Telomestatin-induced Telomere Uncapping Is Modulated by POT1 through G-overhang Extension in HT1080 Human Tumor Cells*\(^\text{5}\)

Received for publication, June 19, 2006, and in revised form, October 16, 2006 Published, JBC Papers in Press, October 17, 2006, DOI 10.1074/jbc.M605828200

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Telomestatin is a potent G-quadruplex ligand that interacts with the 3’ telomeric overhang, leading to its degradation, and induces a delayed senescence and apoptosis of cancer cells. POT1 and TRF2 were recently identified as specific telomere-binding proteins involved in telomere capping and t-loop maintenance and whose interaction with telomeres is modulated by telomestatin. We show here that the treatment of HT1080 human tumor cells by telomestatin induces a rapid decrease of the telomeric G-overhang and of the double-stranded telomeric repeats. Telomestatin treatment also provokes a strong decrease of POT1 and TRF2 from their telomere sites, suggesting that the ligand triggers the uncapping of the telomere ends. The effect of the ligand is associated with an increase of the γ-H2AX foci, one part of them colocalizing at telomeres, thus indicating the occurrence of a DNA damage response at the telomere, but also the presence of additional DNA targets for telomestatin. Interestingly, the expression of GFP-POT1 in HT1080 cells increases both telomere and G-overhang length. As compared with HT1080 cells, HT1080GFP-POT1 cells presented a resistance to telomestatin treatment characterized by a protection to the telomestatin-induced growth inhibition and the G-overhang shortening. This protection is related to the initial G-overhang length rather than to its degradation rate and is overcome by increased telomestatin concentration. Altogether these results suggest that telomestatin induced a telomere dysfunction in which G-overhang length and POT1 level are important factors but also suggest the presence of additional DNA sites of action for the ligand.

Telomeres play an important role in structural chromosome integrity. They cap and protect their extremities from illegitimate recombination, degradation, and end-to-end fusion (1). Telomere replication is sustained in proliferative somatic cells and in most cancer cells by telomerase, a ribonucleoprotein complex that elongates the chromosome ends to compensate losses occurring at each cell division, because of the inability of polymerase to fully replicate telomeric extremities (2). In somatic cells, the absence of telomerase provokes a progressive shortening of telomeric DNA that ultimately leads to replicative senescence, once a critical telomere length has been reached (3). Numerous observations, notably that inhibition of telomerase activity limits tumor cell growth (4), have led to the proposal that telomere and telomerase are potential targets for cancer chemotherapy (3, 5).

In human, telomeres consist of the repetition of the G-rich duplex sequence 5’-TTAGGG-3’. A G-rich 3’-strand extends beyond the duplex to form a 130–210-base overhang (G-overhang) (6, 7). Telomeres may be structurally organized in different conformations together with several telomere-associated proteins, such as TRF1, TRF2, and POT1 (8). The G-overhang is either accessible for telomerase extension in an open state or inaccessible in a capped (or closed) conformation that involves the formation of a putative t-loop structure (8). Although the t-loop structure has not been defined in detail, it may be created by the invasion of the G-overhang into the duplex part of the telomere (9). The t-loop structure is induced in vitro by the binding of TRF2 in the vicinity of the telomeric G-overhang (10).

Telomeric proteins stabilize the telomere by protecting the single-stranded G-overhang from degradation (8). Uncapping of the telomere ends leads to telomeric dysfunction characterized by end-to-end fusion, inappropriate recombination, anaphase bridges, and G-overhang degradation that either lead to apoptosis or senescence (11–13).

A dominant negative mutant of TRF2, TRF2\(^\text{Abram}\), displaces TRF2 and its interacting factors off the telomeres and causes a loss of telomeric overhangs, apoptosis, senescence, and chromosome abnormalities (8). POT1 (protection of telomere 1) binds specifically to the single-stranded G-overhang (14) and has been described as a regulator of telomere length (15, 16). POT1 has been found associated with the double-stranded telomeric DNA protein TRF1 and TRF2 through the bridging proteins PTOP/ TINT1/PIP1 and TIN2 (17). Suppression of POT1 function by RNA interference in human cells leads to the loss of the telo-
meric single-stranded overhang, induced apoptosis, senescence, and chromosomal instability in human cells (18, 19).

