΔG°_{ne}) has been estimated according to the equation:

$$\Delta G^\circ_{ne} = \Delta G^\circ_{obs}(1\ M\ Na^+) - N\Delta G^\circ_{Lys} \quad (2)$$

where ΔG°_{obs} is the standard free energy at $[Na^+] = 1$ M [$\Delta G^\circ_{obs} = RT \ln K_D(1\ M\ NaCl)$] and ΔG°_{Lys} is the standard free energy of formation of a single lysine−phosphate ionic interaction and corresponds to ~0.18 kcal·mol^{-1} at $[Na^+] = 1$ M.

Cooperative Free Energies of Pot1pN−Tel$_2$ and Pot1pN−TelCG$_6$TelC Complexes. The cooperative free energy of the binding of Pot1pN protein to Tel_2 and $TelCG_6TelC$ was determined by fitting the filter-binding data to a model involving two cooperative binding sites:

$$Y = ((K_1 + K_2)[P] + K_1K_2K_c[P]^2)/(1 + (K_1 + K_2)[P] + K_1K_2K_c[P]^2) \quad (3)$$

$$\Delta G_{coop} = -RT \ln K_c \quad (4)$$

where Y is the fraction of DNA bound, [P] is the concentration of Pot1pN protein, K_c is a cooperativity factor, and K_1 and K_2 are the equilibrum association constants for binding to the 3′ and 5′ end telomere repeats of Tel_2 or $TelCG_6TelC$, respectively (*41*).

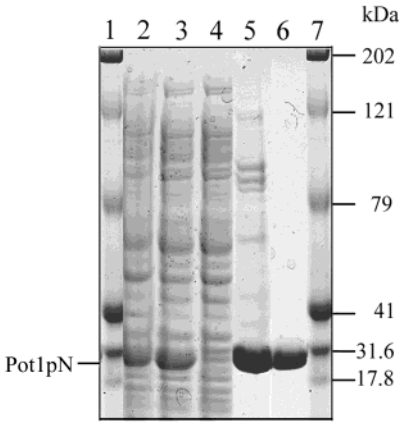

FIGURE 1: Expression and purification of Pot1pN. Protein samples were resolved by SDS−PAGE (4−20%) and stained with Coomassie Brilliant Blue. Lanes: 1 and 7, protein marker (Invitrogen); 2, crude cell lysate from Pot1pN-expressing *E. coli*; 3, soluble fraction after sonication; 4, flow-through after the Ni-NTA−agarose affinity column; 5, bound fraction on the Ni-NTA−agarose affinity column; 6, fraction after the Superdex 75 gel filtration column.

Gel Mobility Shift Assay. Pot1pN in binding buffer (25 mM HEPES/NaOH, pH 7.5, 50 mM NaCl, 40 mM KCl, 7% glycerol, 1 mM EDTA, and 0.1 mM DTT) was mixed with 1 μM ^{32}P-labeled telomeric ssDNAs (20mer, 30mer, and 43mer) in a total volume of 20 μL. The reaction mixtures were incubated at room temperature for 10 min. Then the mixtures were directly loaded onto a 4−20% nondenaturing polyacrylamide gel. Electrophoresis was carried out in TBE buffer at 150 V for 85 min at 4 °C. The gels were dried, and radiolabeled ssDNA was visualized using a PhosphorImager.

RESULTS

Expression and Purification of Pot1pN. A protein consisting of amino acids 1−185 of Pot1p (hereafter called Pot1pN) with six histidines tagged at the N-terminus was produced in *E. coli* BL21(DE3), which expresses T7 RNA polymerase when induced with isopropyl β-D-thiogalactopyranoside (IPTG). Overproduction of Pot1pN was conducted at room temperature to increase the solubility of the protein. Pot1pN accumulated to about 5−10% of total protein in the *E. coli* cells (Figure 1). Two chromatographic steps (a Ni-NTA−agarose affinity column and gel filtration) yielded protein at least 99% pure as judged by Coomassie Brilliant Blue staining of an SDS−polyacrylamide gel (Figure 1). The typical yield of Pot1pN was 50−60 mg of pure protein/L of growth medium.

Pot1pN Binds a Minimum Six-Base Telomeric ssDNA. It has been shown that a 43mer single-stranded telomeric DNA is bound by Pot1p and Pot1pN in gel shift assays (*8*). To determine the minimum binding site of Pot1pN, we synthesized a panel of telomeric ssDNAs of varying length and sequence and examined their Pot1pN binding properties by gel filtration chromatography. In these experiments, highly concentrated protein and a slight molar excess of telomere ssDNAs were used to prevent dissociation of the protein−ssDNA complexes (Figure 2, Table 1).

