DNA-Directed Polymerase Subunits Play a Vital Role in Human Telomeric Overhang Processing

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Abstract

Telomeres consist of TTAGGG repeats bound by the shelterin complex and end with a 3' overhang. In humans, telomeres shorten at each cell division, unless telomerase (TERT) is expressed and able to add telomeric repeats. Effective telomere maintenance, the DNA strand complementary to that made by telomerase must be synthesized. Recent studies have discovered a link between different activities necessary to process telomeres in the S phase of the cell cycle to reform a proper overhang. Notably, the human CST complex (CTC1/STN1/TEN1), known to interact functionally with the polymerase complex (POLA/primase), was shown to be important for telomere processing. Here, focus was paid to the catalytic (POLA1/p180) and accessory (POLA2/p68) subunits of the polymerase, and their mechanistic roles at telomeres. We were able to detect p68 and p180 at telomeres in cells using chromatin immunoprecipitation. We could also show that the CST, shelterin, and polymerase complexes interact, revealing contacts occurring at telomeres. We found that the polymerase complex could associate with telomerase activity. Finally, depletion of p180 by siRNA led to increased overhang amounts at telomeres. These data support a model in which the polymerase complex is important for proper telomeric overhang processing through fill-in synthesis, during S phase. These results shed light on important events necessary for efficient telomere maintenance and protection.

Implications: This study describes the interplay between DNA replication components with proteins that associate with chromosome ends, and telomerase. These interactions are proposed to be important for the processing and protection of chromosome ends. Mol Cancer Res; 13(3): 402–10. ©2014 AACR.

Introduction

Telomeres are structures located at the end of linear chromosomes, essential for their stability and integrity. They consist of stretches of repetitive DNA sequences and the protein complex bound to them, known in mammals as shelterin. Their role is to protect the chromosome ends from being inappropriately recognized by the DNA damage machinery (1). The ends of mammalian telomeres exhibit a 3' overhang of 50 to 300 nucleotides, produced by competing actions of telomerase, extending it by repeat additions, by ExoI and Apollo, which create the 5' resected end (2), and by C-strand fill-in processing. Another complex, called the CST complex, is known to limit telomerase activity in S phase (3), and to associate with the RNA primer synthesizing complex Polε-primase (4), thereby contributing an additional activity likely to participate in overhang processing.

In human cells, telomeres shorten at each cell division, unless telomerase is present and active in adding TTAGGG repeats. During active telomere elongation in telomerase-positive cells, no significant change is observed in mean overhang length (5), suggesting coordination between telomerase and the C-strand synthesis machinery. In humans, the mechanism of C-strand processing is tightly regulated, as demonstrated by the observation that the 5' ends in AATC-5' 80% of the times and in AATCC-5' 15% of the times (6). The terminal 5' nucleotide, however, becomes randomized upon the depletion of POT1 (7), the overhang binding protein in the shelterin complex. Different models for the creation of the 3' G-strand overhang have been proposed and, in general, they invoke an interplay of leading and the lagging strand processing due to the intrinsic properties of DNA replication and telomerase activity (see for example ref. 8). Recently, it has been elegantly shown that the two strands are in fact differentially processed during replication (9). The leading overhang is initially a blunt end that is processed later in S phase for the creation of the overhang. The lagging strand overhang length is first determined by the placement of the last RNA primer, which is then removed before further processing (9). Final overhang processing occurs in late S-G2. This study establishes the basis for a differential processing of the two strands without excluding other additional processing events for the C-strand. Additional indication of differential metabolism for the two terminal strands was described in the mouse system, in which the exonucleases Apollo and Exol, were found to be important for overhang production through 5' end resection at the leading and lagging strands, respectively (2). This work also elucidated an important role for POT1b in both leading and lagging strand overhang formation through the coordination of the nucleases action and the C-strand fill-in through association with the CST complex.