Because of the repetition of guanines, the G-overhang is prone to form a four-stranded G-quadruplex structure that has been shown to inhibit telomerase activity in vitro (20). Small molecules that stabilize the G-quadruplex are effective as telomerase inhibitors (21–24), and several series have been reported to date to induce replicative senescence after long term exposure to tumor cell cultures (25–29). Among them, the natural product telomestatin is one of the most active and selective telomeric G-quadruplex ligands (30–32). We have shown recently that telomestatin impairs the conformation and the length of the telomeric G-overhang, an effect that is thought to be more relevant than double-stranded telomere erosion as a marker for its cellular activity (33). Telomestatin also inhibits POT1 to the telomeric G-overhang in vitro and induces GFP-POT1 dissociation from telomeres in EcR293 cells (34). In tumor cells, telomestatin was shown to completely dissociate TRF2 from telomeres, a result thought to be the consequence of the t-loop disruption (35).

In this study we have investigated the effect of telomestatin treatment in the tumor cell line HT1080. Our results indicate that G-quadruplex stabilization provokes the rapid degradation of both G-overhang and telomere together with the delocalization of GFP-POT1 and TRF2 from telomeres. DNA damage response is induced by telomestatin, which is partially localized at telomeres. The expression of GFP-POT1 in HT1080 induces the lengthening of the G-overhang and partially protects cells from telomestatin-induced G-overhang degradation and senescence induction. These data confirm the importance of POT1 and G-overhang in the action of telomestatin but suggest the presence of additional DNA sites of action.

**EXPERIMENTAL PROCEDURES**

Oligonucleotides and Compounds—All oligonucleotides were synthesized and purified by Eurogentec (Seraing, Belgium). Telomestatin was purified according to Ref. 31. Other compounds were commercially available (Sigma). Telomestatin was prepared at 5 mM in MeOH/Me2SO (50:50). Further dilutions were made in water.

Plasmids—Full-length hPOT1 was cloned into the pET22b expression vector by PCR using the Marathon testis cDNA library (Clontech). The cDNA was completely sequenced and corresponded to the sequence previously released (14). This construct contained an N-terminal T7 sequence allowing its coupled transcription/transcription. The GFP-POT1 plasmid was constructed by insertion of the POT1 cDNA after PCR amplification from pET22bPOT1 vector at BamHI-XbaI of the pEGFP-C1 plasmid (Clontech). The ΔOB-POT1 and TRF2 sequences were cloned by PCR from POT1 and TRF2 cDNAs (a gift from E. Gilson, ENS, Lyon, France), using 5′-CCCTAACCTTACCTAACC-C3′ oligonucleotide (21C) (33). The protein allows detection of the amount of single strand overhang available for hybridization. Experiments were performed either on genomic DNA

3 The abbreviations used are: DMEM, Dulbecco’s modified Eagle’s medium; GFP, green fluorescent protein; PBS, phosphate-buffered saline; ChIP, chromatin immunoprecipitation.
from treated cells or on purified genomic DNA treated in vitro with telomestatin, as indicated in the text.

**TRF Analysis**— Aliquots of 5 µg of undigested genomic DNA were hybridized at 37 °C overnight with 0.5 pmol of [γ-32P]ATP-labeled (5'-CCCTAA)3-CCC3'- oligonucleotide in hybridization buffer (10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 10 mM MgCl2, 1 mM dithiothreitol) in the presence of RsaI and Hinf restriction enzymes in a volume of 20 µl. Reaction was stopped with 2 µl of 1% SDS, 1 mg/ml proteinase K and incubated for 30 min at 50 °C. Hybridized samples were size-fractionated on 0.8% agarose gels in 1× TBE buffer. The gels were stained with ethidium bromide, washed, and dried on Whatman filter paper. Ethidium fluorescence and radioactivity were quantified on a PhosphorImager (Typhoon 9210, Amersham Biosciences). Telomeric smears were revealed by exposure in a phosphorimager (Typhoon 9210, Amersham Biosciences). Telomeric sequences in immunoprecipitates were analyzed on 0.8% agarose gels in 1× TBE buffer. The gels were stained with ethidium bromide, washed, and dried on Whatman filter paper. Ethidium fluorescence and radioactivity were quantified on a PhosphorImager (Typhoon 9210, Amersham Biosciences). The mean length of the TRF corresponds to the peak of the integration curve from three separate experiments.

