THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 45, Issue of November 5, pp. 47264–47271, 2004

Printed in U.S.A.

TIN2 Binds TRF1 and TRF2 Simultaneously and Stabilizes the TRF2 Complex on Telomeres*

Received for publication, August 6, 2004, and in revised form, August 12, 2004
Published, JBC Papers in Press, August 16, 2004, DOI 10.1074/jbc.M409047200

Jeffrey Zheng-Sheng Ye‡§¶‖, Jill R. Donigian‡‖, Megan van Overbeek‡‖**, Diego Loayza‡**, Yan Luo‡‡§§, Andrew N. Krutchinsky¶¶, Brian T. Chait¶¶, and Titia de Lange‡‖‖**

From the ‡Laboratory for Cell Biology and Genetics, the ¶¶Laboratory for Mass Spectrometry and Gaseous Ion Chemistry, and the ‡‡Laboratory for Biochemistry and Molecular Biology, the Rockefeller University, New York, New York 10021 and the §Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

Human telomeres contain two related telomeric DNA-binding proteins, TRF1 and TRF2. The TRF1 complex contains the TRF1 interacting partner, TIN2, as well as PIP1 and POT1 and regulates telomere-length homeostasis. The TRF2 complex is primarily involved in telomere protection and contains the TRF2 interacting partner human (h)Rap1 as well as several factors involved in the DNA damage response. A prior report showed that conditional deletion of murine TRF1 reduced the presence of TRF2 on telomeres. Here we showed that TRF2 is also lost from human telomeres upon TRF1 depletion with small interfering RNA prompting a search for the connection between the TRF1 and TRF2 complexes. Using mass spectrometry and co-immunoprecipitation, we found that TRF1, TIN2, PIP1, and POT1 are associated with the TRF2-hRap1 complex. Gel filtration identified a TRF2 complex containing TIN2 and POT1 but not TRF1 indicating that TRF1 is not required for this interaction. Co-immunoprecipitation, Far-Western assays, and two-hybrid assays showed that TIN2, but not POT1 or PIP1, interacts directly with TRF2. Furthermore, TIN2 was found to bind TRF1 and TRF2 simultaneously, showing that TIN2 can link these telomeric proteins. This connection appeared to stabilize TRF2 on the telomeres as the treatment of cells with TIN2 small interfering RNA resulted in a decreased presence of TRF2 and hRap1 at chromosome ends. The TIN2-mediated cooperative binding of TRF1 and TRF2 to telomeres has important implications for the mechanism of telomere length regulation and protection.

The TTAGGG repeat arrays of mammalian telomeres associate with two related telomeric DNA-binding proteins, TRF1 and TRF2 (1–3). These factors have closely related C-terminal Myb-type DNA binding domains and bind TTAGGG sequences as dimers or higher order oligomers. Dimerization is mediated by the TRF-homology domain, the signature motif of this family of telomeric proteins (4, 5). The crystal structure of the TRFH domains of TRF1 and TRF2 shows that the heterodimerization of TRF1 and TRF2 is impeded by crucial amino acid differences in the main dimerization interface (4, 5), and TRF1/TRF2 heterodimers are not formed *in vitro* or *in vivo* (3). Therefore, the prevailing view has been that TRF1 and TRF2 form two separate complexes at telomeres.

TRF1 recruits a number of other proteins to telomeres (reviewed in Ref. 6). The acidic N terminus of TRF1 binds to tankyrase 1 and 2 which are poly(ADP-ribose) polymerases that can modify TRF1 (7–11). ADP-ribosylation of TRF1 impedes its DNA binding activity *in vitro*, and tankyrase overexpression removes TRF1 from the telomeres and promotes its degradation. The *in vitro* ADP-ribosylation of TRF1 by tankyrase is inhibited by a second TRF1-interacting partner, TIN2 (12, 13). TIN2 appears to protect TRF1 from tankyrase *in vivo*, because RNAi[1]-mediated depletion of TIN2 results in tankyrase-dependent TRF1 loss (12, 13). TIN2 also functions to recruit PIP1 (also referred to as PTOP) to the TRF1 complex (14, 15). PIP1 is a POT1-interacting protein that mediates the binding of POT1 to the TRF1 complex (14, 15). A third direct interacting partner of TRF1 is PINX1 (16), and TRF1 has been shown to bind Ku (17), the BLM RecQ helicase (18, 19), and the ATM kinase (20, 21).

TRF2 also has a number of direct and indirect interacting partners. Most or all TRF2 is in a complex with human (h)Rap1, a direct interacting partner of TRF2 (22). The TRF2-hRap1 complex interacts with the Mre11-Rad50-Nbs1 recombinational repair complex (23), the ERCC1/XPF nucleotide excision repair endonuclease (24), the ATM kinase (21), the WRN and BLM helicases (25), and Ku (26). These interactions are likely to occur only in a fraction of the telomeric TRF2 complex, because none of these DNA damage response factors are abundant at telomeres, whereas TRF2/hRap1 is.

The idea that TRF1 and TRF2 form independent complexes was reinforced by functional studies revealing their distinct roles at telomeres. TRF1, TIN2, PIP1, and POT1 are all implicated in telomere-length homeostasis, a process that regulates the maintenance of telomeric DNA by telomerase (Refs. 12–15, 27–29, and reviewed in Ref. 6). TRF2 on the other hand is crucial for telomere protection (reviewed in Ref. 30). Its inhibition leads to dysfunctional telomeres that are detected and

* This work was supported by Grants GM49069 and RR00862 from the National Institutes of Health (to T. d. L. and B. T. C., respectively). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Supported by a Mentored Clinical Investigator Award K08CA93604 from the NCI, National Institutes of Health and by The Translational and Integrative Medicine Research Fund from Sloan-Kettering Cancer Institute. Current address: Division of Hematology, Depts. of Medicine and Pharmacology, New York University School of Medicine, 550 First Ave., New York, NY 10016.

‖ These authors contributed equally to this work.

** Supported by training grants.

§§ Current address: Laboratory of Gene Expression and Regulatory Biology, Institute of Molecular and Cell Biology, 61 Biopolis Dr., Proteos Building, Singapore 138673.

‖ ‖ To whom correspondence should be addressed: Box 159, The Rockefeller University, 1230 York Ave., New York, NY 10021. Tel.: 212-327-8146; Fax: 212-327-7147; E-mail: delange@mail.rockefeller.edu.

[1] The abbreviations used are: RNAi, RNA interference; h, human; ES, embryonic stem; siRNA, small interfering RNA; IF, initiation factor; HA, hemagglutinin; FH2, FLAG-HA-HA; IP, immunoprecipitation; IVT, *in vitro* translated.

