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Telomere shortening and cell cycle arrest in *Trypanosoma brucei* expressing human telomeric repeat factor TRF1

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Abstract

Trypanosoma brucei has telomeres composed of 15 kb tracts of TTAGGG repeats that end in 3′ overhangs and form t-loops. This structure is also present in mammalian cells and is thought to reflect the presence of telomere-binding proteins. The human TTAGGG repeat-binding factor TRF1 binds to telomeres and regulates their length. We attempted to interfere with the normal function of trypanosome telomeres by expressing human TRF1 in *T. brucei*. TRF1 localized to telomeres in cultured procyclic (midgut-stage) trypanosomes with great fidelity, but not in bloodstream-stage trypanosomes. Procyclic trypanosomes expressing high levels of TRF1 for extended periods of time exhibited shortening and increased size heterogeneity of their telomeres and the cell cycle was arrested in G1–S. These effects were not detected in cells expressing a TRF1 mutant incapable of binding to TTAGGG repeats. We argue that TRF1 displaces putative endogenous trypanosome telomere-binding proteins, not yet identified, and affects telomeres in ways that reflect its role as a negative regulator of telomere length in human cells.

Keywords: *Trypanosoma brucei*; Telomere; TRF1; Telomere-binding protein

1. Introduction

The problems posed by the ends of linear chromosomes are solved in eukaryotic cells by various mechanisms. Telomerase adds repeats to the 3′ overhangs, compensating for telomere loss caused by incomplete DNA replication [1,2]. Homeostatic mechanisms, involving the binding of specific proteins to the duplex telomeric repeats, maintain a constant species-specific telomere length, even in cells with high levels of telomerase activity [3]. The telomere-binding proteins Rap1p in *Saccharomyces cerevisiae*, Taz1 in *Schizosaccharomyces pombe*, and TRF1 and TRF2 in animals, bind duplex telomere repeats and are negative regulators of telomere length [4–7]. Moreover, TRF2 protects the 3′ overhang and prevents chromosome ends from fusing and from activating ATM/p53-dependent DNA-damage-checkpoint pathway and initiating apoptosis [8,9]. Apart from the Myb domains, little else is conserved in the sequences or structural motifs of Rap1, Taz1, TRF1 and TRF2. High expression of Rap1, Taz1, TRF1, or TRF2, however, results in telomere shortening, suggesting a common role as negative regulators of telomerase [4–7]. The negative regulation of telomere length by TRF1 and TRF2 can be explained by their involvement in t-loop formation [10]. In the t-loop, the 3′ overhang is inserted into the duplex part of the telomere. This presumably limits accessibility of telomerase to the 3′ overhang. A role for Rap1 and Taz1 in t-loop formation has not been demonstrated, and t-loops have not been found in yeast. A human ortholog of Rap1 was recently discovered. However, hRap1 associates with telomeres through its interaction with TRF2, not by directly binding to DNA [11].

The complex process of antigenic variation in *Trypanosoma brucei* is centered at telomeres. The infectious stage in the vertebrate host, or bloodstream form (BF) of *T. brucei*, expresses a cell surface coat made of 10 million molecules of a single species of variant surface

Abbreviations: BF, bloodstream form; ES, expression site; IF, indirect immunofluorescence; kb, kilo base pairs; PF, procyclic form; TRF1 and TRF2, telomere repeat-binding factor 1 and 2; VSG, variant surface glycoprotein.

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glycoprotein (VSG) [12,13]. Only one VSG gene (*VSG*), from a repertoire of about 1000, is transcribed at any time [14]. Trypanosomes replicate freely in the bloodstream and provoke the host's immune response. However, a few parasites remain undetected by the immune system by periodically switching the expressed *VSG*. Invariably, the expressed *VSG* is adjacent to a telomere [15]. Telomeric *VSG*s are also the last genes of long polycistronic transcription units known as expression sites (ESs). Only one ES is transcriptionally active at a time, and antigenic switching is achieved either by replacement of the transcribed *VSG* by the duplicated copy of one of the hundreds of *VSG*s distributed throughout the genome, or by concomitant activation and inactivation of two different ESs. In the non-infectious midgut procyclic form (PF), all ESs are transcriptionally inactive and chromatin appears to be remodeled to repress transcription [16].

Trypanosome telomeres, like those of mammals, consist of TTAGGG repeats [17,18]. Because of the presence of telomeric *VSGs*, individual telomeres can be studied easily. Interestingly, *T. brucei* telomeres grow by approximately 7 bp per cell division [18–20], presumably by telomerase-mediated addition of TTAGGG repeats, and telomere length remains very homogeneous within the trypanosome population. Transcriptionally active telomeres grow slightly faster (10 bp per cell division), suggesting a correlation between telomere structure and the transcriptional status of the genes located in their proximity [20]. Telomerase activity with limited processivity has been described in three species of kinetoplastids, including *T. brucei* [21]. In addition, position effects within the ES [22–24], and chromatin remodeling and developmentally regulated repression effects close to telomeres [16], suggest a role for telomeres in regulating antigenic variation and suggest that there are differences in telomeric chromatin structure between BF and PF trypanosomes. BF but not PF trypanosomes convert a portion of their T residues to the glycosylated base J (β-D-glucosyl-hydroxymethyluracil), most of which is located in the telomeric repeats [25,26].

T. brucei contains 11 chromosomes of 1–6 Mb [27,28], which contain all the essential genes, and about 100 minichromosomes of 25–100 kb. Minichromosomes are rich in telomeric repeats and contain *VSGs* that can be translocated into an active ES on a large chromosome, thereby contributing to the repertoire of expressible *VSGs* [29]. As in some other organisms, trypanosome telomeres are situated around the periphery of the nucleus during G1–S phase [30]. As cell division progresses, minichromosomes — and therefore most of the telomeric DNA — redistribute in a few clusters located in the center of the nucleus during mitosis. Minichromosomes locate at opposite poles of the dividing nuclei [31,32]. Whereas minichromosomes segregate along a central intranuclear mitotic spindle, large chromosomes are partitioned by kinetochore microtubules [31]. With an average length of 15 kb, telomeric repeats comprise approximately 10% of the *T. brucei* genome, making this organism an excellent system in which to study telomere structure and function. Trypanosome telomeres have been recently shown to form t-loops [33]. T-loops are also present in *Oxytricha fallax* [34], confirming the evolutionary conservation of this structure. Proteins that bind to the duplex part of the telomere, however, remain unidentified in organisms other than yeast and mammals, hindering evolutionary comparisons of telomere biology.

We have expressed human TRF1 in BF and PF *T. brucei*, hoping to disrupt, and thereby identify, some of the normal functions of trypanosome telomeres. We found effects that are consistent with the role of TRF1 as a negative regulator of telomerase and with the presence of endogenous telomeric proteins in trypanosomes.

