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Identification of Alternative Transcripts of the *TRF1/Pin2* Gene

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Abstract TRF1 and Pin2 play an essential role in telomere homeostasis, by regulating telomere maintenance. They are generated from the same gene, *TRF1/Pin2*, by alternative splicing but no functional differences between these proteins have been demonstrated. We report here the detection of new alternative transcripts of the *TRF1/Pin2* gene in peripheral blood lymphocytes resulting from a 76 nt insertion. Real-time RT-PCR showed that these transcripts were also produced in various normal human cells and tissues and in immortalized cell lines, but at levels lower (by a factor of 8–111) than those for the TRF1 and Pin2 transcripts. These new transcripts are predicted to encode polypeptides identical to TRF1/Pin2 at the C-terminal end but entirely lacking the acid domain and the amino-terminal part of the homodimerization domain of TRF1/Pin2. These proteins, fused at their N-terminal ends to enhanced green fluorescent protein (EGFP), were found to be located at telomeres and to induce apoptosis in cell lines with short telomeres, thereby displaying similar activity to TRF1/Pin2. However, these putative proteins lack regions important for interactions with other proteins and for homodimerization. Unlike TRF1/Pin2, they were unable to interact with tankyrase 1, suggesting that these proteins may play a role in telomere homeostasis different from those of TRF1/Pin2. The production of these alternative transcripts was downregulated in peripheral blood lymphocytes following PHA-p activation, suggesting a possible role in resting lymphocytes. J. Cell. Biochem. 93: 968–979, 2004.

Key words: TRF1; Pin2; telomeres; alternative transcripts

Telomeres are nucleoprotein structures that cap the ends of eukaryotic chromosomes, protecting them from degradation and end-to-end fusion [for reviews: Ancelin et al., 1998; de Lange, 2002]. They consist of G-rich repetitive DNA sequences bound by specific proteins, such as TRF1/Pin2 [for review: Zakian, 1995]. Two forms of this protein, TRF1 and Pin2, have been described but no functional difference between these two forms has been demonstrated. TRF1 and Pin2 are generated from the *TRF1/Pin2* gene by alternative splicing [Shen et al., 1997; Young et al., 1997]. Pin2 is identical to TRF1 except for an internal deletion of 20 residues (residues 296–316 in TRF1). TRF1/Pin2 is a small, ubiquitous protein with an acidic amino-terminus, a conserved homodimerization domain, a nuclear localization signal (NLS), and a Myb-type helix-turn-helix DNA binding domain [Chong et al., 1995; Broccoli et al., 1997a,b]. TRF1/Pin2 binds telomeric DNA predominantly as a homodimer [Bianchi et al., 1997, 1999; Shen et al., 1997; Griffith et al., 1998; Konig et al., 1998; Smogorzewska et al., 2000] although single TRF1/Pin2 Myb domains have been shown to interact with telomeric DNA [Konig et al., 1998].

Changes in telomere length have been implicated in aging [Sedivy, 1998] and cancer [Mu and Wei, 2002]. The loss or disruption of a telomere may cause cellular senescence, genetic instability, cell death, or lead to cellular transformation/immortalization, depending on the cell context. TRF1/Pin2 has been shown to play an essential role in telomere homeostasis, as a regulator of telomere maintenance [for review: Zhou et al., 2003].

Telomeres are maintained by telomerase, a specialized reverse transcriptase [Greider and Blackburn, 1985; Morin, 1989]. In some telo-

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merase-positive cell lines, TRF1/Pin2 overexpression leads to a gradual decline in telomere length, whereas the expression of a dominant negative mutant that eliminates TRF1/Pin2 from telomeres induces telomere elongation [van Steensel and de Lange, 1997]. It has also been suggested that tankyrase 1 (TRF1-interacting ankyrin-related ADP-ribose polymerase), a polyadenosine diphosphate ribose polymerase (PARP), promotes the elongation of telomeres by telomerase. It is thought to do this by removing TRF1/Pin2 from telomeric DNA, by binding to the amino-terminal domain TRF1/Pin2, causing the adenosine diphosphate (ADP)-ribosylation of this protein [Smith et al., 1998; Cook et al., 2002; Rippmann et al., 2002]. Thus, TRF1/Pin2 is thought to regulate the *cis* elongation of telomeres by telomerase negatively by interacting with protein partners in each individual telomere. The partners of this protein include Tin2 (TRF1-interacting nuclear protein 2), Pot1 (protection of telomere 1), and PinX1 (protein interacting with NIMA-interacting factor) [for review: Zhou et al., 2003]. Tin2 interacts with TRF1/Pin2 via a region in the homodimerization domain and colocalizes with TRF1 [Kim et al., 1999]. Tin2 facilitates TRF1-dependent DNA looping and bending on DNA tracks by changing the TRF1 off-rate, thereby increasing the complexity of telomeric structures, preventing telomerase from gaining access to telomeres [Kim et al., 2003]. Pot1 regulates telomerase-mediated telomere elongation by binding to single-stranded telomeric DNA in a process controlled by TRF1/Pin2 [Baumann and Cech, 2001; Colgin et al., 2003; Loayza and de Lange, 2003]. PinX1 is a potent telomerase inhibitor thought to play a role in the TRF1/Pin2-negative regulation of telomerase activity by forming a stable complex with hTERT, the catalytic subunit of telomerase [Zhou and Lu, 2001].

TRF1/Pin2 also interacts with proteins involved in DNA damage repair and cell-cycle control, such as Ku and ATM (ataxia telangiectasia mutated). High-affinity interactions between TRF1/Pin2 and Ku70/80 are responsible for an essential telomere capping function that prevents telomere fusion [Hsu et al., 2000]. TRF1/Pin2 is also a critical downstream target of ATM, a kinase that acts on Ser 219 and is essential for the mitotic checkpoint in response to DNA damage [Kishi et al., 2001a; Kishi and Lu, 2002]. TRF1/Pin2, therefore, appears to be a key molecule connecting telomere maintenance with cell-cycle control.

TRF1/Pin2 may also play an important role in processes other than telomere maintenance. For instance, this protein has been implicated in regulation of the mitotic spindle; it also promotes microtubule formation and interacts with the microtubule regulator EB1 [Nakamura et al., 2001, 2002]. A recent study showed that although a negative allele of hTRF1 does not affect the growth and viability of various primary and transformed human cells, inactivation of the mouse *TRF1* gene causes early embryonic death independently of telomere length regulation [Karlseder et al., 2003].