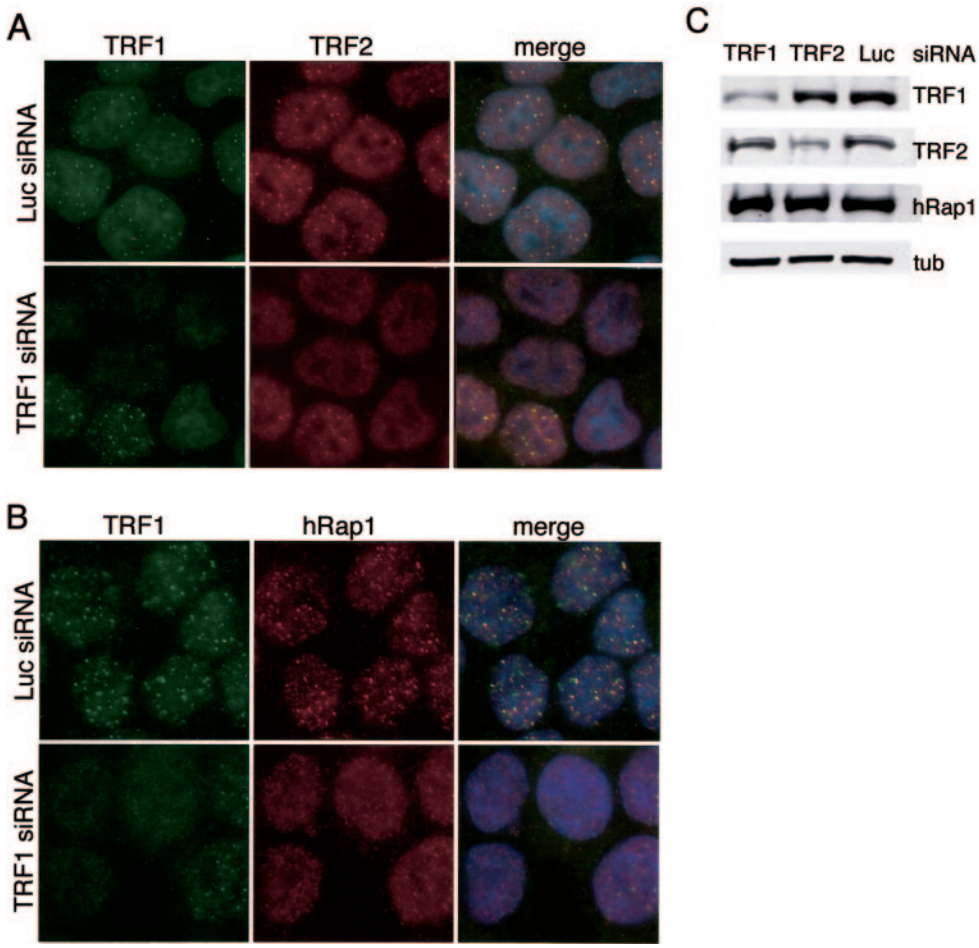

FIG. 1. **TRF1 siRNA reduces the presence of TRF2 and hRap1 on telomeres.** *A*, reduced TRF2 telomeric signals after TRF1 siRNA treatment. The IF of HeLa cells 48 h after introduction of siRNA to TRF1 or luciferase (*Luc*) as indicated. TRF1 was detected with a mouse polyclonal antibody (*green*). TRF2 was detected with number 647 (*red*). DNA is stained with 4,6-diamidino-2-phenylindole and included in the merged panels. *B*, reduced hRap1 telomeric signals after TRF1 siRNA treatment. The experimental set-up was as in *A* except that hRap1 was detected with number 666 (*red*). *C*, depletion of TRF1 with siRNA does not affect TRF2 and hRap1 protein levels. The immunoblot of HeLa cell proteins harvested 72 h after the introduction of siRNAs to TRF1, TRF2, or luciferase is shown. Antibodies are TRF1, number 371; TRF2, number 647; hRap1, number 765; *tub*, α-tubulin detected with DM1α (Sigma).

processed by the DNA damage response machinery (31–34). However, targeted deletion of Trf1 in the mouse resulted in early embryonic lethality and ES cells deprived of Trf1 function die rapidly (35, 36). The cell-lethal phenotype of Trf1 loss would not be expected if Trf1 solely acted to regulate telomere length and suggests that Trf1 may be contributing to telomere protection. Interestingly, Trf1-deficient ES cells show diminished presence of Trf2 at chromosome ends, although the telomeric DNA is preserved (35). Here we report that human TRF1 and TRF2 are linked by a common interacting factor, TIN2, and that this connection is required for the stable binding of TRF2 to chromosome ends. These findings suggest that the lethal phenotype of TRF1 deficiency is because of a defect in telomere protection by TRF2.

EXPERIMENTAL PROCEDURES

siRNA Experiments—Double-stranded siRNA was generated to target human TRF1 mRNA at nucleotides 102–120 (CGACGAGGAGCAGTTCGAA). TIN2 and green fluorescent protein siRNAs have been described previously (12). Sequences for the control siRNA to luciferase and the TRF2 siRNA are available upon request. HeLa cells were transfected using Oligofectamine (Invitrogen) using a protocol supplied by the manufacturer. Specifically, 5×10^5 cells were inoculated to a 6-cm culture dish and after 16–24 h were subjected to two sequential transfections separated by a 24-h interval. Shortly after the second transfection, cells were trypsinized and plated to glass coverslips placed in a 10-cm culture dish. Cells were either fixed at 48 h after the initial transfection for IF, or protein for immunoblots was isolated after 72 h (12).

Mass Spectrometric Analysis of the hRap1 Complex—For isolation of hRap1 complexes from HeLaS3 cells, two retroviral vectors based on pLPC were generated by introducing a FLAG-HA-HA tag (FH2) in a N- or C-terminal position (details available on request). Human Rap1 cDNA was PCR-amplified and cloned separately into either the N or C terminally tagged constructs and sequenced. These constructs were transfected into amphotrophic Phoenix cells using calcium phosphate and the retroviral stocks were prepared for infection of semi-adherent HeLaS3 cells. Clones expressing tagged hRap1 were selected using puromycin (2 μg/ml) and isolated with cloning cylinders. The efficiency of N and C terminally tagged hRap1 in forming a complex with endogenous TRF2 and TRF1, were tested by co-immunoprecipitations (IP). HeLa S3 clones that expressed tagged hRap1 at a level 5–10-fold above that of endogenous hRap1 protein were selected for purification. Cells were grown in suspension culture (20 liters) at 37 °C to a density between 0.9 and 1.2×10^6/ml. Cell harvest, extraction of nuclear proteins, sequential binding to affinity matrix, and peptide elutions were performed according to published procedures (37) except that commercial affinity resins were used (anti-FLAG M2 resin from Sigma and anti-HA 3F10 resin from Roche). Eluted proteins were separated by SDS-PAGE (4–20% gradient, Invitrogen). The entire gel lane was sliced into 29 2-mm pieces, and the proteins in each gel piece were subjected to trypsin digestion. The resulting peptides were extracted and the proteins identified using a combination of two different mass spectrometers. First, tryptic mass maps of proteins from each gel piece were obtained using an in-house-constructed MALDIQqTOF mass spectrometer, and second, fragmentation spectra of all the discernable tryptic

peptides were obtained using an in-house-constructed MALDI-ion trap mass spectrometer (38, 39). Accurate masses of the tryptic peptides and the masses of their fragments were used to identify proteins in each gel piece with the computer search engine XProteo.

Co-immunoprecipitation Analysis—The nuclear extract was prepared from HeLaS3 cells expressing N-FH2-TIN2 and C-FH2-TRF1 (15) and precleared by Sepharose CL-4B for 30 min at 4 °C. A typical IP contained 200 μl of precleared nuclear extract (5–6 mg/ml) and various antibodies (anti-FLAG (M2), 4–6 μg; anti-HA (3F10),1.0–1.2 μg; anti-myc (9E10, Oncogene), 0.6–1.0 μg; polyclonal anti-TRF1 (number 371), 0.6–1.0 μg; polyclonal anti-TIN2 (number 865), 0.6–1.0 μg)). After rotating for 2–3 h at 4 °C, 30 μl of protein G-Sepharose beads (settled volume) were added to each IP (beads were preblocked o/n with 10% bovine serum albumin in phosphate-buffered saline), and the tubes were rotated for another hour. Beads were collected by centrifugation and washed three times with lysis buffer, eluted with Laemmli loading buffer, and analyzed by immunoblotting as described previously (15). Where indicated, ethidium bromide or RNase A was added to 100 μg/ml during the IP. For peptide blocking controls, FLAG or HA peptide was added to a final concentration of 250 μg/ml or 500 μg/ml, respectively, together with M2 or 3F10 antibody. Co-IPs of proteins from 293T cells were performed as described previously (12).