2. Materials and methods

2.1. Trypanosome cell lines and constructs

T. brucei strain 427, antigenic type 1.2 (MITat 1.2), clone 221a [13], had been previously manipulated to create the PF cell line 29-13 and the BF cell line 13-90 that constitutively express T7 RNA polymerase from the tubulin locus and TetR from the RNP1 locus [35]. PF were cultured at 27°C in SDM-79 supplemented with fetal bovine serum and containing 15 μg ml^{-1} G418 (Sigma) and 25 μg ml^{-1} hygromycin (Sigma), to a maximum cell density of 10^7 trypanosomes ml^{-1}. BF were cultured in HMI-9 containing 5 μg ml^{-1} G418 and 5 μg ml^{-1} hygromycin to a maximum cell density of 2×10^6 trypanosomes ml^{-1}. The FLAG-TRF1 fusion was cloned in the *Hin*dIII-digested plasmid pLew111 [36], a derivative of pLew82 [35]. All transfections were done as previously described [37]. A FLAG-tagged version of $TRF1^{R425V}$ was engineered as below. Prior to any experiments, BF were washed in trypanosome dilution buffer, (TDB: 5 mM KCl, 80 mM NaCl, 1 mM $MgSO_4 \cdot 7H_2O$, 20 mM Na_2HPO_4, 2 mM $NaH_2PO_4 \cdot 2H_2O$, 20 mM glucose) and kept on ice. PF were washed and resuspended in phosphate-buffered saline pH 7.3 (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4) and kept on ice.

2.2. TRF1 mutagenesis

A site-directed mutagenesis protocol (Stratagene 'QuickChange' kit) was followed to create FLAG-$TRF1^{R425V}$ from TRF1. Briefly, primers (GTGTCAT-

GTTAAAAGACAGATGGGTGACCATGAAGA) and (TCTTCATGGTCACCCATCTGTCTTTTAACATGACAC), provided by H. Moss and T. de Lange, are complementary to each other and carry the triplets GTG or CAC (underlined), by which a mutation is introduced in the TRF1 sequence that changes codon AGG (arginine) for GUG (valine), abolishing the binding of TRF1 to TTAGGG repeats (H. Moss and T. de Lange, unpublished data). Plasmid pLew111 carrying FLAG-TRF1 was used as template. After denaturation of the template and annealing of the primers, extension by *Pfu* DNA polymerase followed by *Dpn*I digestion of the methylated non-mutated plasmid template were performed. The mutated plasmid was recovered in an *E. coli* recipient cell line.

2.3. Genomic blotting

A total of 5×10^8 trypanosomes were resuspended in TNE (10 mM Tris pH 7.4, 100 mM NaCl, 10 mM EDTA) and lysed in TNES (10 mM Tris pH 7.4, 100 mM NaCl, 10 mM EDTA, 1% SDS) in the presence of 100 μg ml^{-1} of proteinase K. After overnight incubation with proteinase K at 37°C, and phenol/chloroform extractions, DNA was precipitated with isopropanol, and resuspended in TE. Ribonuclease A treatment, phenol/chloroform extractions and isopropanol precipitation followed. For telomere blots, DNA was digested overnight with restriction enzymes *Alu*I/*Hinf*I/*Rsa*I, *Xmn*I or *Eco*RI, under recommended conditions (New England Biolabs). Telomeric restriction fragments were detected using a probe containing TTAGGG repeats and labeled as previously described [38]. The probe for *VSG* 221 was labeled as previously reported [16]. All probes were labeled with α-^{32}P-dCTP.

2.4. Western blotting

10^7 BF or PF were lysed in 100 μl of loading buffer (50 mM Tris Cl pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol). Ten microlitre of these protein extracts (equivalent to 10^6 cells) were separated on a 10% SDS-polyacrylamide gel. TRF1 protein expressed in Baculovirus was provided by T. de Lange and separated under similar conditions. Proteins were transferred to nitrocellulose and blots were blocked in 10% fat-free milk powder and 0.5% Tween 20 in PBS. Antibody incubations were performed in 0.1% fat-free milk powder and 0.1% Tween 20 in PBS (incubation buffer) at 4°C. Baculovirus TRF1 or exogenous FLAG-tagged TRF1 expressed in trypanosomes were detected using rabbit polyclonal antibody 371 [6]. FLAG-tagged TRF1 was also detected using commercially available *anti*-FLAG monoclonal antibodies M2 (Sigma). Blots were then washed three times for 10 min in incubation buffer, then incubated for 1–2 h with goat *anti*-rabbit antibody (Amersham) and washed three times for 10 min in incubation buffer and one time for 2 min in PBS. Antibody-protein complexes were detected using the ECL chemiluminescence system (Amersham).

2.5. In situ hybridization and immunofluorescence

A protocol was adapted for trypanosomes from previously published procedures [39]. BF or PF diluted to 10^6 trypanosomes ml^{-1} in TDB or PBS, respectively were attached to glass coverslips by centrifugation at $3000 \times g$ for 5 min at 4°C. Coverslips were immersed in 2% formaldehyde dissolved in PBS and incubated 5 min at 4°C. Trypanosomes were permeabilized in 4 ml 0.5% NP-40 in PBS for 5 min at 4°C and maintained in PBS at 4°C. For in situ hybridization, DNA was denatured by incubating permeabilized trypanosomes in 70% formamide/2 × SSC for 5 min at 70°C. Coverslips were washed briefly in 2 × SSC at 50°C. DNA denaturation, hybridization and washing conditions were essentially as described [40]. The digoxigenin-labeled $(CCCUAA)_{27}$ RNA probe was synthesized by SP6 polymerase from plasmid pTH5 [41], previously digested with *BamH*I, in the presence of digoxigenin-uridine 5′-triphosphate (Boehringer). Cells were incubated with sheep antibody to digoxigenin (anti-digoxigenin) (Boehringer). Either after hybridization or just after permeabilization, the preparations were incubated for 10 min in PBS with 50 mM glycine, and for 15 min in PBG (PBS with 0.1% cold-water–fish-skin gelatin from Sigma and 0.5% BSA). FLAG-tagged TRF1 was detected by using the *anti*-FLAG monoclonal M2 (Sigma) or rabbit antibody 371 [6]. To detect *T. brucei* flagellar basal body and flagellar rod proteins, monoclonal antibodies BBA4 and ROD1 (provided by K. Gull) were used as previously reported [42]. DNA was stained using DAPI. Antibodies M2, 371, BBA4, and ROD1 were detected with fluorescein isothyocyanate-conjugated (FITC) donkey *anti*-mouse or *anti*-rabbit antibodies, respectively (Jackson).

2.6. Measurement of DNA content by flow cytometry

Conditions similar to reported protocols were followed [43]. PF trypanosomes expressing TRF1 or $TRF1^{R425V}$ were washed and resuspended in ice-cold PBS pH 8.0 with 0.5 mM EDTA to a density of 10^6 ml^{-1}. Trypanosomes were fixed by slowly adding ethanol to a final concentration of 70% and storing at 4°C for periods of 24 h or longer. Fixed trypanosomes were pelleted and resuspended to 10^7 ml^{-1} in PBS pH 8.0 containing 50 μg ml^{-1} propidium iodide and 40 μg ml^{-1} RNAse and incubated for 1 h at room temperature. Intensity of red fluorescence was measured using a FACSort flow cytometer (Beckon-Dickinson and Co).