In Saccharomyces cerevisiae, as well as in most other eukaryotes, the CST complex, composed by three RPA-like proteins, Cdc13-Stn1-Ten1 (CTC1-OBFC1-Ten1 in humans), was found...
to be important for telomere protection and replication (10, 11). The role of the CST complex in the coordination of telomere elongation in humans was corroborated by the findings that it contributes to limiting telomerase activity at extending telomeres through complex interactions with telomerase itself and TPP1/POT1 (3). In addition, it was recently shown that CST plays a role in both general telomere replication and overhang processing, particularly for longer telomeres that represent a challenge for the replication machinery. Specifically, C-strand fill-in was affected in OBFC1-depleted cells, delaying the processing that leads to the final, mature C-overhang (12, 13). The human CST complex, in addition, was isolated as a set of accessory factors for the POLε–primase complex (4). In S. cerevisiae, Cdc13 is able to interact with POL1, the POLε homolog, and STN1 interacts with POL12, the regulatory subunit of Polε–primase, which is named POLA2 in humans (14). The POLε–primase complex is believed to be important for replication of the lagging strand and the C-strand fill-in for both strands after resection. The complex is composed by the catalytic subunit, Polε, also termed p180, two primase subunits, and the regulatory subunit POLA2, or p68 (see ref. 15). Although it has recently been established that telomeres, notwithstanding their reported nature as fragile sites of DNA replication on chromosomes (16), do not undergo a telomere-specific replication program, but mainly a chromosomal DNA replication, it is possible that this activity of telomeres through complex interactions with telomerase could be important for telomere protection and replication (17). Additionally, telomeres found physically associated with primase and the lagging strand replication machinery in the ciliate Euplotes crassus (18). This association appeared to be developmentally regulated as it occurred specifically in mated cells, and not in vegetatively growing cells. Genetic evidence in fusion and budding yeast also implicates primase subunits in telomere replication (14, 20, 21), although the mechanisms at play remain unclear.

In addition, telomerase was found physically associated with primase and the lagging strand replication machinery in the ciliate Euplotes crassus (19). This association appeared to be developmentally regulated as it occurred specifically in mated cells, and not in vegetatively growing cells. Genetic evidence in fusion and budding yeast also implicates primase subunits in telomere replication (14, 20, 21), although the mechanisms at play remain unclear.

Our study investigates these possible roles at human telomeres, and focuses on p68 (POLA2) and p180 (POLA1). We show that they are present at telomeres in S phase, interact with the shelterin complex and with the CST subunit OBFC1, as well as with telomerase itself, and that they are important for the regulation of telomeric overhang amounts in human cells.

Materials and Methods

Cell lines and antibodies

The HeLa cell line is a subclone of HeLa S3 (ATCC CCL-2.2), with telomere length in the 3- to 6.5-kb range (22), and used in ref. (23). The HTCl75 cell line is a HT1080 derivative described in ref. 24. The cells were grown in DMEM/10% BCS, and the retroviral transduction protocol was identical to that described in ref. 25. Cells were selected for the plasmid with 2 μg/ml puromycin, where applicable. All rabbit sera used were generated against a peptide conjugated to KLH and used for immunization into rabbits, as per the protocol set by the manufacturer (BioSynth). The peptides were: NH2-GCKGRQEALERLKAKAGEK-OH for p180, and NH2-GCTGRYRPAADGAQRP-OH for p68. The peptide for FEN1 NH2-GCKSKKAKRTAAGKF-KRK-OH, for TRF1 NH2-GGCSIEKEDHLKHEEIQNI-OH (as described in ref. 24), for POT1 NH2-GCYGRYRPLSNSDQNLKDEL (as described in ref. 26), for TPP1 NH2-GCTGPRAGRPRA-QARVGR-OH, and for OBFC1 NH2-GCKTIEGIDTRVRRG-SIRT-OH. The p53BP1 antibody was purchased from Novus (NB100-304). The TRF2 antibody used for immunofluorescence was purchased from Millipore, clone 4A794 (05-521). The Chk1-p-Ser345 antibody was purchased from Cell Signaling Technology (#2348). The p68 and p180 antibodies used for Western blot analyses and TRAP assays were purchased from Abcam (Ab57002 and Ab5609). The POT1 (25) and FLAG-TRF1 constructs and cell lines are described in refs. 24 and 26.

Plasmids

The CDNA for p68 (gene name POLA2) was purchased as a full-length clone from the EST collection maintained by Invitrogen. The full-length cDNA was amplified by PCR using primers with appropriate cloning sites (5′ BamHI and 3′ EcoRI) and cloned into pLPC-MYC (25) to generate a MYC-tagged version driven by the CMV promoter. The PCR oligonucleotides were: 5′-TGCCTAG-GATCGCCGATCGGCCAGCAGT-G3′ and 5′-TGAGAGAAAATTCGAGCTCCAGCAA-3′ corresponding to target sites for codons 2-7 at the 5′ end and the last 7 codons of the cDNA, including the stop codon. The OBFC1 cDNA was PCR cloned from a complete EST purchased from ATCC as a template, and with the following two oligonucleotides: 5′-ATAACAGA-GATCTCAGCGCCTGGACGCGTCCGACG-3′ and 5′-TCCACCTC-TGCACTGCAAGCTGGTGTATATCAG-3′, yielding a BglII-XhoI fragment as a PCR product. The TPP1 EST was purchased from Invitrogen and PCR cloned with the following two oligonucleotides: 5′-AGGAGATCCCTGGCGGGCTGCAG-3′ and 5′-GAGGCACGTCGCATATCATCGAGAAGG-3′, yielding a BamHI-XhoI fragment as a PCR product.

