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Synergism of the Two Myb Domains of Tay1 Protein Results in High Affinity Binding to Telomeres*[S]

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Background: In contrast to mammalian TRF1 and TRF2, yeast telomeric protein *Yl*Tay1 possesses two Myb domains.
Results: Kinetic and thermodynamic analyses revealed binding properties of individual Myb domains of *Yl*Tay1p.
Conclusion: The combined presence of the two Myb domains synergistically increases the affinity of *Yl*Tay1p to telomeric DNA.
Significance: The study demonstrates evolutionary tinkering with telomere-associated proteins.

Double-stranded regions of the telomeres are recognized by proteins containing Myb-like domains conferring specificity toward telomeric repeats. Although biochemical and structural studies revealed basic molecular principles involved in DNA binding, relatively little is known about evolutionary pathways leading to various types of Myb domain-containing proteins in divergent species of eukaryotes. Recently we identified a novel type of telomere-binding protein *Yl*Tay1p from the yeast *Yarrowia lipolytica* containing two Myb domains (Myb1, Myb2) very similar to the Myb domain of mammalian TRF1 and TRF2. In this study we prepared mutant versions of *Yl*Tay1p lacking Myb1, Myb2, or both Myb domains and found that *Yl*Tay1p carrying either Myb domain exhibits preferential affinity to both *Y. lipolytica* (GGGTTAGTCA)$_n$ and human (TTAGGG)$_n$ telomeric sequences. Quantitative measurements of the protein binding to telomeric DNA revealed that the presence of both Myb domains is required for a high affinity of *Yl*Tay1p to either telomeric repeat. Additionally, we performed detailed thermodynamic analysis of the *Yl*Tay1p interaction with its cognate telomeric DNA, which is to our knowledge the first energetic description of a full-length telomeric-protein binding to DNA. Interestingly, when compared with human TRF1 and TRF2 proteins, *Yl*Tay1p exhibited higher affinity not only for *Y. lipolytica* telomeres but also for human telomeric sequences. The duplication of the Myb domain region in *Yl*Tay1p thus produces a synergistic effect on its affinity toward the cognate telomeric sequence, alleviating the need for homodimerization observed in TRF-like proteins possessing a single Myb domain.

The ends of linear chromosomes are capped by nucleoprotein complexes (telomeres) providing a solution to the end-replication problem and shielding the chromosomal termini from recognition by exonucleases and DNA repair machinery (1, 2). The major players in mediating telomeric functions are proteins directly associated with chromosomal ends. These include (i) telomerase, a unique reverse transcriptase carrying its own RNA template representing the major molecular tool for maintaining telomere length (3), (ii) single-stranded (ss) DNA-binding proteins (*e.g.* human and fission yeast Pot1 or budding yeast Cdc13) tightly attached to 3′ single-stranded telomeric overhang through 1–3 OB-folds (4), and (iii) Myb/homeodomain-containing proteins (*e.g.* mammalian TRF1, TRF2, fission yeast Taz1, and budding yeast Rap1) binding to the double-stranded (ds) repetitive part of telomeres (1, 5). Other proteins (*e.g.* Rap1, Tin2, and Tpp1 in Metazoa, Rap1, Poz1, Tpz1, and Ccq1 in fission yeasts) associate with telomeres indirectly via protein-protein interactions, and together with DNA-binding proteins they form a protective complex called shelterin (6). Finally, some proteins (*e.g.* tankyrase, various nucleases, and DNA repair proteins) form transient contacts with telomeres, whose frequency and duration depend on the state of a particular telomere (1).

The sequences of telomere-associated proteins undergo relatively fast evolutionary diversification (7), thus hampering the identification of their counterparts in distantly related organisms needed to uncover general principles of telomere maintenance. These efforts are made easier by the fact that despite their dissimilarities at the level of amino acid sequences, telomere-associated proteins share common structural elements (8). All known ds telomeric DNA-binding proteins contain at least one conserved Myb/homeodomain, although the rest of the proteins have very different sequence and domain topology (1, 8). Thus, Myb domains of known telomeric proteins can be used effectively as queries for searches within whole genomic sequences of organisms distantly related to established models for telomere biology (9–11).

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Yeasts, especially *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, and *Candida albicans* have proved to be invaluable models for the studies of telomeres (12). The ascomycetous fungi are a highly heterogeneous group of microorganisms comprising more than 1000 known species resulting from diverse evolutionary trajectories (13), making them ideal for comparative analysis of telomeres. With this concept we initiated studies of nuclear telomeres in nonconventional yeast species including *Yarrowia lipolytica*. We characterized *Y. lipolytica* mutants lacking telomerase and found that they rapidly lose telomeric repeats and survive due to structural changes at the chromosomal ends (14). Characterization of proteins associated with the telomeres of this yeast species led to identification of a protein Tay1 (telomere associated in *Yarrowia lipolytica* 1), which (with the exception of its two Myb domains) does not resemble any known dsDNA-binding telomeric protein (15). *Yl*Tay1 protein and its homologs found in *S. pombe* (Mug152) and filamentous fungi thus represent a novel group of proteins protecting the ds portion of telomeres. It was shown that *Yl*Tay1p exhibits a preference for telomeric DNA *in vitro* and seems to bind to the substrate DNA as a dimer (15). Interestingly, the sequences of both Myb domains (Myb1, Myb2) are more similar to the Myb domains of mammalian TRF1/TRF2 proteins than to Myb domains of yeast telomeric proteins Rap1 (*S. cerevisiae*, *C. albicans*, *K. lactis*) or Taz1 (*S. pombe*). However, the contribution of individual Myb domains to a high affinity binding to telomeric repeats was not examined, and the binding properties of *Yl*Tay1 were not compared with those of its mammalian counterparts.

Here, using various biochemical and biophysical methods, we investigated the contribution of each Myb domain to the specificity of *Yl*Tay1 toward telomeric DNA. We found that although the individual Myb domains exhibit preferential binding to telomeric sequences, their combined presence dramatically increases the affinity of the protein for telomeric repeats. Interestingly, *Yl*Tay1 protein exhibits a substantially higher affinity for human telomeric repeats than either TRF1 or TRF2, raising both evolutionary and technical implications.

EXPERIMENTAL PROCEDURES

Microbial Strains—*S. cerevisiae* W303–1A (*MATa*, *ade2-1*, *his3-11*, *his3-15*, *leu2-3*, *leu2-112*, *trp1-1*, *ura3-1*, *can1-100*). Yeast cultures were grown in YPD medium (1% (w/v) yeast extract (Difco), 2% (w/v) Bacto-peptone (Difco), 2% (w/v) glucose) at 28 °C. *Escherichia coli* strain DH5α (Invitrogen; F^- Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK^-, mK^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1) was used for cloning of recombinant DNA, and strain BL21- (DE3) (Invitrogen; F^- *omp*T *hsd*SB(rB^-, mB^-), *gal*, *dcm* (DE3)) was used for production of recombinant *Yl*Tay1–6HN proteins.