3. Results

To express TRF1 in *T. brucei*, we employed the inducible construct pLew111 [36], in which the FLAG-TRF1 fusion was inserted between a T7 promoter and a gene conferring resistance to phleomycin. Fig. 1A depicts this construct and the salient features of TRF1. Plasmid pLew111 containing TRF1 was transfected into PF 29-13 and BF 13-90 cells [35]. Upon low induction with doxycycline (0.1 μg ml^{-1}) and selection with phleomycin (2.5 μg ml^{-1}), 11 PF clones and six BF clones expressing TRF1 were obtained from two independent transfections. All PF and BF clones were analyzed by western blotting, which revealed equal levels of TRF1 in all clones (data not shown). Comparing with known quantities of baculovirus-expressed TRF1, it was established that, upon induction with high levels of doxycycline (1 μg ml^{-1}), trypanosomes

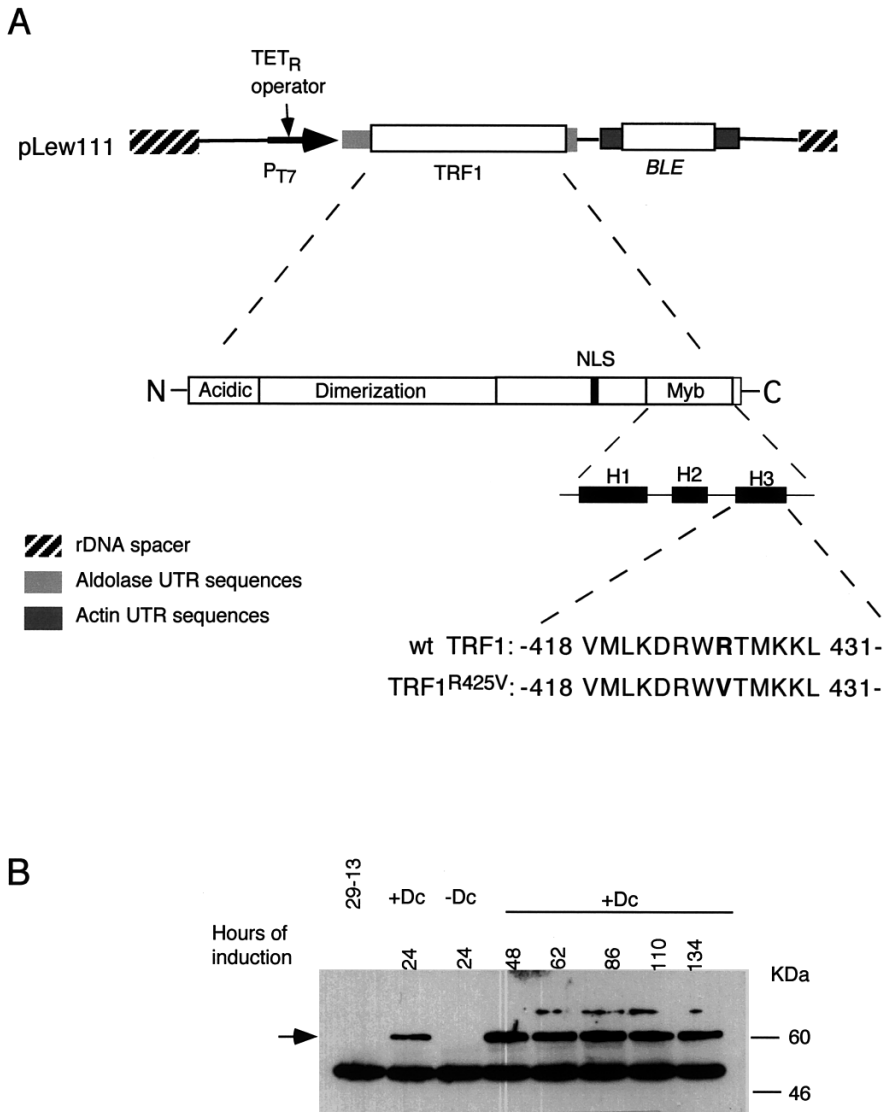


Fig. 1. Inducible expression of TRF1 and TRF1^{R425V} in *T. brucei*. (A) FLAG-tagged *TRF1* was inserted into pLew111 immediately downstream of a T7 promoter containing a TetR operator. The construct was transfected into BF 13-90 or PF 29-13 cell lines, which express T7 RNA polymerase and TetR. The site of insertion is a ribosomal DNA spacer. The untranslated regions flanking *TRF1* and *BLE* are depicted. In the presence of tetracycline (or doxycycline), the T7 promoter is de-repressed and *TRF1* and the bleomycin resistance gene (*BLE*) are transcribed. The salient characteristics of the *TRF1* gene are identified. H1, H2 and H3 are the three helices of the Myb domain. A point mutation was introduced in the third helix of the Myb domain that changes an arginine for a valine at position 425, creating TRF1^{R425V}. (B) A western blot using antibodies for FLAG shows inducible expression of TRF1 in the PF 29-13 strain. As indicated by the arrow, robust expression of TRF1 was obtained 24 h after induction with doxycycline (+ Dc) and increased to a constant level after 48 h. The upper band probably corresponds to traces of TRF1 dimers. An endogenous 50 kDa band is also detected by the *anti*-FLAG antibody.

