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Positive and Negative Regulation of *Tetrahymena* Telomerase Holoenzyme▽†

Keren L. Witkin,‡ Ramadevi Prathapam, and Kathleen Collins*

Department of Molecular and Cell Biology, University of California at Berkeley, Berkeley, California 94720-3204

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Telomerase replenishes the telomeric repeats that cap eukaryotic chromosome ends. To perform DNA synthesis, the active site of telomerase reverse transcriptase (TERT) copies a template within the integral telomerase RNA (TER). In vivo, TERT and TER and additional subunits form a telomerase holoenzyme capable of telomere elongation. We previously purified epitope-tagged *Tetrahymena thermophila* TERT and characterized two of the associated proteins. Here we characterize the remaining two proteins that were enriched by TERT purification. The primary sequence of the p75 polypeptide lacks evident homology with other proteins, whereas the p20 polypeptide is the *Tetrahymena* ortholog of a conserved multifunctional protein, Skp1. Genetic depletion of p75 induced telomere shortening without affecting the accumulation of TER or TERT, suggesting that p75 promotes telomerase function at the telomere. Affinity purification of p75 coenriched telomerase activity and each other known telomerase holoenzyme protein. On the other hand, genetic depletion of Skp1p induced telomere elongation, suggesting that this protein plays a negative regulatory role in the maintenance of telomere length homeostasis. Affinity purification of Skp1p did not detectably enrich active telomerase but did copurify ubiquitin ligase machinery. These studies reveal additional complexity in the positive and negative regulation of *Tetrahymena* telomerase function.

In most eukaryotes, nuclear chromosomes are capped by a tandem array of simple-sequence repeats. These repeats and their associated proteins protect chromosome ends from unfavorable recombination and end-joining reactions (6, 16). If the number of repeats at any given telomere becomes insufficient to form an insulating chromatin structure, checkpoint activation can halt cell division and signal for cellular senescence or apoptosis (43). Some loss of telomeric repeats is inherent in the process of chromosome replication by DNA-templated DNA polymerases. However, cells can balance this telomeric repeat loss with new repeat synthesis. A balanced equilibrium of repeat loss and gain results in telomere length homeostasis.

New telomeric repeat synthesis is carried out by the ribonucleoprotein (RNP) reverse transcriptase telomerase (9, 21). The telomerase RNA subunit (TER) carries an internal template complementary to the strand of telomeric repeats with 5′-3′ polarity towards the chromosome end. TER also harbors critical nontemplate motifs that scaffold regulatory factors and improve DNA synthesis processivity (10, 49). The active site for DNA synthesis is provided by telomerase reverse transcriptase protein (TERT), which has a central region of homology with viral reverse transcriptase active sites as well as unique N- and C-terminal extensions (5, 26). The TERT-specific motifs are important for its interactions with TER and single-stranded DNA substrates (5, 11). Recombinant TER and TERT from *Tetrahymena thermophila* and some other species can be coassembled in heterologous cell extracts such as rabbit reticulocyte lysate to reconstitute a catalytically active enzyme.

In vivo, telomerase complexes are generated by specific pathways of RNP biogenesis. All endogenously assembled telomerase holoenzyme complexes harbor RNA binding proteins that fold and package TER into a biologically stable RNP (11). In vertebrate and yeast (*Saccharomyces cerevisiae*) cells, these RNA binding proteins are not telomerase specific. Human TER assembles with proteins that recognize the hairpin-hinge-hairpin-ACA (H/ACA) motif characteristic of a large family of small nucleolar and Cajal body RNAs. Yeast TER assembles with a heteroheptameric complex of Sm proteins shared by many small nuclear RNAs (35, 42). In contrast, ciliate TER is folded and stabilized by assembly with a telomerase-specific RNA binding protein. In *Tetrahymena*, cellular accumulation of TER and TERT requires the telomerase holoenzyme protein p65 (53). Both p65 and the orthologous *Euplotes aediculatus* telomerase p43 bind TER directly and specifically in vivo and in vitro (2, 3, 38, 53). Reconstitution assays using purified p65, TER, and TERT have shown that p65 initiates the hierarchical assembly of a p65-TER-TERT ternary complex (36, 38).

Additional telomerase-associated proteins that do not alter TER accumulation in vivo or telomerase catalytic activity in cell extract have been described. The best-characterized proteins of this class are the *Saccharomyces cerevisiae* proteins Est1p and Est3p, which endow a biologically stable and catalytically active telomerase RNP with the ability to elongate telomere substrates (33). The association of Est1p with the yeast TERT Est2p occurs through TER and is regulated with the cell cycle (37, 47, 55). The specificity of Est3p association

* Corresponding author. Mailing address: Department of Molecular and Cell Biology, 16 Barker Hall #3204, University of California at Berkeley, Berkeley, CA 94720-3204. Phone: (510) 643-1598. Fax: (510) 643-6334. E-mail: kcollins@berkeley.edu.

† Supplemental material for this article may be found at http://mcb.asm.org/.

‡ Current address: Laboratory of Molecular and Cellular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892.

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with active enzyme is not yet elucidated, but Est3p interaction with Est2p is dependent on Est1p (37). Est1p contributes one of the physical links between telomerase and the telomere by binding to the single-stranded telomeric-repeat DNA binding protein Cdc13p (6, 50). However, both molecular and genetic lines of evidence indicate that there are additional roles for Est1p as well (48).

Tetrahymena thermophila is a genetically tractable model organism that is rich in telomeres and telomerase (13). We engineered a strain of *Tetrahymena* deleted for the endogenous TERT locus, *TRT*, but expressing fully functional epitope-tagged TERT from a transgene integrated at the *BTU1* locus (53). Affinity purification of the epitope-tagged TERT, fused at its C terminus to the tandem affinity purification (TAP) tag (39), coenriched four other proteins described by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) apparent molecular weights as p75, p65, p45, and p20 (53). The primary sequence of p45 did not reveal any known structural motifs, while the primary sequence of p65 suggested putative RNA binding domains. Gene disruption experiments revealed that p65 and p45 are essential for telomere maintenance and that depletion of p65 but not p45 reduced the cellular levels of TER and TERT. In vitro, purified recombinant p65 binds to the TER terminal stem and 3′ polyuridine tail and enhances TERT-TER interaction (36, 38). These biochemical activities are likely to underlie the biological requirement for p65 in TER and TERT accumulation.

