Regulation of Telomeric Repeat Binding Factor 1 Binding to Telomeres by Casein Kinase 2-mediated Phosphorylation*

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Mi Kyung Kim1, Mi Ran Kang1, Hyung Wook Nam5*, Young-Seuk Bae1, Yu Sam Kim5*, and In Kwon Chung5‡§1

From the 1Department of Biology and Molecular Aging Research Center, 2Department of Biochemistry, and 3Protein Network Research Center, Yonsei University, Seoul 120-749 and the 1Department of Biochemistry, College of Natural Sciences, Kyungpook National University, Daegu 702-701, Korea

Telomere maintenance is essential for continued cell proliferation and chromosome stability. Telomeres are maintained by telomerase and a collection of associated proteins. The telomeric protein telomeric repeat binding factor 1 (TRF1) negatively regulates telomere length by inhibiting access of telomerase to telomere termini. Here we report that TRF1 interacts with the β subunit of casein kinase 2 (CK2) and serves as a substrate for CK2. CK2-mediated phosphorylation is required for the efficient telomere binding of TRF1 in vitro and in vivo. Inhibition of CK2 by the CK2 inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole decreased the ability of TRF1 to bind telomeric DNA. The resulting telomere-unbound form of TRF1 was then ubiquitinated and degraded by the proteasome. Partial knockdown of CK2 by small interfering RNA resulted in removal of TRF1 from telomeres and subsequent degradation of TRF1. Mapping of the CK2 target site identified threonine 122 as a substrate in TRF1. A threonine to alanine change at this position led to a diminished DNA binding due to reduced dimerization of TRF1. In addition, phosphorylation of threonine 122 seemed critical for TRF1-mediated telomere length control. Our findings suggest that CK2-mediated phosphorylation of TRF1 plays an important role in modulating telomere length homeostasis by determining the levels of TRF1 at telomeres.

Telomeres are specialized nucleoprotein complexes that protect the ends of eukaryotic chromosomes (1). Without functional telomeres, chromosomes are prone to nucleolytic degradation and end-to-end fusion leading to cell death or possibly genetic recombination or survivor selection (2, 3). Most human normal somatic cells show a progressive loss of telomeric DNA during successive rounds of cell division due to a DNA end-replication problem (4, 5). Thus, telomere shortening functions as a control mechanism that regulates the replicative capacity of cells and cellular senescence (6, 7). In humans, telomerase activity is expressed in a majority of immortalized cells, but undetectable in normal somatic cells (8). The introduction of the telomerase catalytic subunit gene into normal somatic cells prevents telomere erosion and extends their proliferative life span (9). Conversely, inhibition of telomerase results in telomere shortening and subsequent growth arrest of cancer cells followed by senescence (10). These observations suggest that telomerase is necessary for the proliferation of primary and transformed cells, and that the activation of telomerase may be a critical step in human carcinogenesis (11).

Telomere homeostasis is regulated by telomerase and a collection of associated proteins, including TRF12 and TRF2 (12–14). Both proteins contain a C-terminal DNA binding motif that is closely related to the Myb domain and an internal conserved TRF homology domain that mediates dimerization (15–17). TRF2 is required to protect chromosome ends (18) and stabilizes a terminal loop structure called the t-loop (19). Overexpression of TRF1 in telomerase-positive cells results in a gradual telomere shortening, whereas a dominant negative mutant induces inappropriate telomere elongation (20, 21). These results indicate that TRF1 negatively regulates telomere length by inhibiting the interaction between telomeres and telomerase.

TRF1 and its interacting proteins such as tankyrases, TIN2, and Pinx1 have been shown to control telomere length. Tankyrases, originally identified as TRF1-interacting proteins, catalyze the formation of long chains of poly(ADP-ribose) onto TRF1 using NAD+ as a substrate (22). Overexpression of tankyrase 1 in the nucleus results in removal of TRF1 from telomeres (23) and subsequent degradation of TRF1 via the ubiquitin-proteasome pathway (24). Consistent with these findings, overexpression of tankyrase 1 leads to telomere elongation, a phenotype similar to that upon TRF1 inhibition (23, 25). The inhibition of TRF1 by tankyrase 1 is in turn controlled by a second TRF1-interacting protein, TIN2 (26). TIN2 forms a ternary complex with TRF1 and tankyrase and appears to stabilize the TRF1-tankyrase interaction. TIN2 protects TRF1 from poly(ADP-ribose)ylation by tankyrase 1 without affecting tankyrase 1 automodification. Moreover, partial knockdown of TIN2 by small interfering RNA results in loss of TRF1 from telomeres, leading to subsequent telomere elongation (27). Thus, TIN2 appears to protect TRF1 from being modified by