Depletions by siRNA or shRNA

HeLa cells were maintained in DMEM (Invitrogen) supplemented with 1% penicillin and streptomycin and 10% FBS. The siRNAs used were synthesized by Dharmacon RNA Technologies. For p68 RNAi, double-stranded siRNA were designed to target the following sequences: p68-1 siRNA 5′-UCCAGGAAAGAGGA-AAAUC3′ (target in the 5′ UTR, exon 3) and p68-2 siRNA 5′-UAUCUGCUGCUUAAGAGCAAUU3′-3′ (coding sequence, exon 7). For p180: p180-1 siRNA 5′-CUAGAUACCUUAAGCAGUUA3′ (coding sequence, exon 13); p180-2 siRNA 5′-CAGAACAUUGUGUGAGCUAA3′ (coding sequence, exon 21); p180-3 siRNA 5′-AGACGUGAAGUGUAUA3′ (3′ UTR, exon 37). HeLa cells were transfected using Lipofectamine (Invitrogen) according to the manufacturer’s instructions. Briefly, cells at a confluency of approximately 50% to 60% were plated in a 6-well plate 18 to 24 hours before transfection. Transfections were done once and cells were processed 48 hours after transfection for protein extraction or immunofluorescence. As a control, siGFP (Dharmacon) was used. For shRNA, the LMP vector from Open Biosystems was used, which is based on the miR30 miRNA. The target sequences were PCR cloned according to the manufacturer’s protocol based on the NM_002689.2 sequence for the p68 cDNA. The target sequences...
were: sh5’UTR, 5’-CTCTGGGACCTGACTGAGATGTAGTGAA-GCCACAGTGATCTTICAGTGGAGGCTACGAGA-3’; sh664, 5’-CCCTTCTGGACTCTCTAACACCTAGTGAAGCCACAGATGTAA-GTCCTGAGAAGATTCGTTACCGAGA-3’; sh1088, 5’-CCCTTCTGGACTCTCTAACACCTAGTGAAGCCACAGATGTAA-GTCCTGAGAAGATTCGTTACCGAGA-3’. sh3’UTR, 5’-AATGTTCGGTGTCCAGAA-GTAATAGTGAAGCCACAGATGTATAATTCACTCTGGACACGGAGC-ATG-3’.

Immunostaining for p53BP1 and TRF2 was performed for HeLaII cells plated onto glass coverslips and processed for RNAi. After the 48-hour transfection period, cells were extracted with Trx buffer (0.5% Triton X-100, 20 mmol/L Hepes-KOH, pH 7.9, 50 mmol/L NaCl, 3 mmol/L MgCl₂, 300 mmol/L sucrose) for 10 minutes at room temperature (RT). After two PBS washes, the cells were fixed with PBS/3% paraformaldehyde, 2% sucrose for 10 minutes at RT. After two more PBS washes, cells were permeabilized with Trx buffer for 10 minutes at RT, washed twice with PBS, and blocked with PBS (PBS/0.2% fish gelatin, 0.5% BSA) for 30 minutes. Coverslips were then incubated with the rabbit anti-p53BP1 antibody (Novus NB100-304A-1), at a concentration of 1:500 in PBS overnight. Coverslips were then rinsed three times with PBS solution and incubated with secondary TRITC-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch) in PBS at a concentration of 1:500 for 45 minutes at RT. Coverslips were rinsed twice times with PBS. Coverslips were then incubated with PBS and DAPI at 100 ng/mL to visualize the nuclei. Coverslips were mounted onto slides with embedding media. Images were collected with an Olympus BX61 fluorescence microscope using a 60× objective connected to a Hamamatsu ORCA-ER CCD camera, controlled by the SlideBook 5.1 image capture software. The telomere FISH shown in Supplementary Fig. S1C was performed as described in ref. 2.