Here we describe the molecular identification and functional characterization of the remaining proteins isolated by TAP-tagged TERT (TERT-TAP) affinity purification. The sequence of p75 is novel. Depletion of the essential gene encoding p75 induced telomere shortening, as previously observed upon depletion of the essential genes encoding p45 and p65. Like depletion of p45 but unlike that of p65, depletion of p75 did not affect the cellular levels of TER and TERT. Affinity purification of p75 enriched telomerase activity as well as the other holoenzyme proteins TERT, p65, and p45. The sequence of p20 revealed it to be the *Tetrahymena* ortholog of Skp1, a multifunctional protein best characterized as a component of SCF (Skp1/Cullin/F-box protein) ubiquitin ligases (7, 52). Curiously, depletion of the essential *Tetrahymena* gene encoding Skp1p induced telomere overelongation rather than shortening. Affinity purification of tagged Skp1p did not enrich active telomerase; instead, it predominantly copurified components of an SCF ubiquitin ligase complex. This suggests that Skp1p could contribute to telomere homeostasis by playing a role beyond the context of the telomerase holoenzyme. Overall, our studies reveal that despite the relatively simple subunit composition of ciliate telomerase complexes, the ciliate telomerase-telomere interaction has a complexity of positive and negative regulation.

MATERIALS AND METHODS

***Tetrahymena* growth and strain construction.** *Tetrahymena* cultures were grown at 30°C in 2% proteose peptone, 0.2% yeast extract, 10 μM $FeCl_3$ supplemented with 150 μg/ml ampicillin and streptomycin and 1.25 μg/ml amphotericin B (Fungizone). Cells were starved overnight (12 to 24 h) in 10 mM Tris-HCl (pH 7.5) prior to transformation by particle bombardment (8). We generated gene disruption constructs similar to the previously generated telomerase subunit gene disruption constructs (34). An expression cassette encoding paromomycin resistance was flanked with *SKP1* genomic regions of between 600 and 950 bp such that homologous recombination would remove the entire open reading frame. Gene targeting was performed in strain CU522 as described previously (34). Selection for the maximal extent of gene replacement was performed by daily passaging of cultures into media with doubled drug concentration until the culture failed to double in 24 h, at which point cultures were passaged into media with increasing intermediate drug concentrations to reach the maximum drug concentration permissive for doubling in 24 h. Cultures were passaged daily at this drug concentration for several weeks, and then single cells were isolated to establish independent clonal cultures. To test whether a gene is essential, cells released from selection were passaged daily in media without drug for ~2 weeks. This allows an essential gene to back-assort from the limiting, possibly very low copy number present at maximal selection to a higher copy number optimal for growth and more readily detected by Southern blot analysis.

Transgenes were integrated at the *BTU1* locus in strain CU522 under control of the endogenous *BTU1* promoter as described previously (53). The N-terminal ZZ tag consisted of a six-histidine sequence and tandem protein A domains followed by a tobacco etch virus (TEV) protease cleavage site subcloned from the TAP tag. Skp1p C-terminal epitope tagging at the endogenous locus was performed in strain CU522 by use of a targeting construct that contained the epitope tag followed by a stop codon, an adjacent polyadenylation signal, and an expression cassette conferring resistance to paromomycin, flanked on each side by *SKP1* genomic DNA.

Extract preparation and affinity purifications. Knockdown cell extracts were made using cultures grown overnight without drug to obtain large-scale volumes of cells doubling at a wild-type rate. Whole-cell extracts were generated by cell lysis with 0.2% NP-40 in T2MG buffer (20 mM Tris-HCl at pH 8.0, 1 mM $MgCl_2$, 10% glycerol) supplemented with approximately 10 mM beta-mercaptoethanol and protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml leupeptin). Cell lysates were centrifuged at 130,000 × *g* for 1 h. Affinity purifications were performed using lysates from cells in mid-log-phase growth (2×10^5 to 4×10^5/ml) or starved cells cultured as described above with no obvious difference in protein associations. Tagged proteins were enriched by binding to immunoglobulin G (IgG) agarose (Sigma) in extracts adjusted to 50 to 100 mM NaCl. Resin was washed in binding buffer and in TEV protease elution buffer (10 mM Tris-HCl at pH 8.0, 0.1 M NaCl, 0.1% NP-40, 0.5 mM EDTA) before elution with recombinant TEV protease (24) for approximately 30 min at room temperature. Endogenous p75 immunopurification employed rabbit polyclonal antibody raised and affinity purified against denatured six-histidine-tagged p75 (unpublished data). Antibodies were prebound to protein A-Sepharose (Amersham), and then resin was incubated for 1 h at 4°C with whole-cell extract supplemented to 50 mM NaCl. Resin was washed three times in binding buffer with additional 0.2 mM EGTA, 0.1% NP-40, and 0.1 mM phenylmethylsulfonyl fluoride.

Assays for DNA, RNA, protein, and activity. For Southern blot assays of gene disruption, genomic DNA was digested with restriction enzymes as indicated, resolved by agarose gel electrophoresis, and transferred to membrane prior to hybridization. Each hexamer-labeled probe was designed to recognize the genomic region immediately 5′ of the integration site. Southern blot assays of telomere length were performed as previously described (34) using cultures grown to large-scale volume in drug-free media and harvested in mid-log phase after the same number of doublings at 30°C. For experiments analyzing telomere length over a time course of continuous doublings, cultures were maintained in continuous growth by daily dilution into fresh drug-free media.

RNA was prepared by standard methods (4). Northern blots to detect small RNAs were probed with 5′ end-labeled complementary oligonucleotides. Immunoblots used affinity-purified rabbit polyclonal antibodies raised against a peptide from the TERT motif AB region (12) or against full-length recombinant p65 or p45 (53). Nonspecific rabbit IgG was used to detect the protein A domains of tagged proteins. Activity assays were performed by direct primer extension using reaction buffer with final concentrations of 50 mM Tris-acetate (pH 8.0), 10 mM spermidine, 5 mM beta-mercaptoethanol, 2 mM $MgCl_2$, 0.4 mM dTTP, 0.3 μM [^{32}P]dGTP (800 Ci/mmol; PerkinElmer), and 400 nM primer $(G_4T_2)_3$. Assay mixtures were incubated for 45 to 60 min at 30°C, and incubation was followed by DNA precipitation and product analysis by denaturing gel electrophoresis.