For co-IPs of TRF1 and TRF2 from BJ/hTERT cells, cells were retrovirally infected with FLAG-tagged TRF1 or vector alone and were expanded on 15-cm plates. At confluency, cells were trypsinized, collected, washed in 10× pellet volume phosphate-buffered saline, washed in 10× pellet volume resuspension buffer (10 mM Tris-HCl (pH 7.4), 60 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA, and 0.1 mM EGTA), and resuspended in 10× pellet volume lysis buffer (resuspension buffer with 0.2% Nonidet P-40, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and a complete protease inhibitor mixture tablet (Roche)). The cell lysate was kept on ice for 10 min with occasional mixing, and the nuclei were collected by centrifugation, washed in resuspension buffer, and then resuspended in 3× pellet volume nuclear extraction buffer (20 mM Tris-HCl (pH 7.4), 400 mM NaCl, 0.5 mM EDTA, 0.2% Nonidet P-40, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and a complete protease inhibitor mixture tablet). The nuclear extract was kept on ice for 30 min with occasional vortexing, the lysate (derived from ~3×10^8 cells) was centrifuged, and the supernatant was diluted with an equal volume of water. The diluted supernatant was incubated with 100 μl (settled volume) of bovine serum albumin-blocked Sepharose 6B beads for 30 min at 4 °C, centrifuged, removed from the beads, incubated with 100 μg/ml ethidium bromide for 20 min on ice (yielding the IP input), and then incubated with 100 μl (settled volume) of bovine serum albumin-blocked FLAG beads overnight at 4 °C. Beads were washed four times with 1 ml of wash buffer (20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 0.2 mM EDTA, 0.1% Nonidet P-40, 15% glycerol, and 0.5 mM phenylmethylsulfonyl fluoride) and then incubated with 120 μl of elution buffer (wash buffer with 0.2 mg/ml FLAG peptide).

Gel Filtration—Nuclear extract from HeLaS3 cells (10 ml, 8 mg protein/ml) was dialyzed overnight at 4 °C against BC150/40% glycerol (20 mM Tris (pH 7.3), 150 mM KCl, 0.2 mM EDTA, 40% glycerol, 0.025% Nonidet P-40, and 0.5 mM dithiothreitol) and cleared by ultracentrifugation at 25,000 rpm for 30 min. The dialyzed sample (~5 ml) was loaded to a Sephacryl S-300 (Amersham Biosciences) column (2.5 cm × 70 cm, ~350 ml of packed volume) that was equilibrated with BC150/20% glycerol. Proteins were fractionated with BC150/20% glycerol at a linear flow rate of 25 ml/h, and 5-ml fractions were collected. Blue dextran (2 MDa) appears at the end of the void volume (approximately one-third of column volume), and bovine serum albumin (67 kDa) appears at approximately two-thirds of the column volume).

Two-hybrid Interactions—Yeast two-hybrid assays were performed as described previously (15).

Far-Western Analysis—Two micrograms of purified protein derived from Sf21 insect cells or *Escherichia coli* were subjected to SDS-PAGE and then blotted onto nitrocellulose. The blots were incubated in blocking buffer (10 mM HEPES (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 5% milk) for 3 h at 4 °C. Following the blocking step, the blots were probed overnight at 4 °C with ^{35}S-labeled *in vitro* translated (IVT) protein prepared using the TNT T7 Coupled Reticulocyte Lysate System (Promega) (a 50-μl reaction mixture in 5 ml of blocking buffer). The next morning, the blots were washed five times every 30 min in wash buffer (10 mM HEPES (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 0.25% milk) and then incubated with Amplify (Amersham Biosciences) for 10 min. The blots were exposed on a PhosphorImager screen overnight. For the modified Far-Western experiment, the blots were incubated with 4 μg of baculovirus-derived TIN2 in 5 ml of blocking buffer after the blocking step, washed three times every 5 min in wash buffer, and processed as described above.

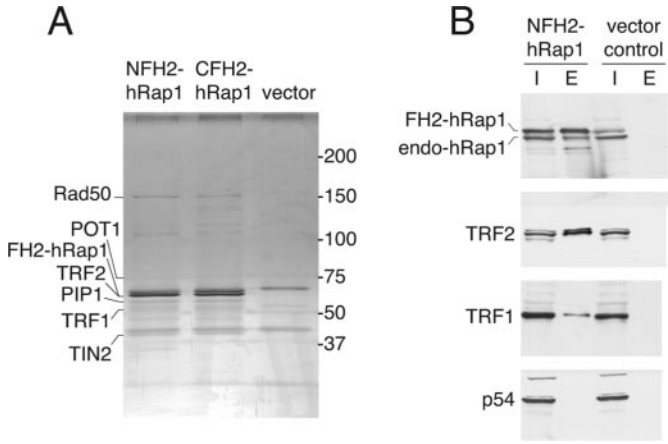

FIG. 2. **Identification of TRF1 complex components in the hRap1 complex.** *A*, proteins present in purified hRap1 complex. Shown is a silver-stained gel of the indicated affinity-purified hRap1 complexes derived from HeLaS3 cells expressing FH2-tagged hRap1 (N or C terminally tagged as indicated) and material derived from vector control cells processed in parallel. Relevant interacting proteins that were identified by mass spectrometry are indicated next to the lanes. Peptides derived from TRF1 were not identified by mass spectrometry of the hRap1 complex. The presence of TRF1 in the indicated band was deduced from immunoblotting analysis. *B*, immunoblotting analysis of the purified hRap1 complex. The affinity-purified hRap1 complex from HeLaS3 cells expressing N terminally tagged hRap1 or vector control cells analyzed for the presence of hRap1 (number 765), TRF1 (number 371), TRF2 (number 647), or a control protein (anti-p54) is shown. Input (*I*) lanes contained 0.1% of input lysate, elution (*E*) lanes contained 2% of the eluate.

RESULTS

TRF1 RNAi Results in Reduced TRF2 and hRap1 Signals at Telomeres—In mouse ES cells, a conditional deletion of the *Trf1* gene results in partial loss of Trf2 from chromosome ends. To verify that this effect also takes place in human cells, we used RNAi to TRF1. HeLa cells were transfected with TRF1 or control siRNAs and processed for immunofluorescence after 48 h. At this time point, TRF1 protein levels and the TRF1 IF signals are severely reduced (Fig. 1). Concomitant with the loss of TRF1 from telomeres, we observed a reduction in the IF signals of TRF2 and hRap1 (Fig. 1, *A* and *B*). Approximately 80% of the nuclei showed diminished TRF1 signals ($n = 200$), and of these 95% also showed a loss of the typical punctate pattern of TRF2 and hRap1 at telomeres. TRF1 RNAi did not affect the levels of TRF2 and hRap1 proteins as detected by immunoblotting (Fig. 1*C*), suggesting that TRF2/hRap1 are still present but have a diminished ability to accumulate at chromosome ends. When IF was performed without treatment with Triton X-100, which removes nucleoplasmic proteins, TRF2 and hRap1 were detected throughout the nucleus in cells treated with TRF1 siRNA, whereas control cells showed a punctate telomeric pattern under these conditions (data not shown). These data showed that TRF1 inhibition affects the accumulation of the TRF2 complex on telomeres in the human cells as it does in mouse ES cells.