Chromatography of Pot1pN relative to protein standards revealed a molecular mass expected for a protein monomer (Figure 2A). The elution positions of Pot1pN−ssDNA complexes were earlier than that of Pot1pN itself, as expected for entities with increased molecular mass. Moreover, the ratio of the absorbance at 260 nm versus 280 nm (A_{260}/A_{280}) reflects the composition of the peaks of gel filtration chromatography. The protein alone has $A_{260}/A_{280} = 0.58$,

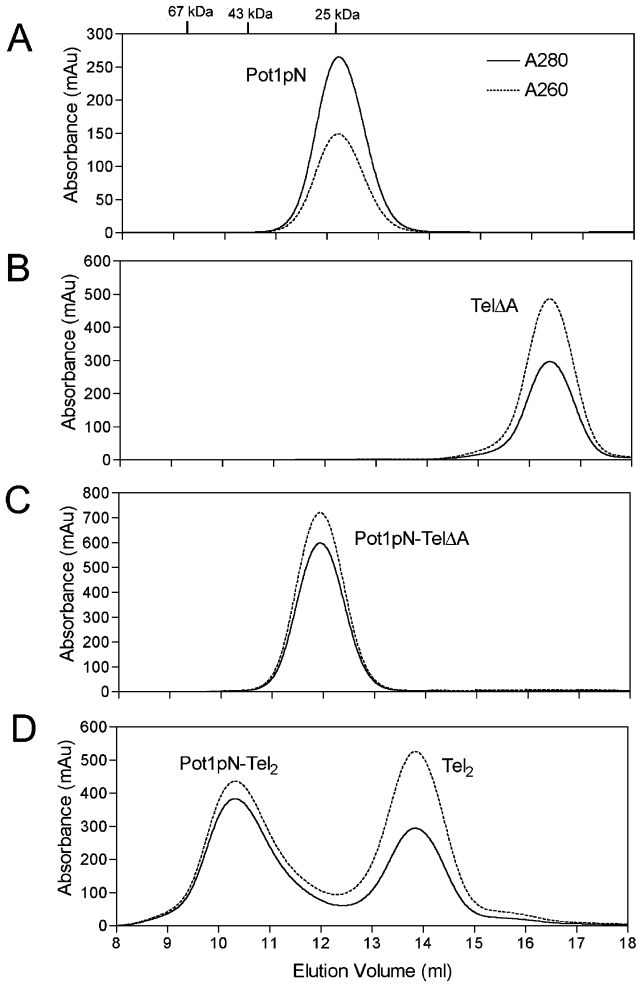

FIGURE 2: Binding of Pot1pN to telomeric ssDNA revealed by gel filtration chromatography. The absorbances at 260 nm (A_{260}) and at 280 nm (A_{280}) are shown in dashed and solid lines, respectively. The elution volumes of three marker proteins (albumin, 67 kDa; ovalbumin, 45 kDa; chymotrypsinogen, 25 kDa) are indicated. Panels: (A) Pot1pN; (B) telomere ssDNA TelΔA; (C) the Pot1pN−TelΔA complex; (D) the Pot1pN−Tel_2 complex. Excess Tel_2 was mixed with the purified Pot1pN protein so that a peak corresponding to free ssDNA could also be observed.

Table 1: Pot1pN Binds a Minimum Six-Base Telomeric ssDNA, GGTTAC, As Assessed by Gel Filtration Chromatography

oligonucleotide	sequence	elution position (mL)	A_{260}/A_{280}	binding[a]
Tel_2	GGTTACAGGTTACA	10.19	1.14	yes
TelGG	GGTTACAGG	11.88	1.16	yes
TelΔA	GGTTAC	11.84	1.16	yes
permute 1	GTTACG	11.96	0.84	yes*
permute 2	TTACGG	12.16	0.58	no
permute 3	TACGGT	12.16	0.58	no
permute 4	ACGGTT	12.12	0.58	no
permute 5	CGGTTA	11.92	0.96	yes*
	no ssDNA	12.2	0.58	no

[a] Summary of telomeric ssDNA-binding ability of Pot1pN. Yes, no, and yes* represent binding, no binding, and binding with high dissociation rate, respectively.