Mass spectrometry. Samples were precipitated with trichloroacetic acid, resuspended in 40 μl of 100 mM Tris (pH 8.5) with 8 M urea, reduced in 5 mM Tris [2-carboxyethyl] phosphine (Pierce), carboxyamidated in 10 mM iodoacetamide (Sigma), diluted to 2 M urea in 100 mM Tris (pH 8.5), and digested with 0.5 μg of sequencing-grade trypsin (Promega). Peptides were purified on Spec PT C18 pipette tips (Varian). Mass spectrometry was performed on a Thermo Finnegan LCQ Deca XP Plus instrument at the Cancer Research Laboratory mass spectrometry facility (http://biology.berkeley.edu/crl/mass_spec/index.htm). Spectra were analyzed with the SEQUEST algorithm (18) against the initial release of

Tetrahymena macronuclear gene predictions. Protein identifications in purified samples and parallel mock purifications were compared using DTASelect and Contrast software (46) to eliminate nonspecific purification background.

Nucleotide sequence accession numbers. GenBank accession numbers for sequences reported here are AY522576 and AY522588.

RESULTS

Isolation of cDNAs encoding p75 and p20. Sequencing of p75 and p20 peptides obtained in a previously described large-scale TERT-TAP affinity purification (53) was performed by Edman degradation. Peptide sequences were used to design degenerate PCR primers to amplify cDNA or genomic DNA protein-coding sequence. From the initial peptide sequences, we were unable to isolate a gene fragment that was validated by a known peptide sequence not used in primer design. To obtain additional peptide sequences, we performed a second large-scale TERT-TAP affinity purification, omitting the final step of chromatography on calmodulin agarose, which had greatly decreased the recovery yield in the previous large-scale affinity purification. Polypeptides that comigrated with p75 and p20 from a complete purification could be clearly resolved by SDS-PAGE from the nonspecific background. The SDS-PAGE gel was transferred to membrane, and slices that contained p75 or p20 were excised. The proteins were then processed to obtain peptide sequence by Edman degradation as previously described (53).

Degenerate primers were again designed based on peptide sequences and used to amplify putative cDNA or genomic DNA protein-coding regions. Sequencing of cloned PCR products did reveal fragments of coding region for each protein that harbored additional peptide sequence not used in primer design (see the supplemental material for additional details). The full-length p20 cDNA sequence was compiled from overlapping fragments obtained by standard methods of 5′ and 3′ rapid amplification of cDNA ends. The partial cDNA sequence of p75 was compiled in a similar manner, but the extreme 5′ end of the full-length cDNA was repeatedly recalcitrant to cloning. Following the online release of shotgun sequencing reads from the *Tetrahymena* macronuclear genome project (17), we could amplify the missing p75 cDNA region by reverse transcription and PCR with specific-sequence primers. The genomic locus encoding each protein was also amplified as overlapping PCR fragments, cloned, and sequenced to support the deduced open reading frame (see the supplemental material for additional details).

BLAST searches using the p75 amino acid sequence did not reveal obvious homology with known or predicted proteins, and no conserved functional domains were identified. For consistency with *Tetrahymena* gene nomenclature and previous studies, we designated the gene encoding p75 as *TAP75* for *t*elomerase-*a*ssociated (or *T*ERT-*a*ssociated) *p*rotein of 75 kDa. BLAST searches using the p20 amino acid sequence revealed extensive homology with the eukaryotic protein Skp1 spanning the entire length of the coding region (see Fig. S1 in the supplemental material). Overall, p20 is ~50% identical to human Skp1 in its primary sequence. Because reciprocal BLAST searches indicated that p20 is the human Skp1 ortholog, we designated the *Tetrahymena* gene encoding p20 as *SKP1* and subsequently refer to the protein as Skp1p.

Studies with many eukaryotes have established that Skp1 functions as a subunit of SCF ubiquitin ligases, which catalyze ubiquitin modification of target proteins that are subsequently degraded by the proteasome. In SCF, Skp1 bridges Cullin and the F-box protein specificity factor for substrates (7, 52). In addition to its role in SCF complexes, *S. cerevisiae* Skp1p serves unrelated roles as a component of the centromere binding factor 3 (CBF3) kinetochore complex (14, 44), the vacuolar ATPase complex RAVE (41), and a complex implicated in recycling of the SNARE Snc1p (20). Furthermore, even within CBF3, Skp1p has genetically separable activities in complex assembly, complex disassembly, and checkpoint signaling (28, 31, 40).

Genetic depletion phenotypes of p75 and Skp1p. The genes encoding the *Tetrahymena* telomerase holoenzyme subunits TER, TERT, p65, and p45 are essential for growth (34, 53). In order to determine if the genes encoding p75 and Skp1p are similarly essential, we targeted the genomic loci encoding p75 and Skp1p for disruption. Through homologous recombination, the entire coding region of *TAP75* or *SKP1* was replaced with a cassette conferring resistance to the drug paromomycin. Initial transformants selected at low drug concentration have only a few of the 45 copies of a macronuclear chromosome disrupted. These cells were passaged into media with progressively increasing drug concentrations to select for an increase in copy number of the disrupted chromosome. After selecting for maximal gene replacement, we released isolated cells into drug-free media to allow back-assortment of the endogenous locus to a copy number not limiting for growth (see Materials and Methods). Southern blots of genomic DNA revealed the persistence of wild-type chromosomes in wild-type cells (Fig. 1A, lanes 1 and 4), two clonal isolates of cells selected for *TAP75* disruption (lanes 2 and 3), and two clonal isolates of cells selected for *SKP1* disruption (lanes 5 and 6). The extents of knockdown of *TAP75* or *SKP1* showed little variation when independent clonal isolates of selected cells that were grown in parallel were compared (compare the ratios of wild-type and disrupted chromosomes in Fig. 1A, lanes 2 and 3 or 5 and 6 to normalize for DNA loading), but we note that the extent of knockdown will vary with differences in culture conditions across experiments. These results demonstrate that p75 and Skp1p are encoded by essential genes.

We exploited the strains depleted for p75 or Skp1p to investigate the role of each protein in vivo. Depletion of p65 reduces the steady-state accumulation of TER and TERT, but depletion of p45 does not (53). To extend this analysis, we measured the steady-state levels of TER and TERT in knockdown strains with reduced levels of p75, Skp1p, or TERT (encoded by the *TRT* locus). Genetic depletion of TERT (Fig. 1B, lanes 2 and 3) but not of p75 or Skp1p (lanes 4 to 7) reduced TER accumulation relative to other small nuclear RNAs such as U2 and U6 (Fig. 1B) or the cytoplasmic signal recognition particle RNA (data not shown). We were unable to directly measure the extents of protein depletion in all of the knockdown strains due to the limited sensitivity of our antibodies, but the altered telomere lengths of these strains suggest that each protein was depleted to a functionally limiting extent (see below). TERT level was reduced in the *TRT* knockdown strain (Fig. 1C, lanes 1 and 2), compared to the level of a nonspecific cross-reacting polypeptide used as the loading control. In contrast, the level of TERT was unaltered by de-