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† To whom correspondence should be addressed: Dept. of Biology, Yonsei University, 134 Shinchon-dong, Seoul 120-749, Korea. Tel.: 82-2-2123-2660; Fax: 82-2-364-8660; E-mail: topoviro@yonsei.ac.kr.
2 The abbreviations used are: TRF1, telomeric repeat binding factor 1; CK2, casein kinase 2; GST, glutathione S-transferase; RFP, red fluorescent protein; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; EMSA, electrophoretic mobility shift assay; siRNA, small interfering RNA; ESI-MS/MS, electrospray ionization tandem mass spectrometry; ATM, ataxia telangiectasia mutated; PD, population doubling; LC, liquid chromatography.
tankyrase 1. A third TRF1-interacting protein, Pinx1, has been proposed to affect telomere length control by inhibiting telomerase activity (28).

In this study, we identified CK2β as a TRF1-interacting protein using a yeast two-hybrid screen. CK2 interacts with and phosphorylates TRF1 in vitro and in cells. CK2-mediated phosphorylation is required for the efficient telomere binding of TRF1. Inhibition of CK2 resulted in release of TRF1 from telomeres and led to subsequent degradation of TRF1 via the ubiquitin-proteasome pathway. Our findings suggest a novel role of CK2 that functions as a positive regulator for determining the levels of TRF1 at telomeres.

**EXPERIMENTAL PROCEDURES**

_Yeast Two-hybrid Screening—_Yeast two-hybrid screening was performed as described previously (29). Briefly, the TRF1 cDNA was fused to the LexA DNA binding domain and transformed by the lithium acetate method into the EGY48 yeast strain. Expression of the LexA-TRF1 fusion protein was verified by Western blotting using anti-LexA antibody. The stable strain was transformed again with an HeLa cDNA library fused to the activation domain vector pB42AD (Clontech).

**GST Pulldown Assay, Immunoprecipitation, and Immunoblot—**GST pulldown assay, immunoprecipitation, and immunoblotting were performed as described previously (29). Briefly, relevant proteins were expressed in cells, followed by lysis. For GST pulldown assay, lysates were precleared with glutathione-Sepharose 4B (Amersham Biosciences) and incubated with glutathione-Sepharose beads containing GST fusion proteins for 2 h at 4 °C. For immunoprecipitation, lysates were preincubated with protein A-Sepharose (Amersham Biosciences) and incubated with primary antibodies precoupled with protein A-Sepharose beads for 2 h at 4 °C. The precipitated proteins were washed extensively and subjected to immunoblot analyses. Immunoprecipitation and immunoblotting were performed using anti-TRF1 (Sigma), anti-TRF2 (Upstate), anti-CK2α (Santa Cruz Biotechnology), anti-FLAG (Sigma), anti-V5 (Invitrogen), anti-HA (Santa Cruz Biotechnology) antibodies.

**In Vitro Kinase Assays—**Human CK2 holoenzyme was expressed and purified from bacteria to homogeneity as described previously (30). In vitro kinase assays were performed with [γ-32P]ATP (Amersham Biosciences) and GST fusion proteins as described before (31). The samples were separated on an 8% SDS-polyacrylamide gel and subjected to autoradiography. In some experiments, the reactions were supplemented with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB, Calbiochem) as indicated.