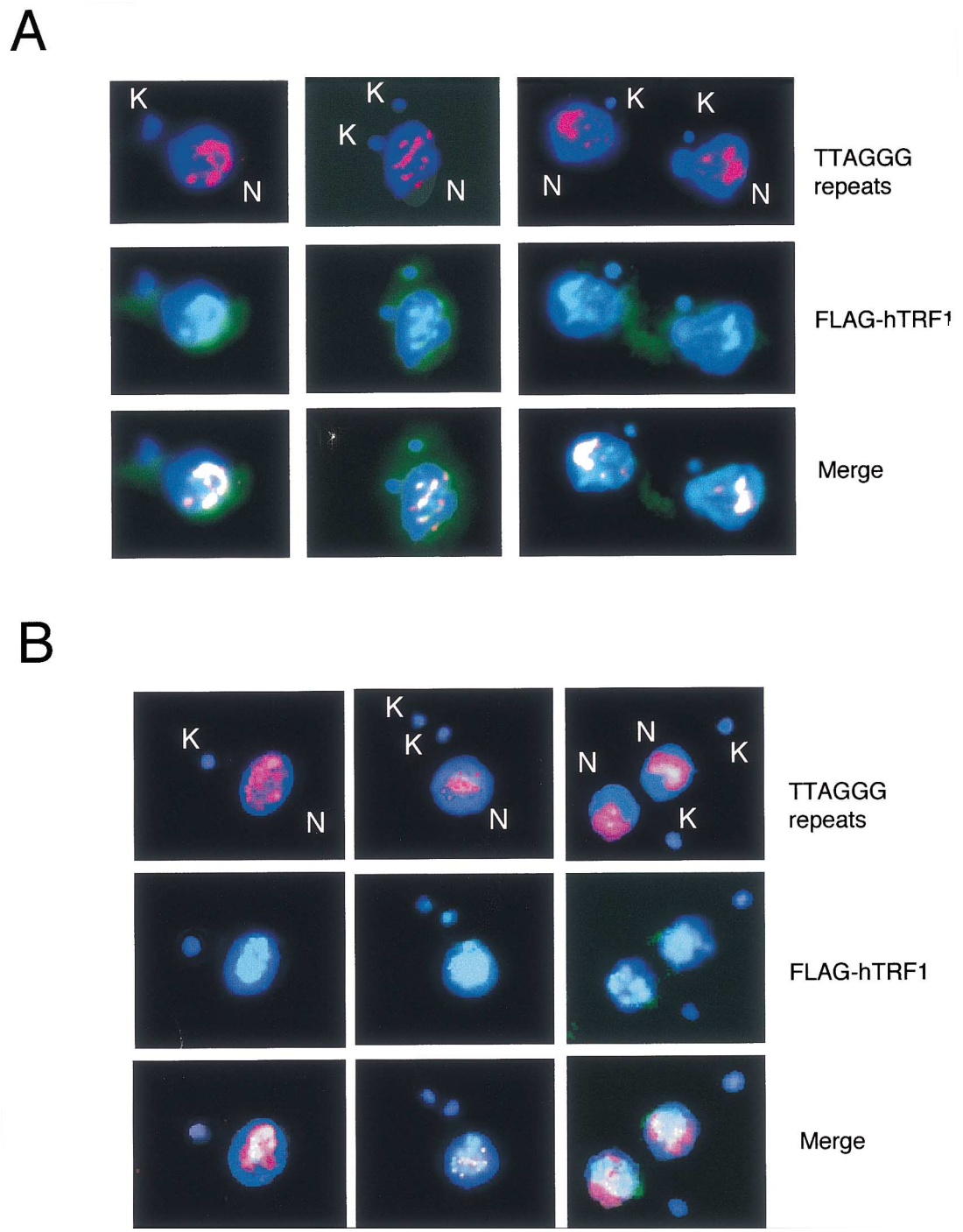


Fig. 2. TRF1 binds to telomeres in PF but not in BF trypanosomes. Nuclear and kinetoplast DNA are detected by DAPI staining (blue fluorescence). An in situ hybridization for TTAGGG repeats (red fluorescence) indicates where the telomeric DNA is located and an indirect immunofluorescence for FLAG-TRF1 (green fluorescence) shows distribution of TRF1in PF (A) and BF (B). The left panels show a trypanosome with one kinetoplast (K) and one nucleus (N). In the middle panels, a trypanosome in S-phase with 2 K and 1 N exhibits telomeres aligned in the center of the nuclei. The right panel depicts a post-mitotic trypanosome with 2 K and 2 N, where telomeres are situated at opposite poles of the nuclei. The merge between the red and the green fluorescence (yellow) reveals complete co-localization of TRF1 and telomeric repeats in PF throughout the cell cycle. In contrast, BF exhibited incomplete localization of TRF1 to telomeres.

express $\sim 5 \times 10^5$ molecules of TRF1 per cell (data not shown). Moreover, trypanosomes expressed high levels of TRF1 for prolonged periods (Fig. 1B). An endogenous cross-reacting band of 50 kDa is seen throughout the blot, and does not vary between induced and non-induced cells.

3.1. TRF1 binds to trypanosome telomeres

To examine the possible binding of TRF1 to trypanosome telomeres, we performed fluorescent in situ hybridization (FISH) coupled with indirect immunofluorescence (IF) in PF (Fig. 2A) and BF trypanosomes (Fig. 2B) expressing TRF1. The red fluorescence indicated the presence of the digoxygenin-labeled RNA probe specific for telomeric repeats. The green fluorescence reveals the binding of rabbit antibody 371 to TRF1 [6]. The merge between the top and middle panels revealed complete co-localization of TRF1 and telomeres in the PF. In contrast, in the BF TRF1 does not uniformly co-localize with the telomere probe and does not condense to punctate spots as in PF. We also observed that the division rate of PF expressing TRF1 declined after 48 h (see below). We observed this in all PF clones. In contrast, BF trypanosomes carrying the TRF1 gene grew normally and independently of the presence of TRF1. This indicated a possible correlation between the binding of TRF1 to telomeres and an effect on cell division. In non-transfected as well as in non-induced trypanosomes, no immunofluorescence staining is detected with antibody 371.

3.2. Binding of TRF1 to telomeres causes cell division arrest

To further study the correlation between the binding of TRF1 to telomeres and the effects of this binding on cell division, we employed a TRF1 mutant allele, $TRF1^{R425V}$, deprived of telomere-binding activity. $TRF1^{R425V}$ carries a point mutation that changes the argenine for valine in the Myb DNA-recognition helix 3 of TRF1 (Fig. 1A). This mutation abolishes the binding of TRF1 to duplex TTAGGG repeats in vitro and in mammalian cells (H. Moss and T. de Lange, manuscript in preparation). By site directed mutagenesis, the same point mutation was introduced into the TRF1 copy previously FLAG-tagged and cloned in pLew111. PF were transfected with pLew111 carrying FLAG-$TRF1^{R425V}$ and 10 phleomycin-resistant clones expressing $TRF1^{R425V}$ were obtained. By protein blotting, we established that the levels of expression of TRF1 and $TRF1^{R425V}$ were identical (data not shown).

In Fig. 3A, the growth rates of induced and non-induced PF carrying TRF1 or $TRF1^{R425V}$, and the parental procyclic line 29-13 (PF 29-13), are compared. Trypanosomes expressing TRF1 suffered a reduction of their duplication rate. After 62 h expressing TRF1, cell growth was almost completely arrested. Nonetheless, most of the cells in the culture were alive, as evidenced by their motility. After 72 h, when cell density in most of the cultures was $1–2 \times 10^7$ trypanosomes ml^{-1}, and $5–6 \times 10^6$ for the cells expressing TRF1, all cultures were diluted in fresh medium to 8×10^5 trypanosomes ml^{-1} in order to allow continuous exponential growth. In addition, trypanosome cultures previously growing in the presence of doxycycline were split and grown in the presence and in the absence of doxycycline. Trypanosomes previously expressing TRF1 but deprived of doxycycline at 72 h (rescued), began to divide at a normal rate of 6–8 h, comparable to the normal division rate exhibited by the $TRF1^{R425V}$-expressing cells. The growth rate of trypanosomes expressing TRF1 also increased gradually after 72 h. Altogether, trypanosomes expressing high levels of TRF1 exhibit a cell division arrest phenotype after 48–62 h of continuous induction (6–8 divisions). This cell division arrest extends for 24–48 h, after which trypanosomes began to divide at a normal rate of 1 duplication per 6–8 h. Similar results were found with two different trypanosomes originating from two independent transfection experiments and expressing similar levels of TRF1.

