Tetrahymena POT1a Regulates Telomere Length and Prevents Activation of a Cell Cycle Checkpoint[∇]

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The POT1/TEBP telomere proteins are a group of single-stranded DNA (ssDNA)-binding proteins that have long been assumed to protect the G overhang on the telomeric 3' strand. We have found that the *Tetrahymena thermophila* genome contains two POT1 gene homologs, POT1a and POT1b. The POT1a gene is essential, but POT1b is not. We have generated a conditional POT1a cell line and shown that POT1a depletion results in a monster cell phenotype and growth arrest. However, G-overhang structure is essentially unchanged, indicating that POT1a is not required for overhang protection. In contrast, POT1a is required for telomere length regulation. After POT1a depletion, most telomeres elongate by 400 to 500 bp, but some increase by up to 10 kb. This elongation occurs in the absence of further cell division. The growth arrest caused by POT1a depletion can be reversed by reexpression of POT1a or addition of caffeine. Thus, POT1a is required to prevent a cell cycle checkpoint that is most likely mediated by ATM or ATR (ATM and ATR are protein kinases of the PI-3 protein kinase-like family). Our findings indicate that the essential function of POT1a is to prevent a catastrophic DNA damage response. This response may be activated when nontelomeric ssDNA-binding proteins bind and protect the G overhang.

In order to maintain genome integrity, the telomeric DNA from cells with linear chromosomes is packaged into a protective nucleoprotein complex (10). In the absence of this complex, the telomeres are recognized as DNA damage and subject to repair by nonhomologous end joining (33). The resulting chromosome fusions lead to genome instability (36). The protective telomeric complex is composed of a series of unique telomere proteins that bind the double-stranded region of the telomeric DNA and/or the single-strand overhang on the 3' G-rich strand (37). Although the exact composition of the complex varies between species, vertebrates, yeasts, plants, and ciliates all use a series of structurally related proteins to protect their telomeres. The G-overhang binding proteins all bind G-strand DNA through a conserved OB fold motif, while the double-stranded DNA-binding proteins bind via a conserved myb motif (23, 37, 46).

In vertebrate cells, telomeres are packaged by a core complex of six proteins which function both in telomere protection and in telomere length regulation (10). This core complex (which is sometimes called shelterin) contains two double-stranded DNA-binding proteins, TRF1 and TRF2, which anchor the complex along the length of the telomere, and the TRF1/2 interaction partners Rap1, TIN2, and TPP1. The G-strand binding protein POT1 is also a component of the com-

plex. Although POT1 is secured into the complex through interactions with TPP1 (17, 31, 50), POT1 molecules are also thought to bind the G-strand overhang via their OB fold-containing DNA-binding domain (45, 46). Additional telomere-associated proteins include a number of DNA damage response factors (33). However, these factors appear to bind only transiently during replication of a functional telomere, and they do not activate a full DNA damage response (43). In contrast, when a telomere becomes dysfunctional due to telomere shortening or removal of telomere proteins, a full DNA damage checkpoint is activated via ATM or ATR protein kinases, members of the PI-3 protein kinase-like family. Activation of the checkpoint leads to accumulation of γ H2AX and other DNA repair proteins (3, 9, 15).

Members of the POT1 protein family were first isolated from the ciliates *Oxytricha nova* and *Euplotes crassus* because these cells contain literally millions of telomeres and hence large amounts of telomere protein (46). The ciliate proteins were named TEBPs (telomere end-binding proteins) because they bind telomeric G-strand DNA and show a preference for the 3' terminus. The POT1 proteins from vertebrates, yeasts, and plants were later identified based on their sequence identity to the OB fold motifs of the TEBP DNA-binding domain (2, 39). The *Schizosaccharomyces pombe* protein was named POT1 (protection of telomeres) because gene disruption results in complete loss of the telomeric DNA. However, removal of the vertebrate POT1 protein(s) has a more minor effect on telomere integrity (5, 16, 47). Although telomeres lacking POT1 elicit a DNA damage response, they do not lose their G-strand overhangs or undergo telomere shortening or rampant chromosome fusions. This lack of a severe telomere-uncapping phenotype, together with the unexpected finding that POT1 is a component of the core telomere protein complex, suggests that the function of POT1 is more com-

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plex than first envisioned. Interestingly, while humans have only one POT1 protein, *Euplotes*, plants, and mice have two or more TEBP/POT1 paralogs which show some separation of function (16, 39, 44). In particular, the mouse POT1b seems to regulate resection of the telomeric C strand, while POT1a plays a greater role in preventing a telomeric DNA damage response (16).

Unlike *Oxytricha* and *Euplotes*, the ciliate *Tetrahymena thermophila* can be manipulated genetically and so provides an excellent opportunity to study the in vivo function of ciliate TEBP/POT1 proteins. *Tetrahymena* cells contain two distinct nuclei: the germ line micronucleus, which contains five pairs of diploid chromosomes, and the transcriptionally active macronucleus, which contains 250 to 300 smaller chromosomes with a copy number of ~45 plus ~9,000 ribosomal DNA (rDNA) minichromosomes (22, 49). The resulting ~40,000 macronuclear telomeres are composed of 250- to 350-bp G₄T₂/C₄A₂ repeats. These telomeres have a very precise terminal DNA structure, as the C strands end with the sequence 5' C₃A₂- or 5' C₂A₂-, while most G strands extend another 14 or 15 nucleotides (nt) or 20 or 21 nt and terminate with the sequence 5'-T₂G₄T (20). Studies with telomerase-deficient cells have revealed that this precise G-overhang structure is generated by a series of specific G- and C-strand processing reactions (21). Although the nucleases responsible for telomere processing remain unknown, studies with yeast and vertebrate cells suggest that they are likely to be general DNA repair nucleases rather than dedicated sequence-specific telomere nucleases (4, 29). If this is the case, the specificity of overhang processing may come from G-overhang binding proteins providing the boundaries for nuclease digestion (19).