**Electrophoretic Mobility Shift Assays—**Nuclear extracts were prepared from MCF7 cells expressing the FLAG-TRF1, and EMSA was performed as described (32). Briefly, DNA probes were prepared by annealing the two oligonucleotides (TTAGGG)6 and (CCCTAA)6 end-labeled with [γ-32P]ATP (Amersham Biosciences) and T4-poly nucleotide kinase (New England Biolabs), and purified by free nucleotide removal spin column (Qiagen). Labeled DNA probes (0.5 ng) were incubated with nuclear extracts (6 μg of protein) and poly(dI·dC) (0.5 μg) as the nonspecific competitor (Amersham Biosciences) for 20 min at 25 °C and were fractionated on an 8% nondenaturing polyacrylamide gel. For supershift analysis, nuclear extracts were preincubated with anti-FLAG antibody (Sigma) prior to EMSA.

**Chromatin Immunoprecipitation—**Chromatin immunoprecipitations were performed as described (33) with the following modifications. Briefly, cells were fixed with 1% formaldehyde in phosphate-buffered saline and lysed, followed by sonication to obtain chromatin fragments with an average size of 600 bp. Lysates were immunoprecipitated with anti-FLAG antibody and supplemented with protein A-Sepharose beads. The immunocomplexes were heated at 65 °C for 4 h to reverse the cross-links. Slot blots were hybridized with a 300-bp random-labeled TTAGGG probe or an Alu probe. The quantification of the percent precipitated DNA was done with ImageQuaNT software, and the percentage of each immunoprecipitation sample was calculated based on the signal relative to the corresponding total DNA signal.

**siRNA Transfections—**The siRNA target sequences specific for CK2α and CK2β were 5′- AUGUCUGCCAGUGAUGUCUGAUG-3′ and 5′-ACACGAGGACAGUAACCAAAGUCUC-3′, respectively. Transient transfections of siRNAs were performed using Lipofectamine (Invitrogen). 72 h after transfection, nuclear extracts were immunoblotted using anti-TRF1 (Sigma), anti-TRF2 (Upstate), anti-CK2α, and anti-CK2β (Santa Cruz Biotechnology) antibodies.

**Indirect Immunofluorescence—**Cells were fixed in 5% paraformaldehyde and permeabilized with 0.5% Triton X-100 in phosphate-buffered saline. Cells were blocked in phosphate-buffered saline containing 2% bovine serum albumin and incubated with mouse anti-TRF1 (Sigma) or mouse anti-TRF2 (Upstate), and rabbit anti-CK2β (Santa Cruz Biotechnology). After washing, cells were incubated with Alexa Fluor 568 goat anti-mouse immunoglobulin and Alexa Fluor 488 goat anti-rabbit immunoglobulin (Molecular Probes). DNA was stained with 4,6-diamino-2-phenylindole (Vector Laboratories).

**Nano-LC and ESI MS/MS Analysis—**In gel protein digestion was performed as described previously (34). Ultimate Nano-LC systems, combined with a FAMOS autosampler and a Switchos II column switching valve (LC-Packings), were used as described previously (35). Briefly, the samples were loaded onto a pre-column, and then the Switchos II column switching device transferred the flow paths to the analytical column. The column outlet was directly coupled to the high voltage ESI source, which was interfaced to the QSTAR Pulsar quadrupole time-of-flight mass spectrometer (Applied Biosystems).

**RESULTS**

**Identification of CK2β as a TRF1-interacting Partner—**To identify TRF1-interacting factors, we screened a HeLa cell cDNA library using the yeast two-hybrid system. With the full-length TRF1 as bait, 26 positive clones were obtained and sequenced. One of the isolated clones was identified as CK2β (Fig. 1A). CK2 is a protein serine/threonine kinase involved in a variety of cellular functions, including cell proliferation and transformation (36). The enzyme is a heterotetrameric protein complex composed of two identical regulatory β subunits and
two catalytic α or α′ subunits. Because the functional importance of phosphorylation in regulating the ability of TRF1 to bind telomeric DNA has not been elucidated, we were particularly interested in identification of the protein kinase that can bind telomeric DNA has not been elucidated, we were particu-
larly interested in identification of the protein kinase that can
associate with and phosphorylate TRF1.