Cell synchronization and FACS

HeLaII were plated 10⁶ cells in 10-cm plates. After 24 hours, thymidine to a final concentration of 2 mmol/L was added to the media. The cells were treated with thymidine for 14 hours, and then they were rinsed three times with warm PBS and fresh medium without thymidine. The cells were released for 11 hours and then thymidine, 2 mmol/L final concentration, was added to the medium again for the second block. After 14 hours the cells, were released as above and collected at the appropriate time points for ChiP and FACS. For FACS, cells were collected and rinsed twice in cold PBS, resuspended in 0.2 mL of PBS/2 mmol/L EDTA, 2 mL of cold 70% ethanol was added dropwise and the cells were kept at 4°C for 24 hours for fixation. The cells were then spun down and resuspended in 0.5 mL of PBS/2 mmol/L EDTA. Of note, 10 µL of heat-inactivated RNase A (10 mg/mL) and 25 µL of propidium iodide (1 mg/mL) were added and the cells were incubated at 37°C for 30 minutes. The samples were then analyzed using a FACSCalibur Flow Cytometer.

Cell extracts and immunoprecipitations

Cell extracts and immunoprecipitations were performed as described in ref. (26). The quantitations shown in Fig. 4 were done using the ImageJ analysis software. For siRNA and shRNA Western blot analyses, the relative ratios were obtained by normalizing the p180 and p68 signal to GAPDH and by dividing by the untreated sample signal. For the coimmunoprecipitation experiments, the enrichment values were obtained by subtracting the signal from the preimmune from the signal for each antibody.

TRAP assays

The TRAP assays were performed as per the manufacturer’s protocol (Millipore, S7710). For the IP-TRAP assays, the immunoprecipitations were performed as described above, and the beads were washed six times in cold lysis buffer. The beads were then resuspended in 40 µL of lysis buffer, and 2 µL of the resuspended beads were used as input for the TRAP assay. For RNase-treated controls, 20 µL of resuspended beads were removed and treated with 10 µg of RNase A at 37°C for 30 minutes and washed twice with cold lysis buffer.

Chromatin immunoprecipitations

The chromatin immunoprecipitations (ChiP) were performed, as described in ref. 26, on HeLaII cells synchronized by double thymidine block as described.

In-gel hybridization

Genomic DNA was isolated from cells as described in ref. 25 and processed for in-gel hybridization. The DNA was digested with AluI and Mbol, and control samples were further digested with ExoI to digest the signal derived from the 3’ overhang. Of note, 4 µg of DNA for each sample was loaded on a 0.7% agarose gel in 0.5X TBE. Following electrophoresis, the gels were dried at RT for at least 3 hours, and then prehybridized with in Church mix (0.5 mol/L Na₂PO₄, pH 7.2, 1 mmol/L EDTA, pH 8.0, 7% SDS, 1% BSA) for 30 minutes at 50°C and hybridized overnight with end labeled (CCCTAA)₄ or (TTAGGG)₄ oligonucleotides at 50°C. After hybridization, the gels were washed three times with 4 × SSC and one time with 4 × SSC, 0.1% SDS at 55°C, and exposed overnight to a PhosphorImager screen. To detect the total telomere signal, the gels were then denatured in 0.5 mol/L NaOH and 1.5 mol/L NaCl for 30 minutes and neutralized (3 mol/L NaCl, 0.5 mol/L Tris-HCl, pH 7.0) twice for 15 minutes, rinsed with dH₂O and hybridized with the end-labeled oligonucleotides. The gels were then exposed overnight to a PhosphorImager screen, and the G-overhang signal was calculated by dividing the native (CCCTAA)₄ signal by that obtained with the denatured gel.

Telomeric 3’ overhang analysis by double-strand specific nuclease (DSN)

The DSN reaction was performed as previously described (27). Four micrograms of genomic DNA in 1 × DSN buffer was digested with 0.2 µg of DNA of DSN (Axxora cat. EVN-EA001-KI01) at 37°C for 2 hours. As a control, 10 U of ExoII were added to the genomic DNA before DSN and incubated at 37°C for 1 hour to digest terminal single-stranded DNA. The digestions were stopped with 0.5 µL of 0.5 mol/L EDTA and an equal volume of formamide before heating the samples at 65°C for 5 minutes. The samples were run on a 6% denaturing polyacrylamide gel containing 8 mol/L urea. Electrophoresis was performed at 15V/cm in 0.5× TBE. The gel was then electroblotted onto a Hybond N membrane (Amersham-GE) in 0.5× TBE buffer at 4°C and 30V for 90 minutes. The membrane was then air dried, UV cross-linked, and hybridized to a C-rich telomeric probe at 42°C. The membrane was washed with 0.2 mol/L wash buffer (0.2 mol/L
Progression, in the case of p180, with a slight delay in S/G2 yielded a higher level of depletion, of 65% (Fig. 1B and D). These results could indicate a possible role for Polα in the cell-cycle progression or induction of DNA damage in the case of our p68 depletion, while no overt effects on cell-cycle progression or induction of DNA damage in the case of our p68 knockdown experiments were observed.