Given the potential for the *Tetrahymena* POT1/TEBP homolog to function in various aspects of telomere replication and protection, we set out to examine the phenotype of cells in which the POT1 gene is disrupted. We found that *Tetrahymena* has two POT1 genes that appear to have arisen by gene duplication and evolved different functions. We show here that POT1a is not required for correct overhang processing or G-overhang protection but that it is required to prevent activation of a cell cycle checkpoint and to regulate telomere length.

MATERIALS AND METHODS

***Tetrahymena* growth.** *Tetrahymena* cells were grown in 1.5× PPYS at 30°C as previously described (20). CdCl₂ was added to 2 µg/ml to maintain the expression of the rescuing POT1a allele. Cells were washed in 10 mM Tris (pH 7.5) prior to resuspension in medium without cadmium. To obtain the growth curves, the culture was adjusted regularly to keep the cells in log phase (a concentration of <2 × 10⁵ cells/ml). Caffeine was added to 0.3 mM. Growth curves were performed with multiple POT1a clones.

Generation of POT1a cell lines. Cells expressing tandem affinity purification (TAP)-tagged POT1a (TAP-POT1a) were generated using biolistic transformation to introduce the TAP-POT1a gene replacement construct into the native POT1a gene locus. The construct contained a TAP-tagged version of the synthetic POT1a gene and a cycloheximide marker cassette flanked by genomic DNA from 5' and 3' of the native POT1a gene. The 5' genomic sequence terminated at the initiating ATG, so targeted integration would result in expression of the TAP-POT1a from the native POT1a promoter. Transformed cells were selected with increasing amounts of cycloheximide until about half of the wild-type (WT) genes were replaced with the tagged version. EcoRI digestion of genomic DNA gave rise to a 4.8-kb band from the native gene locus and a 3.2-kb band from the TAP-POT1a gene.

The conditional POT1a knockout was generated essentially as described for

FIG. 1. Identification of *Tetrahymena* POT1 paralogs. (A) Sequence identity between *Tetrahymena* POT1a, *Tetrahymena* POT1b, and the *Oxytricha nova* TEBP α subunit. (B) ChIP experiment showing that POT1a is present at rDNA telomeres. Panel I, schematic of the macronuclear rDNA telomere. Arrows indicate positions of PCR primers used to amplify subtelomeric (Tel) and internal (Int) segments. Panel II, multiplex PCR products obtained from a representative ChIP assay. Lanes: 1 to 3, threefold serial dilutions of the input DNA delineating the linear range of the PCR; 4 to 6, chromatin from WT cells; 7, chromatin from TAP-POT1a cells. Lane 4, no antibody; lane 5, histone H3 antibody; lane 6, purified POT1a antibody; lane 7, IgG beads. Panel III, average enrichment of telomeric versus internal sequences from ≥ 3 independent experiments. ID, identity; Sim, similarity; CDS, coding sequence.

the TERT knockout (19). CU522 cells were transformed with a gene-targeting construct that replaced exons 2 to 5 of the endogenous POT1a gene with a neomycin resistance cassette (exon 1 encodes only seven amino acids), and cells were selected in paromomycin to obtain partial gene replacement. The rescuing synthetic POT1a allele was then introduced into the BTU1 (*beta tubulin*) gene locus, and transformants were selected in 20 µM paclitaxel (12). To obtain the full gene replacement, cells were further selected in paromomycin in the presence of 2 µg/ml CdCl₂.

Chromatin immunoprecipitation (ChIP). Cells were fixed with 1% formaldehyde for 3 min and then washed with wash buffer I (phosphate-buffered saline, pH 6.5), wash buffer II (10 mM HEPES, pH 6.5, 25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA), and wash buffer III (10 mM HEPES, pH 6.5, 200 mM NaCl, 10 mM EDTA, 0.5 mM EGTA). The final pellet was resuspended in 50 mM Tris, pH 8.0, 2% sodium dodecyl sulfate (SDS), 10 mM EDTA plus protease inhibitor cocktail (5 µg/ml antipain, 2 µg/ml aprotinin, 16 µg/ml benzamide, 6 µg/ml chymostatin, 1 µg/ml E64, 5 µg/ml leupeptin, and 1 µg/ml pepstatin A); sonicated to shear DNA to 500 to 1,000 bp; and clarified by centrifugation at 16,000 × g. Fifty microliters of supernatant (extract from 2.5 × 10⁶ cells) was mixed with antibody to histone H3 or full-length POT1a in 1 ml lysis buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate [NaDoc] plus protease inhibitor cocktail) overnight at 4°C. The antibody complexes were precipitated with protein A Sepharose for the wild type, or with immunoglobulin G (IgG) Sepharose for TAP-POT1a, for 1 h at 4°C. Precipitates were washed sequentially with 0.5 ml radioimmunoprecipitation assay buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% NaDoc, 1% NP-40), 0.5 ml high-salt solution (50 mM Tris, pH 8.0, 500 mM NaCl, 0.1% SDS, 1% NP-40), 0.5 ml LiCl solution (250 mM LiCl, 50 mM Tris, pH 8.0, 0.5% NaDoc, and 1% NP-40), and 0.5 ml Tris-EDTA, pH 8.0, twice. Antigens were eluted with 1% SDS and 0.1 M NaHCO₃, and cross-linking was reversed by heating to 65°C for 4 h. The DNA was purified and analyzed by multiplex PCR using the Int and Tel primers (Fig. 1B). To determine the relative amount of each product, one oligonucleotide of each primer pair was

5' end labeled. The products were then separated in 3% agarose gels with Tris-borate-EDTA buffer, the gels were dried, and the products were quantified by PhosphorImager analysis.