To confirm the direct interaction between TRF1 and CK2β, we
performed GST pulldown experiments. GST-TRF1, but not
GST-TRF2 or the control GST, precipitated endogenous CK2β
from MCF7 cell extracts (Fig. 1B), indicating that TRF1 inter-
acts with CK2β in vitro. To determine whether CK1 and CK2β
associate in vivo, MCF7 cells were transfected with FLAG-
TRF1 or FLAG-TRF2 and subjected to immunoprecipitation.
CK2β was detected in anti-FLAG or anti-TRF1 immunopre-
cipitates when FLAG-TRF1 was expressed, but not in anti-

**FIGURE 1. Physical Interaction between TRF1 and CK2β.** A, analysis of the
physical interaction between TRF1 and CK2β using the yeast two-hybrid
assay. RAP1 and unrelated FKBP52 were used as TRF2-binding and negative
controls, respectively. The blue signal on the SG-HWUL plate and the growth
on the SG-HWUL plate indicate activation of the reporter genes, LacZ and
LEU2, respectively. S, synthetic; G, galactose; H, histidine (−); W, tryptophan
(−); U, uracil (−); L, leucine (−); X, X-Gal (5-bromo-4-chloro-3-indolyl-β-D-
galactopyranoside). B, interaction between TRF1 and CK2β in vitro. GST-TRF1,
GST-TRF2, or GST were immobilized on glutathione-Sepharose and incubated
with MCF7 cell extracts, followed by detecting endogenous CK2β by
immunoblotting. C, coimmunoprecipitation of CK2β and TRF1. MCF7 cells
were transfected with FLAG-TRF1 or FLAG-TRF2 and then subjected to immu-

**FIGURE 2. Phosphorylation is required for the efficient telomere binding
of TRF1.** A, EMSA of nuclear extracts prepared from MCF7 cells expressing
FLAG-TRF1. Gel-shift assays were performed with nuclear extracts (6 µg of
protein) and a labeled double-stranded DNA probe (0.5 ng) containing six
tandem TTAGGG repeats. Supershift of FLAG-TRF1 complex with anti-FLAG
antibody is shown in lane 3. Lanes 4 and 5 contained nuclear extracts incu-
bated with λ-phosphatase and reaction buffer, respectively. Lanes 6–8 con-
tained nuclear extracts prepared from cells that were treated for 4 h with 200
µM DRB or 10 µM MG132, or a combination of the two as specified. B, chroma-
tin immunoprecipitation of telomeric DNA by TRF1. MCF7 cells expressing
FLAG-TRF1 or the empty vector were either treated for 4 h with 200 µM DRB or
left untreated as a control, followed by chromatin immunoprecipitation with
anti-FLAG antibody. Total DNA and immunoprecipitated DNA were applied
to nitrocellulose in a slot blot manifold with 2-fold dilutions of DNA. Duplicate
slot blots were hybridized with a telomeric probe or an Alu probe.
Phosphorylation Is Required for the Efficient Telomere Binding of TRF1—To determine whether phosphorylation of TRF1 affects its telomere-binding activity, we performed EMSAs with nuclear extracts prepared from MCF7 cells expressing FLAG-TRF1. Nuclear extracts gave rise to a discrete protein-DNA complex that was supershifted by anti-FLAG antibody (Fig. 2A, lanes 2 and 3). When nuclear extracts were pretreated with a λ-phosphatase that directs toward serines, threonines, and tyrosines, TRF1 binding was markedly reduced compared with untreated extracts, suggesting that the complex formation is TRF1 phosphorylation-dependent (Fig. 2A, lanes 4 and 5).

When cells were treated with DRB, the complex detected in TRF1 significantly decreased (Fig. 2A, lane 6). Reduction in TRF1 binding could be due to degradation or turnover of the protein. To address this issue, we pretreated cells with a proteasome inhibitor MG132 and subsequently incubated with DRB. Incubation of cells with MG132 rescued the DRB-induced down-regulation of TRF1 binding activity (Fig. 2A, lane 7). In the absence of DRB, incubation of cells with MG132 alone did not cause a change in the level of TRF1 binding (Fig. 2A, lane 8). These results suggest that DRB-induced reduction in TRF1 complex is due to proteasome-mediated degradation of TRF1.