Depletion of p180 by siRNA, but not of p68, leads to a DNA damage response

To study the roles of p180 and p68, we performed depletions of both proteins by siRNA and p68 by shRNA in HeLaII cells (Fig. 1 and Supplementary Fig. S4). Among the three siRNA targets used for p180, the depletion was consistent down to about 30% of control endogenous levels (Fig. 1A and C). For p68, the first target site resulted in slightly over 50% depletion, and the second one yielded a higher level of depletion, of 65% (Fig. 1B and D). These levels of depletion correlated with mild effects on cell-cycle progression, in the case of p180, with a slight delay in S/G2–M (Supplementary Fig. S1A and S1B), and no obvious effect was seen in the case of p68. The depletions we report in this study are milder than those previously reported by others (15), and may explain our capacity to detect the telomeric effects reported here without major inhibition of cell-cycle progression elicited with more significant reduction of Polα activity. Knockdown of p180 generated a broad DNA damage response in the cell, as judged by the induction of p53BP1 foci (Fig. 2A). This effect was not observed upon depletion of p68. The average number of nuclei with three or more p53BP1 foci went from below 8% in control or p68 siRNA, to 35% with our best p180 siRNA targets (Fig. 2C). This observation likely corresponds to a broad induction of DNA damage, and not telomere deprotection, because no colocalization with telomeres was observed in this case (Supplementary Fig. S1C). The DNA damage response seen with p180 depletions is compatible with replication stress, as a significant activation of Chk1 was observed (Fig. 2B), known to result from induction of ATR. Upon quantitation of the signal detected for Chk1-Ser-345 phosphorylation, the levels of activated Chk1 increased by at least 5-fold upon p180 depletion (Fig. 2D). We did not detect a significant effect with p68 depletion, as the signal obtained was not significantly different from the siGFP-negative control (Fig. 2B and D).

Thus, disruption of Polα activity led to an apparent ATR-dependent DNA damage response as well as a mild S phase delay in the case of p180 depletion, while no overt effects on cell-cycle progression or induction of DNA damage in the case of our p68 knockdown experiments were observed.

POLα–primase components can be detected at telomeres by chromatin immunoprecipitation

To determine whether p180 and p68 could be detected at telomeres, we performed ChIP, a technique widely used to study proteins localization at telomeres. The assay was carried out on asynchronous as well as synchronized cell lines using anti-peptide rabbit sera against p180, p68, and OBFC1, the latter being a subunit of the CST complex. ChIP done on asynchronous HTC75 control cell lines yielded a low but reproducible signal for both p68 and p180 (Fig. 3A and B). We were able to visualize shelterin components at telomeres in these cells, such as TRF1 and POT1, as previously reported, as well as FEN1, with a yield of 4% of the total telomeric signal, likely representing an S phase population (28, 29). In these cells, we were able to detect a low but reproducible signal for p68, with a yield of 3%. The p180 subunit was not detected at telomeres in this setting. We also performed the ChIP on HTC75 cells with normal or elongated telomeres (through POT1405 expression) and found that OBFC1 and p68 were also present at elongated telomeres (not shown). As the roles of Polα–primase are primarily related to DNA replication in S phase, we sought to determine whether a telomeric association would be occurring for p68, and perhaps also p180, during this phase, as has been reported for FEN1 (28). HeLaII cells were synchronized through a double thymidine block, and the cells were collected at 0, 2, 4, 6, and 8 hours after release (Fig. 3C and D). We found that, in staged S phase cells, both p68 and p180 could be reliably detected at telomeres, albeit at low levels. Although p180 peaked in the early S phase and in late S–G2, p68 associated with telomeres more stably throughout S phase. These results could indicate a possible role for Polα at telomeres specifically during S phase.

The POLα–primase complex interacts with shelterin

We then sought to determine whether an association between the Polα complex and the telomeric shelterin complex could be detected. We also examined possible interactions with OBFC1,
given the known relationship with the Polα-primase complex (4). To that end, we used HTC75 cells expressing tagged versions of OBFC1, POT1, TRF1, or TPP1 and asked whether each component could individually be immunoprecipitated with anti-p68 or anti-p180 antibodies.