Indirect immunofluorescence. *Tetrahymena* cells were permeabilized for 3 min in PHEM buffer {60 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)], 25 mM HEPES, 10 mM EGTA, and 2 mM MgCl₂, pH 6.9} plus 0.5% Triton X-100 and then fixed for 2 h at 4°C in PHEM buffer plus 3% formaldehyde as described by Morrison et al. (35). The POT1a antibody was made by injecting rabbits with full-length recombinant POT1a protein. The antibody to *Tetrahymena* pericentrin was a gift from Mark Winey, University of Colorado.

Flow cytometry. *Tetrahymena* cells were lysed in a buffer containing 0.25 M sucrose, 10 mM MgCl₂, and 0.5% Triton X-100 at a concentration of 1×10^6 cells/ml (35). Propidium iodide was added to 10 µg/ml, and nuclei were stained for 30 min prior to analysis using a FACSCalibur instrument. Data were plotted using CellQuest software. To ensure visualization of the normal macronuclear G₂ DNA content, the cadmium-grown cells were harvested from log-phase cultures.

Telomere analysis. Telomere length was determined by Southern hybridization to restriction-digested genomic DNA using previously described subtelomeric probes to the rDNA and macronuclear telomeres 8 and 10 (21). Overhang lengths and 3' terminal sequences were analyzed using the oligonucleotide ligation and primer extension assay described by Jacob et al. (19, 20). Oligonucleotide duplexes were prepared by heating to 85°C and slow cooling, and 1 pmol duplex was then ligated to 4 µg of genomic DNA. The DNA was separated on 0.7% agarose gels and blotted to nylon membrane, and the signal was quantified using a PhosphorImager. To normalize for DNA loading, the membrane was decayed and then hybridized with a probe to the rDNA gene and requantified. The signal for each ligation reaction was then divided by the corresponding rDNA signal. To compare the relative amounts of overhang present at different times after cadmium removal, the signals obtained with the six different guide oligonucleotides were summed and divided by the sum of the corresponding rDNA hybridization signals. G-overhang length was measured as described previously (19, 20). Briefly, oligonucleotide duplex was ligated to Alu1/HinfI-digested DNA, and the DNA was purified on a QIAGEN PCR purification column to remove unligated duplex. The guide oligonucleotide was primer extended with T4 DNA polymerase in the presence of dCTP and dATP. Primer extension products were separated in sequencing gels, and the signal was quantified by use of a PhosphorImager.

RESULTS

Identification of *Tetrahymena* POT1 paralogs. Searches of the *Tetrahymena* genome database revealed two genes encoding proteins with sequence identity to the α subunit of the *Oxytricha* TEBP and the vertebrate POT1 proteins. The *Tetrahymena* genes, named POT1a and POT1b, lie 1.3 kb apart on the same macronuclear chromosome, suggesting that the second gene arose by a gene duplication. To determine the full protein sequence encoded by each gene, we used reverse transcriptase PCR (RT-PCR) to amplify the cDNA. We were able to obtain the complete sequence for POT1a, but the low abundance of the POT1b mRNA prevented us from amplifying the extreme 5' end. We therefore used the TIGR gene prediction, together with sequence alignment to the *Oxytricha* and *Euplotes* TEBPs, to deduce the sequence of the terminal ~78 amino acids. Based on this, the two *Tetrahymena* POT1 proteins are 44% identical and 57% similar to each other and 24 to 25% identical to the *Oxytricha* TEBP (Fig. 1A). The predicted OB fold motifs in the DNA binding domain are 20 to 26% identical to those of human POT1.

We next used ChIP to investigate whether the POT1 proteins bind to macronuclear telomeres. Initially we performed the ChIP with an antibody raised against full-length POT1a which has over 200-fold-greater affinity for POT1a than for POT1b (data not shown). *Tetrahymena* cells were cross-linked with formaldehyde and sonicated, and the soluble chromatin was immunoprecipitated with POT1a antibody or a control

antibody to histone H3. Following reversal of the cross-links, the precipitated DNA was amplified by multiplex PCR using primer pairs designed to amplify either a subtelomeric region of the rDNA molecule that lies immediately adjacent to the telomere or an internal region located 3.1 kb from the telomere (Fig. 1B). As shown in Fig. 1B, lane 5, the anti-histone antibody preferentially precipitated the internal region of the rDNA. This was expected because nucleosomes are largely excluded from *Tetrahymena* telomeres (6). In contrast, precipitation with the POT1a antibody resulted in a 7.5-fold enrichment of the PCR product from the subtelomeric region over what was seen for the internal region (lane 6). No enrichment was observed for either region in the absence of antibody or when the sheared chromatin was prepared without formaldehyde cross-linking. We therefore conclude that POT1a is located at macronuclear telomeres.