Detection of TIN2, PIP1, and POT1 in Association with TRF1 and TRF2—A possible explanation for the effect of TRF1 siRNA on TRF2 is the presence of a proteinaceous link between these two complexes that stabilizes TRF2 on telomeres. To find TRF1- and TRF2-interacting factors that might represent such a linking factor, we performed mass spectrometry on isolated TRF1 and TRF2 complexes. To identify components of the TRF2-hRap1 complex, HeLa cells with moderate overexpression of hRap1 tagged with a dual N-terminal FLAG-HA-HA cassette (FH2-

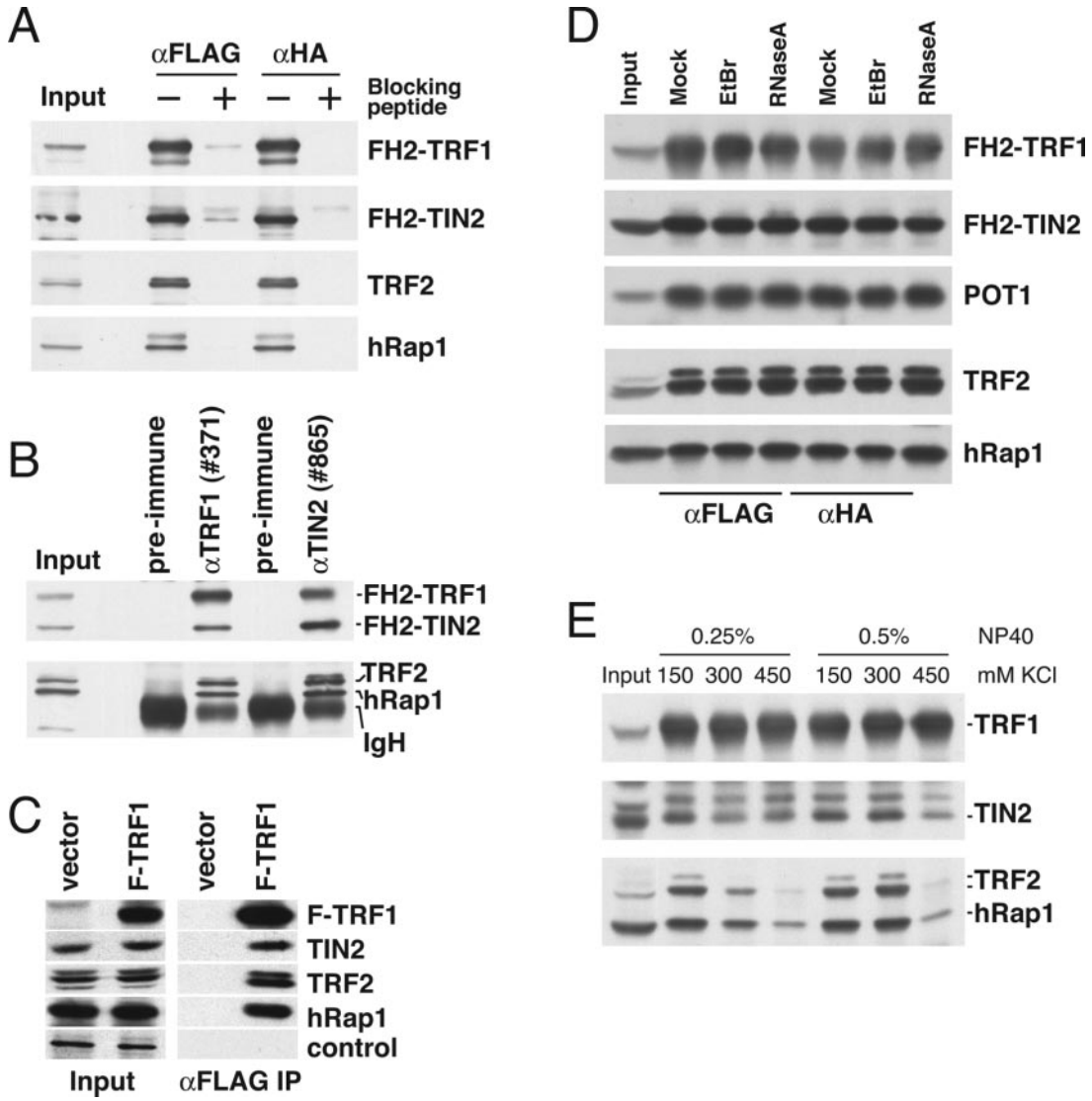

FIG. 3. **Co-immunoprecipitation of the TRF1 and TRF2 complexes.** *A*, co-IP of endogenous TRF2/hRap1 with tagged TRF1/TIN2. IP of nuclear extract from HeLaS3 cells expressing C terminally tagged FH2-TRF1 and N terminally tagged FH2-TIN2 with anti-FLAG (M2) or anti-HA (3F10) antibody in the absence or presence of blocking peptides. Antibodies used for immunoblotting were anti-TRF1 (number 371), anti-TIN2 (number 864), anti-TRF2 (number 647), and anti-hRap1 (number 765). *B*, co-IP of endogenous TRF2/hRap1 with TRF1/TIN2. IP and immunoblotting were the same as in *A* except anti-TRF1 (number 371) or anti-TIN2 (number 864) were used in IP. Pre-immune sera were used as control. *C*, immunoblot of extracts from retrovirally infected BJ/hTERT cells expressing FLAG-tagged TRF1 or vector alone immunoprecipitated with FLAG beads and eluted with FLAG peptide. Antibodies used for the immunoblot are indicated to the *right* (*control* is a nonspecific band that reacted with the hRap1 antibody). *D*, no effect of ethidium bromide (*EtBr*) or RNase on the TRF1-TRF2 association. Ethidium bromide or RNase A were added to nuclear extract (both to 100 μg/ml) during preclearance and throughout the IP reaction. Immunoblotting was done as in *A* except anti-POT1 antibody (number 978) was also used. *E*, TRF1-TRF2 complex dissociation in 450 mM KCl. IP and immunoblotting were done as in *A* except KCl concentration varied and two Nonidet P-40 (*NP40*) concentrations were used as indicated. Anti-TRF1 (number 371) was used for IP.

hRap1) were used for the affinity purification of the hRap1 complex following a protocol established for telomeric complexes (15) (Fig. 2). As expected from previous work (23, 24), mass spectrometry identified TRF2 and Rad50 in association with hRap1. In addition, peptides derived from TRF1, PIP1, and POT1 were detected in two independent isolates of the hRap1 complex but not when vector control cells were subjected to the same procedure. Similarly, we identified peptides derived from TRF2 and hRap1 in a TRF1-TIN2 complex that was isolated from HeLa cells using the same dual affinity purification scheme (15). These data are consistent with the report of Songyang and co-workers (14), who identified TRF2 and hRap1 in association with TIN2 by mass spectrometry.

The presence of TRF1 in the purified FH2-hRap1 complex was verified by immunoblotting (Fig. 2*B*), which showed that ~5–10% of the endogenous TRF1 could be recovered in association with the hRap1 complex. Extracts from vector control cells did not yield TRF1, and as an additional control, an irrelevant nuclear protein (the RNA-binding protein p54(nrb); (40)) was not present in the hRap1 complex. Immunoblotting also verified the specific association of TRF2 and hRap1 with affinity purified TRF1-TIN2 complexes from cells expressing FH2-TRF1 and FH2-TIN2 (Fig. 3, *A* and *B*). The association between TRF1 and the TRF2 complex was not a specific feature of the HeLa cells used for these experiments, because it was also noted in a different cell line, BJ/hTERT cells, expressing FLAG-tagged TRF1 (Fig. 3*C*).

We next tested whether the association between the TRF1 and TRF2 complexes was because of tethering by nucleic acid. The addition of ethidium bromide to cell extracts at a concentration that releases TRF2 from DNA *in vitro* did not affect the interaction of TRF2/hRap1 with the TRF1 complex (Fig. 3, *C* and *D*). Similarly, RNase A treatment did not affect the recovery of TRF2 and hRap1 in the TRF1/TIN2 IPs (Fig. 3*D*), argu-

ing against tethering by RNA. However, when the TRF1-TIN2 complex was treated with high salt (450 mM KCl), the association with TRF2 and hRap1 was severely diminished (Fig. 3*E*). The salt sensitivity of the interaction between the TRF1 and TRF2 complexes can explain why TRF1 and its associated factors were not recovered in a previously analyzed TRF2 complex that isolated from a high salt heparin chromatography fraction (23, 24).