Cell-division arrest was only observed after high and prolonged induction of TRF1. For this reason, and because we used a lower resolution camera, the punctate pattern of TRF1 is not as easily seen in Fig. 3B as it is in Fig. 2A. However, the nuclear distribution of $TRF1^{R425V}$ was homogeneous and readily distinguishable from that of wild-type TRF1. Some cells expressed less TRF1 than others, and the punctate pattern was more obvious in cells expressing lower levels. After 110 h, when cells have escaped from cell division arrest, more cells expressed lower levels of TRF1 than at previous time points. Trypanosomes not induced, or previously induced but deprived of doxycycline at 72 h, do not express detectable levels of TRF1. These findings indicate that the DNA binding of TRF1, and not just the presence of the protein, arrests cell division.

Fig. 3. Binding of TRF1 to telomeres correlates with transient cell division arrest. (A) PF 29-13 carrying TRF1 or $TRF1^{R425V}$ in the presence and in the absence of doxycycline, as indicated, were counted during exponential growth. After 72 h, as indicated by the arrow, cultures were diluted to 8×10^5 trypanosomes ml^{-1} to continue exponential growth. A portion of trypanosomes previously growing in the presence of doxycycline were then grown in the absence of the drug (rescued). Trypanosomes continuously expressing TRF1 exhibited a transient cell division arrest between 48 and 84 h. (B) At high levels of induction with doxycycline, TRF1 (green fluorescence) localizes to the nucleus (blue fluorescence) in a punctate pattern that reflects its telomeric binding. After 8 h of induction, 85–90% of the cells expressed high levels of TRF1, whereas the rest of the cells expressed low levels. After 110 h, 65% of the cells expressed intermediate levels of TRF1 and 45% exhibited low or undetectable levels of TRF1. In contrast, $TRF1^{R425V}$ exhibited a broad pattern of distribution in the nuclei, which is maintained throughout extended periods of time. Cells not induced with doxycycline, and rescued cells previously induced but deprived of doxycycline at 72 h, do not show detectable levels of TRF1. The inserts show the trypanosomes indicated by the arrows at higher magnification. The images are less detailed than those shown in Fig. 2, owing to the four-fold lower resolution of the available camera.

3.3. Trypanosomes expressing TRF1 arrest in G1–S

We next investigated whether cell division arrest occurs at a specific phase of the cell cycle. We collected trypanosomes carrying TRF1 or TRF1^{R425V} after 8, 62 and 110 h of continuous growth in the presence or in

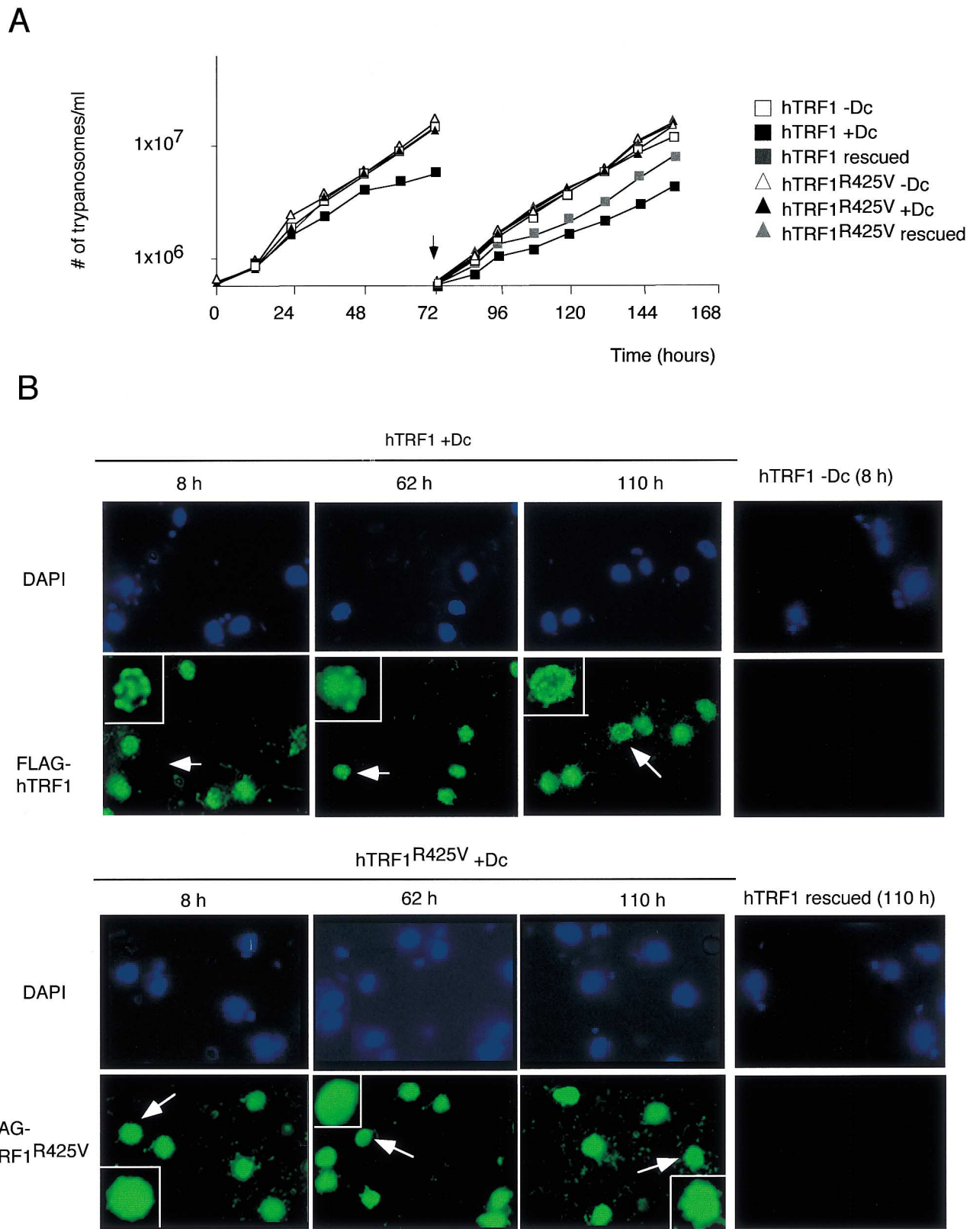


Fig. 3. (*Continued*)

the absence of doxycycline, and performed IF using the primary antibodies BBA4 for the flagellar basal body and ROD1 for the paraflagellar rod proteins PFR180 and PFR200 [42] (Fig. 4A). We co-stained with the DNA-intercalating dye DAPI, to detect trypanosome nuclei and kinetoplasts. During the exponential growth of PF 29-13, approximately 73% of the cells exhibited one nucleus, one kinetoplast, one basal body, and one flagellum (Fig. 4B). These trypanosomes are considered to be in G1. Trypanosomes in nuclear S-phase are distinguished by having two basal bodies, one flagellum and one nucleus. Towards the end of the nuclear S-phase, the second paraflagellar rod appears. Trypanosomes in S-phase represented 9% of the non-TRF1-expressing cells. Cells in G2, prior to cell division, exhibit an elongated nucleus and an incipient second flagellum. These also accounted for 9% of the cells in the culture. The remaining 9% of the cells consisted of mitotic (M) or post-mitotic (PM) cells, with two kinetoplasts, two flagellae and two nuclei or one elongated nucleus. The percentages of cells at each phase of the cell cycle are consistent with previous reports [44]. Parental cells in stationary phase, or cells expressing TRF1^{R425V}, or uninduced TRF1 cells, were also found in all phases of the cell cycle (Fig. 4 A, B).