Construction of Yeast and Bacterial Expression Vectors Carrying Versions of the YlTAY1 Gene—Recombinant DNA techniques were carried out by standard procedures (16). The oligonucleotides (supplemental Table 1) were synthesized by MWG Operon, Metabion, or Sigma. For expression in *S. cerevisiae*, *YlTAY1* was amplified with primers YlTay1_pYES_UP and YlTay1_pYES_DOWN using pTay1–6HN plasmid DNA (15) as a template. The primers carried HindIII and XhoI restriction sites in regions flanking the start and the stop codons of the *YlTAY1* open reading frame, respectively. To facilitate efficient translation initiation, the primer YlTay1_pYES_UP contained the Kozak sequence (5′-AAAAAA-3′) immediately upstream of the initiation ATG codon. The resulting PCR product was gel-purified using the Zymoclean Gel Recovery kit (Zymo Research), digested with HindIII and XhoI, ligated into the pYES2/CT vector linearized with the same restriction enzymes, and transformed into *E. coli* DH5α grown on LB solid media with 100 μg/ml ampicillin. The resulting plasmid pYES-Tay1 carries *YlTAY1* under the control of the *GAL1* promoter.

The mutant versions of pYES-Tay1 lacking Myb1 (Δ1) and Myb2 (Δ2) were prepared by inverse PCR with primers ΔMyb1_UP and ΔMyb1_DOWN (for Δ1) or ΔMyb2_UP and ΔMyb2_DOWN (for Δ2) using pYES-Tay1 DNA as a template. The resulting PCR fragments were gel-purified, ligated using T4 DNA ligase, and transformed into *E. coli* DH5α grown on LB solid media with 100 μg/ml ampicillin. The mutant lacking both Myb domains (ΔΔ) was amplified by inverse PCR using ΔMyb2_UP and ΔMyb2_DOWN using pYES-Tay1-Δ1 as a template and cloned analogously as the single mutants. The mutant versions of pYES-Tay1 carrying substitutions at selected sites within either Myb1 or Myb2 domains were prepared by inversion PCR with the corresponding couple of primers (their names indicate the mutated amino acids; supplemental Table 1) using pYES-Tay1 DNA as a template. The bacterial expression vectors carrying mutant versions of *Yl*Tay1 lacking Myb domains (pTay1–6HN-Δ1, -Δ2, and -ΔΔ) were prepared analogously as described for pYES-Tay1 plasmids with the exception of using pTay1–6HN DNA as a template.

All PCR reactions were performed using 1 unit of Phusion Hot Start High-Fidelity DNA polymerase (Finnzymes) and contained each dNTP at 200 μM, corresponding primers at 1 μM and 10 ng of template DNA. The conditions of PCR reactions were adjusted according to the sequences of the primers and lengths of the final PCR product.

Yeast Growth Conditions and Transformation—Plasmids carrying WT (pYES-Tay1) or mutant versions of *YlTAY1* gene (pYES-Tay1-Δ1, -Δ2, and -ΔΔ) were transformed into *S. cerevisiae* strain W303–1A using the lithium acetate method (17), and transformants were selected for uracil prototrophy on solid SD media (0.17% (w/v) yeast nitrogen base, 0.5% (w/v) $(NH_4)_2SO_4$, 2% (w/v) glucose) containing corresponding amino acids and bases with the exception of uracil. Cells from single colonies were grown on SD media, then inoculated into liquid SGal (the same composition as SD except that instead of glucose, 2% (w/v) galactose was used as a sole carbon source) media and cultivated for 6 h at 28 °C. Three microliters were then spotted from 10-fold serial dilutions on either SD (repressible conditions) or SGal (inducible conditions) solid media, and the growth was inspected after a 3-day cultivation at 28 °C.

Purification of Recombinant YlTay1 Proteins—The expression and purification of various versions of *Yl*Tay1 protein was performed essentially as described in Kramara *et al.* (15) with several modifications. Bacterial expression plasmids carrying wild-type (pTay1–6HN-WT) as well as mutant versions (pTay1–6HN-Δ1, -Δ2, and -ΔΔ) of *YlTAY1* were transformed into One Shot BL21(DE3) cells, and the transformants were

grown on LB plates containing 100 μg/ml ampicillin. The cells were then inoculated into 30 ml of 2×YT media (1.6% (w/v) Bacto-Tryptone, 1% (w/v) Bacto-peptone, 1% (w/v) NaCl (pH 7.1)) containing 2% (w/v) glucose, 100 μg/ml ampicillin and cultivated overnight (15 h) at 37 °C and 225 rpm. The cells were centrifuged for 5 min at 3000 rpm (Sorvall RT 7 Plus) at 25 °C, washed once with 2×YT, inoculated into 1 liter of 2×YT containing 100 μg/ml ampicillin, and cultivated at 37 °C and 275 rpm until the A_{600} reached a value of 0.7–0.8. The culture was cooled to 28 °C followed by the addition of isopropyl β-D-1-thiogalactopyranoside (final concentration 1 mM) and cultivation for additional 3 h at 28 °C. The culture was then centrifuged for 15 min at 5000 rpm at 4 °C (F10–6 × 500y rotor in Sorvall RC 6+), the cells were washed once with 200 ml of ice-cold phosphate-buffered saline, and the pellet was frozen at −20 °C. The pellet was thawed on ice (30–45 min) and resuspended in a final volume of 30 ml of buffer A (20 mM HEPES-NaOH (pH 7.3), 300 mM NaCl, 1 mM DTT) containing 1× Complete (EDTA-free) protease inhibitors (Roche Applied Science), 10 mM $MgCl_2$, 50 units of DNase I (Invitrogen), and 2 μg of PureLink RNase A (Invitrogen). Lysozyme (Sigma) was added to a final concentration of 1 mg/ml, and the suspension was incubated for 15 min on ice with occasional shaking. The cells were broken by sonication (5 × 20 s at a setting of 7 (Branson Sonifer 450)). Each cycle of sonication was followed by 40 s of incubation on ice. Triton X-100 was added to a final concentration of 0.1% (v/v), and the suspension was incubated for an additional 15 min on ice. The insoluble material was pelleted by 30 min of centrifugation at 15,000 rpm at 4 °C (F21–8 × 50y in Sorvall RC 6+). The supernatant was mixed with a 0.5-ml bed volume of the His-Select(R) Cobalt Affinity Gel (Sigma) equilibrated with 3 × 10 volumes of buffer A. The whole suspension was transferred to a 50-ml Falcon tube and incubated for 60–90 min rocking at 7 °C. The beads were then washed 3 times with 20 volumes of buffer A containing 0.1% (v/v) Triton X-100 followed by 5 × 10 volumes of the wash buffer I (20 mM HEPES-NaOH (pH 7.3), 300 mM NaCl, 10 mM imidazole (pH 7.7)), and 5 × 10 volumes wash buffer II (50 mM sodium phosphate buffer (pH 7.0), 50 mM NaCl, 10 mM imidazole (pH 7.7)). The beads were then transferred to a chromatographic column, and the bound proteins were eluted with 6 × 1 ml of elution buffer (50 mM sodium phosphate buffer (pH 7.0), 50 mM NaCl, 500 mM imidazole (pH 7.7)). The fractions containing *Yl*Tay1 protein (or its mutant versions) were loaded onto 5-ml PD MidiTrap G-25 columns (GE Healthcare) prewashed three times with elution buffer without imidazole. The presence and purity of proteins were verified by 10% SDS-PAGE stained with Coomassie Brilliant Blue R-250. Concentrations of proteins were determined by the Bradford assay (Bio-Rad), and proteins were stored in 100-μl aliquots at −80 °C.