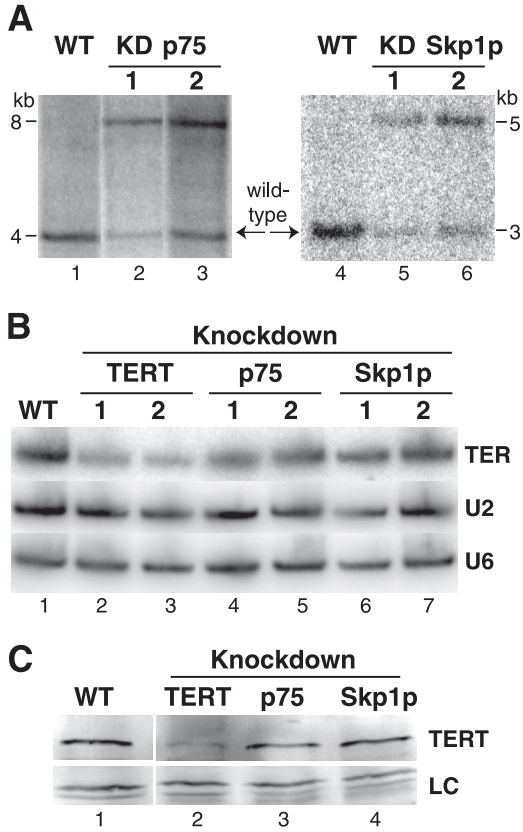


FIG. 1. Genetic depletion of p75 and Skp1p. (A) Cells transformed with the *TAP75* or *SKP1* gene disruption construct were selected for maximal gene disruption and then released from selective pressure. Genomic DNA was isolated from wild-type cells (WT) and two clonal isolates of p75 or Skp1p knockdown cells (KD). DNA was digested with BbsI (lanes 1 to 3) or NdeI and BglII (lanes 4 to 6) for Southern blots that were hybridized with a DNA probe equally complementary to the wild-type and disrupted loci. A separate lane of the same nondenaturing gel was loaded with a DNA ladder. (B and C) Whole-cell extracts from wild-type cells and TERT, p75, and Skp1p knockdown cell cultures were normalized by total protein concentration prior to RNA extraction for Northern blot hybridization (B) and protein analysis by immunoblotting (C). The Northern blot was probed for TER and for U2 and U6 small nuclear RNAs to control for loading. A nonspecific cross-reacting polypeptide from the immunoblot with anti-TERT antibody is also shown as a loading control (LC).

pletion of p75 or Skp1p (Fig. 1C, lanes 3 and 4). These results suggest that p75 and Skp1p are not determinants of the stability of telomerase RNP in vivo.

Genetic depletion of TER, TERT, p65, or p45 induces telomere shortening (34, 53). For comparison, we assayed for a change in telomere length induced by genetic depletion of p75 or Skp1p. The palindromic chromosome encoding rRNA (the ribosomal DNA [rDNA]) is present at 9,000 copies per macronucleus and harbors half of the total macronuclear chromosome ends. Genomic DNA was collected from wild-type and knockdown strains and digested to liberate rDNA terminal restriction fragments with a precise length of subtelomeric sequence. The net length of the GT-rich strand of telomeric repeats was determined by Southern blotting of a denaturing gel hybridized with an oligonucleotide probe complementary to the subtelomeric region. Subtracting the subtelomeric ~350 nucleotides (nt), the telomeric repeat array in wild-type cells was a typical ~300 nt (Fig. 2A, lanes 1 and 3). Cells depleted for p75 had short telomeres with only ~100 to 150 nt of telomeric repeats (Fig. 2A, lane 2). In contrast, cells depleted for Skp1p had atypically long telomeres with an extra ~200 nt of telomeric repeats (Fig. 2A, lane 4).

Tetrahymena macronuclear telomere lengths increase with continuous growth at 30°C (1, 29). To investigate whether the altered telomeres of the knockdown strains remained sensitive to this change in telomere length homeostasis, we monitored telomere length in wild-type and knockdown strains grown in continuous culture in parallel. Telomere length in wild-type cells increased over 5 days of continuous growth, as expected (Fig. 2B, lanes 10 to 12). Telomeres in cells depleted for TERT or p75 were shorter than wild-type telomeres at each time point, but they also increased in length over 5 days of continuous growth (Fig. 2B, lanes 1 to 9). Similarly, although telomeres in cells depleted for Skp1p were longer than wild-type telomeres at each time point, they increased in length over the time course of continuous growth as well (Fig. 2B, lanes 13 to 18). These findings suggest that the mechanisms underlying balanced homeostasis are intact in each knockdown strain but that the equilibrium set point for telomere length is altered.

Antibody immunopurification of telomerase activity. We next investigated whether p75 and Skp1p were associated with catalytically active telomerase in *Tetrahymena* cell extract. To test for an association of the endogenous proteins with telomerase enzyme activity, we raised and affinity purified rabbit polyclonal antibodies against recombinant proteins expressed in bacteria (unpublished data). Recombinant p75 was severely degraded and largely insoluble under a variety of expression conditions, while recombinant Skp1p was abundantly expressed, soluble, and readily purified. Rabbits immunized with denatured p75 generated antibodies that could detect the protein in immunoblots of bacterial expression extracts (Fig. 3A) and *Tetrahymena* overexpression strain extracts (data not shown) but not in wild-type *Tetrahymena* whole-cell extracts. Rabbits immunized with soluble Skp1p failed to produce antibodies that could recognize the endogenous protein in *Tetrahymena* wild-type or overexpression strains, and even bacterially expressed Skp1p was inefficiently recognized by affinity-purified antiserum (data not shown). We suspect that the poor antigenicity of recombinant *Tetrahymena* Skp1p and the inability to detect endogenous protein derive from the high sequence conservation of eukaryotic Skp1 proteins and Skp1 posttranslational modifications (45, 51).

Affinity-purified p75 antibody was immobilized on protein A resin and incubated with *Tetrahymena* whole-cell extract. As a control, a mock purification was performed using antibodies from preimmune serum. The antibody resins and associated complexes were washed and then split to detect TER by Northern blot hybridization (Fig. 3B) or telomerase activity by the direct primer extension assay (Fig. 3C). The antibody against p75 specifically enriched both TER and telomerase activity. Even though telomerase complexes remained bound to p75 antibody resin, long product DNAs that are characteristic of the endogenously assembled *Tetrahymena* telomerase holoenzyme were synthesized.