**Chromatin Immunoprecipitations (ChIP)**—ChIP was performed according to the manufacturer’s procedure (Upstate Biotechnology) using TRF2 antibody (H-300; Santa Cruz Biotechnology). Telomeric sequences in immunoprecipitates were evidenced by PCR amplification according to a method described previously (37). The final telomere primer concentrations were 270 nM (tel1) and 900 nM (tel2), and PCR amplification was subjected to 35 cycles of 95 °C for 15 s, 54 °C for 2 min. The primer sequences were as follows: tel1 5'-GGTTTTGTGAGGTGAGGGTGAGGTGAGGTGAGGTGAGGT-3' and tel2 5'-TCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTATCCCTATCCCTA-3'.

**RESULTS**

**Telomestatin Induces a Decrease of the Telomeric G-overhang in HT1080 Cells**—Recent studies have indicated that the telomeric G-overhang represents one of the direct targets of quadruplex ligands (33, 38). We analyzed the effect of telomestatin on the telomeric G-overhangs from HT1080 cells. As shown before, hybridization of a telomeric C-rich probe (21C) under nondenaturing conditions allowed the measurement of the relative single-stranded G-overhang signal in undigested genomic DNA samples (33).

Treatment of HT1080 cells with 1, 2, and 5 µM telomestatin for 48 h induces a dose-dependent decrease of the G-overhang signal, which represents 32, 15, and 10% of the untreated control, respectively (Fig. 1, A and C).

Previous results with telomestatin in A549 cells (33) indicate that the apparent decrease in G-overhang signal may result from the stabilization of the quadruplex, making it less prone to hybridization to its complementary C-rich probe. To exclude this possibility, we performed the following experiment: telomestatin (1–5 µM) was added to purified DNA just prior to the hybridization reaction. This results in a nearly complete inhibition of the G-overhang signal (Fig. 1, B and C). The inhibition is almost completely reversed in the presence of another G-quadruplex competitor (Pu22myc) that traps the ligands, leaving the overhang free for hybridization with 21C probe (Fig. 1, B and C).

**FIGURE 1.** Effect of telomestatin on the telomeric G-overhang in HT1080 cells and in vitro on purified genomic DNA, evaluated by nondenaturing solution hybridization with 21C telomeric probe (CCC(TAACCC)3). A, telomestatin (1–5 µM) induced a strong decrease of the G-overhang signal in HT1080 cells treated with the ligand for 48 h, as compared with control untreated cells. A competition with Pu22myc (10 µM) barely reverses the hybridization inhibition, indicating a degradation of the G-overhang in HT1080-treated cells. G-overhang, signal of the gel with the CCC(TAACCC)3 probe; Et-Br, ethidium bromide staining of the gel. B, telomestatin (1–5 µM) inhibits in vitro the hybridization reaction to the G-overhang from purified HT1080 DNA. A competition with Pu22myc (10 µM) reverses the hybridization inhibition. G-overhang, signal of the gel with the CCC(TAACCC)3 probe; Et-Br, ethidium bromide staining of the gel. C, quantification of the telomestatin effect against purified HT1080 DNA in HT1080 DNA and in HT1080-treated cells, with or without competition with Pu22myc. G-overhang hybridization signal is normalized relative to the EtBr signal. The results are expressed relative to untreated DNA (defined as 100%) and corresponded to the mean ± S.D. of three independent experiments, including those presented in B and C.

To determine the real degradation of the G-overhang induced by telomestatin in HT1080 cells, in this study we used the competition with Pu22myc on DNA samples from telomestatin-treated HT1080 cells. Results indicated a limited reversion (~15%) of the G-overhang signal decrease (Fig. 1, A and C, compare with reactions in the absence of Pu22myc). Therefore, we concluded that the G-overhang signal loss in HT1080-
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FIGURE 2. Telomestatin alters the GFP-POT1 and TRF2 localization at telomeres in HT1080 cells. Effect of telomestatin (2 \( \mu M \)) on GFP-POT1 in HT1080 cells treated for 48 h. Fluorescence for GFP-POT1 (green), TRF2 (red), and Hoechst (blue) was determined on fixed cells. GFP-POT1 and TRF2 signals colocalize in control untreated cells. Telomestatin treatment induces a strong decrease of the telomeric sites of GFP-POT1 fluorescence and also induces a decrease of the TRF2 fluorescence.

Treated cells mainly corresponds to an effective degradation of the telomeric G-overhang in vivo.