To ensure that the above-described results did not reflect cross-reactivity of the POT1a antibody with POT1b, we also performed ChIP using a cell line expressing TAP-tagged POT1a. The cell line was made by integrating a TAP-tagged POT1a cDNA into the POT1a gene locus (Fig. 2A and B). Following drug selection, the extent of gene replacement was monitored by Southern hybridization with a probe 5' to the POT1a-coding sequence (Fig. 2C, panel I). Once partial gene replacement was achieved (~50%), we performed a ChIP analysis using IgG beads to precipitate the TAP-tagged protein. These experiments confirmed that POT1a is present at rDNA telomeres, as we again saw enrichment of the subtelomeric versus internal PCR products (Fig. 1B, lane 7). We also generated a cell line expressing hemagglutinin-tagged POT1b (data not shown), but to date we have been unable to detect either the hemagglutinin-tagged or the wild-type POT1b protein and have thus been unable to confirm its telomeric location.

Generation of a conditional POT1a knockout cell line. To learn more about the function of the *Tetrahymena* POT1 proteins, we set out to disrupt the macronuclear version of each gene. We were able to obtain a full macronuclear gene knockout for POT1b (data not shown), indicating that this gene is not essential. However, preliminary studies did not indicate an obvious phenotype, so we postponed further studies of this cell line. When we transformed cells with a POT1a gene disruption construct (Fig. 2A) and selected with increasing concentrations of paromomycin, we were unable to disrupt all the copies of the endogenous gene. Since this indicated that POT1a is essential, we introduced a cadmium-regulated version of the POT1a gene into the knockdown cells (Fig. 2B). The conditional gene consisted of a synthetic POT1a cDNA under control of the metallothionein (MTT1) promoter (19, 40). The synthetic gene encoded the same protein sequence as the native POT1a gene but had different codon usage, so it could be expressed in either *Escherichia coli* or *Tetrahymena* (8). Expression of this gene in the presence of cadmium was expected to provide a level of POT1a protein sufficient to allow the disruption of all copies of the endogenous POT1a gene.

Southern hybridization was used to verify the introduction of the synthetic POT1a gene and the disruption of the endogenous gene (Fig. 2C, panel II). Hybridization with a probe to the synthetic POT1a gene gave rise to bands of ~2.8 and 4.2 kb with restriction-digested DNA from the conditional cell line

FIG. 2. Generation of POT1a knock-in and knockout cell lines. (A) Gene-targeting constructs used to generate the TAP-POT1a and conditional POT1a gene replacement construct (I) and POT1a gene knockout construct (III) were recombined into the native POT1a gene locus (II). The MTT1-synPOT1a rescuing allele (V) was integrated into the beta tubulin gene locus (IV). Exons are labeled as E1 to E8. Southern blot probes are shown as black bars. Restriction sites are marked as follows: E, EcoRI; X,

but not from the parental cells, indicating the successful introduction of the conditional gene. Likewise, a probe to the POT1a gene locus gave the expected 2.0-kb band with DNA from the parental cells and a 2.7-kb band with DNA from the knockout cells. The wild-type 2.0-kb band was almost gone from samples from the knockout cells, and the residual signal was at the level expected for hybridization to the transcriptionally inactive micronuclear POT1a gene. Moreover, when RT-PCR was used to detect endogenous POT1a expression, mRNA could be detected in the wild-type cells but not in the knockout cells (Fig. 2D, panel I). We therefore conclude that the endogenous macronuclear POT1a gene had been successfully disrupted.

Loss of POT1a leads to growth arrest and a monster cell phenotype. To check that the synthetic POT1a gene showed the expected cadmium-dependent regulation, we used RT-PCR to monitor mRNA levels after growth with and without cadmium. Although POT1a mRNA could be detected in cells grown with cadmium, the amount of PCR product was decreased ~20-fold 20 h after cadmium was withdrawn (Fig. 2D, panel II), indicating that gene expression had been repressed. We also performed indirect immunofluorescence with the POT1a antibody to monitor the distribution of the POT1a protein. As predicted for a telomere protein, we observed macronuclear staining when cells were grown in the presence of cadmium (Fig. 3A). However, this staining started to disappear within a few hours after cadmium removal and was completely gone by 20 to 24 h. It is interesting that we were unable to see micronuclear staining in either wild-type or knockout cells grown with cadmium. Thus, POT1a must either be absent from or present at very low levels in the micronucleus.

XbaI; B, BamHI; P, PstI. (B) Strategy used for gene targeting and phenotypic assortment. The knock-in or knockout gene-targeting constructs recombine with a few of the ~45 macronuclear chromosomes carrying the targeted gene to confer a low level of drug resistance. Continuous culturing with increasing drug concentrations then drives phenotypic assortment so that a growing number of chromosomes carry the gene disruption/replacement. If the disrupted gene is non-essential, all the copies of the wild-type gene are eventually replaced (cell I). If it is essential, the wild-type chromosomes will be reduced to the minimum required for viability (cell II). A complete knockout of an essential gene is generated by introducing a rescuing copy of the wild-type gene under the control of the regulated MTT1 promoter (cell III). (C) Southern blots showing POT1a gene replacement and gene disruption. Panel I, TAP-POT1a cells. EcoRI digested DNA was hybridized with a probe to the POT1a gene locus. endPOT, 4.8-kb band from the WT gene locus; TAP-POT, 3.2-kb band from the gene replacement. Panel II, conditional POT1a cells. PstI/BglII-digested DNA from WT and POT1a knockout cells was hybridized with probes to synthetic POT1a and the endogenous POT1a gene locus. endPOT, band from the WT POT1a gene; KO, band from the gene disruption; synPOT, bands from synthetic POT1a gene. M, molecular size marker. (D) RT-PCR showing loss of endogenous POT1a expression and cadmium regulation of the synthetic POT1a gene. mRNA was amplified by RT-PCR following treatment with (+) or without (–) reverse transcriptase. Panel I, RNA from wild-type cells (lanes 1 and 2) or POT1a cells (lanes 3 and 4) was amplified with primers to endogenous POT1a (top) or telomere protein p80 (bottom). Panel II, RNA from conditional POT1a cells grown with (+ Cd) or without (– Cd) cadmium for 20 h was amplified with primers to synthetic POT1a (top) or telomere protein p80 (bottom). Lanes 1 to 3 and 5 to 7 show threefold serial dilutions of the input cDNA.