Electrophoretic Mobility Shift Assay (EMSA)—The double-stranded YlTEL50 probe (carrying 5 *Y. lipolytica* telomeric repeats)) used for EMSA was prepared by digestion of 15 μg of pMH25 plasmid (15) in a final volume of 50 μl using 50 units of EcoRI (New England Biolabs) followed by dephosphorylation using 50 units of calf intestinal phosphatase. The resulting 50-bp fragment was gel-purified using the ZymoClean gel extraction kit (ZymoResearch). The gel-isolated YlTEL50 DNA was labeled with 10 units of T4 polynucleotide kinase (Fermentas) and 50 μCi of [γ-^{32}P]ATP (final concentration 0.5 μM) for 60 min at 37 °C in a final volume of 20 μl. Reactions were diluted to 50 μl with water, and the labeled oligonucleotide was purified using G-50 Sephadex (GE Healthcare). The binding reaction and gel electrophoresis were performed as described (15).

Electron Microscopy—The typical DNA binding reaction for electron microscopy was performed in 10 μl of 1× HN buffer containing 5 ng/μl substrate DNA and 7–10 ng/μl purified *Yl*Tay1p. The reactions were carried out at room temperature for 15 min followed by the addition of 10 μl of 1.2% glutaraldehyde and incubation at room temperature for additional 6 min. To remove the unbound proteins and fixative, the samples were diluted to 50 μl in HN buffer and passed over 2-ml columns of 6% agarose beads (ABT Inc., Burgos, Spain) equilibrated with TE buffer (10 mM Tris-HCl, (pH 7.4), 0.1 mM EDTA-NaOH). Aliquots of the fractions containing the complexes were mixed with a buffer containing spermidine and adsorbed onto copper grids coated with a thin carbon film glow-charged shortly before sample application. After adsorption of the samples for 2–3 min, the grids were dehydrated through a graded ethanol series and rotary shadowcast with tungsten at 10^{-7} torr (18). Samples were examined in an FEI T12 TEM equipped with a Gatan 2kx2k SC200 CCD camera. Dimensions of particles in the images saved from the CCD cameras were analyzed using Digital Micrograph software (Gatan, Inc.). Adobe Photoshop software was used to arrange images into panels for publication.

Isothermal Titration Calorimetry (ITC)[2]—ITC measurements were performed on a VP-ITC instrument (Microcal; GE Healthcare) at 25 °C. Solutions of protein and DNA were prepared with the same batch of buffer (50 mM sodium phosphate, 50 mM NaCl (pH 7.0)) to minimize artifacts due to minor differences in buffer composition. The duplex portion of the telomeric DNA was prepared by hybridizing the complementary strands at a 1:1 molar ratio. The proper formation of the duplex was checked by ion-exchange chromatography using Mono Q HR 5/5 column (GE Healthcare) with a NaCl gradient from 0.1 to 2.0 M. The protein solution (11 μM) was degassed and placed in the cell (1.423 ml). The DNA solution (50 μM) in a syringe was gradually added to the protein solution in 25 injections of 10 μl at intervals of 5 min while stirring at 242 rpm. The experimental data were fitted in Origin 7.0 software supplied with the instrument using a one-site binding model. From the fit, the binding enthalpy change (ΔH), association constant (K_a) and binding stoichiometry (n) were obtained. Binding free energy (ΔG) and entropy (ΔS) contributions were determined from the standard equation $\Delta G = -\mathrm{RT}\ln K_a = \Delta H \times T\Delta S$. The uncertainties in the parameters obtained are given by standard deviation of three independent measurements and the minor inaccuracy in determination of protein and DNA concentrations (relative error contribution 10%).

Fluorescence Anisotropy—The equilibrium binding of *Yl*Tay1 protein variants to DNA oligonucleotide duplexes containing 1.5 telomeric repeat was analyzed by fluorescence anisotropy. The DNA oligonucleotide labeled with the Alexa Fluor 488 (Invit-

[2] The abbreviations used are: ITC, isothermal titration calorimetry; FA, fluorescent anisotropy.

rogen) was allowed to hybridize with the complementary oligonucleotide at an equimolar ratio. The complete formation of the duplex was verified by PAGE. The measurements of fluorescence anisotropy were conducted on a FluoroMax-4 spectrofluorometer (Horiba Jobin-Yvon, Edison, NJ) equipped with a thermostable cell holder and magnetic stirrer. Samples were excited with vertically polarized light at 490 nm, and both vertical and horizontal emissions were recorded at 520 nm. The integration time was 3 s. All measurements were conducted at 25 °C in 50 mM sodium phosphate buffer (pH 7.0) containing 50 mM NaCl. A fixed delay of 120 s was set between each protein aliquot addition and start of the measurement to allow the binding reaction to reach equilibrium. This delay was sufficient, as no further change in anisotropy was observed. Each data point is an average of three measurements. The experimental binding isotherms were analyzed by nonlinear least squares regression in SigmaPlot 11 software (Systat Software) using a single-site binding model according to Heyduk and Lee (19) and confirmed by numerical approach using DynaFit software (20).

RESULTS

Heterologous Expression in S. cerevisiae Is a Convenient System for Assessing the Role of Myb Domains on the Activity of YlTay1p—In silico analysis of the amino acid sequence of *Yl*Tay1 protein revealed that it contains two putative Myb domains (Ref. 15, Fig. 1*A*). To investigate their contribution to the biological effects of the protein, we took advantage of a serendipitous finding that the expression of the *YlTAY1* gene from a strong (*ADH1*) promoter inhibits growth of *S. cerevisiae*.[3] To test the possibility that the inhibitory effect of *YlTAY1* expression is due to its DNA binding activity, we prepared an expression vector with the *YlTAY1* gene and its mutant versions under the inducible *GAL1* promoter. As expected, whereas the growth of the transformants carrying the wild-type *YlTAY1* (WT) on glucose was similar to the control strain, their growth was dramatically reduced on media containing galactose as a sole carbon source (Fig. 1*B*). Next, we tested the mutant version of *Yl*Tay1p lacking both putative Myb domains (ΔΔ) and found that their growth on galactose was restored, indicating that the DNA binding activity of *Yl*Tay1p is responsible for the growth inhibition (Fig. 1*B*). Importantly, expression of the mutant versions of *Yl*Tay1p lacking either Myb1 (Δ1) or Myb2 domain (Δ2) did not interfere with the growth of *S. cerevisiae*, demonstrating that both Myb domains are essential for inhibition of growth mediated by the expression of *Yl*Tay1p on *S. cerevisiae*.