Telomeric Complexes Analyzed by Gel Filtration—Gel filtration was used to gain further insight into the interaction between the TRF2 complex and the TRF1 complex. Endogenous telomeric protein complexes of HeLa cells were size-fractionated and constituent proteins were identified by immunoblotting (Fig. 4). We detected a TRF2-hRap1 complex (complex III) in the lower molecular mass range that appeared to lack the other telomeric proteins. This is consistent with the direct interaction between these two factors (22). The fractions in the 2 MDa range (the exclusion limit of the column) contained tankyrase 1, TRF1, TIN2, POT1, TRF2, and hRap1. Although the co-elution of these proteins could be because of the presence of a single large complex, it is also possible that these high molecular mass fractions contain multiple complexes that each have a molecular mass in the ~2-MDa range or that they are held together by DNA. The most informative complex was recovered in fractions 24–26 (complex II), which contained TRF2, hRap1, TIN2, and POT1 but lacked TRF1 and tankyrase 1. The reduced presence of TRF1 in these fractions suggested that the association of TIN2 and POT1 with TRF2/hRap1 is not mediated by TRF1. The lack of requirement for TRF1 in this association further confirms that DNA tethering is an unlikely source of the connection between the telomeric complexes and points to an association of either TIN2 or POT1 or one of their interacting partners with the TRF2-hRap1 complex.

Direct Binding of TIN2 to TRF2 and TRF1—Because TIN2 and POT1 appeared to be shared between the TRF1 and TRF2 complexes, we tested these factors and their common binding partner PIP1 for interactions with TRF2 and hRap1. A previous two-hybrid analysis indicated that PIP1 does not bind TRF1, TRF2, or hRap1 (15). Similarly, POT1 did not interact with TRF2 or hRap1, and TIN2 did not interact with hRap1 (data not shown). However, TIN2 showed a robust interaction with TRF2 (Table I). The interaction could be observed with full-length TIN2 as well as with an N-terminal fragment of TIN2. The C terminus of TIN2 also showed significant interaction with TRF2, but this interaction is very weak. In contrast, the interaction of TRF1 with TIN2 is not affected by a deletion of the N-terminal half of the protein, indicating that TIN2 binds TRF1 and TRF2 through different domains.

The interaction of TIN2 with TRF2 was further tested by a Far-Western assay in which baculovirus-derived purified TRF2 and other relevant proteins were probed with [^{35}S]methionine-labeled *in vitro* translated TIN2 (Fig. 5*A*). Using this assay, we detected a robust interaction of TIN2 with TRF2 (Fig. 5*A*). The binding of TIN2 to TRF2 was not observed with TIN2–13, a C-terminal fragment of TIN2 that retains its TRF1 interacting domain (data not shown). Thus, these data corroborate the yeast two-hybrid data indicating that TIN2 interacts with TRF2 and that this interaction requires sequences present in the N terminus of TIN2.

Because TIN2 binds TRF1 and TRF2 with distinct domains, we considered that the TIN2 interacting domains in TRF1 and TRF2 might be different as well. The interaction with TIN2 has been mapped within the TRFH domain of TRF1, a region similar to the TRF2 TRFH domain. TRF1 and TRF2 differ most prominently in their N termini, which is acidic in TRF1 and basic in TRF2. We therefore tested whether the basic N terminus of TRF2 was responsible for the binding of TIN2. However, in the Far-Western assay, TIN2 bound efficiently to a form of TRF2 lacking this region (Fig. 5*A*).

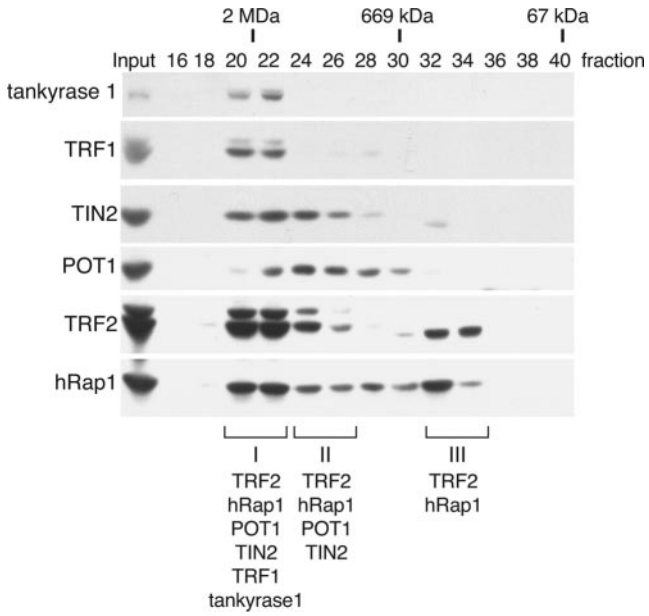

FIG. 4. **Gel-filtration analysis of telomeric complexes.** Immunoblotting analysis of endogenous telomeric proteins in HeLa nuclear extract fractionated on Sephacryl S-300. 10 μl of the indicated fractions were loaded/lane. Antibodies used: anti-tankyrase 1, number 465; anti-TRF1, number 371; anti-TIN2, number 864; anti-POT1, number 978; anti-TRF2, number 647; and hRap1, number 765. Molecular mass markers used are blue dextran (*2 MDa*), thyroglobulin (*669 kDa*), and bovine serum albumin (*67 kDa*).

Having established that TIN2 binds to TRF2 as well as to TRF1, we asked whether TIN2 can link TRF1 to TRF2. To address this issue, we performed a modified Far-Western assay in which unlabeled TIN2 is tested for its ability to mediate binding of labeled TRF1 to filter-bound TRF2 (Fig. 5*B*). As expected, in the absence of TIN2, *in vitro* translated TRF1 associated with filter-bound TIN2 but did not bind to TRF2. However, when the same assay with labeled TRF1 was performed in the presence of (unlabeled) TIN2 in a binding mixture, TRF1 had the ability to associate with TRF2. This result suggested that TIN2 can tether TRF1 to TRF2.

TIN2 Affects the Presence of TRF2 on Telomeres—As shown here for human cells and reported previously for mouse cells, a depletion of TRF1 from telomeres leads to partial removal of TRF2 as well. The finding that TIN2 can form a proteinaceous link between TRF1 and TRF2 suggested that TIN2 loss would also lead to the removal of TRF2. To test this, we used siRNA depletion of TIN2. We reported previously on two siRNAs to TIN2 that strongly reduced the TIN2 levels and removed TIN2 and TRF1 from telomeres (12). The TIN2 depletion also diminished the abundance of TRF1. These effects were in part because of the tankyrase-mediated modification of TRF1, because they were reversed by the PARP inhibitor 3AB or overexpression of a PARP-dead allele of tankyrase. The same siRNAs to TIN2 were used here to examine the effect of TIN2 depletion on the telomeric accumulation of TRF2 and hRap1. Although the proteins levels of TRF2 and hRap1 were not affected, the telomeric signals of these two factors were strongly reduced by TIN2 knockdown (Fig. 6). The same effect was observed for the second TIN2 siRNA (data not shown). The TIN2 siRNA led to loss of TIN2 signal in ~80% of the cells (n = 200) and of these greater than 90% of the nuclei showed diminished TRF2 telomeric signals. Thus, loss of TIN2 affects the presence of TRF2 at chromosome ends.