FIG. 3. Growth phenotype after POT1a depletion. (A and C) Microscopy of POT1a conditional cells. Fixed cells were stained with purified POT1a antibody or serum and DAPI (A) or pericentrin antibody (C). Cells were grown with (+Cd) or without (–Cd) cadmium for 24 h. The POT1a serum cross-reacts with cytoplasmic components, so the whole cell can be visualized. (B) Growth curves for four POT1a clones grown with or without cadmium. (C) FACS analysis showing DNA content of POT1a cells grown with Cd (panel I) or without Cd (panel II) for 24 h. A mix of cells grown with and without Cd is shown in panel III.

In addition to the change in POT1a expression, cadmium removal caused a dramatic change in the growth rate and cell size. To monitor the change in growth rate more carefully, we isolated cells at intervals after cadmium removal and counted them. As shown in Fig. 3B, the cells divided two or three times during the first 10 to 12 h but then underwent a growth arrest. This growth arrest was quite robust, as the cells remained viable but unable to divide for 2 to 5 days. With prolonged culturing, the cell number gradually dwindled as some cells died. Following the initial growth arrest, the size of the cells increased so that by 24 h the cells were extremely large and almost round (Fig. 3A and C). DAPI (4',6'-diamidino-2-phenylindole) staining combined with light microscopy revealed that both the macronucleus and the cytoplasm were greatly enlarged (Fig. 3A). Despite their large size, staining with an-

tibody to pericentrin (Fig. 3C) and tubulin (data not shown) indicated that the cells had a normal number of ciliary rows.

DAPI staining of POT1a-depleted cells indicated that most cells contained one normally sized micronucleus and one greatly enlarged macronucleus. This led us to ask whether the cells had undergone additional rounds of macronuclear replication (endoreduplication) following the inhibition of cell division or whether the large size of the macronucleus reflected decondensation of the macronuclear chromatin. To answer this question, we used fluorescence-activated cell sorter (FACS) analysis to examine the DNA content of cells grown with and without cadmium. We did not attempt to separate the macronuclei from the micronuclei prior to the analysis because the micronuclear DNA content is only 1/20 of that of the macronucleus and in *Tetrahymena* the micronucleus and macronucleus remain firmly associated throughout most of the cell cycle. When FACS analysis was performed on propidium iodide-stained cells, it was apparent that the POT1a-depleted cells contained significantly more macronuclear DNA (Fig. 3D). Although the increases in DNA content varied between clones, the median DNA content of cells grown without cadmium for 24 h was on average double that of asynchronous cultures grown with cadmium, and it was also much greater than that expected for a normal G₂ macronucleus. We therefore conclude that the macronuclear DNA of the POT1a-depleted cells had undergone endoreduplication after the growth arrest. Moreover, the endoreduplication appeared to occur soon after arrest, because there was no further increase in DNA content between 24 and 30 h.

POT1a depletion does not cause telomere fusions or alter G-overhang structure. The strong growth arrest after POT1a depletion suggested that loss of POT1a from the telomere might have resulted in catastrophic deprotection of the telomeric DNA. Since depletion of POT1 is known to cause an alteration in G-overhang structure and telomere fusions in some plant and animal cells (2, 39), we examined *Tetrahymena* POT1a cells for both these events. To detect telomere fusions, we used Southern blots to look for dimerization of the rDNA minichromosome. As the rDNA has a copy number of ~9,000 and contributes approximately half of the telomeres in a cell, frequent telomere fusions should result in the appearance of rDNA dimers. However, no such dimers could be observed with DNA isolated from POT1a-depleted cells (data not shown). This finding suggested that POT1a depletion does not cause frequent G-overhang loss, as chromosomes lacking an overhang tend to be fused end to end by nonhomologous end-joining reactions (3). We also attempted to detect low-frequency fusions by PCR, but control reactions with constructs mimicking an rDNA telomere fusion were unsuccessful.

Tetrahymena telomeres have a very precise G-overhang structure that is generated by a series of 3' and 5' DNA-processing steps. Since these processing steps may require G-overhang binding proteins to define the processing boundaries (19), we next examined whether the removal of POT1a affects the structure of the G overhang. To achieve this, we used an established oligonucleotide ligation and primer extension assay that can determine the most 3' sequence of the overhang and the overall overhang length at a nucleotide resolution (19, 20). In this assay, a duplex is formed between a unique oligonucleotide and a complementary guide oligonucleotide that has an

FIG. 4. G-overhang structure after POT1a depletion. (A) Diagram showing ligation and primer extension assays to monitor the 3' sequence and length of the G overhang. A duplex formed from the unique-sequence oligonucleotide and one of six guide oligonucleotides is ligated to the 3' overhang. The 5'-end-labeled unique oligonucleotide is used to identify the ligation products. To measure overhang length, the 5'-end-labeled guide oligonucleotide is primer extended with T4 DNA polymerase, and the extension products are separated in sequencing gels. (B) Ligation assay showing that POT1a removal does not alter the 3'-terminal nucleotide. Upper panels show ligation products obtained with each guide oligonucleotide at various times after POT1a depletion. The corresponding G-overhang terminus is shown above each lane. Lower panels show loading controls where upper