We report here the detection of new alternative transcripts of the *TRF1/Pin2* gene in various human cells and tissues and in immortalized cell lines. These transcripts encode two proteins identical to TRF1 and Pin2 but with the acidic domain and the amino-terminal part of the homodimerization domain deleted. These structural differences might result in properties different from those of TRF1/Pin2. Indeed, we found that these proteins did not interact with tankyrase 1. Their functions in telomere homeostasis and cell-cycle regulation may thus differ from those of TRF1/Pin2.

MATERIALS AND METHODS

Cell Cultures

Mononuclear cells were isolated from blood samples from normal volunteers (CEN-FAR infirmary) by centrifugation with Histopaque (Sigma, St. Quentin Fallavier, France). They were cultured at a density of 10^6/ml in RPMI-1640 supplemented with 10% decomplemented fetal calf serum (FCS; Invitrogen, Cergy Pontoise, France), 20 U/ml interleukin 2 (Roche Diagnostics, Meylan, France), 2 mM glutamine (Sigma), penicillin–streptomycin (Sigma), and PHA-p (1/500; Becton-Dickinson, Le Pont de Claix, France). We cultured hTERT-BJ1 fibroblasts (Clontech Laboratories, Le Pont de Claix, France) in DMEM supplemented with 4 mM glutamine, penicillin–streptomycin, 1 mM sodium pyruvate (Sigma), 20% medium 199 (Invitrogen), and 10% FCS. Primary fibroblast cultures were kindly provided by Dr. C. Laurent and Dr. J.P. Poujet (IRSN). The T-cell leukemia cell lines CEM-CCRF (ATCC) and CEM 1301 (derived from CEM-CCRF; DAKO, Trappes, France) were cultured in RPMI-1640 (Sigma)

supplemented with 2 mM glutamine and 10% FCS. The glioma cell lines, T98G (ATCC) and CB193 (provided by Dr. Gras, CEA) were cultured in DMEM (Invitrogen) supplemented with 2 mM glutamine, penicillin–streptomycin and 10% FCS.

Conventional RT-PCR

Total RNA was extracted from 1 to 2×10^6 cells in Trizol reagent (Invitrogen) according to the manufacturer's instructions. It was then treated with RNase-free DNase (Roche Diagnostics). Reverse transcription reactions were carried out for 2 h at 42°C on 3 μg total RNA in 60 μl of a reaction mixture containing 300 U MuLV (murine leukemia virus) reverse transcriptase (Invitrogen), 1× buffer, 60 nmol dNTP (Invitrogen), 0.75 μg random hexamers (Promega, Charbonnières les bains, France), and 100 U RNase inhibitor (Promega). PCR was performed in a PTC-200 Peltier thermal cycler (MJ Research, Waltham, MA) on 2 μl of cDNA in a 50 μl reaction mixture containing 1.25 U *Taq* DNA polymerase (Amersham Biosciences, Orsay, France), 1× PCR buffer, 10 pmol dNTP (Amersham Biosciences) and 12.5 pmol primers, in a 35-cycle reaction (each cycle consisted of 45 s at 94°C, of 2 min at 59°C, of 1 min at 72°C).

An alternative exon (E2) in the *TRF1/Pin2* gene was detected with the primers *P1F* (5′-TGC GGA TGG TAG GGA TGC-3′) and *P1R* (5′-AGG GCT GAT TCC AAG GGT GTA-3′) (Fig. 1). Alternative combinations of *TRF1/Pin2* exons were assessed using the following combinations of primers (Fig. 2): Pin2 mRNA: *P2F* (5′-CGC AGA AAC TAT TAT TCA T-3′) and *P2R* (5′-GAC CTT TTT CTT GTA TCC A-3′); TRF1 mRNA: *P2F* and *P3R* (5′-AGG ATT CAG TTA CCG CAG AC-3′); t-Pin2 mRNA: *P3F* (5′-CAT CAC CTC CTA ACA CAG-3′) and *P2R*; t-TRF*1*mRNA: *P3F* and *P3R*. GAPDH mRNA was detected using the primers: 5′-ACC ACC ATG GAG AAG GCT GG-3′ and 5′-CTC AGT GTA GCC CAG GAT GC-3′ and hTERT mRNA was detected using the primers: 5′-CCT GCG TTT GGT GGA TGA TT-3′ and 5′-GGC TGC TGG TGT CTG CTC TC-3′.

Telomerase Assay

Telomerase activity was evaluated with the TRAPeze ELISA Telomerase Detection Kit (Intergen, Edinburgh, UK), according to the manufacturer's instructions. Briefly, 2×10^6 cells were lysed in CHAPS containing 150 U/ml of RNasin (Promega). After 30 min of incubation on ice, lysates were centrifuged at 12,000*g* for 20 min at 4°C, and supernatants were frozen and stored at −80°C. We assayed 0.5 μg of protein extract in a 50 μl reaction mixture containing 10 μl 5× TRAP reaction mix and 2 U of *Taq* DNA polymerase. The reaction mixture was incubated for 30 min at 30°C for telomerase extension, and was then subjected to PCR amplification for 33 cycles of 94°C for 30 s, of 55°C for 30 s on a PTC-200 thermal cycler. The amplified products were visualized by electrophoresis in a 12.5% non-denaturing polyacrylamide gel and staining with Gelstar stain (FMC-Bioproducts, Rockland, ME).

Plasmids

We constructed pEGFP-Pin2, encoding Pin2 with the enhanced green fluorescent protein (EGFP) sequence fused at its amino-terminal end, as follows. The full-length Pin2 cDNA fragment was obtained by RT-PCR from lymphocyte RNA from a normal donor, using the primers *P4F* (5′-ACA TGG CGG AGG ATG TTT CC-3′) and *P0R* (5′-TTT GGG GGT GGG GAT ACT AAA-3′) (Fig. 2B). PCR products were subjected to electrophoresis, purified from the gel (QIA-quick, Qiagen, Courtaboeuf, France) and cloned into the pMOS-Blue vector (pMOSBlue-Pin2), according to manufacturer's instructions (Amersham Biosciences). The Pin2 sequence was then amplified by PCR from this plasmid, using primers with additional *Eco*R1 and *Bam*H1 sites: *EcoR1P4F* (5′-GGA ATT CCA TGG CGG AGG ATG TTT CC-3′) and *BamH1P4R* (5′-CGC ATC CGC GTC AGT CTT CGC TGT CTG AGG-3′). The PCR products were then digested with *Eco*R1 and *Bam*H1 and ligated into pEGFP-C1 (Clontech Laboratories) previously digested with the same enzymes.

pEGFP-t-Pin2 was obtained from pMOSBlue-Pin2 by PCR, using the primer *EcoR1P5F* (5′-GGA ATT CCA TGT TCA CTA TTA TTC ATG GAC TAT CC-3′) (Fig. 2B), which contains a restriction site for *Eco*R1 and the ATG of t-Pin2/t-TRF1, and *BamH1P4R*. The PCR products were then digested with *Eco*R1 and *Bam*H1 and inserted into pEGFP-C1.