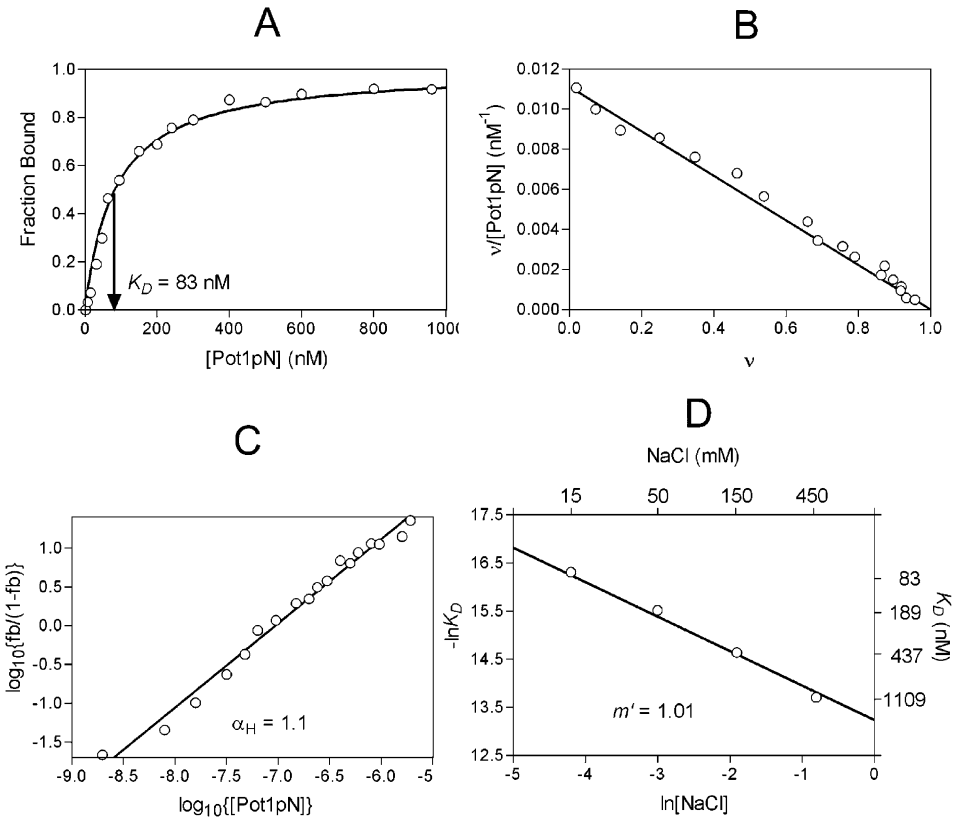

FIGURE 3: Characterization of the interaction of Pot1pN with TelΔA by filter-binding assay. (A) Determination of the dissociation constant K_D of the Pot1pN−TelΔA complex. The telomere ssDNA TelΔA (GGTTAC) was incubated with the indicated concentration of Pot1pN under the conditions described in Experimental Procedures. The solid curve represents a theoretical curve with K_D = 83 nM. (B) Scatchard plot (ν/[Pot1pN] versus ν, where ν is the moles of Pot1pN bound per mole of ssDNA) for the formation of the Pot1pN−TelΔA complex showing noncooperative binding (a linear plot) and the 1:1 stoichiometry of the binding (intercept of the abscissa). (C) Hill plot of Pot1pN binding to TelΔA. The Hill coefficient of 1.1, derived from the slope of the Hill plot, indicates noncooperative binding between Pot1pN and TelΔA. fb = fraction bound. (D) Dependence of the binding of Pot1pN to TelΔA on the NaCl concentration. The data were analyzed as described in Experimental Procedures. m' is the estimated number of ion pairs formed in the complex.

while ssDNA has A_{260}/A_{280} = 1.8 (Figure 2A,B). The protein−ssDNA complexes had unique, intermediate values of A_{260}/A_{280}, a feature which differentiated them from protein or ssDNA.

Although the *S. pombe* telomeric repeat sequence is somewhat heterogeneous, it has been summarized as TTAC(A)(C)G$_{1-8}$ (*42*, *43*). However, Table 1 clearly shows that a circular permutation of this sequence, GGTTAC, is in fact the "core telomeric sequence" with respect to Pot1pN binding, as other permutated versions such as TTACGG gave no binding. All of the telomeric ssDNAs, six bases or longer, that included the core telomeric sequence GGTTAC were capable of binding Pot1pN (Table 1). To the contrary, binding efficiency dropped rapidly with ssDNAs of length under six bases. Two five-base ssDNAs (GGTTA and GTTAC) showed intermediate results in terms of both the elution position and the ratio of A_{260}/A_{280}. The simplest explanation is that these elution peaks represented mixtures of the protein (Pot1pN) itself and the protein−ssDNA complex. For simplicity, hereafter we define Tel as GGTTACA, so TelΔA represents the core sequence GGTTAC.

Dissociation Constant, Stoichiometry, and Salt Dependence. To determine the equilibrium dissociation constant (K_D) of the Pot1pN−TelΔA complex, the dependence of the ratio of the bound to free ssDNA ([Pot1pN−TelΔA]/[TelΔA]) on protein concentration ([Pot1pN]) was measured (Figure 3). Assuming Pot1pN binds TelΔA at a molar ratio of 1:1, then K_D = [Pot1pN][TelΔA]/[Pot1pN−TelΔA], where [Pot1pN], [TelΔA], and [Pot1pN−TelΔA] are the respective equilibrium concentrations. A filter-binding assay (Figure 3A) shows a typical hyperbolic curve for the interaction of various concentrations of protein with a fixed concentration of DNA. The shape of the binding curve is not sigmoidal, and the corresponding Scatchard plot is linear (Figure 3B), indicating that Pot1pN binds to TelΔA in a simple binding equilibrium. This is further confirmed by the Hill constant (α_H = 1.1) (Figure 3C). The stoichiometry of the Pot1pN−TelΔA complex obtained from the abscissa intersept corresponds to one Pot1pN molecule bound per TelΔA (Figure 3B). The apparent dissociation constant K_D for the Pot1pN−TelΔA complex (15 mM NaCl, 23 °C) is calculated to be 83 nM, which is similar to that of TEBPα−TTTTGGGG of *O. nova* (*36*) and is almost 80-fold higher (weaker) than that of Cdc13−TG(1−3) of *S. cerevisiae* (*44*).

To obtain some indication of the sort of protein−DNA interactions that contribute to binding, the salt concentration dependence of the dissociation constant for Pot1pN−TelΔA was examined. Increasing the NaCl concentration from 15 to 450 mM reduced the apparent affinity by a factor of 13.3, revealing a relatively weak contribution of electrostatic interactions to the stability of the Pot1pN−TelΔA complex under our experimental conditions. When the data were plotted as −ln K_D versus ln [NaCl], a linear dependence of the binding on NaCl concentration was observed (Figure 3D).

Table 2: Telomeric ssDNA Binding Specificity of Pot1pN

telomeric sequence	elution position (mL)	A_{260}/A_{280}	binding[a]	K_D (nM)
GGTTAC	11.84	1.14	yes	83
CGTTAC	11.98	0.89	yes*	~18000
GCTTAC	12.21	0.66	no	>100000
GGATAC	12.27	0.55	no	>100000
GGTAAC	12.29	0.58	no	>100000
GGTTTC	12.03	0.82	no	~35000
GGTTAG	11.88	1.01	yes*	1550
GTTAC	12.01	0.75	yes*	~50000
GGTTA	11.95	0.94	yes*	~20000

[a] Yes, no, and yes* represent binding, no binding, and binding with high dissociation rate, respectively.

