Cell Cycle Regulation of G-Quadruplex DNA Structures at Telomeres

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Abstract: DNA and RNA regions containing tracts of guanines can form very stable secondary structures called G-quadruplex (G4). Genomic sequences with the potential to form G4 (G4-motifs) are abundant across species. In all analyzed genomes G4 motifs are found near promoter regions and double strand break sites and at telomeres. Telomeres are very G-rich and prone for G4 formation. Therefore they are routinely used in *in vitro* and *in vivo* experiments to elucidate the function of G4 structures in telomere metabolism. Recently various labs demonstrated that telomere length maintenance is mediated *via* G4 structures. Telomere-binding proteins specifically bind to G4 structure and regulate this structure throughout the cell cycle.

Keywords: Ciliate, helicase, higher-order DNA structure, telomere-binding protein.

INTRODUCTION

Nucleic acids can adopt a variety of different structures. The observation that guanine residues can establish hydrogen bonds to each other was observed more than 50 years ago [1]. Biochemical experiments demonstrated that repeated runs of guanines can form a secondary structure, named G-quadruplex (G4). The core elements of this structure are G-quartets, a square planar arrangement of four guanines bound together by hydrogen bonds. The stacking of these G-quartets results in a higher ordered structure, the G4. G4 structures are stabilized by monovalent or divalent cations such as Na^+, K^+, Ca^{2+}, or Mg^{2+}. G4 structures can form from DNA and RNA molecules (reviewed in [2, 3]) and are very polymorphic. They can be inter- or intramolecular, form parallel or anti-parallel, and contain one to four different nucleic acid strands. Most sequences, which are able to adopt G4 structures, have a conserved sequence motif. In most cases they consist of four G-tracts, in which three or more guanines are next to each other, and a loop region of various nucleotide content and length (1-7 nt loop lengths are possible). Shorter runs of G-tracts (2 nt) and longer loops (up to 25 nt) decrease the stability significantly. G4s are stable structures; numerous factors contribute to their stability: the numbers of guanines in the G-tracts, the loop length and the loop composition as well as the surrounding monovalent cations. It is documented that more stable G4 arise in the presence of K^+ vs. Na^+ or Li^+[4]. Consequently spontaneous formation or unfolding of G4 would be very slow or not even possible under physiological conditions [5] so that proteins, which promote their formation or unwinding, are required to resolve G4 structures.

In recent years knowledge of G4 structure has increased exponentially. Various reports provide strong evidence that G4 structures are present in RNA and genomic DNA. G4 DNA structures are an obstacle for DNA replication, telomere elongation, and transcription, and are currently discussed as a novel class of anti-proliferation drug for cancer treatment [6]. G4 RNA structures, which are more stable than G4 DNA structures [7], are implied to play a diverse role in numerous processes, for example: structural function [8], intronic splicing [9, 10], protein binding [11-13], and post-transcriptional regulation of mRNAs and their targeting [14-17].

In computational screens candidate sequence motifs with the potential to form G4 structure were identified in every studied genome. G4 motifs occur at distinct regions in the genome and their composition and location is conserved through evolution [18, 19], indicating that these structures have regulatory roles during multiple biological processes (reviewed in [20]). They are found at telomeres and transcriptional control regions (e.g. promoters) and have been implicated in regulating transcription, translation, and replication (reviewed in [2]). This observation lead to the predication that G4 structures are a mixed blessing for the cell; they constitute a regulatory element, which is needed for certain events like telomere protection or transcription blockage but, on the other hand, presents a challenge for the cell because G4 stall and block replication and possibly result in DNA breakage and mutation. Here, we review recent studies aimed at uncovering the regulation of G4 at telomeres during the cell cycle. In particular we focus on the results from studies in ciliates, which provided the first direct evidence for the occurrence and regulation of G4 structures at telomeres *in vivo*.