We were able to detect an association between shelterin subunits POT1 and TPP1 and OBFC1 (Fig. 4A). In addition, we confirmed the association between OBFC1 and p180 reported previously (ref. 4; Fig. 4A). We then extended our studies to possible interactions between p180 and p68 with shelterin components. HTC75 cells overexpressing MYC-TPP1 (Fig. 4B) and both HTC75 and HeLa 1.2.1.1 overexpressing MYC-p68 (Supplementary Fig. S3A and S3B) were used to test the potential interaction. MYC-TPP1 was found to coimmunoprecipitate with p180 (Fig. 4B), suggesting an interaction between the Polα and shelterin complexes. Similarly, MYC-p68 could be immunoprecipitated in HTC75 and HeLa 1.2.1.1 with POT1 or TRF2 antibodies (Supplementary Fig. S3A and S3B). We confirmed these findings by immunoprecipitating endogenous p68 in HeLa cells with antibodies against POT1, TRF1, TRF2, and TPP1 (Fig. 4C). In addition, we found that FLAG-TRF1 could be pulled down with p68 or p180 antibodies, confirming the interactions between shelterin and Polα in a different cell line. Therefore, it is possible that the amounts of p180 and p68 observed at telomeres by ChIP (Fig. 3) result from interactions at the protein level with the CST and shelterin complexes. The protein interactions found did not
require cell synchronization as the ChIPs did. Our results therefore demonstrate interactions between three known complexes, the shelterin, CST, and Polα–primase complexes, in three different human cell lines, which are likely to reflect associations occurring at telomeres.

The Polα–primase complex interacts with telomerase.

Our observation that Polα–primase is associated with telomeres and shelterin places p180 and p68 in prime position to interact with the telomerase complex. We thus asked whether an association could be detected. To this end, a HeLaII extract was prepared for immunoprecipitations with p180 antibodies, and the resulting pulled down material was used as input for TRAP activity assay to assess a possible association with telomerase. In Fig. 5A, we show that p180 antibodies can specifically pull down telomerase activity. As controls, no TRAP activity was either detected with preimmune serum, or with RNase-treated material (Fig. 5A and Supplementary Fig. S4B). We found similar yields for FEN1, which was previously shown to associate with telomerase activity (30), and could precipitate TRAP activity with p68 antibodies as well (Supplementary Fig. S4A). Such an interaction was also described by others for TPP1 (31). OBFC1, in our hands, did not pull down TRAP activity (Supplementary Fig. S4A). We could confirm the association between Polα–primase and telomerase by coimmunoprecipitation between p180 and hTERT, the catalytic subunit of telomerase (Fig. 5B): p180 antibodies could precipitate MYC-hTERT from a HTC75 extract, whereas OBFC1 antibodies did not. Thus, we are able to show the telomerase–Polα–primase interaction in two ways, by the IP-TRAP assay and by coimmunoprecipitation.

Figure 3.
Association of p68 and p180 with telomeres by ChIPs. A, ChIP in unsynchronized HTC75 cells. The Alu probe was used as a non-telomere control. The antibodies used are indicated on top; PI, preimmune serum. B, quantitation of the DNA yields for the HTC75 ChIP. The yield for each sample was divided by the total DNA value after subtraction of the preimmune background value. C, ChIP in synchronized HeLaII cells. Cells were subjected to a double thymidine block, and processed for ChIP at 2-hour intervals after release. Probes are indicated at the bottom of the blots. D, quantitation of the yields for p180, p68, and OBFC1 at indicated time points after release. STY, values obtained with the TTAGGG (telomeric) probe. An Alu probe was used as a control.
Depletion of p180 leads to increased telomeric overhang amounts

As both shelterin and CST play important roles in telomere protection through overhang processing, we looked at the telomeric overhang amounts in conditions where p180 or p68 were depleted. We examined both short-term depletion (48 hours) via siRNA for both p68 and p180 (Fig. 6) and long-term depletion using shRNAs for p68 (Supplementary Fig. S4). This assay involves separating restricted genomic DNA by size on an agarose gel and probing the gel in native conditions with a labeled C-rich oligonucleotide. We found that the telomeric overhang amounts increased in amounts in p180-depleted cells (Supplementary Fig. S6). The DSN assay allows the detection of undigested single-stranded telomeric overhangs by hybridization to a labeled C-rich oligonucleotide. We found that the telomeric overhangs increased in amounts in p180-depleted cells (Supplementary Fig. S6A), to an extent similar to that seen with the native hybridization assay (Supplementary Fig. S6B).