Tagged protein copurification of telomerase holoenzyme. To extend the analysis of p75 and Skp1p association with telome-

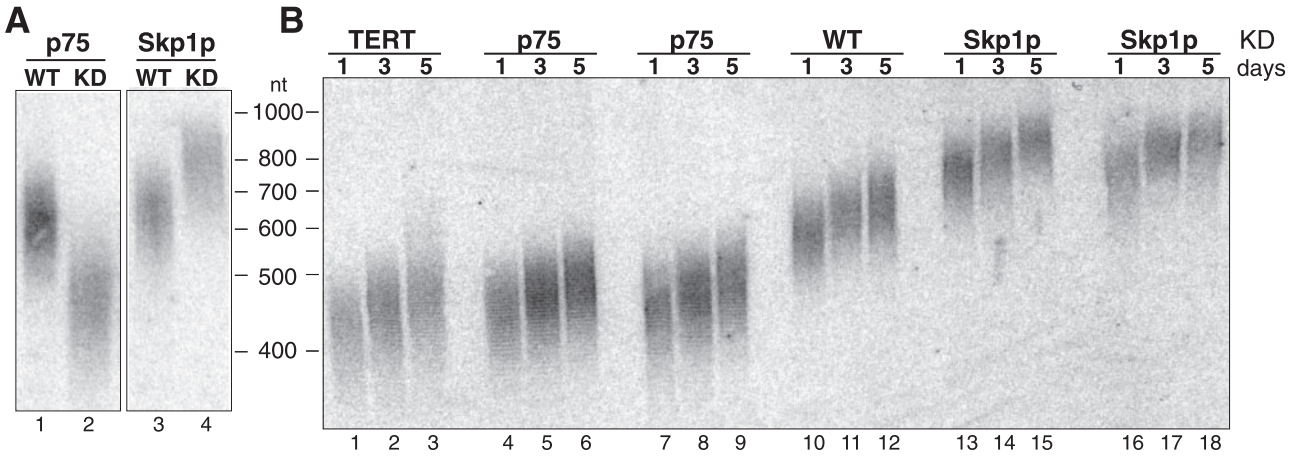


FIG. 2. Altered telomere length in TERT, p75, and Skp1p knockdown strains. (A) Genomic DNA was digested with HindIII and analyzed by Southern blotting for the terminal restriction fragment of macronuclear rDNA chromosomes. A separate lane of the denaturing polyacrylamide gel was loaded with an end-labeled DNA ladder. (B) Wild-type cells, TERT knockdown cells, and two independent clonal isolates of p75 or Skp1p knockdown cells were grown continuously for 5 days by daily dilution into fresh media. Genomic DNA was analyzed after days 1, 3, and 5 of continuous growth as described for panel A. A DNA ladder was run in parallel with identical samples. WT, wild-type cells; KD, knockdown cells.

rase beyond what was possible using antibodies raised against recombinant proteins, we constructed *Tetrahymena* strains expressing epitope-tagged versions of the proteins. Following the same transgene strategy used to express epitope-tagged TERT, p65, and p45, we generated strains that express tagged p75 or Skp1p at the *BTU1* locus. Transgene integration replaces a nonessential beta-tubulin gene that confers hypersensitivity to paclitaxel (Taxol) (19). In several unrelated experiments, we have observed that the calmodulin binding peptide portion of the TAP tag reduces recombinant protein accumulation in *Tetrahymena*. We therefore tagged p75 and Skp1p using only the tandem protein A domains of the TAP tag (the ZZ tag), with an adjacent cleavage site for TEV protease (see Materials and Methods).

Successful expression of ZZ-tagged p75 and Skp1p was confirmed for several independent strains with immunoblots of whole-cell extracts performed using nonspecific IgG, which binds avidly to the protein A modules of the tag (Fig. 4A). A minor degradation product of ZZ-p75 was also detected as a tag-containing, lower-molecular-weight polypeptide specific to the ZZ-p75 expression strains. The accumulation level of ZZ-Skp1p was much greater than that of ZZ-p75 (Fig. 4A); also, much more tagged Skp1p than tagged p75 was recovered per volume of cell extract by affinity purification on IgG agarose (data not shown). Affinity purification of ZZ-p75 enriched both TER (Fig. 4B, lane 1) and telomerase activity (Fig. 4C, lane 1). In contrast, affinity purification of ZZ-Skp1p did not enrich TER or telomerase activity relative to background levels monitored by mock affinity purification from wild-type cell extract (Fig. 4B and C, lanes 2 and 3).

Primer extension activity assays of the resin-bound ZZ-p75 telomerase complexes generated some long product DNAs characteristic of the endogenous *Tetrahymena* telomerase holoenzyme and short products as well (Fig. 4C, lane 1). For comparison, we examined the profile of catalytic activity following p75 elution from affinity resin by TEV protease cleavage between p75 and the ZZ tag. Primer extension assays were conducted on fractions from IgG agarose chromatography of wild-type and ZZ-p75 extracts (Fig. 4D) by comparing protease elutions, material remaining associated with the resin after elution, unbound extract, and input extract. ZZ-p75 purification but not mock affinity purification from wild-type cell extract enriched for catalytically active telomerase as expected, and release of p75 from the resin and the ZZ tag reproducibly improved holoenzyme repeat addition processivity. We cannot calculate the absolute percentage of telomerase holoenzyme bound to p75 by use of the ZZ-p75 expression strain due to competition from endogenous p75, but the specific and robust enrichment of telomerase activity by immunopurification of endogenous p75 (Fig. 3) and affinity purification of ZZ-p75 (Fig. 4) demonstrate that p75 is stably associated with the telomerase holoenzyme.

The predominant *Tetrahymena* telomerase holoenzyme in cell extract harbors both p65 and p45 subunits (53). To confirm that the holoenzyme complex containing p65 and p45 is the same holoenzyme complex containing p75, we assayed for copurification of endogenous p65 and p45 with ZZ-p75. We performed IgG agarose chromatography in parallel from whole-cell extracts containing TERT-TAP, no tagged protein, or ZZ-p75. Specifically bound complexes were eluted from the purification resin by use of TEV protease, which also clipped the ZZ tag module from TERT-TAP and ZZ-p75. Eluted samples were then resolved by SDS-PAGE and queried for the presence of TERT, p65, and p45 by immunoblotting using previously developed antibodies that are more sensitive than the p75 and Skp1p antibodies (Fig. 4E). TERT recovered from the TERT-TAP extract remained fused to the calmodulin binding peptide of the TAP tag (TERT-CBP). TERT recovered from the ZZ-p75 extract instead migrated with the slightly faster mobility of the endogenous protein. Importantly, both TERT-TAP and ZZ-p75 enriched endogenous p65 and p45. In some experiments, we observed a doublet of polypeptides specifically cross-reactive with anti-p65 antibody, which we suspect reflects the extract-induced proteolysis noted in studies of *Euplotes* p43 (3).