TABLE 1. Sequences of guide and unique oligonucleotides^a

3' end of telomere	Guide oligonucleotide sequence
5'-TTGGGG	ACGACTCACTATAGGGCCCCA
5'-GTTGGG	ACGACTCACTATAGGGCCCCA
5'-GGTTGG	ACGACTCACTATAGGGCCCAAC
5'-GGGTTG	ACGACTCACTATAGGGCAACC
5'-GGGGTT	ACGACTCACTATAGGGAACCC
5'-TGGGGT	ACGACTCACTATAGGGACCCC
Unique oligonucleotide	CCCTATAGTGAGTCGTATTA

^a Boldface type indicates regions of hybridization.

additional 5 nt of telomeric sequence at the 3' end (Fig. 4A). The resulting 5-nt overhang can act as an adaptor that allows ligation of the unique oligonucleotide to the G overhang. However, ligation will take place only if the permutation of the telomeric sequence on the guide oligonucleotide matches the 3' end of the chromosome (Table 1). Thus, by performing ligation reactions with guide oligonucleotides corresponding to all six permutations of the telomeric repeat, one can determine the sequence at the 3' end of the chromosome. The length of the overhang is then determined by extending the guide oligonucleotide to the junction between the G overhang and duplex telomeric DNA by use of T4 DNA polymerase. As this polymerase lacks strand displacement activity, the length of the end-labeled primer extension product reflects the length of the overhang.

To examine the sequence at the terminus of the overhang, we performed ligation reactions with each of the six possible guide oligonucleotides and genomic DNA from the POT1a cells. The reaction products were then analyzed by electrophoresis in agarose gels. As previously observed for wild-type cells (20), the predominant reaction product came from ligation reactions using the guide oligonucleotide that was complementary to overhangs terminating in the sequence 5'-G₄T (Fig. 4B). This was true for POT1a cells grown both with and without cadmium. There was a slight increase in the reaction product corresponding to overhangs terminating in 5'-G₄T₂, but PhosphorImager analysis indicated that the fraction of 5'-G₄T₂ increased only to 10 to 15%, which is less than the ~20% of 5'-G₄T₂ telomeres observed after telomerase deple-

panels were decayed and then hybridized with an rDNA probe. Numbers at the bottom indicate the percentage of overhangs (O/H) terminating in each sequence. Arrows indicate products from rDNA; arrowheads mark products from chromosomal telomeres. (C) Histogram comparing the total amounts of ligation product obtained with all six guide oligonucleotides at different times after POT1a depletion. (D) Primer extension assay showing that POT1a removal does not alter G-overhang length. The gel shows reaction products obtained with DNA from POT1a cells grown with (+Cd) or without (-Cd) cadmium for 6 to 48 h. Products corresponding to overhangs of 14, 20, 26, and 32 nt are marked at the right; positions of marker oligonucleotides are shown on the left. The diagram illustrates the products expected from a 14-nt overhang. (E) Histogram showing the relative abundances of the different lengths of overhang before and after POT1a depletion. The amount of reaction product was quantified for each set of bands by PhosphorImager analysis and then normalized to the total amount of signal in that lane. Data are averages of two independent experiments.

tion (19). We therefore conclude that the loss of POT1a from the telomere does not significantly affect the processing of the chromosome 3' end.

As the total amounts of reaction product obtained with the six guide oligonucleotides reflect the amount of G-overhang DNA available for ligation, we were also able to assess whether POT1a depletion left the overhangs more susceptible to degradation. When we compared the total amounts of ligation product obtained at different times after cadmium removal we did find a slight decline, indicating that some telomeres either lacked overhangs or had shorter overhangs (Fig. 4C). However, as almost 80% of the telomeres had completely normal overhangs (Fig. 4B; also see below), it appears that a protein(s) other than POT1a can protect the overhang from degradation.

We next used the primer extension reaction to assess whether the depletion of POT1a alters G-overhang length and hence C-strand processing. As before, the unique oligonucleotide-guide oligonucleotide duplex was ligated to DNA from cells harvested at various times after cadmium depletion, the guide oligonucleotide was then primer extended with T4 DNA polymerase, and the reaction products were analyzed in sequencing gels. Since most of the telomeres from POT1a cells terminate in 5'-G₄T, the ligation was performed only with duplexes containing the guide oligonucleotide corresponding to this sequence. Examination of the primer extension products revealed that POT1a depletion had little effect on overhang length (Fig. 4D). As previously observed for wild-type cells, the products differed in length by multiples of 6 nt, with the most prominent products corresponding to overhangs of 14 or 15, 20 or 21, 26 or 27, or 32 or 33 nt. When the relative abundances of the various lengths of reaction product were measured for different experiments, it became apparent that the general distributions of longer versus shorter overhangs also remained quite similar. We therefore conclude that POT1a is not required for correct processing of either the telomeric G or C strands; nor is it essential to protect the overhang from degradation.

POT1a removal results in telomere elongation. As POT1 binding to the G-strand overhang has the potential to regulate telomerase access to the telomere (27, 41), we next examined whether the removal of POT1a affects telomere length. We first analyzed the rDNA telomeric restriction fragments from cells isolated at various time points after cadmium removal. During the first 10 h, the telomeres remained essentially unchanged, but by 20 h, telomere length had increased, and this increase continued over the next several days (Fig. 5A). The increase in length appeared to have two modes. For most telomeres, the median increase in length was 400 to 500 bp, and the elongation occurred quite gradually. The magnitude and gradual nature of this increase is reminiscent of the telomerase-mediated telomere growth that occurs when *Tetrahymena* cells are maintained in log-phase culture (1, 21). However, a subpopulation of telomeres showed a dramatic elongation, reaching lengths in excess of 10 kb, which is 30- to 40-fold longer than normal. Although this rapid telomere elongation resembles the recombination-based ALT (alternative lengthening of telomeres) elongation pathway observed in yeast and mammalian cells that lack telomerase (14, 25), recombination-based telomere elongation has not previously been observed in *Tetrahymena*. Thus, the mechanism for the rapid elongation is unclear.