The slope of the linear regression of the data enables the calculation of the apparent number of counterions (Na^+) released from backbone phosphates upon binding of the protein (*40*, *45*, *46*). A value of $\partial(\ln K_D)/\partial(\ln [Na^+]) = 0.72$ was obtained. Given that Ψ (the fraction of a counterion bound per phosphate for single-stranded DNA) is 0.71 (*40*), m' (the number of ion pairs formed between basic residues of Pot1pN and the phosphate backbone) is equal to 1.01. In the case of the MS2 coat protein−RNA interaction, the number of ion pairs inferred from this sort of analysis agrees very well with the number observed in the X-ray crystal structure of the same complex (*47*, *48*). The nonelectrostatic contribution to the free energy of binding can also be estimated (eq 2; see Experimental Procedures) (*49*). In our case, the value of ΔG°_{obs} is about −7.84 kcal·mol^{-1} (from the plot of $\ln K_D$ versus $\ln[Na^+]$ extrapolated to 1 M). Estimating that the ion pair destabilizes binding at 1 M NaCl by $\Delta G^\circ_{Lys} = \Delta G^\circ_{Arg} = 0.18$ kcal·mol^{-1} (*45*), the nonelectrostatic contribution ΔG°_{ne} corresponds to −8.02 kcal·mol^{-1}. This indicates that under our filter-binding conditions about 83% of the total free energy is contributed by nonelectrostatic interactions at physiological salt concentration.

Sequence Specificity of Binding. To identify sites critical for formation of stable Pot1pN−TelΔA complexes, a series of telomeric ssDNAs containing substitution of each nucleotide in the parental GGTTAC sequence was evaluated for interaction with Pot1pN. Using the filter-binding assay (Table 2), substitution at any of the six nucleotides caused a dramatic decrease in binding. G2, T3, and T4 appear to be the most important nucleotides for the Pot1pN−TelΔ*A* interaction, with very little (~2%) binding of ssDNA with substitutions at these sites even at Pot1pN concentrations as high as 100 μM.

Gel filtration chromatography of these substituted ssDNAs confirmed the filter-binding experiments (Table 2). Pot1pN did not bind four of the substituted ssDNAs; two separate elution peaks were observed, with positions corresponding to those of Pot1pN and free ssDNA, respectively. Furthermore, the ratio of A_{260}/A_{280} also indicated the absence of ssDNA in the protein elution fraction (Table 2). Substitutions at G1 and G6 resulted in intermediate values for both the elution position and the A_{260}/A_{280}, indicating a weaker interaction between Pot1pN and these substituted ssDNAs as seen in the filter-binding experiments. Although C6 is the least important nucleotide for specificity, the presence of some nucleotide at position 6 remains an important factor for the binding, since the pentanucleotide GGTTA had an almost 200-fold reduction in binding affinity, another 12-fold decrease compared with that of GGTTAG.

Table 3: Telomeric ssDNA with Multiple Telomeric Repeats Binds Multiple Pot1pN Molecules

oligonucleotide[a]	elution volume (mL)	A_{260}/A_{280}	Pot1pN/ssDNA[b]
Tel_4 (28)	12.34	1.16	4
Tel_3 (21)	13.04	1.17	3
Tel_2 (14)	13.57	1.18	2
Tel (7)	15.43	1.14	1

[a] Numbers in parentheses denote the number of nucleotides in the telomeric ssDNA. [b] In all four cases $A_{260}/A_{280} \approx 1.14$, so there must be one PotpN per Tel repeat or *n* Pot1pN/Tel_n.

Table 4: Each Telomeric Sequence Binds One Pot1pN Protein with 3′ End Preference

oligonucleotide[a]	elution volume (mL)	A_{260}/A_{280}	Pot1pN/ssDNA[b]	K_D (nM)
Tel_2 (14)	10.19	1.14	2	19
$TelA_7$ (14)	11.51	1.6	1	1590
A_4TelA_3 (14)	11.63	1.57	1	1580
A_7Tel (14)	11.54	1.62	1	420
Tel (7)	11.87	1.15	1	88

[a] Numbers in parentheses denote the number of nucleotides in the telomeric ssDNA. [b] For $TelA_7$, A_4TelA_3, and A_7Tel, $A_{260}/A_{280} \approx 1.6$, so there must be fewer Pot1pN molecules per 14 nt than with the Tel_2 complex.

Multiple Pot1pN Molecules Bind Longer Telomeric ssDNAs with 3′ End Preference. The length of the 3′ end telomeric overhang varies from species to species. In *S. pombe*, the single-stranded region is 50−75 bases long (P. Baumann, unpublished data). To ascertain whether such a long telomeric ssDNA can provide multiple Pot1p binding sites, Pot1pN protein was mixed with telomeric ssDNAs of various lengths (Tel, Tel_2, Tel_3, and Tel_4) (Table 3). In these experiments, both protein and telomeric ssDNA concentrations were far above the dissociation constant with the condition of DNA excess maintained. By calibrated gel filtration chromatography of Pot1pN−ssDNA complexes, we found that the elution positions of Pot1pN−Tel_n ($n = 1-4$) corresponded to apparent molecular masses of 25, 60, 80, and 100 kDa, respectively (Table 3); because the incremental increase in molecular mass due to the DNA is only 2 kDa, we infer that Tel_n binds *n* Pot1pN molecules. Moreover, all of the protein−ssDNA complexes have the same value of the ratio of A_{260}/A_{280} (Table 3), indicating that all of the complexes have the same ratio of "protein/Tel", which in turn suggests that each Pot1pN−Tel_n complex represents *n* Pot1pN molecules binding adjacent to one another along an oligonucleotide with *n* telomeric repeats.