FIG. 3. Immunopurification of endogenous telomerase. (A) Bacterial extracts without or with recombinant p75 were probed by immunoblotting with anti-p75 antibody (αp75). (B and C) Whole-cell extract from wild-type cells was incubated with immobilized anti-p75 antibody or preimmune serum (Mock). Bound samples were split for analysis of RNA by blot hybridization (B) or for telomerase activity assay by primer extension (C). A truncated recombinant TER was added prior to RNA extraction as a recovery control (RC).

Skp1p assembles into SCF complexes. As described above, affinity purification of ZZ-Skp1p did not enrich catalytically active telomerase (Fig. 4C). Because *Tetrahymena* Skp1p has sequence conservation with its orthologs that extends very close to the N and C termini of the full-length protein (see Fig. S1 in the supplemental material), appending an epitope tag could have interfered with its function or disfavored competition with the endogenous Skp1p protein. In order to tag Skp1p in a manner conclusively not disruptive of its essential function(s), we epitope tagged Skp1p at its endogenous locus. Targeting was performed such that integration of the drug resistance marker obliged C-terminal fusion of Skp1p to a TAP or ZZ tag. Because *SKP1* is essential (Fig. 1A), the recombinant chromosome bearing the drug resistance marker will completely replace the wild-type chromosome only if the tagged protein is functional. We examined the extent of *SKP1* substitution by performing a Southern blot of genomic DNA from cells subject to maximal selective pressure and then grown without selection to allow back-assortment (see Materials and Methods). A complete replacement of *SKP1* was observed for all independent clonal isolates of Skp1p-TAP and Skp1p-ZZ strains (Fig. 5A). These results indicate that Skp1p can be tagged at its C terminus and retain its genetically essential function(s).

Cell extracts were examined for accumulation of Skp1p-TAP or Skp1p-ZZ by immunoblot detection of the tandem protein A domains of the tags. Polypeptides of the expected SDS-PAGE mobilities were indeed accumulated (Fig. 5B), with a detection level that indicated high abundance (data not shown). Affinity purification of either tagged version of Skp1p by use of IgG agarose did not detectably enrich for TER or telomerase activity, despite substantial recovery of the tagged protein itself (data not shown). It was possible that tagging Skp1p at its C terminus retained Skp1p essential function(s) but abrogated a nonessential role of the protein in telomerase regulation. If so, strains expressing Skp1p-ZZ and Skp1p-TAP would be expected to possess altered telomere length. We examined telomere lengths of Skp1p-ZZ and Skp1p-TAP strains using the same Southern blot protocol employed for analysis of telomere length in the gene knockdown strains (Fig. 2A). Telomere length was increased in Skp1p-TAP cells but not in Skp1p-ZZ cells (Fig. 5C), suggesting that at least Skp1p-ZZ retained wild-type function in telomere length homeostasis.

To identify the most abundant interaction partners of *Tetrahymena* Skp1p, we affinity purified Skp1p-ZZ from whole-cell extracts by use of IgG agarose. Specifically bound complexes were eluted by the addition of TEV protease. SDS-PAGE revealed the coenrichment of several proteins with Skp1p-ZZ that were not similarly enriched by mock affinity purification from wild-type cell extract (Fig. 6A). The entire eluted mixture of Skp1p-associated proteins and the eluted mock affinity purification were used for peptide sequence analysis by mass spectrometry (see Materials and Methods). Preliminary gene predictions from the *Tetrahymena* macronuclear genome sequencing project (17) and additional annotation in the *Tetrahymena* Genome Database (http://www.ciliate.org) were used for protein identification. Automated searching identified a Cullin family protein, an F-box domain protein, and an F-box-associated domain protein specifically in the affinity-purified Skp1p sample (Fig. 6B). The preliminary nature of the *Tetrahymena* gene predictions precludes matching the molecular weights of identified proteins with the polypeptides detected by SDS-PAGE, but we note that the size of the largest major Skp1p-associated protein matches that of Cullin proteins in other organisms (Fig. 6A). These results indicate that like Skp1 proteins in other eukaryotes, *Tetrahymena* Skp1p assembles into SCF complexes.

DISCUSSION

This work extends the fundamental characterization of endogenous telomerase holoenzyme complexes. Ciliates have been a rich source of insights about telomeres and telomerase, owing in large part to the high chromosome number of the