Eight hours after induction of TRF1, cells at different phases of the cell cycle were present. After 62 h, however, 94% of TRF1-expressing cells exhibited only one set of organelles (G1–S), and only 6% had two basal bodies (S). No cells were found that had duplicated their paraflagellar rod, kinetoplast or nucleus. After 110 h, all cultures once again contained a normal distribution of cells in different phases of the cell cycle.

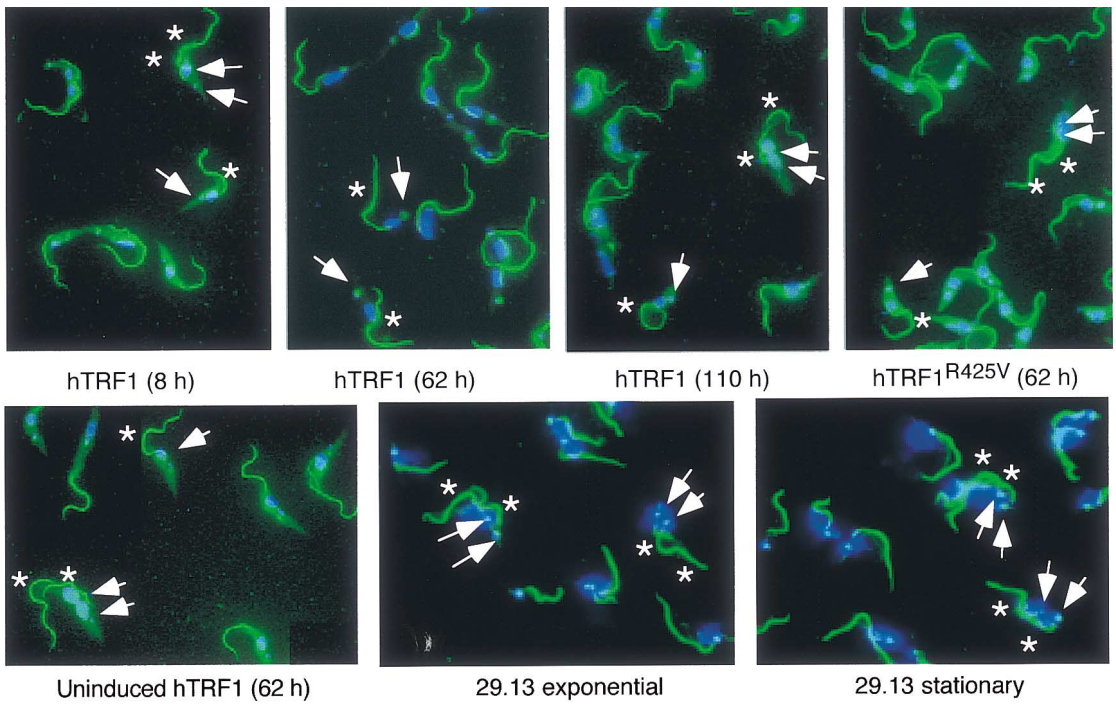
We also evaluated the DNA content of cells expressing TRF1 and TRF1^{R425V} for the same periods of time by flow cytometry (Fig. 4C). Cells dividing normally showed two peaks. The first peak represents approximately 80% of the cells that are in G1 and therefore contain one nucleus (2n). The second peak represents the remaining 20% of the cells in G2–M (4n). After 8 h expressing TRF1 or TRF1^{R425V}, the same 2 peaks were observed. After 62 h, however, cells expressing TRF1 differed from cells expressing TRF1^{R425V}. TRF1 expressors showed only one clear peak, corresponding to cells in G1–S. After 110 h, cells in G2-M reappeared. Altogether, the data are consistent with a transient G1–S cell division arrest after 62 h.

3.4. Trypanosomes expressing TRF1 exhibit telomere shortening

To examine the possible effects of TRF1 on trypanosome telomere stability, we investigated the length distribution of telomeres from cells expressing TRF1 and TRF1^{R425V}. On genomic blots of trypanosome DNA digested with frequent-cutting endonucleases *Alu*I, *Hinf*I and *Rsa*I, telomeric DNA repeats can be visualized between 4 and 23 kb. In Fig. 5A, we compare the distribution of telomeric DNA from trypanosomes induced and not induced carrying either the TRF1 or TRF1^{R425V} constructs. After 62 h, telomeric DNA from trypanosomes expressing TRF1 became increasingly heterogeneous and shorter, whereas telomeres from trypanosomes not expressing TRF1 or expressing TRF1^{R425V} were unchanged. Using the telomeric *VSG* 221 as a marker, a specific telomeric fragment of 18 kb can be visualized on a Southern blot of genomic DNA digested with *Eco*RI (Fig. 5B). We found that the telomere carrying *VSG* 221 was unchanged in trypanosomes not induced with doxycycline or in trypanosomes expressing TRF1^{R425V}. However, in trypanosomes expressing TRF1, the *VSG* 221 telomeric fragment became heterogeneous. Predominantly, this fragment shortened by 3 kb, and fragments were also detectable that were up to 10 kb shorter. This corroborates the finding in Fig. 5A, and proves that telomeres in the cells expressing TRF1 get shorter with time, presumably due to binding of TRF1, which is highly specific for TTAGGG repeat units [6,7,39,45,46] and co-localized with trypanosome telomeres, within the limits of optical resolution (Fig. 2A). To analyze the possibility that telomere shortening was due to random chromosome cleavage, we analyzed the status of non-telomeric copies of *VSG* 121. By digesting DNA with *Xmn*I, three chromosome-internal copies of *VSG* 121 can be detected, in addition to a large fragment con-

Fig. 4. Cells expressing TRF1 arrest at G1-S. (A) Using antibodies for the flagellar basal body (arrows) and the paraflagellar rod (asterisks), both shown by green fluorescence, trypanosomes at different phases of the cell cycle can be identified. Dividing and non-dividing trypanosomes can be seen after 8 h expressing TRF1. After 62 h, only non-dividing forms are present. After 110 h, dividing forms reappeared. Trypanosomes lacking the TRF1 or TRF1^{R425V} constructs (the parental 29-13 line) were also collected in exponential and stationary phases. Cells growing in the absence of doxycycline, or cells expressing TRF1^{R425V}, or parental cells, can be found as dividing and non-dividing forms at all times. (B) For each time point, 100 cells were counted under the fluorescence microscope. The data are summarized as the percentage of cells at a specific phase of the cell cycle for trypanosomes carrying TRF1 or TRF1^{R425V} either induced (+Dc) or not induced (−Dc). After 62 h, most trypanosomes expressing TRF1 contained one flagellum and 1 basal body (some contain 2 basal bodies), and all contained one nucleus. 29-13 cells in exponential (E) or stationary (S) phase were counted only in the absence of Dc. (C) Cells expressing TRF1 or TRF1^{R425V} were fixed in ethanol and DNA stained with propidium iodide prior to flow cytometry. After 8 h of induction, 2 peaks were detected. The first peak represents a population of cells in G1, whereas the second smaller peak represents dividing cells (G2–M), with twice the intensity of the first peak. After 62 h, cells expressing TRF1 showed only the first peak, whereas cells expressing TRF1^{R425V} showed both peaks. After 110 h both TRF1- and TRF1^{R425V}-expressing cells divided normally.