As an alternative way to assess whether long telomeric DNA can bind to multiple Pot1pN molecules, we synthesized another set of telomeric ssDNAs ($TelA_7$, A_7Tel, and A_4TelA_3) of identical size harboring the telomeric sequence at various locations (Table 4). Pot1pN proteins were mixed with them, and the protein−ssDNA complexes were again analyzed by gel filtration chromatography. As expected, if only a single protein were bound, all three oligos containing a single Tel unit eluted later than Pot1pN−Tel_2, but similar to Pot1pN−Tel. In addition, the ratio of A_{260}/A_{280} was significantly higher than that of the PotpN−Tel_2 complex (Table 4), indicating a higher DNA/protein ratio. These results indicate that

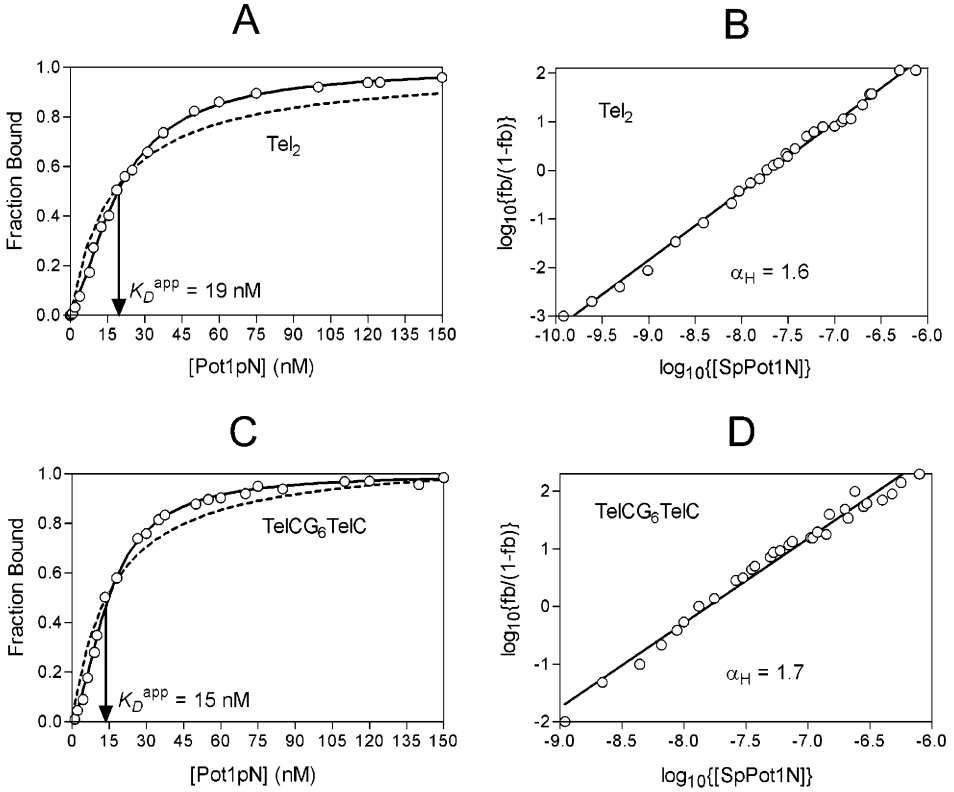

FIGURE 4: Pot1pN binds to Tel_2 and $TelCG_6TelC$ with high cooperativity. (A, C) Equilibrium binding curves for Tel_2 and $TelCG_6TelC$ binding to Pot1pN. The solid lines represent the theoretical binding curves with Hill coefficients of 1.6 and 1.7, respectively. The dotted lines represent the theoretical binding curves with a Hill coefficient of 1, which do not fit the experimental data. (B, D) Hill plots of Tel_2 and $TelCG_6TelC$ binding to Pot1pN.

binding of two Pot1pN molecules rather than the effect of seven extra nucleotides explains the elution position and A_{260}/A_{280} ratio of the Pot1pN−Tel_2 complex.

To explore the 3′ end specificity of the Pot1pN−ssDNA interaction, the dissociation constants of Pot1pN complexes with $TelA_7$, A_7Tel, and A_4TelA_3 were measured by the filter-binding assay. As shown in Table 4, extra nucleotides at the 3′ end interfere with the interaction between the protein and telomeric ssDNA, causing a 4-fold reduction in affinity (1590/420). Interestingly, the dissociation constant of Pot1pN−A_7Tel is almost 5-fold higher than that of Pot1pN−Tel (420/83). This can be ascribed to interference of the seven A's at the 5′ end.