G4 STRUCTURE REGULATION AT TELOMERES

Telomeres are protein-DNA structures at the termini of linear chromosomes. They prevent chromosomal ends from being recognized as sites of DNA damage and being targeted by the cellular repair machinery. Telomeres are essential for genome integrity, because they protect the chromosome ends from homologous recombination, non-homologous end joining, and nucleolytic degradation [21, 22]. Telomeric DNA consists of tandem arrays of short repeated sequences in which the strand running 5'-3' from the centromere towards the chromosome end is usually guanine-rich. Telomeres consist of a double-stranded region, which varies in length between species and organisms and a single-stranded 3' overhang [23, 24]. Telomeric DNA is maintained by an unusual replication mechanism that involves a specialized enzyme, the telomerase [25]. For protection and function of telomeres they are bound by a core group of proteins that form the shelterin complex. This complex is composed of three types of proteins: single-stranded telomeric DNA-binding proteins (for example Pot1 in mammals), double-stranded telomeric DNA binding protein (for example TRF proteins in mammals) and proteins that bind indirectly to the telomeres *via* protein-protein interaction [24, 26, 27]. Because of the repetitive, guanine-rich nature of telomeres (many GGG tracts), telomeres have a high probability to form G4 structures *in vivo*. It is well documented that telomeres form G4 structures under appropriate *in vitro* conditions [28-30]. *In vitro*, the G-rich single-stranded telomeric overhang can form intra- and intermolecular G4 structures. *In vitro* data showed that telomerase activity is influenced by G4 structures: intramolecular antiparallel telomeric G4 structures inhibit telomerase-catalyzed primer extension *in vitro*, whereas intermolecular parallel G4 can be extended by telomerase [31, 32]. In recent years this knowledge has been used for drug development

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[33, 34]. The current working model is to design specific ligands that bind and stabilize telomeric G4s, which results in an inhibition of telomerase, hence preventing the immortalization of cancerous cells.

In addition to G4s at telomeric DNA the telomerase RNA itself also exhibits a G4 motif, which is implied to regulate telomerase activity [35] and TERRA (telomeric repeat–containing RNA [36-38]). Secondary structures at telomeres such as G4 structures or T-loops [39, 40] contribute to telomere function, but have to be tightly controlled as they also present a difficulty for telomere maintenance. In this review we will focus on telomeric G4 structure and will not go into detail of T-loop formation and regulation.

G4 STRUCTURE REGULATION AT TELOMERES IN CILIATES

The *in vivo* observation of telomeric G4 structures has been initially reported in ciliates [41, 42]. Unicellular ciliated protozoa, e.g. *Stylonychia, Oxytricha, Tetrahymena*, or *Paramecium*, have a unique nuclear morphology (reviewed in [43]). Each cell has two distinct and specialized nuclei, the macronucleus and the micronucleus. Macronuclear DNA consists of a large number of short nanochromosomes (up to 10^8 per macronucleus), which each are capped by telomeres of constant length (telomeric repeats are T_4G_4). In the ciliates *Stylonychia* and *Oxytricha* each telomere consists of a 16 nucleotide long single-stranded 3'-dG_4T_4 overhang followed by 20 basepairs of double-stranded 3'-dG_4T_4 [44]. The single-stranded overhang is bound by two OB-fold containing telomere end-binding proteins TEBPα and TEBPβ [45, 46]. These two proteins are conserved from ciliates to vertebrates; POT1 is the homologue of TEBPα [47-49] and TTP1 shares homology to TEBPβ [50, 51]. Experiments utilizing specific antibodies directed against telomeric G4 structures [52] in combination with *in vitro* binding assays provided the most direct evidence that G4 structures form at telomeres *in vivo* and how these structures form and become regulated in the cell cycle [41, 42].

TEBPα binds to the telomeric overhang whereas TEBPβ binds indirectly to the telomeres *via* TEBPα [53, 54]. Very specific antibodies directed against either parallel or antiparallel G4 structure (affinity <5 nM) made it possible to analyze telomeric G4 in more detail [55]. Only antiparallel G4 structures were visualized by standard *in situ* immunohistochemistry. This data supported earlier *in vivo* and *in vitro* observations where two telomeres form intermolecular, antiparallel G4 structure and that extracted ciliate nanochromosomes were linked *via* their ends and formed long DNA aggregates [29, 56, 57]. In stichotrichous ciliates (*Oxytricha*, *Stylonychia*) replication takes place in a distinct morphological region, the replication band. No staining with the anti-G4 antibody was observed in the replication band indicating that G4 structures were resolved during telomere replication and telomerase action [55]. Using RNAi to silence the expression of the two TEBPs revealed that both proteins are required for G4 formation and that TEBPα binds the telomeric overhang and recruits TEBPβ to promote G4 formation. Deletion studies showed that TEBPβ's highly charged C-terminus is required for TEBPβ to promote antiparallel G4 structure formation between two adjacent telomeres [42]. From a combination of *in vitro* and *in vivo* data could be concluded that TEBPβ acts as a chaperone for G4 formation [42, 58, 59]. The mechanism of G4 structure unfolding is less well understood, but several key elements that allow G4 unfolding in a timely fashion have been identified and are depicted in Fig. (**1**).