FIG. 5. Effect of POT1a depletion on telomere length. Southern blots showing the length of the rDNA telomere (A) and non-rDNA telomere 8 (B) after growth with (+Cd) or without (-Cd) cadmium for 6 to 142 h. Telomeric restriction fragments were released with HindIII and identified with chromosome-specific subtelomeric probes. Brackets mark the telomeres that decreased in size. M, molecular size marker.

Although most telomeres became longer after POT1a depletion, a small fraction actually decreased in size (Fig. 5A and data not shown). This suggests that in the absence of POT1a, the DNA terminus had become more accessible to both lengthening and shortening activities.

To determine whether telomere growth was restricted to the abundant rDNA telomere or was a general phenomenon shared by all the macronuclear telomeres, we examined the length profiles of telomeres from two different non-rDNA chromosomes (telomeres 8 and 10) (21). In each case, the telomeric restriction fragments were identified by hybridization to a chromosome-specific subtelomeric probe. As observed for the rDNA telomere, each of the non-rDNA telomeres increased in length after POT1a depletion (Fig. 5B and data not shown). While the extents of the increase varied between chromosomes, in all cases the length increase occurred between 10 and 20 h after cadmium removal. Moreover, while many of the telomeres increased in length by only a few hundred base pairs, some underwent the same dramatic increase in length observed for the rDNA telomeres.

Examination of samples isolated at more closely spaced time points indicated that the length change first became visible between 10 and 15 h after cadmium withdrawal (data not shown). Growth curves for the same cultures indicated that this was right around the time that the cells stopped dividing. Thus, the telomere growth observed after POT1a depletion occurs in nondividing cells and hence is different from the progressive elongation that occurs over many cell divisions when *Tetrahymena* cells are kept in continuous culture (30). It also differs

FIG. 6. Reversal of the growth arrest. Growth curves showing POT1a cells grown with (+ Cd) or without (– Cd) cadmium and with (+ caf) or without (– caf) caffeine. (A) Cd or caffeine was added 24 h after the original cadmium removal. (B) Caffeine was added at the time of Cd removal. Data shown in panels A and B are averages of two experiments.

from the telomere elongation observed in various yeast mutants (28, 34) and mammalian cells expressing mutant forms of POT1 (7, 32), as in these situations the elongation also occurs in dividing cells.

POT1a removal activates a reversible cell cycle checkpoint.

The prolonged viability of POT1a-deficient cells together with the lack of a catastrophic telomere phenotype (e.g., telomere loss or telomere fusions) led us to ask whether reexpression of POT1a could reverse the growth arrest that occurred after POT1a depletion. We found that this was indeed the case, as the readdition of cadmium 24 h after the initial cadmium withdrawal allowed the cells to resume growth at a normal rate (Fig. 6A). This finding suggested that the growth arrest and consequent lack of viability of POT1a-deficient cells might result from POT1a-depleted telomeres activating a DNA damage checkpoint. To explore this possibility, we tested whether cell cycle arrest could be prevented by the addition of caffeine. Although caffeine has pleiotropic effects on cells, it is an effective inhibitor of ATM and ATR and has been used to abrogate DNA damage checkpoints in a variety of organisms, including *Tetrahymena* (24, 26, 48). For example, the addition of caffeine to *Tetrahymena* cells that have suffered DNA damage during S phase abrogates the intra-S-phase checkpoint and allows the cells to resume proliferation (48). However, many cells then die due to uneven macronuclear division. Although ciliate ATM and ATR have not been studied directly, the *Tetrahymena* genome contains obvious homologs of ATR, Chk1, and Chk2.

When we added caffeine to POT1a-depleted cells, we saw a reversal of the growth arrest and cell division (Fig. 6A and B). If the caffeine was added 24 h after cadmium removal, the very large POT1a-depleted cells divided and the average cell size became much smaller (Fig. 6A and data not shown). The cells then continued to divide, but as is observed after DNA damage, many of the cells underwent unequal macronuclear division and died, so the total number of live cells doubled only two or three times. A similar result was observed when the caffeine was added at the time of cadmium removal, as the cells no longer arrested after two or three population doublings but instead underwent several more divisions despite the lack of POT1a (Fig. 6B). Thus, even though the addition of caffeine cannot sustain long-term growth in the absence of POT1a, it clearly reverses the growth arrest that occurs upon POT1a depletion. We therefore conclude that the growth arrest

caused by POT1a depletion is most likely caused by an ATM- and/or ATR-mediated checkpoint. This finding suggests that a major function of POT1a is to hide the telomere from the DNA damage-sensing machinery.

DISCUSSION

We have found that *Tetrahymena* cells resemble those of *Arabidopsis thaliana*, *Euplotes*, and mice in that the genome harbors multiple POT1 genes. While the two *Tetrahymena* genes appear to have arisen from a gene duplication, they have diverged more than the two murine genes, as the *Tetrahymena* proteins are only 44% identical, whereas the murine proteins are 72% identical (16). By analyzing the phenotype of *Tetrahymena* POT1 knockout cells, we have shown that the evolution of the two paralogs has resulted in a separation of function. The POT1b gene is not essential in vegetative cells, and depletion of the POT1b protein has no effect on cell growth or telomere length (R. Lescasse, unpublished data). In contrast, deletion of POT1a causes a dramatic cell cycle arrest and telomere elongation.