Long Telomeric ssDNA Binds Multiple Pot1pN Molecules Cooperatively. If the interaction between Pot1pN and ssDNA were noncooperative, gel filtration chromatographic experiments performed at approximately equimolar protein and ssDNA would yield multiple peaks for Tel_n ($n = 2-4$). Yet, as shown in Table 3, we only observed one protein−ssDNA peak for each Tel_n whose apparent size was about $n \times 22$ kDa, suggesting that multiple Pot1pN molecules bind Tel_n ($n = 2-4$) cooperatively. The remaining ssDNA remained protein free. The cooperativity was confirmed by examining the formation of Pot1pN−Tel_2 at very low [ssDNA] (<0.05 nM) and increasing protein concentration using a filter-binding assay. The Pot1pN−Tel_2 complex gave an apparent dissociation constant K_D^{app} of 19 nM and a sigmoidal binding curve (Figure 4A), diagnostic of a cooperative interaction with the level of cooperativity reflected in the slope of the Hill plot (Figure 4B). Tel_2 binding showed a slope of 1.6, which indicates relatively high cooperativity. The cooperative free energy, $\Delta G_{coop}(Tel_2)$, can also be calculated (eqs 3 and 4; see Experimental Procedures) (*41*). Considering Pot1pN's 3′ end binding preference to telomeric ssDNA [$K_D(A_7Tel)/K_D(TelA_7) = 1590/420 = 3.7$] (Table 4) and K_D of Pot1pN−TelΔA being 83 nM, K_1 and K_2, the equilibrium association constants for Pot1pN binding to the 3′ and 5′ end telomere repeats, can be estimated as 0.012 nM^{-1} and 0.0033 nM^{-1}, respectively. Equation 3 accurately fits the binding data of Pot1pN−Tel_2 with a cooperative factor, $K_c = 39$, corresponding to cooperative energy of −2.2 kcal·mol^{-1} (eq 4). The cooperative binding energy therefore makes a substantial contribution to the overall binding affinity of Tel_2 to Pot1pN protein, since under these conditions the free energy for Pot1pN binding to TelΔ*A* is −9.7 kcal·mol^{-1}.

As an alternative means of assessing cooperative binding, a DNA gel shift assay was conducted in which the ssDNA concentration was fixed. In these experiments, both protein and oligonucleotide concentrations were above the dissociation constant, with reaction conditions proceeding from ssDNA excess to protein excess. These conditions are ideally suited for revealing a successive ladder of complexes if the interaction were noncooperative. Instead, for 20mer, 30mer, and 43mer, Pot1pN bound ssDNA in an "all-or-none" manner (Figure 5A). There were only two bands observed, corresponding to Pot1pN-bound and unbound states of ssDNA. This suggests that long telomeric ssDNA binds multiple Pot1pN proteins highly cooperatively.

To test whether one Pot1pN molecule can bind to more than one oligonucleotide, two different size telomeric ssDNAs (20mer and 43mer) were mixed together with Pot1pN. Complexes were resolved on a nondenaturing