Treatment with Telomestatin Delocalizes Telomeric POT1 in Human Cell Lines—To examine the effects of telomestatin treatment on the binding of POT1 to telomeres in cultured cells, we have designed a GFP-POT1 vector that was transfected in HT1080 cells. As reported previously (15, 34), GFP-POT1 overexpression in telomerase-positive cells results in telomere length elongation (supplemental Fig. S1), suggesting that the N-terminal fusion with GFP does not alter the functional property of the fusion protein to transduce telomere extension.

To localize GFP-POT1 in HT1080 cells, a co-localization experiment has been performed on fixed cells by confocal microscopy using a TRF2 antibody. As shown in Fig. 2, GFP-POT1 colocalizes with almost all the TRF2 dots, suggesting that GFP-POT1 protein is present at telomeres in HT1080 cells. Thus, cells expressing GFP-POT1 fusion protein may be used as a model to investigate the effect of telomestatin on POT1 localization. HT1080 cells expressing GFP-POT1 have been treated for 48 h with 2 \( \mu M \) telomestatin (Fig. 2), a concentration and time exposure with the ligand, at which most of the cells are still viable, because the IC\(_{50}\) values for 2 and 4 days of treatment were equal to 5 and 1.5 \( \mu M \), respectively. Microscopic examination of treated cells indicated a dramatic change in the nuclear organization of GFP-POT1. Telomestatin strongly reduced the GFP-POT1 punctuated signal associated with telomeres to nearly undetectable levels, as compared with untreated controls (Fig. 2).

The dose-dependent effect of telomestatin was also studied in HT1080GFP-POT1 cells after 48 h of treatment (supplemental Fig. S2). At 0.5 \( \mu M \) telomestatin, no obvious decrease or modification of the GFP-POT signal was detectable in >90% of the cells. At 1 \( \mu M \), a decrease of telomeric GFP-POT1 fluorescence was observed in 20–30% of the cells. The main effect of the ligand, i.e. a decrease of the telomeric GFP-POT1 fluorescence, was detectable at 2 \( \mu M \) in about 50–60% of the cells. In addition, a significant fraction of the cells (25–30%) presented a nucleolar localization of GFP-POT1. At 5 \( \mu M \), a telomestatin concentration that significantly impaired the growth of the cells and induced apoptosis (supplemental Fig. S3), nearly all surviving cells presented a complete loss of the GFP-POT1 telomeric signal and a strong nucleolar accumulation, as well as a marked cytoplasmic GFP-POT1 accumulation (supplemental Fig. S2).

Telomestatin Impairs TRF2 Binding at Telomeres and Decreases Telomere Length in HT1080 Cells—To examine whether the delocalization of POT1 is a consequence of a general effect on the telomere structure, we have determined the effect of telomestatin on TRF2 localization. Telomestatin treatment of HT1080 cells (2 \( \mu M \), 48 h) induced a noticeable decrease of the TRF2 signal at telomeres (Fig. 2) that paralleled the effect of telomestatin on GFP-POT1.

The telomestatin effect was also evaluated by ChiP experiments using TRF2 antibodies. In these experiments, the immunoprecipitated telomere sequences were evaluated by specific PCR amplification, as described previously (37). ChiP experiments indicate that telomestatin (2 \( \mu M \), 48 h) provokes the removal of an important fraction of TRF2 from telomeric sequences, in agreement with the immunofluorescence results (supplemental Fig. S4).

The effect of the ligand on TRF2 suggests that either the double-stranded telomeric repeats or the t-loop conformations have been altered. We have thus determined the effect of telomestatin to decrease the length of the double-stranded telomere. Interestingly, the exposure of HT1080 cells to telomestatin (2 \( \mu M \)) induces a rapid telomere shortening detectable after short term treatment (supplemental Fig. S5A). The TRF decrease corresponds to 300 and 600 bases after 4 and 8 days, respectively (supplemental Fig. S5B). These results indicate that telomestatin also induces a dramatic and rapid alteration of the double-stranded telomere repeats and TRF2 binding to telomeres in addition to the G-overhang degradation and the GFP-POT1 delocalization.