Although the molecular mechanism responsible for the inhibitory effect of *Yl*Tay1p on *S. cerevisiae* is not clear (see "Discussion"), the fact that it is dependent on the two Myb domains enables studies assessing the importance of specific amino acid residues for DNA binding of *Yl*Tay1p. With this aim we constructed a series of mutant versions of *YlTAY1* carrying mutations in the conserved positions of the Myb domains. The mutations were designed based on the phenotypes of the versions of hTRF2 mutated in different positions of its Myb domain (21, 22) (Table 1). The mutated genes were then expressed under the *GAL1* promoter to assess their effect on growth of *S. cerevisiae*. We found that although two mutant versions of *Yl*Tay1p (E159K and E246K) inhibited growth of *S. cerevisiae* cells on galactose, four versions (I178A, R189E, I264A, and R283E) lost this ability (Fig. 1*C*, Table 1). The same phenotype observed for deletion mutants and mutants carrying point mutations indicates that the deletion mutants (Δ1, Δ2, ΔΔ) can be used for a detailed biochemical analysis of the DNA binding properties exhibited by individual Myb domains. Furthermore, these results demonstrate that this heterologous experimental system has a potential for fine mapping of the

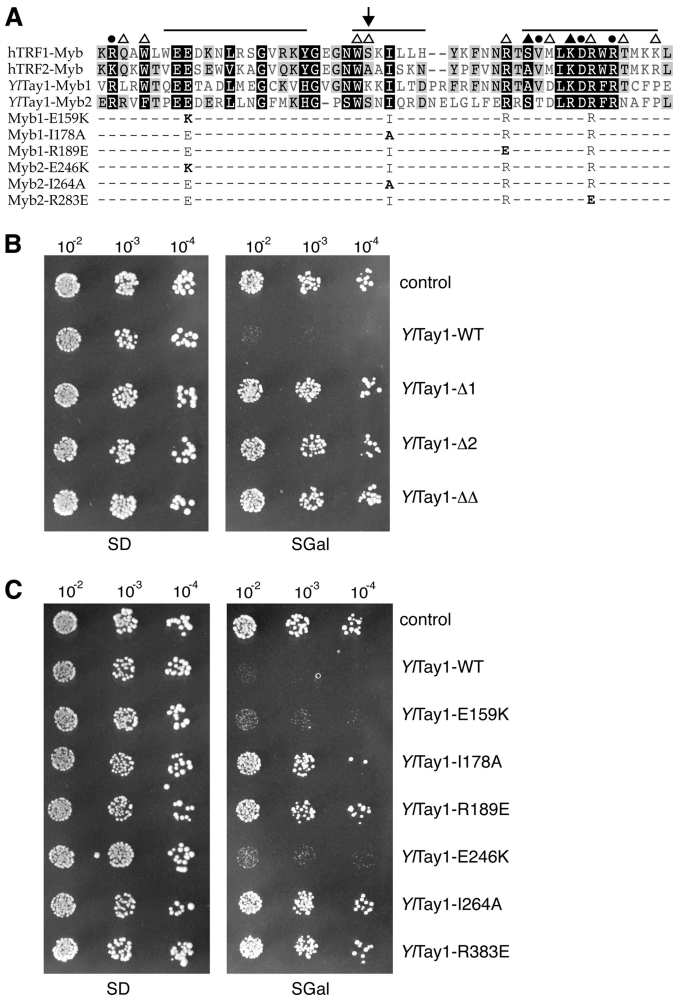

FIGURE 1. **YlTay1p contains two TRF-like Myb domains, both necessary to confer growth inhibition in *S. cerevisiae*.** *A*, shown is conserved features of Myb domains of human TRF1 and TRF2 proteins and two Myb domains of *Yl*Tay1p. Amino acid alignment was produced by ClustalW. Indicated are the positions mutated in the corresponding mutant variants of *Yl*Tay1p, chosen according to the results of Hanaoka *et al.* (21) and Konishi and de Lange (22). *Closed circles*, amino acids that interact with DNA bases; *open triangles*, amino acids that interact with the sugar-phosphate; *closed triangles*, amino acids that interact with both DNA bases and sugar-phosphate; *solid lines*, helical regions (positions of the residues in human TRF1 are adopted from Hanaoka *et al.* (21)). *Arrow*, see "Discussion." *B*, the inhibitory effect of galactose-induced expression of *YlTAY1* gene from *GAL1* promoter on growth of *S. cerevisiae* can be alleviated by deletion of Myb1, Myb2, or both Myb domains. The *scheme on left* illustrates the nomenclature of the deletion mutants lacking Myb1 and/or Myb2 used in this study. *C*, the inhibitory effect of expression of *YlTAY1* gene on growth of *S. cerevisiae* is alleviated by point mutations in some of the conserved amino acids.

[3] A. Mihalikova, S. Kinsky, and L. Tomaska, unpublished results.

TABLE 1

The positions mutated in *Yl*Tay1p and corresponding mutations in hTRF1/hTRF2

The positions were selected based on the studies of Hanaoka *et al.* (21) and Konishi and de Lange (22).

Mutation in *Yl*Tay1p	Corresponding mutation in hTRF2/mTRF2	Inhibition of growth in *S. cerevisiae*
WT		Yes
Δ1		No
Δ2		No
ΔΔ		No
Myb1_E159K	E454K/E449K	Yes
Myb1_I178A	I473A/I468A	No
Myb1_R189E	R482E/R477E	No
Myb2_E246K	E454K/E449K	Yes
Myb2_I264A	I473A/I468A	No
Myb2_R283E	R490E/R485E	No

*Yl*Tay1p protein (for example by mutagenic PCR) because it provides a convenient read-out for mutations disabling DNA binding.

Each Myb Domain of YlTay1p Mediates Preferential Binding to Telomeric Repeats—The experiments in *S. cerevisiae* indicated that both Myb domains of *Yl*Tay1p are important for mediating the effect of the protein, but they did not reveal whether loss of a single Myb domain results in the inability of *Yl*Tay1p to bind DNA, or alternatively, if it is due to changes in its specificity toward telomeric sequences. To address this issue, we prepared a set of bacterial expression vectors carrying the three-deletion versions of *Yl*Tay1p (Δ1, Δ2, ΔΔ). The WT as well as mutant proteins were produced in *E. coli*, purified by affinity chromatography (Fig. 2*A*), and assayed for their DNA binding activities *in vitro* using EMSAs. These experiments demonstrated that, similar to WT protein, both single mutants (Δ1, Δ2) exhibit DNA binding (Fig. 2*B*), indicating that a single Myb domain is fully competent for DNA binding. The double mutant (ΔΔ) did not exhibit any observable binding, demonstrating that at least one Myb domain is necessary for DNA binding *in vitro* (Fig. 2*B*).

To assess the ability of various versions of *Yl*Tay1p to bind to telomeric repeats, the purified recombinant proteins were incubated with the plasmid pYLTEL81 digested with *Bfu*AI, thus producing linear DNA molecules carrying 81 *Y. lipolytica* telomeric repeats at one end (15). Visualization of the DNA-protein complexes by electron microscopy clearly showed that in contrast to the double-mutant lacking both Myb domains (ΔΔ), all three versions of the *Yl*Tay1 protein carrying at least one Myb domain (WT, Δ1, Δ2) bound almost exclusively to the telomeric tract, with only a low binding activity observed toward the internal, non-telomeric region of the plasmid (Fig. 2*C*). Thus, single Myb domains (Myb1, Myb2) of *Yl*Tay1p not only have the ability to bind DNA but also exhibit preferential binding to telomeric sequences.