We obtained pEGFP-t-TRF1 by replacing the *Xmn*I/*Van*91-fragment of Pin2 by that of TRF1 in pEGFP-t-Pin2. The *Xmn*I/*Van*91 fragment of TRF1, containing E8, was obtained by enzymatic digestion of the higher molecular weight RT-PCR product obtained with the primers *P6F* (5′-GAT ACA TTT CAT TCC TTT TT-3′) and

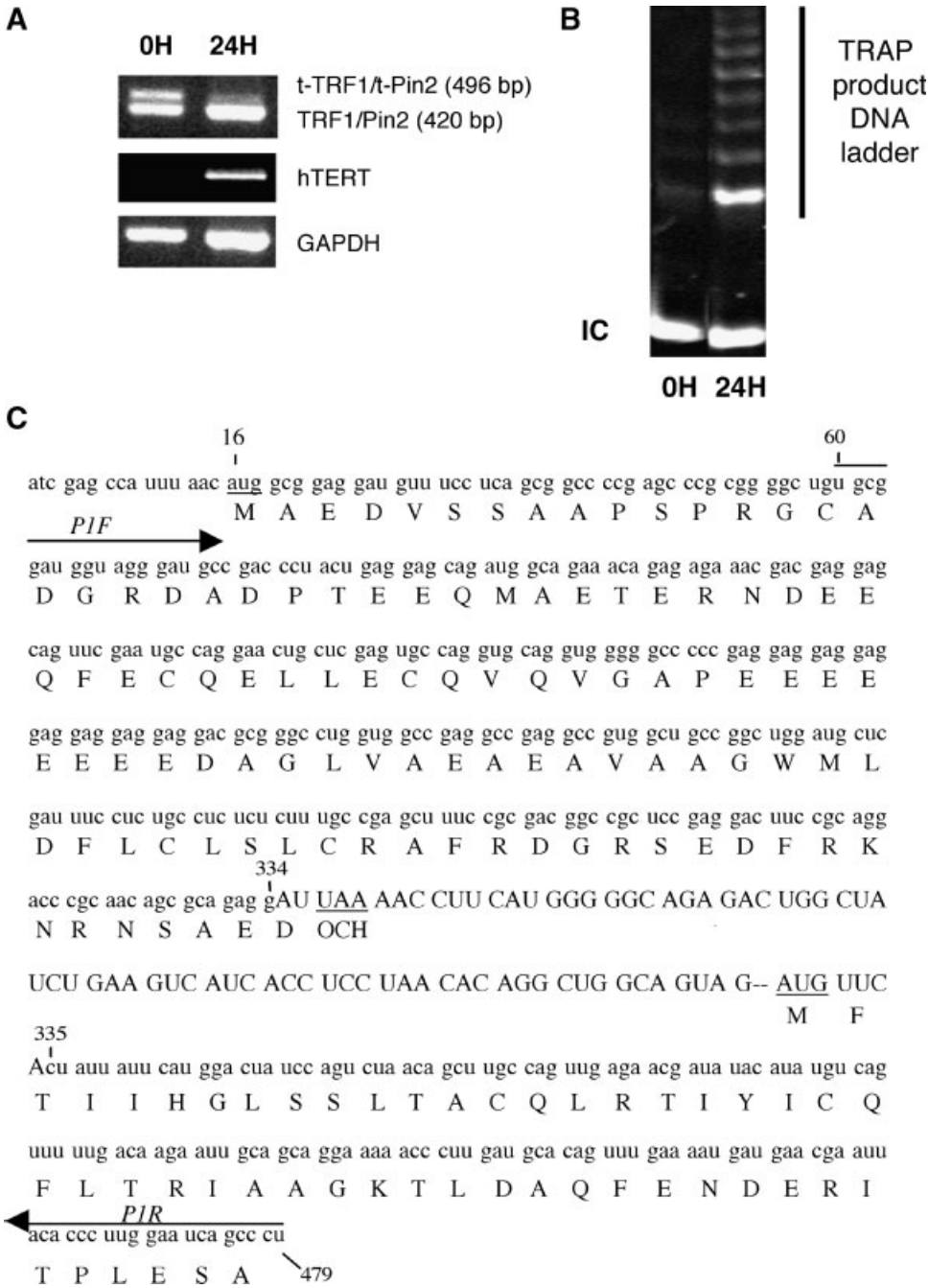

Fig. 1. Detection of alternative transcripts of the *TRF1/Pin2* gene. **A**: Agarose gel electrophoresis of RT-PCR products from TRF1/Pin2 mRNAs, using the *P1F* and *P1R* primers, and from the hTERT and GAPDH mRNAs, for human peripheral blood lymphocyte (PBL) samples. Two RT-PCR products (496 and 420 bp) were obtained with the *P1F* and *P1R* primers. The relative abundance of the 496 bp RT-PCR product amplified from t-TRF1/t-Pin2 was lower in lymphocytes activated by incubation with PHA-p for 24 h, whereas hTERT mRNA levels were high after activation. **B**: Detection of the typical ladder of TRAP products in telomerase-positive samples by acrylamide gel electrophoresis indicating that PHA-p increases telomerase activity in cultured PBL. IC, internal control. **C**: RNA sequence and predicted amino-acid sequence of the corresponding proteins for alternative transcripts of the *TRF1/Pin2* gene. The inserted RNA sequence, corresponding to E2, is indicated in capital letters. Numbering is as for the TRF1 mRNA sequence (GenBank accession no. U40705).