Telomestatin Induces an Early DNA Damage Response at Telomeres—Telomere-initiated senescence or dysfunctional telomeres have been shown to be associated with a DNA damage response involving factors such as 53BP1 and γH2AX (17, 39). The rapid effect of telomestatin to trigger telomere degradation together with POT1 and TRF2 removal may suggest the induction of a DNA damage response at telomeres. To study such DNA damage, we used γH2AX immunofluorescence after short term treatment with the ligand. As shown in Fig. 3A, telomestatin treatment induces a marked DNA damage response evidenced by a strong increase in the γH2AX foci. The effect started at 0.5 \( \mu M \) telomestatin and reaches nearly all cells in the presence of 2 \( \mu M \) telomestatin (Fig. 3, A and B). We have also determined the colocalization of γH2AX foci in HT1080GFP-POT1 with telomeric GFP-POT1 under telomestatin treatment. As shown in Fig. 3B, treatment with telomestatin 0.5 \( \mu M \) mainly triggers a DNA damage response outside from the telomeric foci. Only a fraction of the...
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FIGURE 3. DNA damage response at telomere after telomestatin treatment in HT1080 cells. A, telomestatin induces a DNA damage response in HT1080 cells. Immunofluorescence for γH2AX (red) and Hoechst fluorescence (blue) in untreated (control) or cells treated for 48 h with 2 μM telomestatin (+ Telo). B, merge fluorescence for γH2AX (red), GFP-POT1 (green), and Hoechst (blue) in untreated (control) or cells treated for 48 h with 0.5 μM telomestatin (+ Telo). Telomestatin induced a partial colocalization of γH2AX and GFP-POT1 foci, as compared with untreated cells. Co-localized sites of DNA damage at telomere are indicated by arrowheads. C, number of γH2AX-GFPPOT1 colocalizing foci in HT1080 cells treated by telomestatin (0.5–2 μM). Telomestatin significantly induced a 2.5-fold increase of colocalizing foci, as compared with untreated control cells by the statistical Wilcoxon test. Number of counted nuclei is 13, 23, 21, and 27 for 0, 0.5, 1, and 2 μM telomestatin, respectively. D, immunofluorescence for γH2AX (red) and Hoechst fluorescence (blue) of an HT1080 cell in metaphase after 48 h of treatment with telomestatin (2 μM). Enlarged image on the right shows the sites of DNA damage at the extremity of a mitotic chromosome.

total γH2AX foci colocalizes with GFP-POT1 in treated cells (indicated by arrowheads in Fig. 3B). The γH2AX and GFP-POT1 colocalization is significantly increased by 2.5-fold (p < 0.01), as compared with controls, in cells treated with 0.5, 1, or 2 μM telomestatin, where the GFP-POT1 telomeric signal is still detectable (Fig. 3C). However, in cells treated with 5 μM telomestatin, the analysis was not possible, because of the complete delocalization of the GFP-POT1 protein (see supplemental Fig. S2). These results have been confirmed by Telo-fluorescence in situ hybridization experiments using a telomeric fluorescein isothiocyanate-peptidic nucleic acid probe to determine the localization of γH2AX foci in telomestatin-treated HT1080 cells (supplemental Fig. S6). Analysis of HT1080 cells treated with telomestatin also showed that in some metaphases the γH2AX response is observed at the extremities of chromosomes, in agreement with a response at telomeres (Fig. 3D).

These results suggest that telomestatin is able to induce an early and massive DNA damage response. Interestingly, only a fraction of this response was observed at telomeres, suggesting other sites of action for the ligand throughout the genome.

Overexpression of GFP-POT1 Increases G-overhang and Partially Protects Cells from Telomestatin Effects—POT1 was shown to be essential for telomere capping and allowed us to regulate potential G-quadruplex structures formed at the telomeric G-overhang in vitro (19, 40). Overexpression of POT1 may protect or modulate the telomere dysfunction induced by G-quadruplex ligands. We have therefore examined whether the overexpression of GFP-POT1 modulates the cellular effects of telomestatin. Treatment of HT1080GFP cells with telomestatin (0.5 μM) induced a delayed cell growth arrest after four population doublings, followed by cell death at day 8 (Fig. 4A). Interestingly, HT1080GFP-POT1 cells presented a noticeable resistance to the effect of telomestatin because the growth arrest is not observed after 16 days corresponding to six additional population doublings (Fig. 4A). In contrast, treatment of HT1080GFP and HT1080GFP-POT1 cells with higher telomestatin concentrations (2, 5, and 10 μM) did not induce noticeable differences in the cytotoxic response after 4 days (result not shown). These results suggest that GFP-POT1 expression partially protects HT1080 cells from the antiproliferative effects of the ligand. As a control, doxorubicin treatment of the cell lines does not indicate significant resistance in HT1080GFP-POT1 cells, as compared with HT1080GFP (supplemental Fig. S7). In contrast, HT1080GFP-POT1 cells displayed resistance to the effect of another G-quadruplex ligand, steroid FG,6 indicating selectivity to this class of agents as compared with other DNA-damaging agents (supplemental Fig. S8).