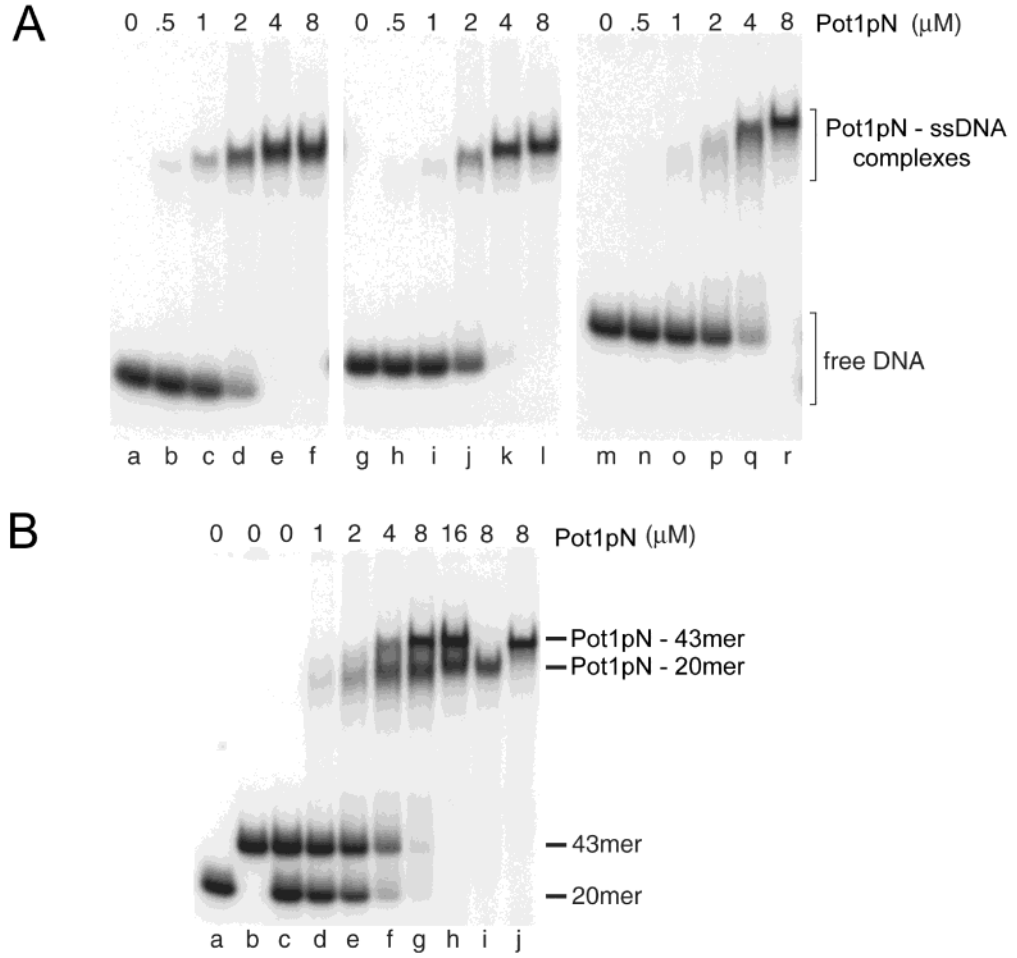

FIGURE 5: Gel mobility shift assay. (A) 1 μM 20mer (lanes a−f), 30mer (lanes g−i), and 43mer (lanes m−r) was incubated with increasing amounts of Pot1pN as indicated. Sequences were based on those of actual cloned telomeres: 20mer, 3 repeats, TelΔATel$_2$; 30mer, 4 repeats, TelCTel$_3$G; 43mer, 6 repeats, TelCTel$_3$G(TelΔA)$_2$G. (B) 1 μM 20mer plus 1 μM 43mer incubated with increasing concentrations of Pot1pN. Complexes were then analyzed by native 4−20% PAGE. The gradual decrease in mobility of the complexes with increasing protein concentration may perhaps be explained by transient binding of additional proteins to the ssDNA−protein complexes at the beginning of the electrophoresis.

polyacrylamide gel. No complexes of intermediate mobility were observed, indicating that each Pot1pN molecule only possesses one telomeric ssDNA-binding site (Figure 5B).

To test whether adjacent binding sites on the DNA are necessary for cooperative interaction among the Pot1pN molecules, another ssDNA was synthesized with two telomeric sequences separated by six G's, the longest linker between two telomeric sequences observed in *S. pombe*. TelCG$_6$TelC (5′-GGTTACACGGGGGGGGTTACAC-3′) was synthesized and analyzed for its binding to Pot1pN by the filter-binding assay. Once more, the data clearly demonstrated highly cooperative binding with an apparent dissociation constant, K_D^{app}, of 15 nM and a Hill constant, α_H, of 1.7. We thereby conclude that the protein−protein interaction among Pot1pN molecules is strong enough to "loop out" intervening nontelomeric DNA and result in highly cooperative binding of multiple Pot1pN molecules.

Similar to Pot1pN−Tel$_2$, the cooperative free energy ΔG_{coop}(TelCG$_6$TelC) can be obtained to be -2.6 kcal·mol^{-1} (eqs 3 and 4). It seems that the cooperativity interaction of Pot1pN−TelCG$_6$TelC is a little bit stronger than that of Pot1pN−Tel$_2$ [ΔG_{coop}(TelCG$_6$TelC) − ΔG_{coop}(Tel$_2$) = -0.40 kcal·mol^{-1}]. This can be independently calculated as the difference in standard free energy between Pot1pN−TelCG$_6$TelC and Pot1pN−Tel$_2$: $\Delta G° = RT\ln(K_D^{app})^2$, $\Delta G°$(TelCG$_6$TelC) = -21.36 kcal·mol^{-1}, $\Delta G°$(Tel$_2$) = -20.97 kcal·mol^{-1}, and $\Delta G°$(TelCG$_6$TelC) − $\Delta G°$(Tel$_2$) = -0.39 kcal·mol^{-1}. This indicates that the cooperative interaction is stronger when there are some nucleotides between the two telomeric repeats. These extra nucleotides may allow the protein−ssDNA complex to adopt an optimal conformation for the cooperative interaction.

Kinetic Stabilities of Pot1pN−ssDNA Complexes. The Pot1pN−TelΔA and Pot1pN−Tel$_2$ or Pot1pN−TelCG$_6$TelC complexes have completely different kinetic stabilities. In a cold chase experiment, Pot1pN was incubated with a trace amount of radioactively labeled DNA to allow complex formation. A 10^3-fold excess of unlabeled TelΔA or Tel$_2$ or TelCG$_6$TelC was then added to the binding reactions to capture any dissociated proteins, and the amount of radioactive telomeric substrates bound to protein was analyzed as a function of time. As revealed in Figure 6, the Pot1pN−TelΔA complex dissociates very quickly ($\ll$0.5 min), which defines a lower limit for the dissociation rate (k_{off} > 10 min^{-1}). Since K_D is 83 nM for TelΔA, the association rate, k_{on}, must be larger than 10^8 M^{-1} s^{-1}, which is within the range for a diffusion-controlled binding event. On the other hand, the decay rates for both the Pot1pN−Tel$_2$ and

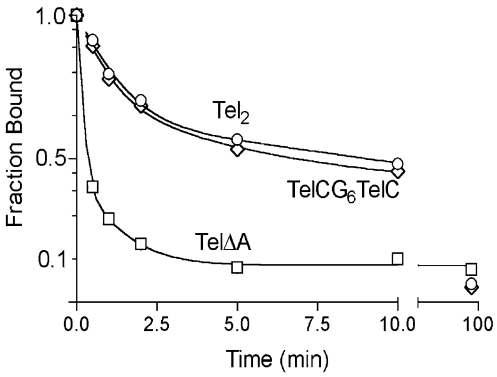

FIGURE 6: Kinetic stability of the Pot1pN−ssDNA complexes. Reactions containing 500 nM protein and 50 nM labeled ssDNA were incubated at room temperature for 30 min prior to challenge with unlabeled ssDNAs. Zero time points were taken prior to competitor addition. Plots: (○) Pot1pN−Tel$_2$; (◇) Pot1pN−TelCG$_6$TelC; (□) Pot1pN−TelΔA.

Pot1pN−TelCG$_6$TelC complexes are biphasic. The fast dissociation occurs within 3 min after the cold chase and accounts for 75% of the complexes. The slow dissociation accounts for 25% of the complexes with a half-life time of 15 min. For either case, the calculated k_{on} is too small for a diffusion-controlled binding event, consistent with a complex assembly and disassembly process.