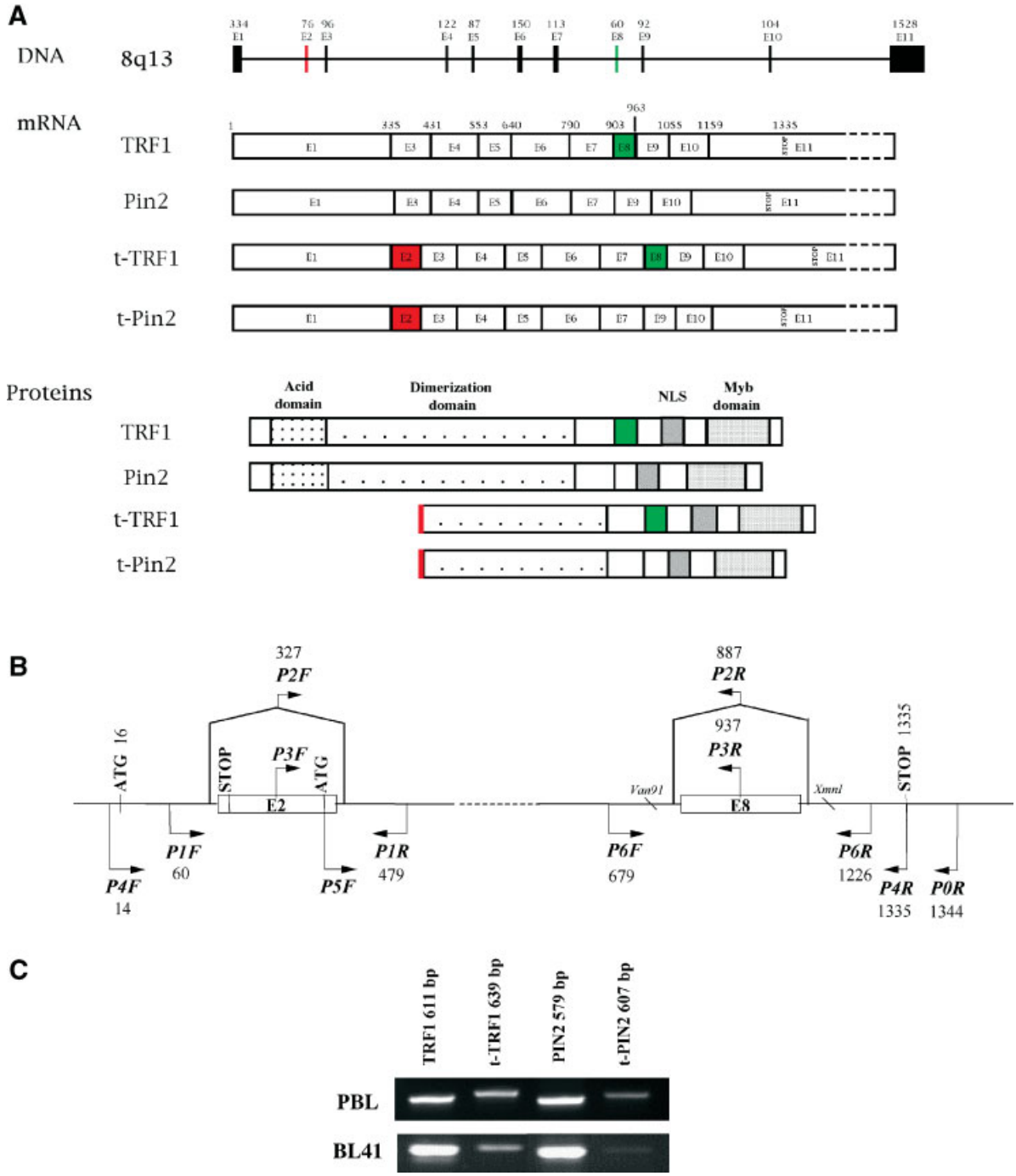

Fig. 2. Alternative transcription of the *TRF1*/*Pin2* gene. **A**: Organization of the *TRF1*/*Pin2* gene and its alternative transcripts and putative polypeptide products. **B**: Locations to which the various primers used in RT-PCR bind on the alternative *TRF1*/*Pin2* cDNAs, as a function of E2 and E8 use. Numbering is as for the TRF1 mRNA sequence (GenBank accession no. U40705) and indicates the 5′-ends of the primers. **C**: Detection of the four alternative transcripts of the *TRF1*/*Pin2* gene in total mRNA extracted from normal PBL and from BL41 Burkitt lymphoma cells.

P6R (5′-GAC CAG TTT CCC TCT CCA TA-3′) from lymphocyte RNA.

We checked that the constructions were correct by complete sequencing (Themis Biofidal, Vaulx en Velin, France).

pcDNA3-FN.tankyrase1, encoding the full-length tankyrase 1 with an N-terminal Flag-epitope tag and a NLS, in the pcDNA3 vector, was kindly provided by Dr. S. Smith.

Real-Time RT-PCR

RNA samples from human tissues were obtained from the Human Total RNA Master Panel II (Becton-Dickinson). We generated cDNAs as described above. Real time RT-PCR was performed in a Light Cycler® thermal cycler (Roche Diagnostics). Reactions were carried out in a 10 μl reaction mixture containing 1× PCR buffer (Light Cycler FAST START DNA Master SYBR-Green; Roche Diagnostics), primers (7 pmol each), $MgCl_2$ (30–50 pmol) and 1–2 μl of diluted cDNA (1/5, 1/10, 1/100). We heated the mixture at 94°C for 10 min, and then subjected it to 45 cycles of 10 s at 94°C, of 10 s at 57–60°C, and of 7–12 s at 72°C. Melting curves were systematically analyzed to determine the specificity of amplification.

The primer pairs used were: for amplification of the TRF1/Pin2 mRNA (E1–E4 exons) sequence, *P2F* and *P1R*; for amplification of the t-TRF1/t-Pin2 (E2–E4 exons) sequence, *P3F* and *P1R*; for the 18S rRNA: 5′-TGT GAT GCC CTT AGA TGT C-3′ and 5′-CTT ATG ACC CGC ACT TAC TG-3′.

The number of target sequences in each sample was determined from a standard curve produced for each run. This standard curve was obtained with serial dilutions of a reference cDNA sample (from the CB193 cell line), in which the number of alternative transcripts of the *TRF1/Pin2* gene had already been determined. Results are expressed with respect to 18S rRNA levels. Alternative transcripts of *TRF1/Pin2* were quantified with respect to E2 usage in the reference sample as follows: PCR was performed on this sample to increase the number of copies of two different pMOS-Blue plasmids, one containing the exons E1, E2, E3, and E4 (corresponding to t-TRF1/t-Pin2 mRNAs), and the other, E1, E3, and E4 (corresponding to TRF1/Pin2 mRNAs) (data not shown). To determine the exact copy numbers of the two plasmids used in this experiment, an additional PCR (primers 5′-AGG AAG GGA AGA AAG CGA AAG GAG-3′ and 5′-AGG GAA TAA GGG CGA CAC GGA AAT-3′ from the pMOS-Blue sequence) was carried out in parallel on the plasmid samples (data not shown).