First it was shown that the C-terminus of TEBPβ, which is required for promoting G4 structures, has two phosphorylation sites [60] that are CDK-dependent. During S phase TEBPβ is phosphorylated at both sites, which leads to its detachment from the telomeric complex (TEBPα). Upon loss of telomeric TEBPβ, telomeric G4 structures can no longer be maintained and are ultimately lost [41, 42]. Although phosphorylation of TEBPβ is a necessary prerequisite for G4 unfolding during S phase, it appears insufficient for telomere replication to proceed in a timely manner. Therefore the current model is that TEBPβ phosphorylation prevents refolding of G4 structures and additional factors are needed for efficient unfolding. Second, telomerase enzyme was identified to play a crucial role in G4 unfolding during S phase [41] as earlier suggested by T. Cech [32]. Telomerase can recognize G4 structures and telomerase activity is stimulated in the presence of G4 DNA [31, 61]. RNAi knockdown experiments targeting either the catalytic subunit of telomerase (TERT) or telomerase RNA (TR) revealed that, in the absence of active telomerase, telomeric G4 structure is no longer unwound in S phase. Subsequent experiments revealed that phosphorylation of TEBPβ is mandatory to recruit telomerase to the telomeric complex and that telomerase is actively involved in displacing TEBPβ from the heterodimer [41]. Interestingly, the human homolog of TEBPβ, TPP1, was also shown to recruit telomerase, demonstrating the high conservation in structure and function of telomere-associated proteins [62, 63]. A third component involved in G4 regulation is a telomerase-associated helicase. Several observations suggest that a RecQ-like helicase is recruited to telomeres by telomerase during S phase and is required to unfold telomeric G4 structure during replication (H. J. Lipps unpublished). Another factor, which might be involved in G4 unfolding, is the state of subnuclear tethering of the telomeric complex to the macronuclear scaffold. The telomeric complex is attached to the subnuclear structure during the cell cycle with the exception of S phase [42]. TEBPα attaches telomeres *via* three subnuclear structure proteins called SNS I-III. At the same time (S phase) as TEBPβ phosphorylation, the three SNS proteins become phosphorylated and telomeres are no longer tethered to the subnuclear structure [58]. This detachment process might be required to restructure telomere into a linear conformation Fig. (**1**).

Studies in ciliates showed that G4 structures form at telomeres *in vivo* and that G4 structures are regulated during the cell cycle. These results support the model that G4 structures form at telomeres as part of the end protection mechanism. In addition they also contribute to telomere metabolism (attachment and detachment of telomeres to subnuclear component and recruitment of telomerase). Ciliates, unlike other organism, have a constant length of telomeric DNA- G4 structures might perform a function for this discrete length regulation. This might also be connected to the observation that inter- and intramolecular G4 structures regulate telomerase in different ways. A hypothesis is that shorter telomeres, which will be elongated by telomerase, form intermolecular G4 structures and long telomeres would form intramolecular G4s (block telomerase). It could be that these two different classes of telomeric G4 may also are regulated by different sets of proteins.

G4 STRUCTURE REGULATION AT TELOMERES IN YEAST

G4 structure occurrence and regulation is so far best understood in ciliates but numerous experiments show strong evidences that G4 structures also exist at the telomeres of higher eukaryotes and also play a crucial a role in telomere maintenance.