DISCUSSION

Pot1p is an essential telomeric ssDNA-binding protein protecting the 3′ end telomeric overhang. Deletion of the *pot1*$^+$ gene in *S. pombe* results in telomere shortening and chromosome end fusions. Its biological importance provides the incentive to elucidate the interaction of this protein with telomeric ssDNA. Toward this end, we have expressed and purified the N-terminal ssDNA-binding domain of Pot1p, Pot1pN, whose sequence shows limited similarity to the N-terminal OB fold of the TEBP α subunit of *O. nova*. With this purified monomeric protein, the telomeric ssDNA-binding properties were characterized. It is evident from our biochemical experiments that Pot1pN binds to telomere ssDNA with moderately high affinity and extraordinary sequence specificity. Additionally, our data reveal highly cooperative interactions between multiple Pot1pN molecules and long telomeric ssDNA, thereby enhancing our understanding of the functional potential of this protein. Strictly speaking, our results pertain only to the N-terminal domain; if full-length Pot1 protein exists as a dimer or higher oligomer, then we would expect that the affinity, specificity, and cooperative protein−protein interactions described here would apply to the individual DNA-binding domains of the oligomer. The studies reported here do not eliminate the need for future studies with the full-length protein.

Comparison of Telomeric ssDNA Binding by Pot1pN, TEBPα, and Cdc13. In the present work, we have shown that Pot1pN possesses sequence specificity for its telomeric ssDNA, a property shared by both TEBPα of *O. nova* and Cdc13 of *S. cerevisiae*. The minimum binding site for Pot1pN is a hexanucleotide telomeric ssDNA (GGTTAC), which is bound with moderate affinity (K_D = 83 nM). This affinity is similar to that of the interaction between TEBPα of *O. nova* and its telomeric ssDNA (TTTTGGGG) (*36*). In contrast, the binding affinity of Cdc13 to its telomeric ssDNA ($K_D \sim 1$ nM) (*44*) is much higher than those of Pot1pN and TEBPα, consistent with a longer minimum Cdc13 binding site (11 nucleotides).

The Cdc13 DNA-binding domain adopts an OB fold that is structurally similar to the OB fold of TEBPα, suggesting that the OB fold may represent an evolutionarily conserved domain for the binding of telomeric ssDNA (*50*). However, there are still some major differences among these OB-fold telomeric DNA-binding proteins. TEBPα needs its first two OB folds for ssDNA binding, and the first 36 residues from the N-terminus preceding the first OB fold are dispensable for this interaction (*27*, *28*). In contrast, Pot1pN is predicted to contain only one OB fold. Furthermore, its first 22 residues are required for its correct folding, as evidenced by the fact that a construct without the N-terminal 22 residues yields insoluble protein products when expressed in *E. coli* (data not shown). Finally, the ssDNA-binding domain of Cdc13 (451−693) is located in the middle of the protein (*44*), whereas those of TEBPα and Pot1p are at the N-terminus.

Cooperative ssDNA Binding of Pot1p and Its Possible Biological Significance. This work also has shown that multiple Pot1p proteins bind to telomeric ssDNA in a highly cooperative manner. Pot1pN is monomeric in solution even at concentrations ∼0.5 mM, yet it oligomerizes readily in the presence of telomeric repeat DNA. This suggests that Pot1 proteins could efficiently bind the entirety of 3′ telomeric overhangs in vivo. Cooperativity reflects the fact that a protein binds with higher affinity to a DNA molecule to which protein is already bound than to an unliganded DNA molecule. This can result from protein−protein interactions between nearest neighbors or protein-induced conformational change in adjacent DNA. Our data with TelCG$_6$TelC, where cooperativity was maintained when two telomeric repeats were separated by a nontelomeric spacer, indicate that protein−protein interactions play an important role in the cooperative binding. Cooperative binding is a rare feature for a sequence-specific single-stranded nucleic acid-binding protein. One example occurs with the *O. nova* TEBP α and *β* subunits (*25*, *36*), although these form a heterodimer rather than the Pot1 homooligomer. Cooperative binding of sequence-nonspecific ssDNA-binding proteins is observed for SSBs that aid DNA replication (*51*−*53*) and DNA recombination proteins such as recA (*54*, *55*).

What insight might cooperative binding provide about functions of the Pot1 protein in *S. pombe*? The 3′ end binding preference as well as the potential for multiple proteins to cooperatively bind to the same strand suggests a potential function as a switch between different telomeric states. During most of the *S. pombe* cell cycle, telomerase is inactive, and the 3′ telomeric overhang must be protected from degradation and from DNA-repair activities. The 3′ G-rich strand overhang could associate with Pot1p molecules to form an unfolded Pot1p-coated state. Alternatively, the protection of the telomere ends could be achieved by the formation of a t-loop structure (*56*−*58*), in which the duplex DNA is invaded by the free 3′ end of the G-rich strand overhang. The transition from t-loop to Pot1p-coated state could proceed via an ordered pathway: a Pot1 protein first competes off part of the t-loop and binds to the 3′ end of the G-rich overhang, and then other Pot1p molecules, because of the highly cooperative binding, help to unfold the t-loop

and efficiently fill in and coat the entire single-stranded overhang. This may allow the later recruitment of telomerase, which elongates the 3′ termini of chromosomes.

NOTE ADDED IN PROOF

The description of Pot1 homologues in other organisms has now been published (*59*).

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