Plasmid Transfection and Immunofluorescence Microscopy

Cells were grown in Labtek (VWR, Fontenay Sous Bois, France) and transfected (1 μg of plasmid DNA for 0.2×10^6 cells) in DMRIE-C reagent (Invitrogen) according to the manufacturer's instructions. For immunofluorescence studies, cells were fixed in 4% paraformaldehyde and were permeabilized with 0.1% Triton X-100. Before staining, cells were incubated in 3% FCS in PBS for blocking. The primary antibodies used were mouse anti-TRF2 (1/200; Clinisciences, Montrouge, France), rabbit anti-EGFP (1/300; Chemicon), rabbit anti-caspase-3 (1/200; Cell signaling, Beverly, MA), and mouse anti-Flag-M2 (1/4,000; Sigma). The secondary antibodies used were anti-mouse antibodies conjugated with Texas-Red (1/200; Southern Biotechnology Associates, Birmingham, Alabama) or Alexa-Fluor 546 (1/2,000; Invitrogen, Cergy Pontoise, France) and anti-rabbit antibodies conjugated with FITC (1/200; Tebu-Bio, Le Perray en Yvelines, France). Nuclei were stained with DAPI and slides were mounted in Fluoromount (Southern Biotechnology Associates).

RESULTS

Characterization of New Alternative Transcripts of the *TRF1/Pin2* Gene

RT-PCR amplification with the *P1F* and *P1R* primers of the 5′ region of the TRF1/Pin2 cDNA (position 60–479, Fig. 1A) from human peripheral blood lymphocyte mRNA from different donors systematically generated an additional product with a molecular weight higher than that of the predicted product. The signal for this additional RT-PCR product was weaker if RT-PCR was performed with mRNA from lymphocytes activated by incubation with PHA-p for 24 h (Fig. 1A). This treatment also dramatically increased mRNA levels for the catalytic subunit of telomerase (hTERT) (Fig. 1A) and telomerase activity (Fig. 1B), as shown in previous studies [Weng et al., 1996].

The cloning and sequencing of this additional product showed it to contain a 76 bp insertion at position 335 of the TRF1/Pin2 mRNA sequence (GenBank accession no. U40705) (Fig. 1C). A human genome sequence database search revealed that this inserted sequence was present only on chromosome 8q13 (GenBank accession no. AC022893), within the *TRF1/Pin2* gene. Furthermore, this sequence was identified as a possible exon by UCSC Genome Browser (chr8: 73,971,070–74,009,688). It was not detected in the sequences of the TRF1/Pin2 pseudogenes

described elsewhere in the human genome [Young et al., 1997]. Moreover, this insertion was present in two EST from a human tissue library, corresponding to the testis (GenBank accession no. AL046407) and endometrium of uterus adenocarcinoma cell lines (GenBank accession no. BE392840). This insertion, therefore, appears to correspond to an additional exon (referred to here as E2) of the *TRF1/Pin2* gene leading to the generation of alternative transcripts (Fig. 2A). The use of E2 results in the generation of a premature TAA stop codon (OCH, at codon 108) followed by a new ATG in the TRF1/Pin2 open reading frame. The use of this new ATG as a translation start codon would result in the production of a polypeptide identical to TRF1/Pin2 at the carboxy-terminal end, but lacking the entire acid domain and 42 amino acids of the homodimerization domain. This putative peptide also contains three new amino acids (MFT) at its amino-terminal end (Fig. 1C).

TRF1 mRNA differs from Pin2 mRNA in that it uses exon E8 (Fig. 2A). We, therefore, selected a set of primers (*P2F*, *P3F*, *P2R*, and *P3R*) making it possible to select by RT-PCR the four mRNAs encoded by the *TRF1/Pin2* gene predicted on the basis of E2 and E8 use (Fig. 2B). All four forms were detected in peripheral blood lymphocytes (Fig. 2C), and in various cell lines, such as BL41 Burkitt lymphoma cells (Fig. 2C). The *TRF1/Pin2* gene may, therefore, encode two other proteins, identical to TRF1 and Pin2 at the carboxy-terminal end but with deletions at the amino-terminal end. We refer to these two proteins as t-TRF1 and t-Pin2.

t-TRF1/t-Pin2 Are Produced in a Wide Range of Cell Types and Tissues

The presence of three new amino acids in t-TRF1 and t-Pin2, but not in TRF1 and Pin2, was not sufficient for the generation of specific antibodies for distinguishing the full-length TRF1/Pin2 proteins from the alternatively spliced forms. Furthermore, the available antibodies directed against the C-terminal end of TRF1 are of such low sensitivity and specificity that they cannot be used to detect small amounts of protein. We, therefore, used real-time RT-PCR to investigate the use of E2 in TRF1/Pin2 mRNAs in different cell types and tissues. For the detection of mRNAs with and without E2, we used two forward primers: one binding within E2 (*P3F*) and the other overlapping the junction between E1 and E3 (*P2F*) (Fig. 2B). These primers were used with a complementary primer binding in E4 (*P1R*), in separate RT-PCRs. We found E2-containing transcripts in all human normal tissues tested (Table I). However, the levels of t-TRF1/t-Pin2 mRNAs were lower than those of TRF1/Pin2 mRNAs by a factor of 12–56 in these samples. Levels of t-TRF1/t-Pin2 mRNAs were higher in the thymus and bone marrow, and in the trachea and small intestine. By contrast, they were lower in the brain, uterus, and spleen. Moreover, we found that the level of t-TRF1/t-Pin2 mRNAs was only one eighth that of TRF1/Pin2 mRNAs in freshly isolated lymphocytes (Table II). However, 3 days of activation by PHA-p significantly decreased the production of these transcripts. Very low levels of the alternatively spliced forms were found in fibroblasts in primary culture and in fibroblasts transfected with a construct encoding hTERT (hTERT-BJ1). We also detected t-TRF1/t-Pin2 mRNAs in various immortalized cell lines, at levels lower than those for TRF1/Pin2 mRNAs by a factor of 13–111, but found no relationship between this ratio and cell type or telomere length (Table II).