Measurements of Thermodynamic Parameters of YlTay1p by Isothermal Titration Calorimetry Reveal Its High Affinity Binding to Telomeric Repeats in Dimeric Form—To gain insight into the affinity and thermodynamics of wild-type *Yl*Tay1p binding to the DNA substrate, we performed measurements of *Yl*Tay1p binding to the 1.5 of the *Y. lipolytica* telomeric repeat (YlTR1.5; 5′-TTAGTCAGGGTTAGT-3′) by ITC. We used 1.5 telomeric repeats because EMSA analysis indicated that it represents the minimal binding site for *Yl*Tay1p (15). A typical ITC profile

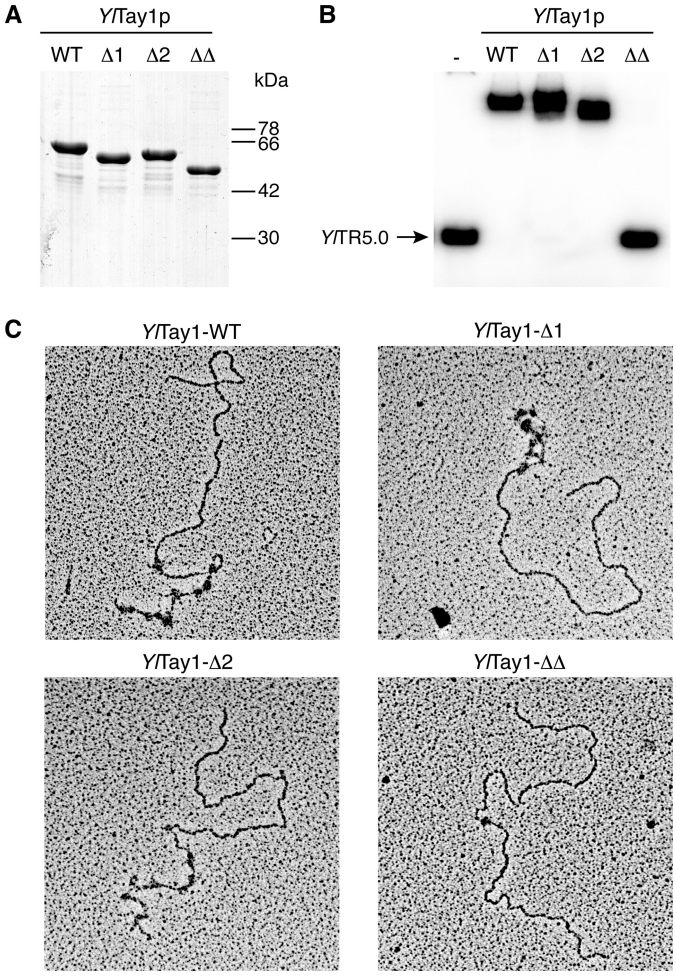

FIGURE 2. **Both Myb domains of *Yl*Tay1p are able to bind DNA and exhibit preference for telomeric repeats.** *A*, shown is SDS-PAGE analysis of purified 6xHN-*Yl*Tay1 protein and its mutated versions lacking Myb1 (Δ1), Myb2 (Δ2), and both (ΔΔ) Myb domains. *B*, electrophoretic-mobility shift assay demonstrates that both Myb domains of *Yl*Tay1p are able to bind telomeric DNA. *YlTR5.0*, double-stranded DNA probe carrying 5 *Y. lipolytica* telomeric repeats. *C*, shown is electron microscopic analysis of the binding of WT and mutant versions of *Yl*Tay1p to a model telomere containing 81 *Y. lipolytica* telomeric repeats. The total length of the linearized plasmid is 3500 bp; the length of the telomeric tract is 810 bp. The bound protein is represented by *dark particles at the terminal parts of DNA molecules*.

obtained at 25 °C is shown in Fig. 3, *top panel*. Exothermic heat pulses were observed after injections of DNA into the protein solution. Each area of the exothermic peak was integrated and corresponded to actual concentrations of the reacting molecules. The values of molar heat of binding for each injection were plotted as a function of DNA:protein molar ratio (Fig. 3, *bottom panel*). The resulting thermogram showed a best-fit according to a model for a single binding site by using a non-linear least squares method. The thermodynamic parameters, indicated in Fig. 3, *bottom panel*, were determined from the fit. The experimental errors of the thermodynamic values shown comprise a standard deviation of the fit for three independent experiments and the accuracy in determination of reactant concentrations.

The ITC experiments revealed that *Yl*Tay1p binds to the double-stranded telomeric DNA with relatively high affinity (K_D^{ITC} = 80 nM) (Fig. 3). Furthermore, the position of the

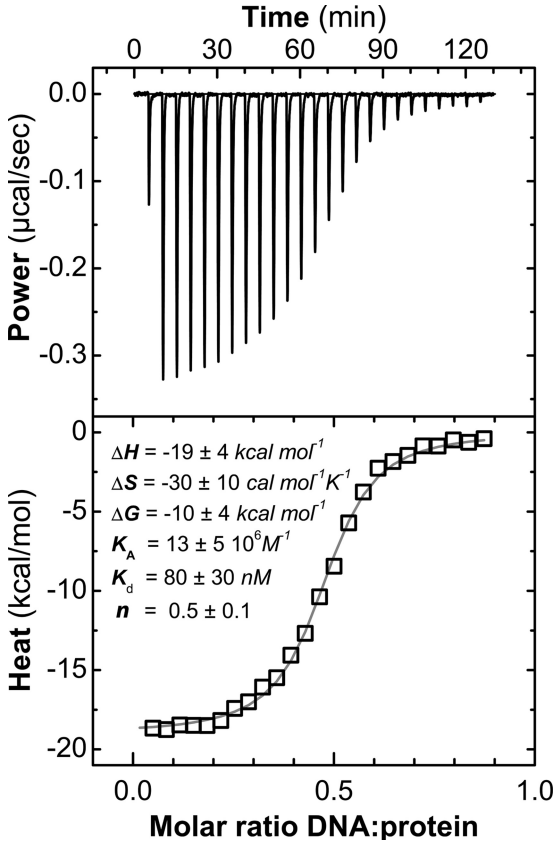

FIGURE 3. **Isothermal calorimetric titration of 15-bp telomeric DNA duplex into full-length *Yl*Tay1 protein.** The measurements were conducted at 25 °C in 50 mM sodium phosphate buffer (pH 7.0) containing 50 mM NaCl. The *top panel* shows the primary titration data; the *lower panel* shows the binding isotherm constructed from the primary data. The model curve (*gray*) represents the result of a nonlinear least squares fitting of the binding isotherm.

inflection point of the thermogram indicates that one molecule of the DNA duplex YlTR1.5 is bound by two molecules of *Yl*Tay1p (Fig. 3). The thermodynamic data show that the relatively high negative binding enthalpy ($\Delta H = -19$ kcal/mol) is partly compensated by entropy contribution ($\Delta S = -30$ cal/mol/K^{-1}; $-T\Delta S = 9$ kcal/mol). The negative binding enthalpy observed by isothermal titration calorimetry demonstrates the formation of hydrogen bonds and van der Waals interactions during protein-DNA complex formation. Additionally, the calculated negative entropy change corresponds to the constraint of the intramolecular vibrational flexibility and the reduction in the translational and rotational degrees of freedom after formation of the nucleoprotein complex (23). The enthalpy and entropy contributions result in a well pronounced free energy of binding ($\Delta G = \Delta H - T\Delta S = -10$ kcal/mol), which corresponds to the observed high binding affinity. From the thermodynamic parameters obtained, it is obvious that the DNA binding of *Yl*Tay1 protein is enthalpy-driven. Overall, the thermodynamic analysis of *Yl*Tay1p binding to *Y. lipolytica* telomeric DNA disclosed the high affinity and dimeric protein binding stoichiometry.