TABLE I
Two-hybrid interaction of TIN2 with TRF1, PIP1, and TRF2

Interactions are expressed in α-galactosidase (Miller) units for the indicated two-hybrid combinations. The numbers represent average values from three independent transformants and S.D.s less than 0.01 are given as 0.01.

	pACT2[a]	TRF1::GAD	TRF2::GAD	PIP1::GAD	POT1::GAD
pBTM116[a]	0.4 ± 0.2	0.5 ± 0.06	0.2 ± 0.02	0.5 ± 0.01[c]	0.2 ± 0.01
TIN2::LexA	0.04 ± 0.01	183 ± 24	70 ± 7	418 ± 30[c]	0.03 ± 0.01
TIN2N::LexA[b]	0.2 ± 0.01	231 ± 39	130 ± 4.6	345 ± 37.5	0.02 ± 0.01
TIN2C::LexA[b]	0.01 ± 0.01	275 ± 36	2.6 ± 0.07	0.02 ± 0.01	0.2 ± 0.01

[a] pBTM116 is the $LexA_{DBD}$ vector; pACT is the GAD vector.
[b] TIN2N comprises the N-terminal 275 amino acids; TIN2C comprises amino acids 196–354 (C terminus).
[c] From Ye *et al.* (12,15).

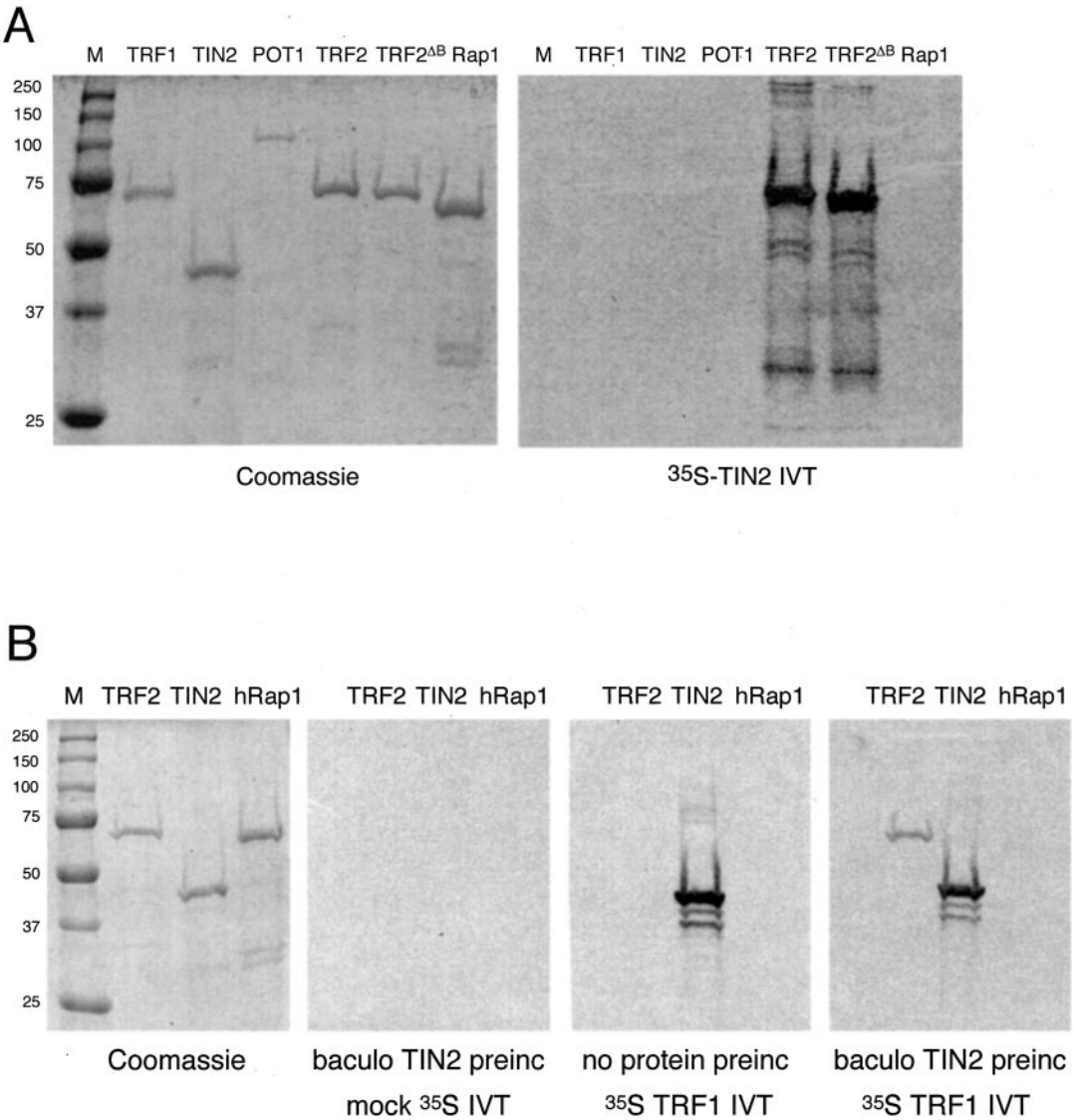

FIG. 5. **TIN2 interacts with TRF1 and TRF2 simultaneously.** *A*, Far-Western analysis of indicated telomeric proteins with [^{35}S]TIN2 IVT protein. Each lane contains ~2 μg of purified protein derived from *E. coli* (using glutathione *S*-transferase; POT1) or Sf21 cells (using a His-tag; all other proteins). The proteins were subjected to SDS-PAGE, stained with Coomassie Blue (*left*), or blotted onto nitrocellulose and incubated with [^{35}S]TIN2 IVT protein (*right*). *B*, modified Far-Western analysis of the ability of TIN2 to bridge TRF1 to TRF2. From *left* to *right*, Coomassie Blue-stained gel loaded with 2 μg of purified baculovirus TRF2, TIN2, and hRap1; nitrocellulose blot of the same proteins preincubated with baculovirus TIN2 followed by incubation with mock ^{35}S-labeled IVT protein (no DNA was added to the IVT reaction); blot preincubated without protein followed by incubation with ^{35}S-labeled TRF1 IVT protein; blot preincubated with baculovirus TIN2 followed by incubation with ^{35}S-labeled TRF1 IVT protein.

DISCUSSION

Here we reported that TIN2 mediates an interaction between the TRF1 and TRF2 telomeric complexes. TIN2 binds to TRF1 and TRF2 and can interact with both factors simultaneously. The removal of TIN2 or TRF1 from telomeres leads to a concomitant loss of TRF2 and hRap1. Thus, the TRF1-TIN2-TRF2 link is important for the stable binding of TRF2 to telomeres. This observation can explain the embryonic lethality of Trf1−/− and Tin2−/− mice and the death of ES cells deprived of Trf1 (35, 36, 41). Furthermore, the data indicate that POT1 is a member of both the TRF1 and the TRF2 complex. The finding of a connection between TIN2 and TRF2 has extensive