We conclude that a decrease in p180 leads to increased overhang amounts, suggesting a role in the regulation of the length of the telomeric overhang without affecting the resection process creating the telomeric 5' end. It is possible that p68 is also involved in this process, although the effect appears weaker.

Discussion

The results presented here provide a link between the Polα-primase complex, and two other telomeric complexes, shelterin and the CST complex. Although shelterin and CST have well-documented roles in telomere function, pertaining to the protection and maintenance of telomeres, the implication of Polα-primase in these processes poses a question: are its roles at telomeres related to conventional chromosome DNA replication, or are there telomere-specific, noncanonical roles for Polα-primase at telomeres? Although the final answer requires additional work, we would argue based on our findings that the observed effect on overhang processing reflects a telomere-specific role for Polα not related to origin-initiated DNA replication. First, we report here for the first time an interaction between p180, and p68 with shelterin. These types of associations were described in budding and fission yeast (20, 21), but never, to our knowledge, in human cells. Second, we did not observe major defects in the cell-cycle profiles of p180- or p68-depleted cells in our study, arguing for mild effects, if any, in chromosomal DNA replication (Fig. 2 and Supplementary Fig. S2). These observations could be simply explained by arguing that the remaining amounts of p180 or p68 in our depletion experiments are sufficient to support DNA replication, but limiting for effective overhang processing. And,
negative approaches could allow for a finer analysis of telomere-specific functions for Pol–primase.

An important future direction will be to understand the mechanism of action of Pol–primase at telomere, if different from or more complex than mere association with the replication fork progressing through the telomere. At present, we think it valuable to interpret these results in light of recent work showing that the CST complex, in particular OBFC1, limits telomerase activity, thereby participating in telomere length homeostasis (3) and has a specific role in C-strand fill-in (12). Knowing that OBFC1 is part of an “alpha activating factor” complex (4), but has no obvious role in the recruitment of the complex to telomeres, leads us to suggest that Pol–primase is recruited through telomere-specific interactions, perhaps involving shelterin, to lay down the terminal DNA primer and restrict the length of the overhang. Depleting amounts of Pol–primase would render this step limiting and result in increasing overhang length overall. It will be interesting to analyze the nature of the potential direct contacts between shelterin and Pol–primase, as well as between Pol–primase and the telomerase complex.

In addition, although we did not observe any significant change in the terminal 5’ nucleotide in our conditions using the STELA assay (not shown), 5’ end resection may be altered upon stronger inactivation of the Pol– complex. This would present an interesting synergy with POT1, which does influence the terminal 5’ nucleotide in human cells (7). As a result of the interactions reported here, the overhang phenotype we describe is predicted to be dependent on telomerase activity, and it would thus be interesting and important to perform the depletion experiments for p68 and p180 in telomerase-negative primary cells. In this context, targeting Pol–primase, or the specific interactions with shelterin, would possibly result in immediate or premature senescence in primary cells, thereby activating an important tumor suppressor system. It is relevant to note that in mouse cells, POT1b is the recruiting activity for the CST complex, itself mediating fill-in synthesis, likely through Pol–primase recruitment (2). Our data are compatible with these findings, and it would be valuable to test the possibility that POT1 is the recruiting factor for CST and Pol–primase in human cells. Two issues remain of note in our opinion. First, as we did not detect obvious telomere deprotection (by looking at telomeric p53BP1 foci), this pathway for overhang processing is not expected to lead to immediate effects such as apoptosis or premature senescence. However, one could hypothesize that lack of effective fill-in synthesis would exacerbate the so-called “end-replication problem” and limit the proliferative potential of cells, and perhaps even of telomerase-positive tumor cells. Longer term experiments than those reported here in primary human cells would be required to examine this possibility. The second issue is whether the overhang phenotype requires cells to be passing through S phase, or whether the specific function of Pol–primase suggested here is independent of actual DNA replication. Even though we did detect an increase in p68 and p180 at telomeres in S phase, it remains possible that some of these interactions occur outside of the context of chromosomal DNA replication and be important for telomere protection. Therefore, it would be valuable to assess the possible effects of Pol–primase in cells that are in G0, which do not experience progression through the cell cycle, S phase, or proliferation.