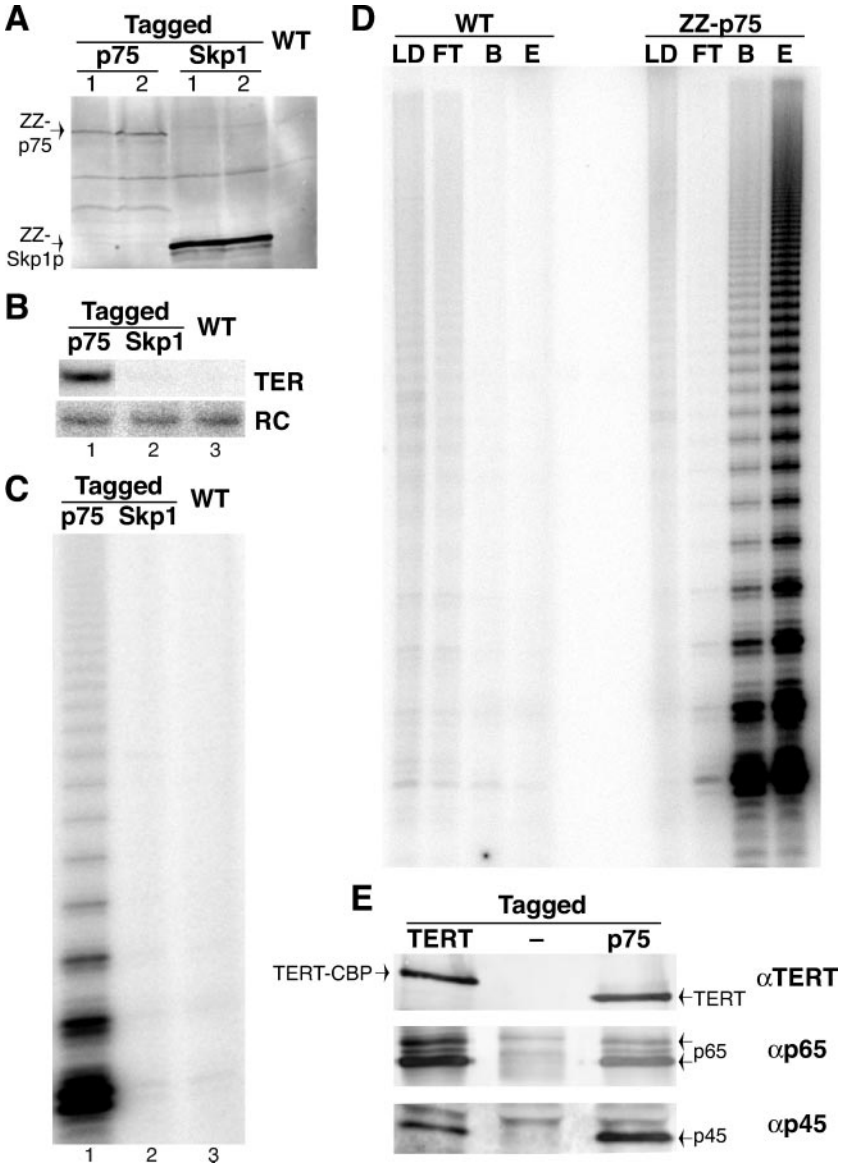


FIG. 4. Telomerase holoenzyme purification by epitope-tagged p75. (A) Whole-cell extracts from wild-type cells (WT) and two clonal isolates of ZZ-p75 or ZZ-Skp1p cells were normalized for total protein concentration before immunoblotting to detect the ZZ tag. Markers were run in a lane of the same gel and transferred to the same blot. (B and C) Normalized whole-cell extracts from wild-type, ZZ-p75, and ZZ-Skp1p strains were incubated with IgG agarose. Bound samples were split for analysis of RNA by blot hybridization (B) or for telomerase activity assay by primer extension (C). Truncated recombinant TER was added prior to the extraction of RNA as a recovery control (RC). (D) Normalized whole-cell extracts from wild-type and ZZ-p75 cell cultures were incubated with IgG agarose. The input (LD) and unbound (FT) fractions as well as the elutions (E) and postelution resins (B) were assayed for telomerase activity. Similar absolute volumes of each fraction were assayed, with the elution representing ~100× the volume concentration relative to input and unbound extracts. Note that the catalytic activity of the LD and FT fractions but not that of the purified samples is subject to nonspecific inhibition from whole-cell extract. (E) Normalized whole-cell extracts from TERT-TAP, wild-type, and ZZ-p75 cell cultures were used for affinity purification. Aliquots of samples eluted from IgG agarose by TEV protease cleavage were analyzed by immunoblotting with antibodies (α) raised against a TERT peptide, full-length p65, and full-length p45. TEV protease cleavage of TERT-TAP releases TERT fused to the calmodulin binding peptide (TERT-CBP). Protein markers and bacterially expressed standards for TERT, p65, and p45 were used to confirm the identity of the cross-reacting polypeptides.

macronucleus (23, 54). The findings here and in previous work reveal that an endogenously assembled *Tetrahymena* telomerase holoenzyme includes TERT, p75, p65, p45, and TER. From an architectural perspective, assembly of the complex begins with p65 binding, stabilizing, and folding TER and promoting TER assembly with TERT. TERT also plays a role stabilizing TER, while p75 and p45 somehow promote the function of a biologically stable telomerase RNP at telomeres. The influence of *Tetrahymena* TERT on TER accumulation was somewhat unexpected, because TERT does not contribute to telomerase RNP stability in yeast and has only marginal impact on telomerase RNP stability in mammals (11). Together, in vivo and in vitro assays suggest that a p65-TER complex recruits TERT to form a catalytically active p65-TER-

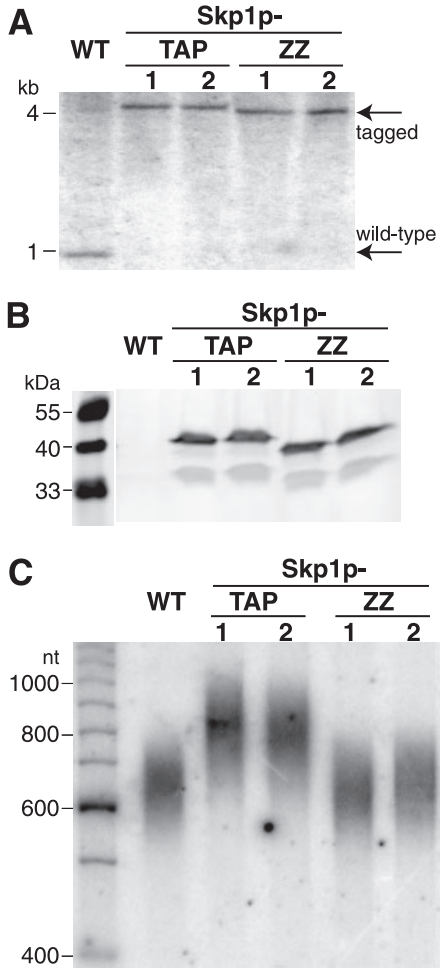
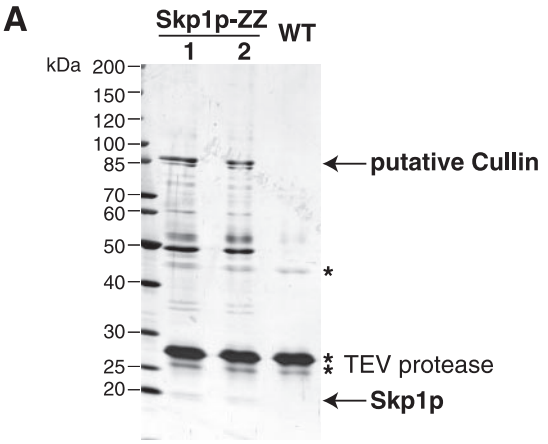


FIG. 5. Tagging of Skp1p at the endogenous locus. (A) Cells transformed to integrate a C-terminal TAP or ZZ tag at *SKP1* were selected to obtain maximal replacement of the wild-type (WT) locus and then released from selective pressure. Genomic DNA was isolated from wild-type cells and cells from independent clonal isolates of each gene replacement, digested with ClaI and HpaI, and used for Southern blot hybridization with a DNA probe equally complementary to wild-type and tagged *SKP1* loci. A separate lane of the same nondenaturing gel was loaded with a DNA ladder. (B) Whole-cell extracts from the same cells were normalized by total protein concentration prior to protein analysis by immunoblotting. Markers were run in the same gel and transferred to the same blot. (C) Genomic DNA from the same cells was digested with HindIII and analyzed by Southern blotting for the terminal restriction fragment of macronuclear rDNA chromosomes. One lane of the denaturing polyacrylamide gel was loaded with an end-labeled DNA ladder.