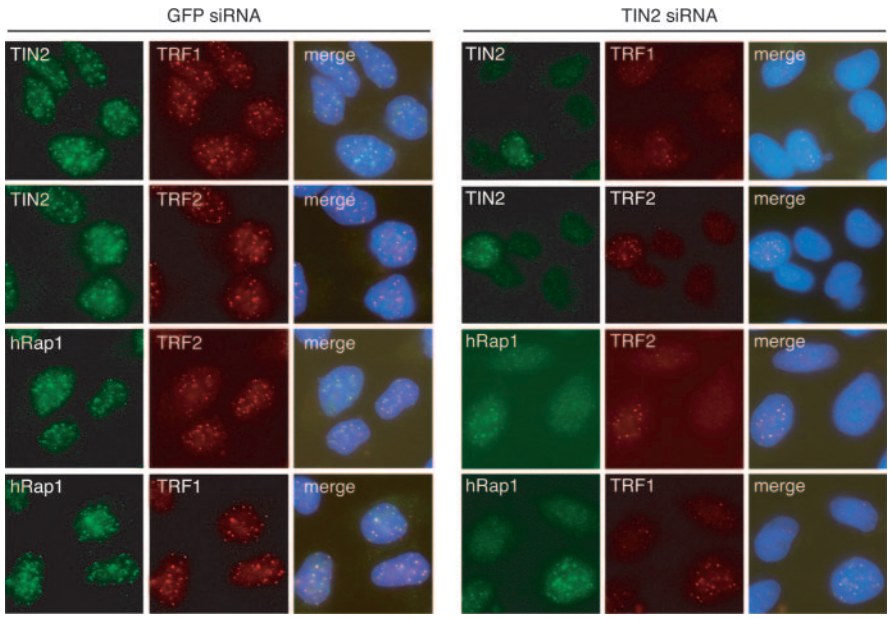

FIG. 6. **Reduced presence of TRF2/hRap1 on telomeres in response to TIN2 siRNA.** HeLa1.2.11 cells were transfected with control green fluorescent protein (*GFP*) siRNA (Dharmacon) or TIN2 siRNA (nucleotides 303–323) and analyzed by immunofluorescence using anti-TIN2 (rabbit polyclonal, number 864), anti-hRap1 (rabbit polyclonal, number 765), anti-TRF1 (mouse polyclonal serum), or anti-TRF2 (mouse monoclonal antibody, Upstate). Polyclonal antibodies are labeled in *red*, and monoclonal antibodies are labeled in *green*. *Merge* includes 4,6-diamidino-2-phenylindole staining of DNA (*blue*).

repercussions for our understanding of telomere function.

TIN2-mediated Cooperative Binding of TRF1 and TRF2 to Telomeres—TRF1 and TRF2 bind to the same sequence and have largely similar DNA binding features. Individually, these proteins bind in a non-cooperative fashion along long arrays of TTAGGG repeats and their off-rate is high (4, 42).[2] *In vivo* photobleaching of overexpressed green fluorescent protein-tagged TRF1 and TRF2 also suggests a short residence time on telomeric DNA *in vivo*, but the behavior of the endogenous proteins is not known (43). Our data suggested that TIN2 plays an important role in stabilizing the TRF2 complex by tethering it to the TRF1 complex. Interactions between two DNA-binding proteins bound to neighboring sites will decrease the off rate of each and increase their affinity for DNA. Thus, the TIN2 link between TRF1 and TRF2 could increase the specificity of both proteins for telomeres. This effect may be particularly important with regard to TRF2, which has interactions with a number of abundant non-telomeric proteins, such as ATM, the Mre11 complex, and ERCC1/XPF. These interactions could lead to an inappropriate localization of TRF2 at non-telomeric sites, for instance, when these factors accumulate at sites of DNA damage.

The stabilization of the TRF2 complex by TIN2 tethering to TRF1 may explain the lethal phenotype of TRF1 and TIN2 deficiency in the mouse. A diminished TRF2 binding to telomeres in the absence of the TRF1/TIN2 stabilizing factor might result in telomere deprotection, which would impede cell proliferation. In tissue culture experiments as well as in conditional deletion of TRF2 from mouse cells, the loss of TRF2 from telomeres results in telomere-telomere fusions (30).[3] However, telomere fusions have not been observed in the TRF1-deficient mouse cells. Although technical challenges could explain this negative result, it is also possible that the telomere deprotection phenotype in the TRF1 knock-out setting is qualitatively different from that of TRF2 loss. Perhaps sufficient TRF2 remains on telomeres to block the non-homologous end-joining pathway, or TRF1 may be required at telomeres for fusions to occur.

TIN2, a Linchpin in the Telomeric Complex—TIN2 has now emerged as a critical element in the telomeric complex. Although it is small, this protein has three separate protein-protein interaction domains. TIN2 binds both TRF1 and TRF2 independently and simultaneously, and TIN2 binds PIP1, which serves to recruit POT1 to the telomeric complex. Because of its protein interactions, TIN2 connects the three main DNA binding activities at telomeres, two double-stranded DNA-binding proteins and the single single-stranded DNA binding factor. Furthermore, TIN2 tethers POT1 to TRF2 independent of its interaction with TRF1 creating two separate protein interaction pathways by which POT1 can arrive at the telomeres. Consistent with this, the binding of POT1 to telomeres is diminished by the inhibition of TRF2 as well as TRF1 (28). We had previously suggested that the loss of POT1 from the telomeres after impaired TRF2 function could be because of the degradation of the single-stranded telomeric overhang (28). However, the current data suggest the loading of POT1 on telomeres could be affected by TRF2 inhibition in a manner that is independent of overhang degradation.

Interestingly, TIN2 and its interacting partner PIP1 are not present in fission yeast, although this eukaryote has Pot1 and one TRF1/2-like protein (Taz1) at its telomeres (44, 45). Indeed, there is no indication that Taz1 and Pot1 of fission yeast are physically connected by a protein factor. Thus, TIN2 and PIP1 may have evolved with the emergence of the second TRF-like factor in vertebrates. A third component found in the vertebrate telomeric complex that is absent from fission yeast chromosome ends is tankyrase (7). TIN2 is connected to tankyrase 1 in that it can control its PARP activity. In this manner, TIN2 can protect TRF1 from being removed from telomeres by tankyrase (12). When tankyrase is overexpressed, the accumulation of TRF1 at telomeres is impaired (46), probably because the higher level of tankyrase can overcome the inhibition by TIN2. However, under these conditions, there is no effect on the binding of TRF2 to telomeres (28). This result is not consistent with a simple model in which the binding of TRF2 to the telomeres is only dependent on the presence of TRF1 and TIN2. One possibility is that the TRF1 in the TRF1-TIN2-TRF2 complex is protected from tankyrase such that the TRF1 that is responsible for TRF2 stabilization remains present on the telomeres.

POT1 as Part of the TRF2 Complex—Gel-filtration data showed that cells contain a TRF2-hRap1 complex that also contains TIN2 and POT1 but not TRF1. PIP1 is likely to be present in this complex as well, because it mediates the TIN2-POT1 interaction (14, 15). POT1 was shown previously to be

[2] A. Bianchi, M. van Breughel, and T. de Lange, unpublished data.
[3] G. Celli and T. de Lange, unpublished data.

associated with the TRF1 complex, and functional studies implicated POT1 in telomere length control by TRF1 (28). The data are consistent with POT1 acting as a negative regulator of telomere length. The interaction of POT1 with TRF2 is consistent with this function, because TRF2, like TRF1, affects telomere length (47). The pathway through which TRF2 controls telomere length has not been established. TRF2 overexpression results in telomere shortening in telomerase-expressing cells (47). However, because TRF2 also accelerates telomere shortening in primary human fibroblasts (48), it is not clear whether its effect on telomere-length homeostasis is through the control of telomerase action, telomere shortening activities, or both. The connection between POT1 and TRF2 would be consistent with an effect on telomerase, but other possibilities are not excluded.

The primary role of TRF2 is in telomere protection. The presence of POT1 in the TRF2 complex is particularly interesting in this regard, because fission yeast POT1 is required for telomere protection (44). Because POT1 has a single-stranded DNA binding domain, it might be required for the protection of the telomeric overhang, a function ascribed to TRF2 (31). However, expression of a POT1 mutant lacking the DNA binding domain or partial knock-down of POT1 with short hairpin RNA has not revealed a deprotection phenotype (15, 28). Further analysis of cells devoid of POT1 will be required to address the possible role of POT1 in the protection of human telomeres.

Acknowledgments—We thank R. G. Roeder for allowing us to collaborate with Yan Luo during a postdoctoral fellowship in the laboratory. We thank Richard Wang for ideas regarding the function and evolution of TIN2. Magda Konarska is thanked for providing us with the antibody to p54(nbr).