Based on the phenotype of the *S. pombe* POT1 gene disruption and the DNA-binding properties of the *Oxytricha* and vertebrate POT1 proteins (2, 45, 46), the disruption of vertebrate and ciliate POT1 genes was expected to lead to degradation of the G-strand overhang and a severe telomere-uncapping phenotype. However, we have found that loss of POT1a, the only essential *Tetrahymena* POT1 protein, has almost no effect on the terminal DNA structure; G-overhang processing is normal, the resulting overhangs are quite stable, and the telomeres do not undergo rampant end-to-end fusions. Instead of being required for G-overhang protection or processing, POT1a is essential because it is needed to prevent a cell cycle arrest that is most likely due to an ATM- and/or ATR-mediated DNA damage checkpoint. A secondary function is to regulate telomere length.

It is striking that loss of POT1a causes such a complete cell cycle arrest even though both G-overhang structure and the length of the telomeric DNA are unperturbed when the arrest first occurs. This observation indicates that other proteins must be responsible for directing correct overhang processing and protecting the overhang from nuclease attack, but also that only POT1a is able to package the DNA in such a way as to prevent the chromosome end from activating a DNA damage

checkpoint. Interestingly, the phenotype of *Tetrahymena* POT1a-deficient cells is rather similar to that observed with the *Saccharomyces cerevisiae* *cdc13-1* mutation. Although the telomeres from Cdc13-1 cells undergo extensive C-strand degradation, the G overhangs are not removed, and they arrest in G₂/M phase (13). The *Tetrahymena* POT1a phenotype is even more similar to what has recently been reported for mouse and chicken cells that lack POT1 (5, 16, 47). In neither vertebrate does loss of POT1 result in G-overhang removal, loss of telomeric DNA, or frequent telomere fusions. In both cases, however, the cells undergo a growth arrest, and in chicken cells, the arrest has been shown to result from the activation of an ATM- and/or ATR-mediated telomeric DNA damage checkpoint (5) (D. Churikov and C. Price, unpublished results). Thus, while the participation of POT1, or POT1 homologs, in processes such as telomere length regulation or C-strand processing may vary between organisms, it appears that the essential function of POT1 is to prevent a catastrophic telomeric DNA damage response and that this function has been conserved in widely diverse organisms.

The telomere length phenotype caused by depletion of POT1a is unusual because unlike most defects in telomere length regulation where the increase or decrease in length occurs gradually over many cell divisions (7, 28, 32, 34), the telomere growth in POT1a-deficient cells happens after the cells have stopped dividing. Moreover, the extent of growth can be quite dramatic, with some rDNA telomeres reaching in excess of 10 kb or >30-fold their normal length. In some ways, the increase in telomere length in the nondividing cells is reminiscent of the kinetics of primer elongation in an in vitro telomerase reaction where, with time, an increasing number of repeats are added to the DNA terminus. It therefore appears that in the absence of POT1a the telomeres are maintained in an open configuration that allows continued access to telomerase and/or the ALT recombination machinery over long periods of time. The prolonged presence of such an open telomere configuration could also explain why a small fraction of the telomeres become shorter after POT1a depletion, as presumably the DNA terminus would also remain accessible to nucleases.

Whether or not the telomere growth in POT1a-deficient cells reflects a direct role for POT1a in telomere length regulation is unclear. On one hand, POT1a might directly limit telomere growth by blocking access to telomerase and/or the ALT machinery, a situation that has been observed during in vitro studies with human POT1 (27). However, it is also possible that the telomere growth simply reflects the lack of cell cycle progression caused by checkpoint activation. Telomere growth might normally be restricted by rapid transition into a stage of the cell cycle where the entire telomere (not just the overhang) becomes packaged into an inaccessible chromatin conformation.

One way that POT1a could simultaneously prevent checkpoint activation and limit telomerase action would be through the simple act of binding the G overhang, thus displacing ssDNA-binding proteins that activate a checkpoint response (e.g., RPA) and preventing telomerase from pairing with the 3' terminal repeat. Given that the G- and C-strand processing reactions required to generate the correct overhang structure occur normally in absence of POT1a, this binding most likely

occurs after telomere replication and processing is complete. If POT1a also interacts with the double-stranded region of the telomeric tract, as has been observed for the vertebrate protein (17, 31, 50), it might also help package the telomere into a closed chromatin conformation. This could again limit access to telomerase, the ALT machinery, and mediators of the DNA damage response.

The next obvious question concerns which protein(s) provides the boundaries for overhang processing and, in the absence of POT1a, protects the overhang from degradation. While overhang protection could be achieved by a number of proteins that are known to bind telomeric G-strand DNA (e.g., RPA or various hnRNPs [11, 18, 38]), POT1b is the most obvious candidate to direct overhang processing. One possibility is that POT1b binds transiently to the overhang during telomere replication and processing but is then replaced by POT1a later in the cell cycle. Such an S-phase-specific function would explain the low levels of POT1b in asynchronous populations of cells and our difficulty in detecting POT1b binding to telomeres. Indeed, a POT1 paralog with such an S-phase-specific function was identified for *Euplotes* some years ago (44). The *Euplotes* replication telomere protein (rTP) is expressed only in replicating cells, and the protein localizes very specifically to replication bands, the sites of DNA replication in the *Euplotes* macronucleus (42). It will be interesting to determine whether the separation of function of POT1 proteins into replication-specific versus non-replication-specific roles is a general phenomenon. Since mouse POT1b limits telomeric C-strand processing, perhaps it also functions predominantly during telomere replication (16).

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