B

TGD ID	Coverage	Annotation
6.m00351	52 %	Cullin family protein
47.m00262	41 %	Skp1 family protein
271.m00034	39 %	Hypothetical protein (FBA domain)
265.m00031	38 %	F-box domain containing protein

FIG. 6. Affinity purification of Skp1p tagged at the endogenous locus. Whole-cell extracts from wild-type (WT) and Skp1p-ZZ cell cultures were normalized by total protein concentration prior to affinity purification with IgG agarose. Aliquots of samples eluted by TEV protease cleavage were used for SDS-PAGE and silver staining or for peptide sequencing by mass spectrometry (2% and 40% of each fraction, respectively). Asterisks indicate exogenous polypeptides from the recombinant TEV protease preparation. *Tetrahymena* Skp1p (marked by an arrow) stains variably and often poorly with silver, perhaps as a consequence of its acidic isoelectric point. Protein identifications are presented using gene numbers and annotation from the *Tetrahymena* Genome Database (TGD). FBA, F-box-associated.

TERT ternary complex, which in turn can incorporate p75 and p45 to gain function at chromosome ends.

It remains to be determined whether an endogenously assembled p65-TER-TERT ternary complex recapitulates all of the catalytic activity features of the holoenzyme assayed in cell extract. It may instead have the severely reduced repeat addition processivity that is observed in assays of the recombinant p65-TER-TERT complex assembled in rabbit reticulocyte lysate (12, 22). Strains overexpressing TERT or TER alone do not have increased levels of telomerase RNP (15, 53), but simultaneous cooverexpression of p65, TER, and TERT might generate enough endogenously assembled ternary complex for future biochemical analysis. No reproducible change in catalytic activity was detected in extracts from *TAP45*, *TAP75*, and *SKP1* knockdown strains (data not shown), but these extracts still contain some wild-type holoenzyme. Conditional expression shutoff has been used to assay depletion phenotypes of some *Tetrahymena* proteins, using strains with transgene expression driven by an inducible promoter and gene knockout at the endogenous locus. This strategy is not universally appropriate and fails in the case of p75 and p45 for two reasons (Q. Tieu and K. Collins, unpublished data). First, even basal expression of most telomerase subunits from the inducible transgene promoter exceeds the endogenous expression level. As a consequence, it is impossible to obtain a shutoff phenotype. Second, transgene expression of telomerase subunits other than TERT or TER results in excess subunit accumulation, which interferes with holoenzyme function and alters telomere length even before promoter shutoff. New technology will be required to investigate the influence of p75 and p45 on the catalytic activity of the endogenously assembled p65-TER-TERT ternary complex.

The smallest protein that was detected in the TERT-TAP affinity purification, Skp1p, is distinct in biochemical, molecular, and genetic terms from the other TERT-TAP enriched proteins, p75, p65, and p45. Skp1p is an abundant, conserved,

multifunctional protein that acts by genetic criteria as a negative regulator of telomerase. Because the predominant Skp1p complexes are vastly more abundant than telomerase RNP, it remains possible that a minor fraction of Skp1p does associate with *Tetrahymena* telomerase holoenzyme. One attractive model for a possible function of Skp1p in telomerase holoenzyme context is provided by analogy to functions of *S. cerevisiae* Skp1p in the centromere DNA binding factor CBF3. Skp1p plays roles in CBF3 assembly, disassembly, and checkpoint signaling (28, 31, 40). Likewise, *Tetrahymena* Skp1p could play a role in telomerase holoenzyme assembly, disassembly, or regulation. In this scenario, *Tetrahymena* Skp1p but not other SCF components should be associated with TERT and/or telomerase holoenzyme.

Alternately, *Tetrahymena* Skp1p could function as part of an SCF complex that targets TERT for degradation. Indeed, recent work has demonstrated that human TERT degradation is regulated by a ubiquitin ligase (27). The *Tetrahymena* strain used for affinity purification may have been sensitized for the detection of TERT complexes involved in protein turnover, because TERT-TAP is expressed in this strain from the *BTU1* promoter instead of the weaker endogenous *TRT* promoter (53). With our current antibody reagents, we have not been able to detect coimmunopurification of endogenous *Tetrahymena* TERT and Skp1p (data not shown). As a third possible model, Skp1p could exert its influence on telomere length by modulating telomere structure. In support of this model, F-box proteins from SCF complexes in fission yeast and mammals target the degradation of telomere proteins (25, 30). However, in these cases, depletion of the F-box protein induces telomere shortening. Clearly, the telomere elongation phenotype resulting from *Tetrahymena* Skp1p depletion could reflect the combined impact of altering several cellular processes. We conclude that *Tetrahymena* Skp1p may not have a direct role in the regulation of TERT or telomerase holoenzyme, and yet the possibility of such a role is not precluded by our results.

Considered from a phylogenetic perspective, it is interesting that our characterization of the *Tetrahymena* telomerase holoenzyme proteins did not reveal any evidence of telomerase subunit conservation between ciliates and yeasts or vertebrates except in TERT. In fact, the *Tetrahymena* telomerase holoenzyme subunits may not even be conserved within ciliates. Affinity purification of *Euplotes aediculatus* telomerase by use of an oligonucleotide complementary to the template region of TER recovered an RNP containing TERT and the p65 ortholog p43, but it did not recover subunits corresponding to p75 or p45 (32). *Euplotes* telomerase holoenzyme equivalents of p75 and p45 could have dissociated during the extraction of macronuclei or as a consequence of affinity purification by oligonucleotide hybridization to TER. Alternatively, p75 and p45 subunits may have been lost in evolutionary adaptation of spirotrichous ciliates such as *Euplotes*, which have short, precise, particularly abundant macronuclear telomeres. While there are no obvious sequence homologs of p75 and p45 in the GenBank database, these *Tetrahymena* proteins are likely to have functional homologs in telomerase holoenzymes of other species. At the functional level, p75 and p45 are most analogous to the Est1p and Est3p telomerase proteins from *S. cerevisiae*. Each of the four proteins, p75, p45, Est1p, and Est3p, acts to give a biologically stable telomerase RNP its function at chromosome ends. Despite large differences between telomere structure in *S. cerevisiae* and that in *Tetrahymena*, our findings indicate that the telomerase-telomere interaction in both organisms is regulated by at least two specialized telomerase holoenzyme proteins. It will be interesting to apply the assays previously used in the characterization of Est1p and Est3p to investigate similarities and differences in the regulation of ciliate and yeast telomerases at telomeres.

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