REFERENCES

1. Chong, L., van Steensel, B., Broccoli, D., Erdjument-Bromage, H., Hanish, J., Tempst, P., and de Lange, T. (1995) *Science* **270,** 1663–1667
2. Bilaud, T., Brun, C., Ancelin, K., Koering, C. E., Laroche, T., and Gilson, E. (1997) *Nat. Genet.* **17,** 236–239
3. Broccoli, D., Smogorzewska, A., Chong, L., and de Lange, T. (1997) *Nat. Genet.* **17,** 231–235
4. Bianchi, A., Smith, S., Chong, L., Elias, P., and de Lange, T. (1997) *EMBO J.* **16,** 1785–1794
5. Fairall, L., Chapman, L., Moss, H., de Lange, T., and Rhodes, D. (2001) *Mol. Cell* **8,** 351–361
6. Smogorzewska, A., and de Lange, T. (2004) *Ann. Rev. Biochem.* **73,** 177–208
7. Smith, S., Giriat, I., Schmitt, A., and de Lange, T. (1998) *Science* **282,** 1484–1487
8. Kaminker, P. G., Kim, S. H., Taylor, R. D., Zebarjadian, Y., Funk, W. D., Morin, G. B., Yaswen, P., and Campisi, J. (2001) *J. Biol. Chem.* **276,** 35891–35899
9. Sbodio, J. I., and Chi, N. W. (2002) *J. Biol. Chem.* **277,** 31887–31892
10. Sbodio, J. I., Lodish, H. F., and Chi, N. W. (2002) *Biochem. J.* **361,** 451–459
11. Cook, B. D., Dynek, J. N., Chang, W., Shostak, G., and Smith, S. (2002) *Mol. Cell. Biol.* **22,** 332–342
12. Ye, J. Z., and de Lange, T. (2004) *Nat. Genet.* **36,** 618–623
13. Kim, S. H., Kaminker, P., and Campisi, J. (1999) *Nat. Genet.* **23,** 405–412
14. Liu, D., Safari, A., O'Connor, M. S., Chan, D. W., Laegeler, A., Qin, J., and Songyang, Z. (2004) *Nat. Cell Biol.*
15. Ye, J. Z., Hockemeyer, D., Krutchinsky, A. N., Loayza, D., Hooper, S. M., Chait, B. T., and de Lange, T. (2004) *Genes Dev.* **18,** 1649–1654
16. Zhou, X. Z., and Lu, K. P. (2001) *Cell* **107,** 347–359
17. Hsu, H. L., Gilley, D., Galande, S. A., Hande, M. P., Allen, B., Kim, S. H., Li, G. C., Campisi, J., Kohwi-Shigematsu, T., and Chen, D. J. (2000) *Genes Dev.* **14,** 2807–2812
18. Lillard-Wetherell, K., Machwe, A., Langland, G. T., Combs, K. A., Behbehani, G. K., Schonberg, S. A., German, J., Turchi, J. J., Orren, D. K., and Groden, J. (2004) *Hum. Mol. Genet.*
19. Opresko, P. L., Von Kobbe, C., Laine, J. P., Harrigan, J., Hickson, I. D., and Bohr, V. A. (2002) *J. Biol. Chem.* **277,** 41110–41119
20. Kishi, S., Zhou, X. Z., Ziv, Y., Khoo, C., Hill, D. E., Shiloh, Y., and Lu, K. P. (2001) *J. Biol. Chem.* **276,** 29282–29291
21. Karlseder, J., Hoke, K., Mirzoeva, O. K., Bakkenist, C., Kastan, M. B., Petrini, J. H. J., and de Lange, T. (2004) *PLoS Biol.* **2,** 1150–1156
22. Li, B., Oestreich, S., and de Lange, T. (2000) *Cell* **101,** 471–483
23. Zhu, X. D., Kuster, B., Mann, M., Petrini, J. H., and de Lange, T. (2000) *Nat. Genet.* **25,** 347–352
24. Zhu, X. D., Niedernhofer, L., Kuster, B., Mann, M., Hoeijmakers, J. H., and de Lange, T. (2003) *Mol. Cell* **12,** 1489–1498
25. Opresko, P. L., Otterlei, M., Graakjaer, J., Bruheim, P., Dawut, L., Kolvraa, S., May, A., Seidman, M. M., and Bohr, V. A. (2004) *Mol. Cell* **14,** 763–774
26. Song, K., Jung, D., Jung, Y., Lee, S. G., and Lee, I. (2000) *FEBS Lett.* **481,** 81–85
27. van Steensel, B., and de Lange, T. (1997) *Nature* **385,** 740–743
28. Loayza, D., and de Lange, T. (2003) *Nature* **424,** 1013–1018
29. Colgin, L. M., Baran, K., Baumann, P., Cech, T. R., and Reddel, R. R. (2003) *Curr. Biol.* **13,** 942–946
30. de Lange, T. (2002) *Oncogene* **21,** 532–540
31. van Steensel, B., Smogorzewska, A., and de Lange, T. (1998) *Cell* **92,** 401–413
32. Takai, H., Smogorzewska, A., and de Lange, T. (2003) *Curr. Biol.* **13,** 1549–1556
33. d'Adda di Fagagna, F., Reaper, P. M., Clay-Farrace, L., Fiegler, H., Carr, P., Von Zglinicki, T., Saretzki, G., Carter, N. P., and Jackson, S. P. (2003) *Nature* **426,** 194–198
34. Karlseder, J., Broccoli, D., Dai, Y., Hardy, S., and de Lange, T. (1999) *Science* **283,** 1321–1325
35. Iwano, T., Tachibana, M., Reth, M., and Shinkai, Y. (2004) *J. Biol. Chem.* **279,** 1442–1448
36. Karlseder, J., Kachatrian, L., Takai, H., Mercer, K., Hingorani, S., Jacks, T., and de Lange, T. (2003) *Mol. Cell. Biol.* **23,** 6533–6541
37. Ogawa, H., Ishiguro, K., Gaubatz, S., Livingston, D. M., and Nakatani, Y. (2002) *Science* **296,** 1132–1136
38. Krutchinsky, A. N., Kalkum, M., and Chait, B. T. (2001) *Anal. Chem.* **73,** 5066–5077
39. Krutchinsky, A. N., Zhang, W., and Chait, B. T. (2000) *J. Am. Soc. Mass Spectrom.* **11,** 493–504
40. Kameoka, S., Duque, P., and Konarska, M. M. (2004) *EMBO J.* **23,** 1782–1791
41. Chiang, Y. J., Kim, S. H., Tessarollo, L., Campisi, J., and Hodes, R. J. (2004) *Mol. Cell. Biol.* **24,** 6631–6634
42. Bianchi, A., Stansel, R. M., Fairall, L., Griffith, J. D., Rhodes, D., and de Lange, T. (1999) *EMBO J.* **18,** 5735–5744
43. Mattern, K. A., Swiggers, S. J., Nigg, A. L., Lowenberg, B., Houtsmuller, A. B., and Zijlmans, J. M. (2004) *Mol. Cell. Biol.* **24,** 5587–5594
44. Baumann, P., and Cech, T. R. (2001) *Science* **292,** 1171–1175
45. Cooper, J. P., Nimmo, E. R., Allshire, R. C., and Cech, T. R. (1997) *Nature* **385,** 744–747
46. Smith, S., and de Lange, T. (2000) *Curr. Biol.* **10,** 1299–1302
47. Smogorzewska, A., van Steensel, B., Bianchi, A., Oelmann, S., Schaefer, M. R., Schnapp, G., and de Lange, T. (2000) *Mol. Cell. Biol.* **20,** 1659–1668
48. Karlseder, J., Smogorzewska, A., and de Lange, T. (2002) *Science* **295,** 2446–2449