DRB-induced reduction in TRF1 complex was further confirmed by chromatin immunoprecipitation experiments (Fig. 2B). Cross-linked extracts prepared from cells expressing FLAG-TRF1 or an empty vector were immunoprecipitated with anti-FLAG antibody. The percentage of telomeric DNA bound to TRF1 was determined in slot blots using a labeled telomeric probe. Anti-FLAG antibody immunoprecipitated roughly 10% of total telomeric DNA in untreated control cells. However, in DRB-treated cells, telomeric DNA immunoprecipitated by anti-FLAG antibody was significantly reduced. These results indicate that the telomere binding of TRF1 requires phosphorylation by a DRB-sensitive kinase. Together with the EMSA data, these results indicate that phosphorylation of TRF1 is important for its telomere binding in cells.

Inhibition of CK2 by DRB Leads to Ubiquitination and Degradation of TRF1—Because inhibition of CK2 by DRB results in a substantial decrease in the levels of TRF1 binding at telomeres, unphosphorylated TRF1 could be dissociated from telomeres and subsequently down-regulated. To investigate this possibility, MCF7 cells were transfected with FLAG-TRF1 and accessed the protein levels in the presence of DRB. FLAG-TRF1 was down-regulated in a time-dependent manner in response to DRB treatment (Fig. 3A). In contrast, FLAG-TRF2 levels were not affected by DRB treatment. To verify the ability of DRB to down-regulate endogenous TRF1 level, MCF7 cells were treated with DRB and immunoblotted using anti-TRF1 antibody. DRB treatment significantly decreased endogenous TRF1 level (data not shown). To examine whether the DRB-mediated reduction of TRF1 proceeds via
the proteasome, cells were pretreated with MG132 and subsequently incubated with DRB. Incubation of cells with MG132 rescued the DRB-induced reduction of TRF1 (Fig. 3A).

To examine the effect of DRB on the half-life of TRF1, MCF7 cells were incubated with cycloheximide in the absence or presence of DRB and analyzed by immunoblotting with anti-TRF1 antibody. As shown in Fig. 3B, and graphically in Fig. 3C, TRF1 was turned over with a half-life of ~2.4 h. In contrast, DRB treatment caused a clear decrease in the half-life of TRF1, indicating the higher turnover of TRF1 in response to the CK2 inhibitor. We next examine whether TRF1 is ubiquitinated in vivo prior to its degradation. MCF7 cells expressing HA-ubiquitin were treated with DRB, MG132, or both. Anti-TRF1 immunoprecipitates were probed with anti-HA antibody to illuminate ubiquitin-modified TRF1 (Fig. 3D). Ubiquitinated TRF1 was significantly elevated in the presence of MG132. This modification was further enhanced in cells treated with both MG132 and DRB, indicating that TRF1 is ubiquitinated in DRB-treated cells. Interestingly, ubiquitination of TRF2 was not observed even when both DRB and MG132 were treated (Fig. 3D).

The effect of DRB on the accumulation of TRF1 on telomeres was further investigated by determining the nuclear localization of TRF1 using anti–TRF1 antibody. TRF1 staining gives a nuclear dot pattern corresponding to telomeres. DRB treatment results in loss of TRF1 from telomeres, which is illustrated by reduced fluorescent dots (Fig. 3E). When cells were incubated with both DRB and MG132, TRF1 was re-associated with telomeres. In contrast, TRF2 expression levels were not affected by DRB treatment (Fig. 3F). Note that the levels of CK2β were decreased when treated with DRB.

**CK2 siRNA Depletion Reduces the Level of TRF1**—To demonstrate the critical role of CK2 in the regulation of TRF1 expression levels in vivo, we used small interfering RNA duplexes (siRNA). Transfection of siRNAs specific for CK2α and CK2β significantly reduced the protein levels of CK2α and CK2β in MCF7 cells (Fig. 4A). We determined the effects of lowered CK2 levels on TRF1 expression. Relative to the controls, CK2α or CK2β siRNA depletion caused a marked reduction in the level of TRF1 (Fig. 4B). However, TRF2 expression level was not affected by CK2α or CK2β siRNA.

Indirect immunofluorescence analysis of MCF7 cells transfected with CK2β siRNA revealed the reduced fluorescent signals of CK2β compared with control cells (Fig. 4C). Moreover, CK2β siRNA resulted in release of TRF1 from telomeres as indicated by decrease of the fluorescent dots of TRF1. We obtained the similar results with CK2α siRNA (Fig. 4D). Taken together, these results indicate that CK2-mediated phosphorylation is important for TRF1 stability and is required for the efficient telomere binding of TRF1.