Our view, based on the results presented here, is that telomere function relies on multiple interactions among three important complexes, shelterin, CST, and Pol–primase. In

Figure 6.
Increased overhang amounts upon siRNA depletion of p180. A, in-gel hybridization for genomic DNA from HeLaII cells treated with siRNA for p68 and p180. Left, native gel probed with the labeled oligonucleotide (CCCTAA)_4 hybridizing to the telomeric overhang. Right, same gel reprobed after denaturation to detect all telomeric sequences. Samples treated with Exol were run alongside to control for detection of single-strand DNA in the native gel. B, overhang intensity is indicated as the ratio of the native signal over the denatured, normalized to the siGFP control. Error bars represent the SE for three independent experiments. The two-tailed Student’s t test for the two significant values (<0.05) is shown.

finally, we documented an association between telomerase and the p180 and p68 subunits of Pol–primase. This association can be detected at the protein level with hTERT, the catalytic subunit of telomerase, and likely reflects a functional role because we can precipitate telomerase activity with primase subunits p68 and p180. This idea is in our view interesting in that it could uncover a telomere-specific set of events implicating Pol–primase, which could be targeted in tumor cells, for instance, to limit their proliferation. Such a role could be highly conserved in evolution, and be related to the finding that a special mutant allele in the budding yeast POL12 gene, which represents the p68 ortholog, displays dysregulated telomere function, including longer telomere length, and telomere deprotection (14). In addition, the yeast POL12 gene interacts genetically with STN1, the OBFC1 ortholog in this organism (14). In human cells, dominant-negative approaches could allow for a finer analysis of telomere-specific functions for Pol–primase.

An important future direction will be to understand the mechanism of action of Pol–primase at telomere, if different from or more complex than mere association with the replication fork progressing through the telomere. At present, we think it valuable to interpret these results in light of recent work showing that the CST complex, in particular OBFC1, limits telomerase activity, thereby participating in telomere length homeostasis (3) and has a specific role in C-strand fill-in (12). Knowing that OBFC1 is part of an “alpha activating factor” complex (4), but has no obvious role in the recruitment of the complex to telomeres, leads us to suggest that Pol–primase is recruited through telomere-specific interactions, perhaps involving shelterin, to lay down the terminal DNA primer and restrict the length of the overhang. Depleting amounts of Pol–primase would render this step limiting and result in increasing overhang length overall. It will be interesting to analyze the nature of the potential direct contacts between shelterin and Pol–primase, as well as between Pol–primase and the telomerase complex.

In addition, although we did not observe any significant change in the terminal 5’ nucleotide in our conditions using the STELA assay (not shown), 5’ end resection may be altered upon stronger inactivation of the Pol– complex. This would present an interesting synergy with POT1, which does influence the terminal 5’ nucleotide in human cells (7). As a result of the interactions reported here, the overhang phenotype we describe is predicted to be dependent on telomerase activity, and it would thus be interesting and important to perform the depletion experiments for p68 and p180 in telomerase-negative primary cells. In this context, targeting Pol–primase, or the specific interactions with shelterin, would possibly result in immediate or premature senescence in primary cells, thereby activating an important tumor suppressor system. It is relevant to note that in mouse cells, POT1b is the recruiting activity for the CST complex, itself mediating fill-in synthesis, likely through Pol–primase recruitment (2). Our data are compatible with these findings, and it would be valuable to test the possibility that POT1 is the recruiting factor for CST and Pol–primase in human cells. Two issues remain of note in our opinion. First, as we did not detect obvious telomere deprotection (by looking at telomeric p53BP1 foci), this pathway for overhang processing is not expected to lead to immediate effects such as apoptosis or premature senescence. However, one could hypothesize that lack of effective fill-in synthesis would exacerbate the so-called “end-replication problem” and limit the proliferative potential of cells, and perhaps even of telomerase-positive tumor cells. Longer term experiments than those reported here in primary human cells would be required to examine this possibility. The second issue is whether the overhang phenotype requires cells to be passing through S phase, or whether the specific function of Pol–primase suggested here is independent of actual DNA replication. Even though we did detect an increase in p68 and p180 at telomeres in S phase, it remains possible that some of these interactions occur outside of the context of chromosomal DNA replication and be important for telomere protection. Therefore, it would be valuable to assess the possible effects of Pol–primase in cells that are in G0, which do not experience progression through the cell cycle, S phase, or proliferation.

Our view, based on the results presented here, is that telomere function relies on multiple interactions among three important complexes, shelterin, CST, and Pol–primase. In
addition, Polτ–primase appears to associate with the telomerase complex, presumably at telomeres. Shelterin is known to be quantitatively associated with telomeres, whereas the CST and Polτ–primase complexes could be viewed as telomerase-associated factors, acting only transiently at telomeres in S phase. We provide evidence here for Polτ–primase as an important complex to consider in the context of telomere protection and maintenance, which potentially offers additional targets to counteract cell transformation and proliferation during tumorigenesis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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