The High Affinity Binding of YlTay1p to Telomeric Repeats Is a Result of Synergic Effect of the Two Myb Domains—If both Myb domains of *Yl*Tay1p are able to bind preferentially to telomeric repeats, what is their contribution to the overall binding characteristics of the protein? To address this question, we employed fluorescent anisotropy (FA) and compared the binding affinity of WT along with the mutant versions of *Yl*Tay1p lacking Myb domains. The binding affinity of *Yl*Tay1p variants to double-stranded DNA was monitored by FA measurement. If the solution contains only free fluorescently labeled DNA molecules, FA is relatively low, due to the fast rotational rearrangement of DNA molecules. If the protein aliquots are added to the solution of labeled DNA, a bulky slower rotating protein-DNA complex is formed, and the anisotropy value increases. We used YlTR1.5 (labeled with Alexa Fluor 488) as a DNA substrate for *Yl*Tay1p binding assays (same substrate as the one used for ITC experiments). The anisotropy change described the extent of *Yl*Tay1p variants binding to telomeric DNA duplex. The equilibrium binding affinity was quantified by analysis of the recorded binding isotherms.

Importantly, both the association (K_a) and dissociation (K_D) constants obtained from the measurements of binding of WT *Yl*Tay1p to the DNA were in excellent agreement with these parameters calculated from the ITC data (Fig. 4, Table 2). The measurements of the binding of the mutant versions of *Yl*Tay1p to the telomeric oligonucleotide revealed that both single mutants (Δ1, Δ2) exhibit almost 4-fold lower affinity to the substrate than the WT protein (Fig. 4*A*; Table 2). The mutant version of *Yl*Tay1p lacking both Myb domains (ΔΔ), in agreement with the experiments described above, exhibited negligible binding to the DNA (Fig. 4*A*; Table 2). Thus, the equilibrium binding data support the cooperative contribution of both Myb domains to the overall high DNA binding affinity of *Yl*Tay1p.

YlTay1 Protein Exhibits Higher Binding Affinity to Both Y. lipolytica and Human DNA Telomeric Repeats Than Human TRF1 and TRF2—As the amino acid sequences of both Myb domains of *Yl*Tay1p are highly similar to the Myb domains of hTRF1 and hTRF2 (15), it was of interest to compare the binding affinity of all three proteins (*Yl*Tay1p, hTRF1, and hTRF2) to either *Y. lipolytica* (YlTR1.5) or human (HsTR2.0) telomeric DNA. The binding of *Yl*Tay1p variants to double-stranded telomeric oligonucleotides was monitored by fluorescence anisotropy. As one would assume, *Yl*Tay1p exhibited much higher (13–23-fold) affinity for the *Y. lipolytica* telomeric DNA (YlTR1.5) than either hTRF1 or hTRF2 (Fig. 4*B*, Table 2).

Surprisingly, the same result was observed when a human telomeric oligonucleotide was used for the binding reaction. Whereas the affinity of hTRF1 ($K_D = 114$ nM) and hTRF2 ($K_D = 135$ nM) for HsTR2.0 was similar to the affinity of *Yl*Tay1p for YlTR1.5 ($K_D = 81$ nM), WT *Yl*Tay1p bound human telomeric oligonucleotide with an ~8-fold higher affinity than either of the human telomeric proteins (Fig. 4*B*, Table 2). Further ITC analysis of WT *Yl*Tay1p binding to human telomeric DNA oligonucleotide HsTR2.0 has shown binding stoichiometry 2:1 (protein:DNA); *i.e.* two molecules of *Yl*Tay1p bind one dsDNA oligonucleotide. Moreover, the ITC measurement confirmed the higher binding affinity ($K_D^{ITC} = 18$ nM) of WT *Yl*Tay1p to human telomeric dsDNA HsTR2.0 (data not shown). Even one of the single mutants lacking the Myb1 domain (Δ1) exhibited more than a 2-fold higher affinity for HsTR2.0 than hTRF1 or hTRF2; similarly, the affinity of Δ2 was 2-fold higher than in the

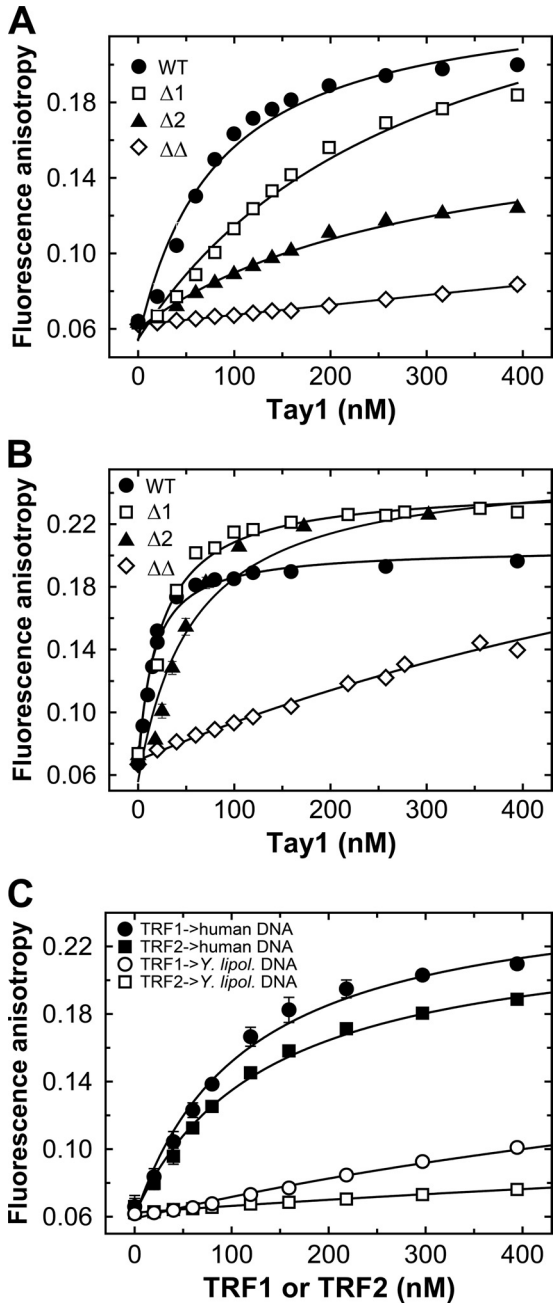

FIGURE 4. **Binding of *Yl*Tay1 protein to telomeric dsDNA.** *A*, shown is equilibrium binding of full-length *Yl*Tay1 (*WT*, *close circle*) protein and its deletion variants lacking Myb1 (Δ1, *open square*), Myb2 (Δ2, *close triangle*), and both Myb domains (ΔΔ, *open diamond*) to fluorescently labeled DNA duplex YlTR1.5 monitored by fluorescence anisotropy. The 15-bp telomeric dsDNA (10 nM) was titrated with protein solution at 25 °C in 50 mM sodium phosphate buffer (pH 7.0) containing 50 mM NaCl. The sequence of the DNA was the same as used in ITC studies. *B*, shown is equilibrium binding of full-length *Yl*Tay1 protein and its deletion variants to human telomeric dsDNA hTR2 monitored by fluorescence anisotropy. The conditions were the same as in *A*. *C*, shown is equilibrium binding of full-length TRF1 and TRF2 to human telomeric DNA HsTR2.0 (*close circle*, *close rectangle*, respectively) or to *Y. lipolytica* telomeric DNA duplex YlTR1.5 (*open circle*, *open rectangle*, respectively). The experimental conditions were the same as described in *A*.