**CK2 Phosphorylates TRF1 on Threonine at Position 122**—To determine CK2-phosphorylation sites, TRF1 was phosphorylated by CK2 in vitro and digested with trypsin (34). The peptide mixtures were separated by nano-LC and then directly introduced to a Qstar Pulsar mass spectrometer using ESI (35). From the peptide sequencing data, one phosphorylated peptide peptide122-TIYICQFLTR131 located in the dimerization domain of TRF1 was detected. Unmodified and phosphorylated peptides were 80 Da different in mass (Fig. 5) and were detected with slightly different retention time in nano-LC. Compared with unmodified peptide MS/MS data, phosphorylated peptide showed the same y-ion patterns (from y1–y8) and different b-ion patterns (Fig. 5), suggesting that the phosphorylation site is located at the N terminus. From these data, we conclude that the phosphorylation site is the first threonine of the N terminus in this peptide (threonine at position 122 of TRF1).

To determine the role of threonine 122 in regulating the telomere binding activity of TRF1, we generated TRF1 mutants in which the threonine residues at positions 122 and 130 were substituted by alanine (Fig. 6A). Moreover, CK2α or CK2β siRNA depletion caused a marked reduction in the level of TRF1 (Fig. 4B). However, TRF2 expression level was not affected by CK2α or CK2β siRNA.

**CK2-mediated Phosphorylation of TRF1**

![CK2-mediated Phosphorylation of TRF1](image-url)
decreased compared with that of wild-type TRF1 when overexpressed.

To measure the effect of threonine to alanine mutations on the telomere binding activity in vivo, we determined the nuclear localization of TRF1 mutants (Fig. 6D). The wild-type and TRF1T130A accumulated at telomeres as indicated by the telomere-like dots. In contrast, TRF1T122A or TRF1T122,130A displayed markedly reduced fluorescent dots of TRF1. Together with the EMSA and immunoblot data, these results indicate that CK2-mediated phosphorylation of threonine 122 but not threonine 130 is important for efficient telomere binding of TRF1.

Threonine 122 Is Important for Dimerization of TRF1 and Regulates Telomere Length—The critical question that remains to be answered is why TRF1 loses its DNA binding activity when it is non-phosphorylated. An obvious possibility would be that TRF1 dimerizes better when threonine 122 is phosphorylated. Dimerization of TRF1 has been known to be a requirement for the efficient telomere binding in vivo and in vitro (37). To test this, coimmunoprecipitation of two differently tagged TRF1 constructs was performed with the wild-type and the mutant proteins. MCF7 cells were cotransfected with RFP-TRF1 and either FLAG-tagged wild-type or mutant TRF1, and subjected to immunoprecipitation (Fig. 7A). Cotransfection of RFP-TRF1 and FLAG-TRF1 resulted in efficient recovery of TRF1 in both anti-RFP and anti-FLAG immunoprecipitates, confirming the homodimerization between differently tagged TRF1 molecules. However, the amounts of immunoprecipitated TRF1 were significantly reduced in cells cotransfected with RFP-TRF1 plus either FLAG-TRF1T122A or FLAG-TRF1T122,130A. In contrast, FLAG-TRF1T130A does not impair its ability to interact with wild-type TRF1. These data indicate that a threonine to alanine mutation at position 122 results in diminished DNA binding due to reduced dimerization of TRF1. However, we do not exclude the possibility that TRF1 carrying the T122A mutation indeed heterodimerizes with wild-type TRF1 but targets it to degradation. According to the structural data of the TRFH domain, the 122 threonine is in the middle of helix 3 of the TRF1 dimerization domain (17). Because deletion of helix 3 does not abolish the ability of TRF1 to dimerize and bind telomeres, this residue may not be directly involved in homo-dimerization but in the folding of the domain. Thus, it is possible that the T122A mutation may impair protein folding independently of blocking a putative phosphorylation site. To test whether inhibition of CK2 indeed decreases TRF1 dimerization, we repeated coimmunoprecipitation experiments with two differently tagged wild-type TRF1 proteins in the presence of DRB. As shown in Fig. 7B, the amounts of immunoprecipitated TRF1 were significantly reduced in the presence of DRB.