To determine whether this partial protection corresponds to a difference in the effect of telomestatin at telomeres, we then analyzed the G-overhang degradation in these two cell lines. We first noticed that the G-overhang signal measured in HT1080GFP-POT1 was found increased by 270%, as compared with HT1080GFP cells, indicating that the overexpression of POT1 has a positive regulatory effect on the G-overhang length (Fig. 4, A and C). Treatment of HT1080GFP-POT1 and HT1080GFP cells with telomestatin for 48 h induces a dose-dependent 2-fold reduction of the G-overhang signal (Fig. 4C). Interestingly, the remaining G-overhang signal in HT1080GFP-POT1-treated cells is 2.1–2.4-fold higher than in treated HT1080GFP cells. Therefore, although the degradation rate induced by telomestatin is nearly equivalent in these two cell lines, the residual value of the G-overhang signal after telomestatin

6 The characterization of this new G-quadruplex ligand will be described elsewhere.
treatment is higher in GFP-POT1-transfected cells, because of its higher initial value. These data suggest a direct relationship between the G-overhang length and the biological activity of G-quadruplex ligands rather than protection induced by GFP-POT1 to explain the partial resistance to telomestatin.

**DISCUSSION**

Our results show that telomestatin induces a rapid degradation of the telomeric G-overhang and the double-stranded telomeric repeats. As a consequence, the telomeric localization of POT1 and TRF2 in HT1080 cells is also dramatically modified by telomestatin treatment. The alteration of the telomeric G-overhang conformation leading to its degradation has been reported recently in different cell lines for several G-quadruplex ligands, including telomestatin (33, 35, 38, 41). Such rapid degradation of the telomeric ends contrasts with the initial concept of action of these ligands, designed to inhibit telomerase activity and to induce a progressive shortening of the double-stranded telomere repeats (42). The growing number of reports of their particular behavior rather suggests that they represent a new class of telomere targeting agents that provokes a telomere dysfunc-
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FIGURE 5. Expression of ΔOB-POT1 but not TRF2 in EcR293 cells induced a protection to senescence induction and G-overhang degradation by telomestatin. A, proliferation curves of EcR293 cells transfected with GFP (control, open circle), TRF2 (open triangle), or DOB-POT1 (open square) in the absence or presence of telomestatin (0.5 μM) (open and closed symbols, respectively). A growth arrest of the culture of EcR293-GFP and EcR293-TRF2 cells appears between days 14 and 17, whereas EcR293-DOB-POT1 cells continue growth up to 32 days, indicating a protection to the antiproliferative effects of telomestatin. B, G-overhang signal of EcR293 cells transfected with GFP, TRF2, or DOB-POT1 in the absence or treated with telomestatin (1 μM) for 48 h. G-overhang signal is normalized relative to the EtBr signal for each loading. Results are expressed relative to untreated DNA samples from HT1080-GFP cells (defined as 100%) and correspond to mean ± S.D. of three independent experiments.

They mimic the effect of the inactivation of key components of the shelterin complex, including POT1 and TRF2. Interestingly, the extent of the telomere dysfunction varies between cell lines. Telomestatin was recently shown to completely dissociate TRF2 from telomere in cancer cells but not in normal or immortalized cells (35). We also showed that telomestatin provokes the delocalization of GFP-POT1, but not of TRF2, from telomeres in an immortalized cell line (34). The effect of telomestatin in this cell line was related to the G-overhang degradation and to the property of the ligand to inhibit POT1 binding to the telomeric G-overhang. Indeed, the G-overhang degradation induced by telomestatin in HT1080 cells is sufficient to explain GFP-POT1 delocalization. However, because TRF2 is a double-stranded telomere-binding protein, its removal from telomeres observed in HT1080 cells not only results in the disruption of the t-loop, where the G-overhang is engaged with a fraction of TRF2, but also in the elimination of double-stranded telomeric sequences, in agreement with the telomere shortening observed with the TRF experiments. Several additional mechanisms other than the G-overhang erosion or the t-loop destruction are possibly involved in order to explain this dramatic telomere and TRF2 loss.