A



B

% of cells at a particular phase of the cell cycle		hTRF1			hTRF1R425V			29.13	
		8h	62 h	110 h	8h	62 h	110 h	E	S
G1	+Dc	73	94	74	73	73	74	73	74
	-Dc	74	73	74	73	74	73		
S	+Dc	9	6	9	9	9	9	9	8
	-Dc	8	9	9	9	9	9		
G2	+Dc	8	0	8	8	8	8	8	8
	-Dc	8	9	9	8	8	8		
M PM	+Dc	10	0	10	10	10	9	10	10
	-Dc	10	9	9	10	9	10		

C

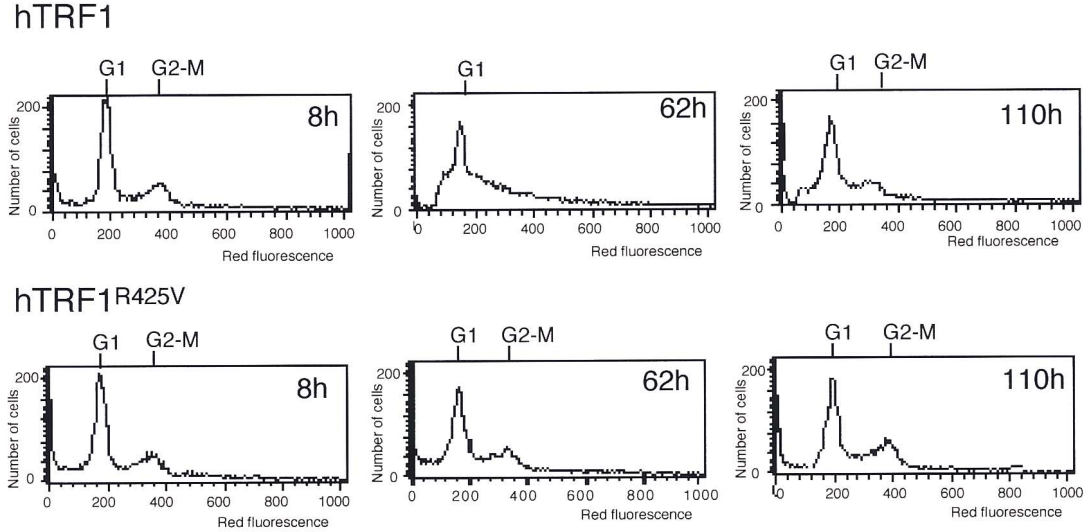


Fig. 4. (*Continued*)

taining a telomeric copy of *VSG* 121 (Fig. 5C). The sizes of the three chromosome-internal copies of *VSG* 121 remained stable in DNA of trypanosomes expressing TRF1 or TRF1^{R425V}, showing no evidence of internal cleavage or degradation of the DNA. The mobility of the *VSG* 121 telomeric band was unchanged in cells expressing either TRF1^{R425V} or TRF1, but small changes would have been undetectable. The location of the *Xmn*I site upstream of *VSG* 121 is not precisely documented, but the fragment is probably longer than 25 kb. The telomere shortening and cell division arrest both occur 48–62 h after induction of TRF1 expression. These results suggest that the binding of TRF1 to trypanosome telomeres destabilizes them.

4. Discussion

The maintenance of telomeres in a variety of organisms is regulated by homeostatic mechanisms in which proteins that bind to the duplex part of telomeres apparently modulate the effects of telomerase. Lack of protection of telomeres by telomeric proteins also results in DNA-damage responses leading to senescence and apoptosis. We expressed the human protein TRF1 in trypanosomes and found that its binding to trypanosome telomeres correlated with two distinct effects: transient cell division arrest and telomere shortening.

Whereas TRF1 can bind to TTAGGG repeats, a mutation in the Myb domain of TRF1 (TRF1^{R425V}) abolishes this binding. The expression of both isoforms in trypanosomes was high and comparable. After few division cycles, PF trypanosomes expressing TRF1 no longer divided, whereas TRF1^{R425V}-expressing cells divided normally. Therefore, it is the binding of TRF1 to the telomeres, and not simply the presence of the protein, which causes these effects. In BF, where TRF1 was as highly expressed but not consistently bound to telomeres, changes in cell division were not observed. As demonstrated by DNA staining and cell sorting, and by IF studies, PF arrest occurs in G1–S, and not haphazardly at any stage of the cycle. This indicates that cells proceeded through the cell cycle until they reached a checkpoint that does not allow progress through S and into G2. We noticed that the arrested cells exhibited elongated flagella and nuclei, which may indicate that cell growth continues despite the lack of progress in the cell cycle. These are characteristics typically ascribed to cells undergoing cell-cycle arrest in other systems (yeast *cdc* mutants, for example) and not to cells that are simply deprived of nutrients or grown to stationary phase. We have corroborated this by growing PF trypanosomes to stationary phase. After 24 h in stationary phase, cells were found in all phases of the cell cycle in proportions similar to those in exponentially dividing cells. In contrast, trypanosomes expressing TRF1 were arrested synchronously in G1–S. We think that the binding of TRF1 alters trypanosome telomeres in ways that result in uncapping of the telomere end and concomitant activation of DNA-damage checkpoints. This reasoning is in accordance with the finding that mammalian cells lacking the DNA-binding activity of TRF2 exhibit cell cycle arrest and p53/ATM-dependent apoptosis [9]. Another possible scenario is that lack of telomere capping function could result in covalent fusion of telomeres [8]. This could result in formation of dicentric chromosomes and ultimate disruption of the chromosomes during mitosis. In this case, a DNA-damage response may be generated from internal DNA breaks and not necessarily by unprotected telomere ends. We found no changes in the telomere blots that would indicate telomere fusion events. In our FISH and IF studies, telomeres showed the expected sub-nuclear distribution expected during the initial 48 h expressing TRF1. During cell division, all the telomeric signal was concentrated in opposite poles of the dividing nuclei, with no evidence of telomeric DNA trapped in the middle, which might indicate telomere fusion events.