Finally, we examined whether phosphorylation of threonine 122 has a role in telomere length regulation. We established MCF7 cell lines stably expressing wild-type or mutant TRF1 proteins. TRF1 proteins were equally expressed in the nuclei as manifested by immunoblotting using anti-FLAG antibody (see Fig. 6C). Cells expressing mutant TRF1 grew normally and exhibited no detectable differences in growth rates or morphology over 70 population doublings. Consistent with the previous findings that TRF1 negatively regulates telomere length (20, 21), overexpression of the wild-type TRF1 or TRF1T130A led to a clear reduction in telomere length after 70 population doublings, although telomere shortening was not evident at 40 population doublings (Fig. 7C). In contrast, the TRF1T122A and TRF1T122,130A mutants caused an increase in telomere length. This result would be consistent with a loss of TRF1 binding to telomeric DNA and subsequent increase in telomere length. Taken together, these observations indicate that
RESULTS

Her we describe a novel function of CK2 for regulating the telomere binding of TRF1. Identification of CK2 as a TRF1-interacting partner raises the possibility that the function of human telomeres is regulated by phosphorylation of TRF1. The phosphorylation of threonine 122 negatively regulates telomere length by enhancing the ability of TRF1 to bind telomeric DNA.

DISCUSSION

The results presented in this work support the hypothesis that CK2-mediated phosphorylation contributes to the efficient telomere binding of TRF1. This provides a novel and important in vivo route for regulation of telomere length homeostasis.

TRF1 negatively regulates the telomere length by inhibiting access of telomerase at telomere termini (20, 21), suggesting that the protein level of TRF1 at telomeres is tightly regulated. Because CK2-mediated phosphorylation enhances the ability of TRF1 to bind telomeric DNA, TRF1 phosphorylation could lead to an increase in the TRF1 level at telomeres, thereby functioning to limit uncontrolled telomere elongation in telomerase-positive cells. Consistent with this idea, the expression level of CK2 is greatly enhanced in a number of human cancers (38). Despite the presence of high levels of telomerase activity in cancer cells, telomeres do not grow in an uncontrolled manner but are, instead, stably maintained within a given size range (39, 40). Increased CK2 activity in human cancer cells may lead to telomerase-inaccessible configuration of telomeres and contribute to maintaining the telomere length at a constant average value.

Inhibition of CK2 results in the non-phosphorylated form of TRF1, which in turn leads to a decrease in its DNA binding activity. This diminished DNA binding could be due to release of TRF1 from telomeres or due to its degradation. In the EMSA data, A-phosphatase treatment of nuclear extracts from cells transfected with FLAG-tagged TRF1 led to a reduced TRF1 binding to telomeric DNA (Fig. 2A). Furthermore, TRF1 mutation with a threonine to alanine change at position 122 resulted in diminished DNA binding under the conditions that the wild-type and mutant TRF1s were equally expressed (Fig. 6). These data indicate that TRF1 loses its DNA binding activity when non-phosphorylated. On the other hand, inhibition of CK2 by DRB yields less TRF1-telomere binding activity due to its degradation by ubiquitin-mediated proteolysis (Fig. 3). However, this effect is rescued by MG132, which suggests that TRF1 can bind DNA even when...
non-phosphorylated by CK2. Based on these data, we conclude that dephosphorylation of TRF1 induced by CK2 inhibitor releases TRF1 from telomeres. The resulting telomere-unbound form of TRF1 is then ubiquitinated and degraded by the proteasome, thereby preventing its rapid re-association with telomeres. However, in the presence of both DRB and MG132, non-phosphorylated TRF1 is not degraded and readily re-associates with telomeres because non-phosphorylated TRF1 is still capable of binding telomeric DNA, although its activity is weak. As shown in the results of EMSA and immunofluorescence experiments (Figs. 2A and 3E), DRB did not completely abolish DNA binding activity of TRF1.