TABLE I. Detection of the Alternative Transcripts of the *TRF1/Pin2* Gene in Normal Human Tissues by Real-Time PCR

Tissue	TRF1 + Pin2 (copies/μl)	t-TRF1 + t-Pin2 (copies/μl)	Ratio
Adrenal gland	5,747 ± 1,692	138 ± 78	42
Placenta	3,742 ± 549	195 ± 53	19
Spleen	7,160 ± 1,391	127 ± 43	56
Trachea	3,049 ± 701	256 ± 67	12
Bone marrow	5,914 ± 1,004	495 ± 77	12
Kidney	8,808 ± 1,325	464 ± 196	19
Prostate	11,950 ± 2,350	453 ± 128	26
Testis	25,628 ± 3,146	1,162 ± 163	22
Uterus	5,511 ± 1,658	105 ± 24	52
Salivary glands	4,399 ± 2,660	187 ± 46	24
Thymus	4,796 ± 1,198	408 ± 288	12
Colon	1,877 ± 602	90 ± 48	21
Skeleton	3,580 ± 475	99 ± 58	36
Thyroid	13,816 ± 2,232	840 ± 82	16
Small intestine	4,588 ± 701	336 ± 63	14
Whole brain	15,077 ± 2,264	271 ± 134	56
Cerebellum	13,022 ± 2,370	291 ± 109	45

Both t-TRF1 and t-Pin2 Localize to Telomeres

We constructed plasmids encoding t-TRF1, t-Pin2, or Pin2 fused at the N-terminal end to EGFP. These constructs were used to transfect cells and, 24 h later, the products of all constructs except the control (pEGFP-C1 alone) were found localized as nuclear speckles during interphase in cells with long telomeres:

TABLE II. Detection by Real-Time PCR of the Alternative Transcripts of the *TRF1/Pin2* Gene in Primary Human Cells and Immortalized Cell Lines

Cells	Type	TRF1 + Pin2 (copies/μl)	t-TRF1 + t-Pin2 (copies/μl)	Ratio
PBL	Primary cells	11,978 ± 355	1,524 ± 265	8
PHA-p-activated (3 days) PBL	Primary culture	19,247 ± 1,853	367 ± 156	52
Fibroblasts	Primary culture	21,444 ± 2,694	334 ± 217	64
hTERT-BJ1	Fibroblasts transfected with the hTERT construct	4,784 ± 806	99 ± 82	60
CEM-CCRF	T-leukemia cell line	41,438 ± 88	1,826 ± 184	23
CEM 1301	T-leukemia cell line derived from CEM-CCRF	18,260 ± 5	1,408 ± 153	13
BL41	Non-Hodgkin lymphoma cell line	9,375 ± 640	458 ± 199	20
K562	Leukemia cell line	10,604 ± 721	265 ± 31	40
CB193	Glioma cell line	14,458 ± 1,130	1,264 ± 484	11
T98G	Glioma cell line	21,128 ± 3,360	190 ± 112	111

Values are calculated from duplicate experiments.

hTERT-BJ1 (data not shown) and CEM 1301 (Fig. 3A,B), a cell line with telomeres exceeding 30 kb in length [data not shown and Hultdin et al., 1998]. We also carried out indirect immunofluorescence assays with an antibody specific to TRF2 to check that EGFP-t-Pin2 (Fig. 3B) colocalized with TRF2 in a similar manner to EGFP-Pin2 in CEM 1301. Our results confirmed the telomeric localization of EGFP-t-Pin2.

TRF1/Pin2 has been shown to induce apoptosis in cell lines with short telomeres [Kishi et al., 2001b]. T98G glioma cells, which have mean TRF (telomeric restriction fragments) lengths of around 3 kb (data not shown), underwent apoptosis within 24–72 h of transfection with the EGFP-t-Pin2 or the EGFP-t-TRF1 construct (Fig. 3C). As previously shown for TRF1/Pin2 [Shen et al., 1997; Kishi et al., 2001a], the apoptotic pathway involved caspase-3 (Fig. 3C). Similar results were obtained with other cell lines with short telomeres: CB193 and CEM-CCRF (data not shown).

Unlike TRF1/Pin2, t-TRF1, and t-Pin2 do not Recruit Tankyrase to Telomeres

Tankyrase 1 is thought to promote telomerase-mediated telomere elongation by removing TRF1/Pin2 from telomeres [Smith et al., 1998; Smith and de Lange, 2000]. Tankyrase 1 has been shown to bind the amino-terminal acidic domain of TRF1/Pin2 [Smith et al., 1998; Sbodio and Chi, 2002]. This region is absent from t-TRF1 and t-Pin2, so we therefore investigated the interactions of t-TRF1 and t-Pin2 with tankyrase 1.

The transfection of CEM 1301 cells with pcDNA3-FN.tankyrase1 resulted in a diffuse nuclear distribution of tankyrase 1 (Fig. 4). However, in cotransfection experiments, tankyrase 1 displayed a punctate staining pattern and colocalized with EGFP-Pin2, indicating that Pin2 recruits tankyrase 1 to telomeres, as previously described [Smith and de Lange, 1999]. By contrast, neither EGFP-t-TRF1, nor EGFP-t-Pin2 significantly modified the subcellular distribution of tankyrase 1, indicating that neither EGFP-t-TRF1 nor EGFP-t-Pin2 recruited tankyrase 1 to the telomeres. Thus, as expected, the lack of the amino-terminal acidic domain in t-TRF1 and t-Pin2 prevented the efficient binding of tankyrase 1 to these proteins.

DISCUSSION

TRF1/Pin2 has been shown to play a crucial role in telomere metabolism and functions and to interfere with cell-cycle machinery [for review: Zhou et al., 2003]. In this study, we identified two new alternative transcripts of the human *TRF1/Pin2* gene produced in most of the human cells, tissues, and immortalized cell lines tested, but to different extents. These new transcripts encode two putative proteins, t-TRF1 and t-Pin2, lacking a large part of the amino-terminal end of TRF1 and Pin2, a region involved in interactions between TRF1/Pin2 and other proteins and in homodimerization. This structural difference may result in functions markedly different from those of TRF1/Pin2.