case of the human proteins (Fig. 4*B*, Table 2). Altogether, comparison of the binding affinity of *Yl*Tay1p variants to *Y. lipolytica* and human telomeric DNA demonstrated that *Yl*Tay1p binds both telomeric sequences significantly tighter than human telomeric proteins TRF1 and TRF2.

TABLE 2

Parameters of binding of *Yl*Tay1p, hTRF1, and hTRF2 to *Y. lipolytica* (YlTR1.5) and human (HsTR2.0) telomeric oligonucleotides obtained by FA measurements

ND, not determined.

	YlTR1.5			HsTR2.0		
Protein	$K_a \times 10^{-6}$	K_D	Fit relative error	$K_a \times 10^{-6}$	K_D	Fit relative error
	M^{-1}	nM	%	M^{-1}	nM	%
WT	12.30	81	11	66.7	15	8
Δ1	3.30	302	12	22.7	44	12
Δ2	3.28	305	11	16.9	59	21
ΔΔ	<0.33	>3000	ND	0.58	1700	30
hTRF1	0.91	1100	18	8.77	114	16
hTRF2	0.53	1900	11	7.41	135	6

DISCUSSION

Telomere binding factors associated with either ss- or dsDNA regions of the chromosomal ends represent an evolutionarily highly divergent group of proteins (7, 8). Their only common feature is the presence of a conserved sequence and/or structural domain (OB-fold or Myb domain) mediating specific binding to telomeric repeats. However, the topology and the number of these DNA binding modules on the corresponding proteins greatly vary. Comparative analysis of the representatives of various types of telomere-binding proteins is the only approach enabling extraction of both specific and general characteristics of telomere protection in eukaryotes (12).

*Yl*Tay1p of *Y. lipolytica* together with its homologues in *S. pombe* (Mug152) and basidiomycetous fungi (15, 24) represent a unique group of telomeric proteins possessing two Myb domains that exhibit high similarity to the Myb domains of mammalian TRF1 and TRF2. The similarities between amino acid sequences of the Myb domains in some cases approaches 50% identity (Myb1 of *Yl*Tay1p and Myb of TRF1; (15)), which is a much higher value than that of the Myb domain of *Sp*Taz1, the most intensively studied fungal telomeric protein considered to be a functional orthologue of TRF1/TRF2. The main difference between *Yl*Tay1p and *Sp*Taz1p, TRF1, or TRF2 is the presence of two Myb domains within the single polypeptide of Tay1p. Although our previous data (gel filtration, electron microscopy) indicate that *Yl*Tay1p forms oligomers *in vitro*, it is possible that in contrast to *Sp*Taz1p, TRF1, and TRF2, where homo-oligomerization is a prerequisite for high affinity DNA binding, the tandem Myb domains of *Yl*Tay1p would allow a single monomer of the protein to bind effectively its target sequences.

The data supporting this hypothesis are derived from an artificial, yet highly informative experimental system. When we tried to test the ability of *Yl*Tay1p to bind *Y. lipolytica* telomeric repeats using the one-hybrid system, we were unable to transform *S. cerevisiae* with an expression plasmid carrying the *YlTAY1* gene under the control of a strong constitutive *ADH1* promoter.[3] When we placed *YlTAY1* under the inducible *GAL1* promoter, we demonstrated that the expression of this gene inhibits growth of *S. cerevisiae* (Fig. 1). We currently do not understand the reason for this inhibitory effect. EMSA assays using *Y. lipolytica*, *S. pombe*, and *S. cerevisiae* telomeric tracts as probes revealed that *Yl*Tay1p is able to bind to telomeres of distantly related yeast species *in vitro* (supplemental Fig. 1).

However, it is unlikely that the inhibition of growth of *S. cerevisiae* cells expressing the *YlTAY1* gene is caused by its binding to telomeres. First, we did not observe any differences in telomere length in the *S. cerevisiae*-expressing *YlTAY1* gene when the cells were transferred from glucose to galactose (supplemental Fig. 2). Second, *YlTAY1* seems to be an essential gene for *Y. lipolytica* (similarly to its *S. pombe* homologue Mug152 (15)), which is able to survive in the absence of telomeric repeats (14). It is more likely that in *S. cerevisiae* *Yl*Tay1p competes for the binding sites with essential DNA-binding proteins, possibly *Sc*Rap1p and/or *Sc*Tbf1p, both telomere-associated proteins that are indispensable not due to their telomeric functions but due to their binding to nontelomeric sites within the *S. cerevisiae* genome (25, 26). Displacement of such proteins by *Yl*Tay1p may thus interfere with their vital functions. Regardless of the reason, the ability of *Yl*Tay1p to inhibit growth in *S. cerevisiae* is dependent on its DNA binding activity (Fig. 1). The fact that mutant versions lacking either Myb1 or Myb2 domain do not affect growth of *S. cerevisiae* indicates that the high affinity binding is achieved by the concerted action of both Myb domains.

Experiments reconstituting the binding of *Yl*Tay1p and its mutant versions to telomeric sequences using EMSA and electron microscopy revealed that the individual Myb domains bind DNA (Fig. 2*B*) and exhibit preference for telomeric sequences (Fig. 2*C*). It is likely that the binding of the mutant proteins containing a single Myb domain results from oligomerization of the protein (see above). On one hand, this would suggest that the primary function of oligomerization for the wild-type protein is not to facilitate the specific binding to the DNA substrate but to mediate formation of telomere-telomere bridges as observed in our previous report (15). On the other hand, when the protein lacks a single Myb domain but retains its ability to form oligomers, it should still bind DNA with some specificity similar as in the case of the single hTRF1 Myb domain, which is able to specifically bind to human telomeres (27, 28). This line of argument together with data from *S. cerevisiae* (Fig. 1) predicts that even though single deletion mutants exhibit a preference for telomeric repeats, their affinity for the substrate should be substantially lower than in the case of wild-type protein.