In addition to TRF1 phosphorylation by CK2, it has been previously reported that ATM interacts with and phosphorylates TRF1 in response to ionizing DNA damage (41). Because cells with ATM mutations display accelerated telomere shortening to ionizing radiation (42, 43), phosphorylation of TRF1 by ATM might be involved in regulation of telomere length. Moreover, overexpression of TRF1 results in accelerated telomere shortening, a phenotype similar to that of ATM mutations (44). Although the effect of phosphorylation of TRF1 by ATM on the telomere binding has not been investigated, ATM may reduce the ability of TRF1 to bind telomeric DNA during DNA damage response. In contrast, phosphorylation of TRF1 by CK2 leads to an increase in TRF1-telomere binding. Recently, it has been reported that Xenopus TRF1 (xTRF1) dynamically associates with mitotic telomere chromatin and dissociates from interphase telomere chromatin (45). Consistent with our results, xTRF1 is phosphorylated by polo-like kinase, and its phosphorylation is required for the efficient xTRF1 association with telomeres. These findings suggest that polo-like kinase regulates telomere configuration in a cell cycle-dependent manner. During S phase, xTRF1 is dephosphorylated and released from telomeres to form a telomerase-accessible configuration, whereas the telomere association of xTRF1 in M phase leads to a telomerase-inaccessible configuration. In a similar way, CK2 may regulate TRF1 recruitment to human telomere chromatin in a cell cycle-dependent manner. Although CK2 participates in the regulation of various stages of the cell cycle progression, the mitotic phosphorylation of CK2 targets, including topoisomerase II and transcription factor Six1, was detected in mammalian cells (46, 47). Thus, cell cycle-dependent TRF1 recruitment to telomeres regulated by CK2 should be addressed in future studies.

Telomere length is regulated by tankyrases through its interaction with TRF1 (21). Tankyrase 1 poly(ADP-ribosyl)ates TRF1 and releases it from telomeres, allowing access of telomerase to telomeres and subsequently telomere elongation (22, 23). Thus, tankyrase 1 is a positive regulator of telomere length. On the contrary, CK2 interacts with and phosphorylates TRF1, and CK2-mediated phosphorylation enhances the ability of TRF1 to bind telomeric DNA, suggesting a role of CK2 as a negative regulator of telomere length. This hypothesis is supported by our findings that overexpression of the wild-type TRF1, but not TRF1T122A or TRF1T122,130A, led to shortened telomeres (Fig. 7B). Based on these results, we propose a model where two counteracting processes may determine the levels of TRF1 binding at telomeres: tankyrase 1 as a releasing factor and CK2 as a binding factor. The dynamic balance between these two proteins may regulate the telomere configurations in a cell cycle-dependent manner by determining the equilibrium level of TRF1 at telomeres, thus maintaining telomere length at a constant average length. TRF2 may constitutively remain at the telomere termini. Subsequent ubiquitination/degradation of TRF1 precludes the ability of TRF1 to bind telomeric DNA, whereas the telomere association of xTRF1 in M phase leads to a telomerase-inaccessible configuration. In a similar way, CK2 may regulate TRF1 recruitment to human telomere chromatin in a cell cycle-dependent manner. Although CK2 participates in the regulation of various stages of the cell cycle progression, the mitotic phosphorylation of CK2 targets, including topoisomerase II and transcription factor Six1, was detected in mammalian cells (46, 47). Thus, cell cycle-dependent TRF1 recruitment to telomeres regulated by CK2 should be addressed in future studies.

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When TRF1 is dissociated from telomeres by either inhibition of CK2 or activation of tankyrase 1, telomerase can gain access to the TRF1-free telomeres to elongate the chromosome termini. Subsequent ubiquitination/degradation of TRF1 prevents its rapid re-association with telomeres. Consistent with a previous report (24), our results suggest that telomeres are re-assembled with newly synthesized TRF1 rather than the dissociated telomere-unbound form to establish telomerase-inaccessible telomeres. Thus, sequential post-translational modifications of TRF1, including phosphorylation by CK2, poly(ADP-ribosylation) by tankyrases, and ubiquitination by
CK2-mediated Phosphorylation of TRF1

Fbx4 (48), play important roles in modulating telomere length homeostasis by determining the levels of TRF1 at telomeres.

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