The levels of mRNA for t-TRF1/t-Pin2 were about 1/10th to 1/100th those for the TRF1 and Pin2 mRNAs, depending on the cell type or tissue considered. The highest levels of t-TRF1/

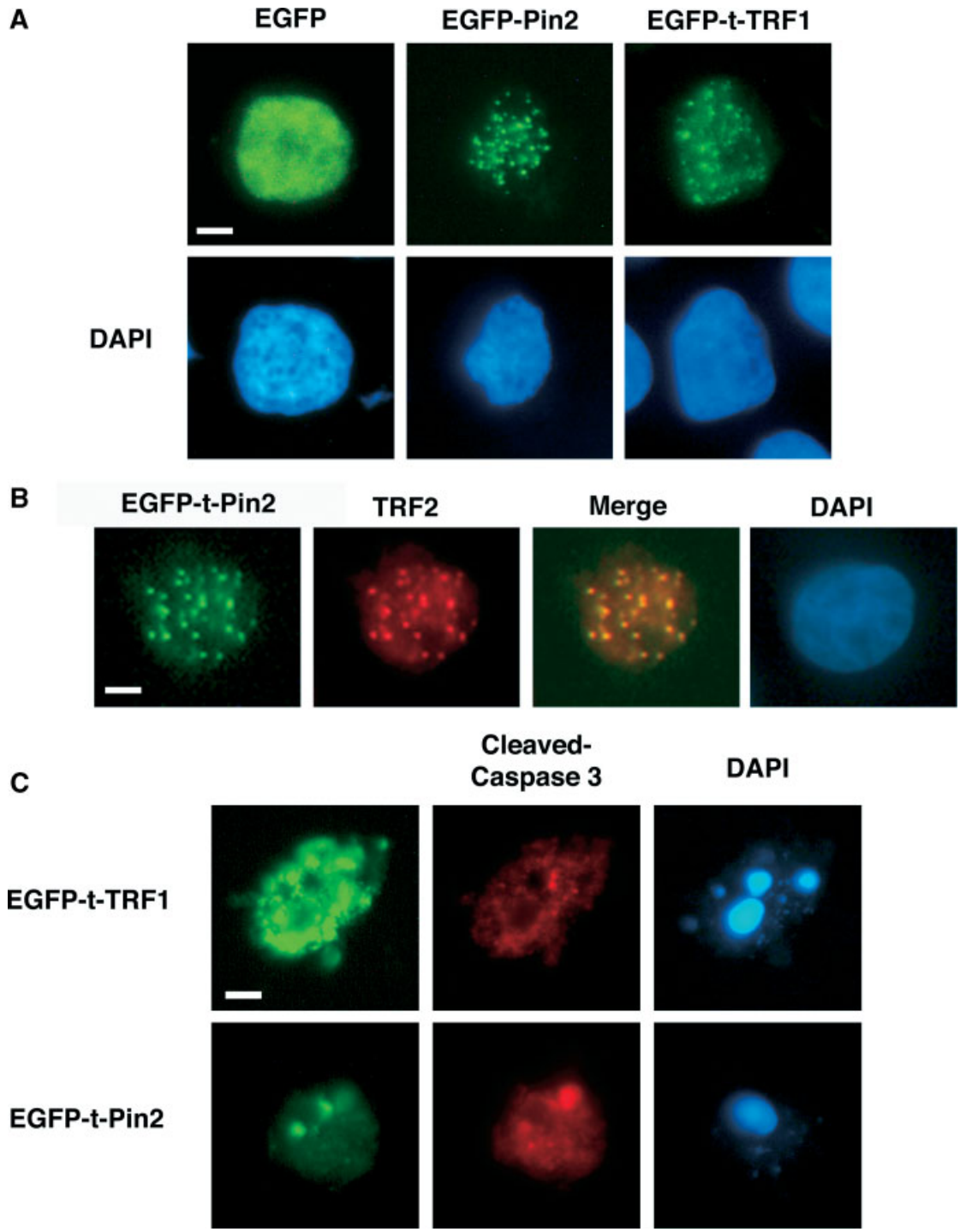

Fig. 3. Production of Pin2, t-TRF1, and t-Pin2 fused to EGFP in the CEM 1301 and T98G cell lines. Scale bars: 10 μm. **A**: Telomeric localization of EGFP-Pin2 and EGFP-t-TRF1, but not EGFP, in CEM 1301. **B**: EGFP-t-Pin2 (green) colocalized with TRF2 (red) in CEM 1301 nuclei stained with DAPI (blue). **C**: EGFP-t-TRF1, like EGFP-t-Pin2, induced the apoptosis of T98G, 24 h after transfection. Cells producing EGFP-t-TRF1 or EGFP-t-Pin2 (green) had cleaved caspase-3 (red) and fragmented, pycnotic nuclei stained with DAPI (blue).

t-Pin2 mRNAs were found in peripheral blood lymphocytes. PHA-p activation enabled lymphocytes to proliferate in response to IL-2 and induced a transient increase in hTERT mRNA levels and telomerase activity [Weng et al., 1996]. Interestingly, the in vitro activation of lymphocytes by PHA-p decreased the relative abundance of the t-TRF1/t-Pin2 mRNAs, sug-