The cell-cycle division arrest seen in trypanosomes is transient. Possible explanations for this are that some trypanosomes are not as seriously affected as others, so a proportion continue dividing, or the majority of cells induce higher expression of endogenous telomeric proteins that compete with TRF1 and overcome its negative effects.

We describe for the first time a protein-mediated telomere shortening effect in trypanosomes. This finding correlates with similar observations in other organisms, where over-expression of telomeric proteins results in telomere shortening [4–7]. The telomere-binding proteins TRF1 and TRF2 appear to hide telomere ends from telomerase by forming t-loops. In particular, TRF1 has activities consistent with that role, such as its capacity to bend and pair duplex telomeric DNA, acting as a force that promotes formation of t-loops [45,47]. Therefore, the ascribed function of TRF1 as a negative regulator of telomerase could by itself explain some of the effects found in trypanosomes. However, the telomere shortening effect found in trypanosomes expressing TRF1 is rather abrupt, not gradual as seen in cells expressing high levels of negative regulators of telomerase in other systems. Reduced accessibility of telomerase normally results in a gradual shortening of telomeres, presumably due to incomplete replication at chromosome ends. Therefore, we favor the hypothesis that high levels of TRF1 destabilize trypanosome telomeres by removal of endogenous proteins.

The difference in the binding of TRF1 to telomeres in PF and BF might reflect differences in the telomeric chromatin of these two life-cycle stages. The only iden-

tified difference between BF and PF telomeres is the conversion of a substantial amount (~ 15%) of T into J in BF telomeres, which does not occur in PF [26]. This results in the presence of bulky glucose residues that would probably restrict the access of telomeric proteins that are highly specific for TTAGGG repeats. In vitro, TRF1 binding requires two units of one and a half repeats and exhibits a remarkable specificity for TTAGGG, tolerating very few changes in that sequence [46]. One J is expected to occur, on average, every two

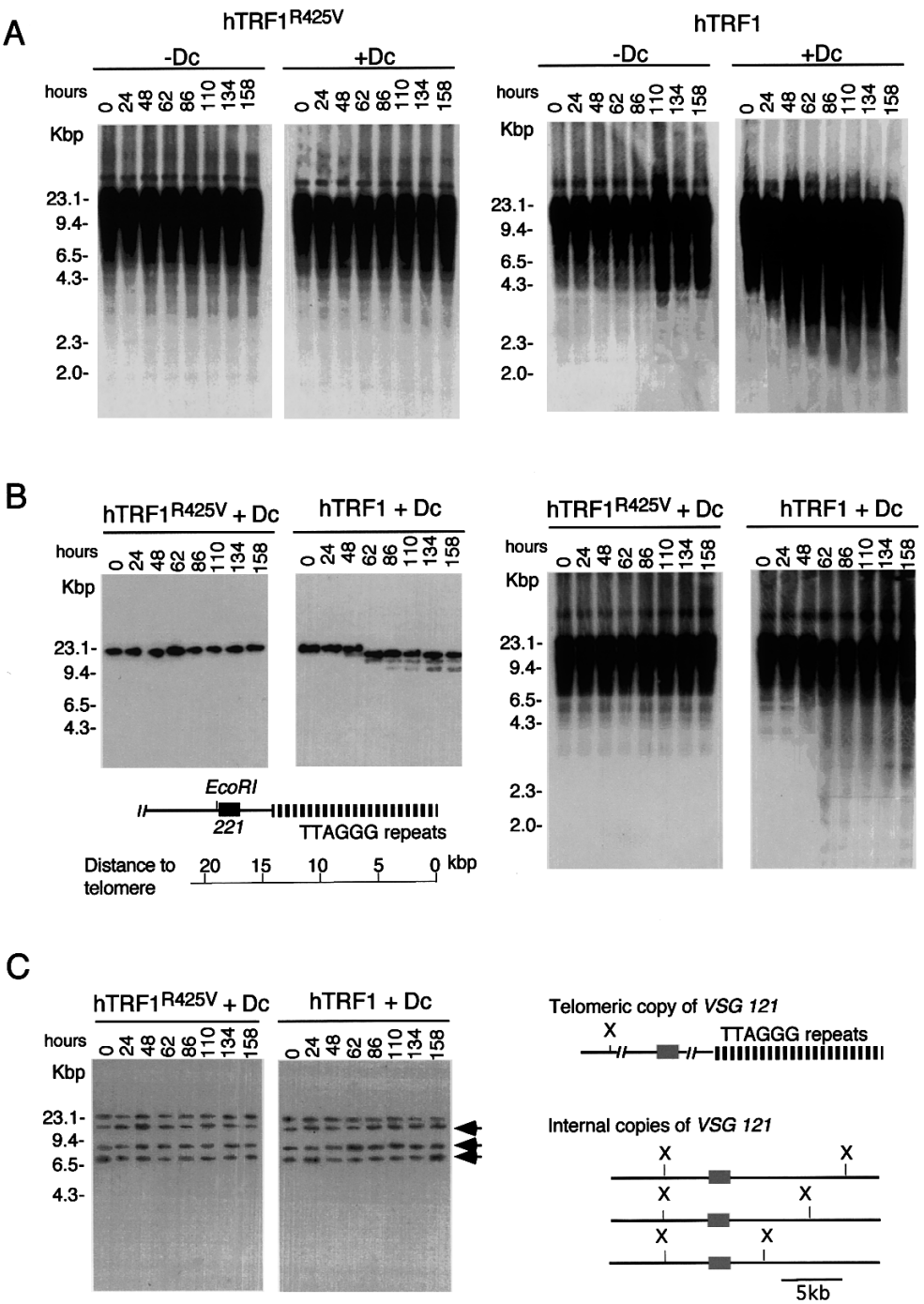


Fig. 5. Telomere shortening in trypanosomes expressing TRF1. (A) Trypanosomes in the presence (+ Dc) or in the absence of (− Dc) carrying either TRF1^{R425V} or TRF1 were collected every 24 h as indicated. Genomic blots of DNA digested with endonucleases *Alu*I, *Hinf*I and *Rsa*I were probed with TTAGGG repeats. Cells not induced with doxycycline, as well as cells expressing TRF1^{R425V}, contained telomeres ranging predominantly between 6 and 23 kb. However, after 24–48 h of TRF1 expression, many telomere restriction fragments become shorter. (B) Because an *Eco* RI site is present next to the *VSG 221* gene, a specific telomere fragment can be visualized in Southern blots. (C) Endonuclease *Xmn*I liberates three internal and one telomeric *VSG* 121 fragments, as indicated.

repeats, which is a level that could account for a substantial reduction in the binding of TRF1. Differential accessibility of TRF1 to BF and PF telomeres could also reflect differences in endogenous telomeric chromatin structure.

Collectively, our results indicate a previously unrecognized function of telomeres in trypanosomes: preventing chromosome ends from triggering DNA-damage responses and cell cycle arrest. Because these effects were triggered by the binding of TRF1 specifically to telomeres, we propose that a protein with similar function is present in trypanosomes. Current work is aimed at identifying components of trypanosome telomeric chromatin.

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