The ITC and FA measurements point to exactly that direction (Figs. 3 and 4). Single deletion mutants lacking either Myb1 or Myb2 domain exhibited an almost 4-fold lower affinity for the *Y. lipolytica* telomeric sequence than the wild-type protein. This indicates that *Yl*Tay1p Myb domains act synergistically to set the affinity of the protein to the values observed by both ITC and FA ($K_D = 70 - 80$ nM). Similar K_D values have been obtained for binding of human TRF1 and TRF2 to human telomeric repeats (Fig. 4*B*, Table 2). These values are in good agreement with data obtained by Hanaoka *et al.* (21), although these authors observed that the DNA binding domain of hTRF1 has an almost 4-fold higher affinity for human telomeric repeats ($K_D \sim 200$ nM) compared with hTRF2 ($K_D \sim 750$ nM). The K_D values for hTRF1 DNA binding domain obtained by König *et al.* (27) were much lower (~3 nM). Furthermore, we compared the K_D values obtained for *Yl*Tay1p lacking either the Myb1 or Myb2 domain with those of plant proteins from the SMH family containing single Myb-like domains (29). We found that the affinities observed for *Yl*Tay1p variants with only one Myb domain fall into the interval observed for the SMH proteins ($K_D \sim 100 - 400$ nM).

The thermodynamic values determined by isothermal titration calorimetry are in good agreement with the values described in the single previous thermodynamic study showing equivalent c-Myb domain interaction with their cognate DNA sequence (30). In this study, Oda *et al.* (30) characterized the specific binding of c-Myb R2R3, containing two Myb domains, to its cognate DNA sequence using isothermal titration calorimetry. The value of binding enthalpy for c-Myb R2R3 at 25 °C ($\Delta H = -15$ kcal/mol) is in good agreement with the value −19 kcal/mol measured for the *Yl*Tay1p interaction with DNA in our study. Similarly, values of free energy of binding ΔG are comparable: −10 kcal/mol for *Yl*Tay1p and −12 kcal/mol for c-Myb R2R3; both values were measured at 25 °C. The comparable values of thermodynamic parameters for DNA binding of c-Myb R2R3 or *Yl*Tay1p, both containing two DNA binding domains, allow us to speculate that the binding is mediated by the same number of Myb domains. So, even though two molecules of *Yl*Tay1p containing a total of four DNA binding domains bind to the target sequence, only two DNA binding domains may take part in the interaction with DNA at any time. It is possible that the remaining two DNA binding domains may contribute to interactions leading to shaping of telomeric DNA into higher-order structures *in vivo*. The comparison of *Yl*Tay1p binding affinity to its cognate *Y. lipolytica* and human telomeric DNA reveals unexpected results. Intriguingly, *Yl*Tay1p exhibits about 8-fold higher affinity for human telomeric repeats than its mammalian counterparts (in fact, it binds to human telomeres with about 4-fold higher affinity than to its native *Y. lipolytica* telomeric substrate) (Fig. 4*B*, Table 2). Hanaoka *et al.* (21) observed that several substitutions at critical positions within the Myb domain substantially increased the affinity of binding of hTRF2, indicating that the natural selection does not necessarily lead to maximization of the binding affinity. In addition to differences in amino acid sequences, the higher affinity binding of *Yl*Tay1p to human telomeric repeats compared with hTRF1/hTRF2 may be caused by the presence of tandem Myb domains on a single polypeptide, making the binding more efficient than in the case of a single Myb domain-carrying protein that provides the two Myb domains in the form of homodimer. The observation that the affinity of Δ1 version of *Yl*Tay1p is within the same range as the TRF1/TRF2 goes in line with this argument (providing it still can form homo-oligomers observed for wild-type protein). Yet it is surprising that Δ1 has higher affinity for the human probe than Δ2, as Myb1 (retained in Δ2) is more similar to the Myb domain of TRF1/TRF2 than Myb2 (retained in Δ1), especially when both single mutants exhibit very similar affinity to *Y. lipolytica* telomeric repeats. At present we do not have an explanation for this discrepancy. Perhaps the presence of a lysine in Myb1, in the position in which serine or alanine mediate the contact with DNA (Fig. 1*A*, *arrow*; Ref 21), is responsible for a decreased binding of the Myb1 domain to the human telomeric sequence. As in the case of other telomere-binding proteins, like TRF1, TRF2, and *Sc*Rap1 (21, 27, 28, 31–33), more detailed structural

analysis of *Yl*Tay1p (or at least its Myb domains) would be highly instrumental in addressing these issues.

The observations that *Yl*Tay1p exhibits higher affinity for human telomeres than to *Y. lipolytica* telomeres and that it shows lower dissociation constants for binding to human telomeres than human telomeric proteins TRF1/TRF2 underline one important general evolutionary principle: natural selection does not necessarily lead to maximization of the affinity of a particular DNA-binding protein to its cognate DNA substrate. Perhaps the binding properties of a DNA-binding protein are tuned to inferior values, thus, enabling its dynamic association with the target DNA loci.

Comparative analyses, similar to that presented in our study, allow one to formulate hypotheses about evolutionary paths leading to contemporary telomeric binding factors. Even when limiting the comparison to fungi and mammals and disregarding proteins like Tbf1 able to bind telomeric repeats *in vitro* but binding to subtelomeric regions *in vivo* (25), the repertoire of Myb domain-containing proteins directly associated with double-stranded regions of telomeres is quite wide. Modern fungal or mammalian cells contain a diverse combination of three major players: (i) TRF-like proteins (*Sp*Taz1, TRF1, TRF2) possessing a single Myb domain and binding to telomeres as homo-oligomers (although a Myb domain of hTRF1 can bind to telomeric sequence (27)), (ii) *Yl*Tay1-like with two tandem Myb domains exhibiting high similarity to Myb domains of TRF-like proteins, and (iii) *Sc*Rap1-like proteins possessing two Myb domains exhibiting a weak similarity to the former two groups of proteins (32). The evolution of these three groups of proteins could have proceeded in two phases (supplemental Fig. 3). During the first phase, the ancestral genomes accumulated precursors of all three types of proteins possibly via the neutral evolutionary ratchet (34) involving gene and domain duplications. This might have generated a complex set of proteins that could have adapted to either general functions related to regulation of gene expression or more specialized functions at telomeres. In some cases the proteins started to play both telomere- and non-telomere-associated roles (*Sc*Rap1p, *Yl*Tay1p) making them essential components of the cell. In other cases, all three types of proteins were adopted for a specialized function(s), leading to loss of DNA binding activity (*Sp*Rap1, hRap1) and/or gene loss (there does not seem to be a *Yl*Tay1-like protein in mammalian cells and conventional yeast models like *S. cerevisiae*, *K. lactis*, *C. albicans*, or *S. pombe*). Are there any advantages of having two single Myb domain-containing proteins *versus* a protein containing two Myb domains within a single molecule? For example, the former is probably more dynamic in its effect (in response to its cellular level) as a certain minimum level of a protein is necessary to form homodimers with a reasonably high binding affinity. In addition, having two separate telomeric proteins forms a basis for higher levels of regulation that may be important for complex mammalian cells. On the other hand, the latter results in formation of more stable complexes independently of the instantaneous concentration of the protein and thus may be more tolerant to changes in environmental conditions as could be expected in single-cell organisms. Future studies on telomere-binding proteins as well as other components involved in telomere maintenance from various phylogenetic groups will fill the gaps in this incomplete picture.

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