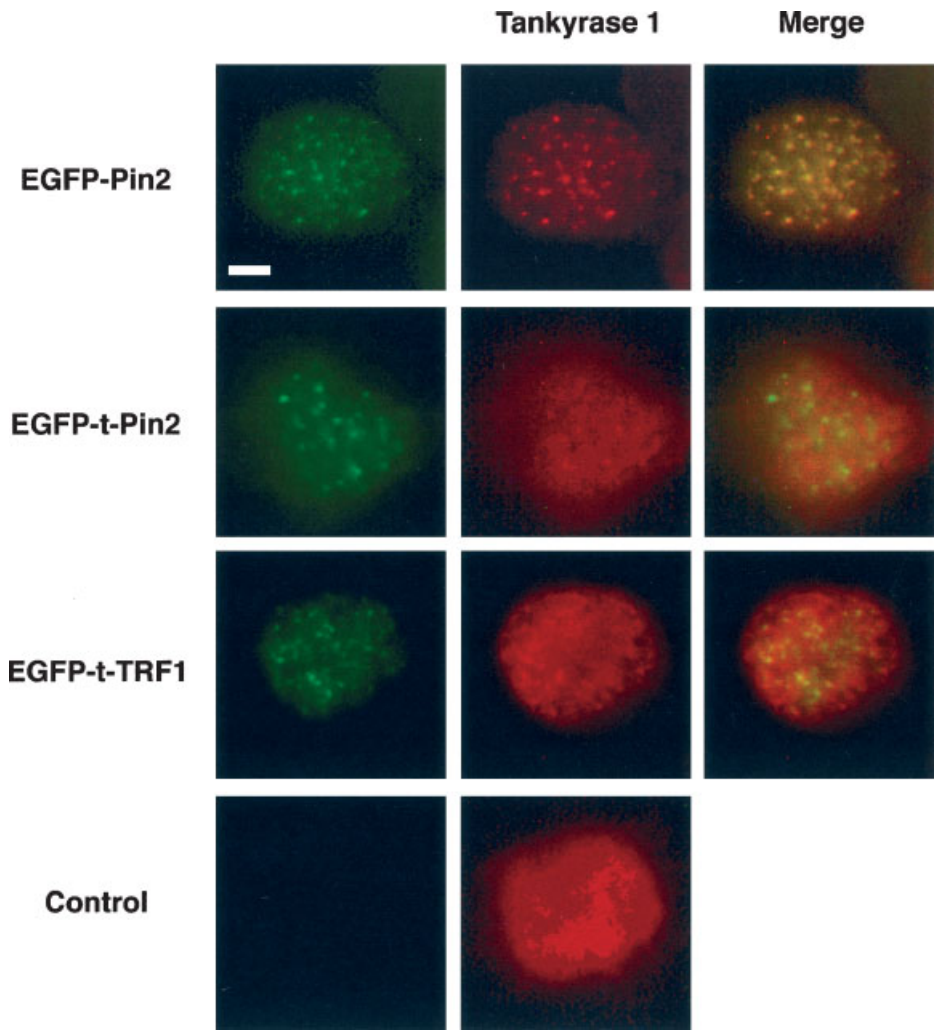

Fig. 4. t-TRF1 and t-Pin2 do not recruit tankyrase 1 to telomeres. CEM 1301 cells were cotransfected with pcDNA3-FN.tankyrase1.WT, together with the EGFP-Pin2, EGFP-t-Pin2, or EGFP-t-TRF1 construct. Immunofluorescence analysis was carried out 24 h later. Control cells were transfected with the pcDNA3-FN-tankyrase1.WT alone. Paraformaldehyde-fixed cells were stained with antiflag antibody (red) to detect tankyrase 1, or anti-EGFP antibody (green). Bar: 10 μm.

gesting that t-TRF1-t-Pin2 may have a role in resting lymphocytes.

TRF1/Pin2 has been reported to bind telomeres preferentially in dimer form, although it has also been shown to bind as a monomer [Konig et al., 1998]. Constructions of TRF1 lacking the acidic domain (TRF1 66–439) bind telomeres, but less strongly than full-length TRF1/Pin2 [Bianchi et al., 1997; Smogorzewska et al., 2000]. We found that the t-TRF1 and t-Pin2 fusion proteins, like TRF1/Pin2 and TRF2, were present in telomeres in interphase nuclei. As deletion of the 83 amino-terminal amino acids has been shown to abolish dimerization with full-length TRF1 [Bianchi et al., 1997], t-TRF1 and t-Pin2, which lack the first 107 amino-terminal amino acids of TRF1/Pin2, probably bind to telomeres as monomers.

The overproduction of t-TRF1 and t-Pin2 rapidly induced caspase-3-mediated apoptosis in cell lines with short telomeres but not in cell lines with long telomeres, as previously shown for TRF1/Pin2 [Kishi et al., 2001b]. It has been shown that the induction of apoptosis by TRF1/Pin2 results from the presence of too much unbound Pin2/TRF1 in transfected cells with short telomeres. The mechanism by which unbound TRF1/Pin2 induces apoptosis is unclear. It has been shown to be linked to a block in cell-cycle progression related to the G_2/M [Shen et al., 1997] and spindle checkpoints [Nakamura et al., 2001, 2002] and to be

inhibited by the ATM-catalyzed phosphorylation of Ser 219 [Kishi et al., 2001a]. The carboxy-terminal domain of TRF1/Pin2 has been shown to be required for the induction of apoptosis, whereas the presence of a functional telomeric binding domain is not [Kishi et al., 2001a]. We show here that the induction of apoptosis also requires neither the acidic domain nor dimerization, as t-TRF1/t-Pin2 dimerization is highly improbable.

TRF1/Pin2 has been reported to repress telomere elongation by telomerase because its stable overexpression reduces telomere length in some cell lines with long telomeres and the overproduction of a dominant negative mutant—a TRF1 lacking the C-terminus—leads to the elongation of telomeres in telomerase-positive cells [van Steensel and de Lange, 1997]. We did not investigate the efficiency of telomere-bound t-TRF1/t-Pin2 for downregulating telomerase-mediated telomere elongation because TRF1/Pin2-mediated telomere shortening can only be observed after many population doublings [van Steensel and de Lange, 1997] and we were unable to obtain stably transfected clones overproducing t-TRF1 or t-Pin2.

The ADP ribosylation of TRF1/Pin2 by tankyrase 1 has been shown to release TRF1/Pin2 from telomeres, thereby facilitating telomerase-mediated telomere elongation [Smith et al., 1998; Smith and de Lange, 2000; Chang et al., 2003]. We showed in cotransfection experiments that the lack of the amino-terminal acidic domain prevented t-TRF1/t-Pin2 from interacting with tankyrase 1, consistent with previous reports indicating that the amino-terminal acidic domain is the predominant binding site for tankyrase 1 on TRF1/Pin2 [Smith et al., 1998]. Thus, tankyrase 1 could not remove t-Pin2 and t-TRF1 from telomeres whereas it is able to do this for TRF1/Pin2. Thus, t-TRF1 and t-Pin2 may play an important role in counteracting the effects of tankyrase 1 activation. Indeed, the persistent binding of t-TRF1 and t-Pin2 may limit the telomerase-mediated elongation of telomeres favored by the removal of TRF1/Pin2. Alternatively, telomere-bound t-TRF1 and t-Pin2 may fulfill other roles of TRF1/Pin2 essential to telomere function or maintenance, but not necessarily related to the regulation of telomere elongation. The interactions of t-TRF1/t-Pin2 with other factors known to interact with TRF1/Pin2, such as Pot1, Tin2, and Ku70, should also be investigated to determine whether these interactions are involved in telomere homeostasis.

The main argument against a role for t-TRF1/t-Pin2 in telomere length regulation is that a dominant negative mutant of TRF1/Pin2 allows the progressive elongation of telomeres [van Steensel and de Lange, 1997] but would be expected to have no effect on t-TRF1/t-Pin2 because this mutant exerts its effects by dimerization, which is not possible for t-TRF1 and t-Pin2. However, t-Pin2 and t-TRF1 may play a role, but only in particular cell types other than those used in these studies, as suggested by the differences in mRNA levels for these proteins in different cell types. Alternatively, t-TRF1/t-Pin2 may slow down the elongation process in cells expressing the dominant negative mutant and may mediate a subset of telomere functions reducing the effects of the mutant. The persistent production of t-TRF1/t-Pin2 may thus explain why cells expressing the dominant negative mutant are less affected than mouse cells in which the gene has been disrupted, demonstrating that murine TRF1 is absolutely required for normal cell growth, telomere structure and chromosomal stability [Karlseder et al., 2003; Iwano et al., 2004]. Further studies are, therefore, required to assess the importance of alternative splicing of the *TRF1/Pin2* gene in telomere homeostasis and function.

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