Double-stranded telomere shortening may result from a stalling of the lagging-strand replication fork by the stabilization of G-quadruplexes. In that case, a failure to solve the stalled replication forks will induce DNA double strand breaks and the loss of telomeric sequences. The inactivation of WRN helicase, a RecQ DNA helicase, in WS cells was reported to induce a preferential loss of the lagging strand of telomeres (43). Because WRN activity on telomeric sequence was also found sensitive to the effect of G-quadruplex ligands, the inhibition of this helicase may induce the telomere loss during replication (44).

We have also found that telomestatin provokes a DNA damage response in HT1080-treated cells. The DNA damage response is induced at telomestatin concentrations where an important fraction (40–50%) of the telomeric G-overhang is degraded. A fraction of the γH2AX foci is localized at telomere in HT1080-GFP-POT1 cells, indicating that a telomeric dysfunction is induced by the ligand. However, because telomestatin provokes the delocalization of GFP-POT1 and TRF2 and because an important fraction of telomeres remains with a significant GFP-POT1 signal, it is difficult to establish a direct relationship between telomere degradation and DNA damage. Our results indicate that an important fraction of the γH2AX response is not colocalized at telomeres. It is possible that one part of these DNA damage foci corresponds to GFP-POT1 uncapped telomeres. On the other hand, recent analysis of the genome composition has identified many G-quadruplex-forming sequences outside telomeres (45, 46). Because telomestatin does not discriminate between telomeric and other species of G-quadruplex, such as c-myc (47), it is possible that these foci correspond to DNA damage during replication of these G-quadruplex-forming loci. In agreement, preliminary experiments indicate that PCR amplification of the rDNA locus is highly sensitive to the effect of telomestatin.

Because telomestatin causes cellular effects analogous to those due to dysfunctional telomeric proteins and because POT1 regulates in vitro the G-quadruplex conformation at telomeric sequences (40), we have speculated that telomestatin effects may be modulated by GFP-POT1 expression in HT1080 cells. Our results indicate that this is partially true at a low telomestatin concentration (0.5 μM), where the growth arrest is delayed after several population doublings. Interestingly, this partial resistance is because of an increased initial G-overhang length in HT1080-GFP-POT1 cells rather than to a difference in the rate of the G-overhang degradation induced by telomestatin.

The use of a dominant negative POT1 lacking the two OB-fold domains that interact with the G-overhang also provokes a

7 P. Della-Gaspera and C. Trentesaux, unpublished results.
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G-overhang signal increase in EcR293 cells, as well as telomere lengthening (Ref. 16 and results not shown). Interestingly, a resistance phenotype to telomestatin is also observed in this transfected cell line, confirming that G-overhang length rather that a direct effect of POT1 is responsible for the long term resistance to the ligand.

The up-regulation of the G-overhang was also observed in a cellular clone (JFD9) selected for resistance to 12429, a G-quadruplex ligand from the triazine series (38, 48). In this cell line, the G-overhang signal corresponds to 150% of that measured in parental A549 cells, thus emphasizing the relationship between the G-overhang length and the biological activity of the ligand. The overexpression of POT1 or ΔOBPOT1 also increases the double-stranded telomere length of the transfected cells. It is possible that the increased TRF may contribute to the resistance to the senescence induced by the ligand, as already observed in the JFA2 cell line selected for resistance to 12459 that presents cross-resistance to telomestatin (36).

Our data also indicate that the G-overhang length is not the unique factor that controls the cytotoxic effect of telomestatin, because increased concentrations of the ligand are able to overcome the resistance at short term and because DNA damage foci are also observed outside of the telomeres.

In conclusion, our results show that the G-quadruplex ligand telomestatin induces a telomere dysfunction associated with telomere erosion that dissociates POT1 and TRF2. However, increased concentrations of the ligand are able to overcompete the resistance at short term and because DNA damage foci are also observed outside of the telomeres.

Acknowledgments—We thank Dr. A. Londono-Vallejo for helpful discussions; Dr. J. M. Millot for help in statistical analysis; P. Koebel for technical help in cell sorting; H. Kaplan for video microscopy facilities; and J. Macadre for technical help in cell culture.

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