Several experiments show strong evidence that G4 structures play a role during telomere metabolism also in yeast (reviewed in [64]). Recently Est1, a subunit of the telomerase complex in yeast, was shown to bind to G4 structures and by this stimulates telomerase activity *in vitro* [65]. Additional experiments on Est1 and G4 regulation showed that Est1 can promote G4 formation and contributes to telomere end capping [66]. Cdc13, a single-stranded telomeric DNA-binding protein, was also linked to G4 structure regulation. Cdc13 is required for telomere end capping and the length of the telomeric overhangs increases in its absence. In the absence of natural capping mechanisms at the telomeres, G4 structures formation has a positive impact on telomere maintenance [67]. Sgs1 helicase, a RecQ homolog in yeast, plays an important role during ho-

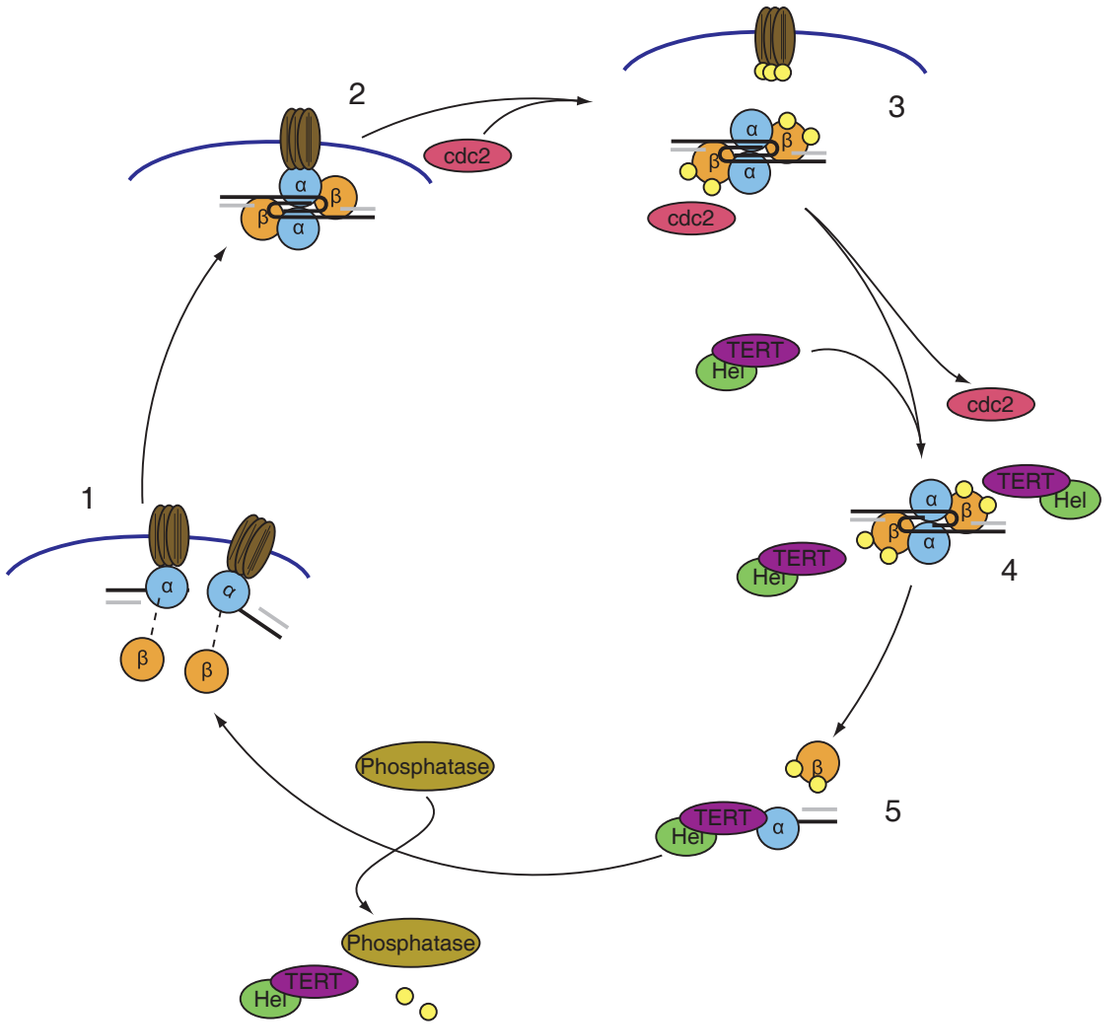

Fig. (1). A model of telomeric G4 DNA structure regulation during the cell cycle in *S. lemnae*. (1) The TEBPα (α) binds to the telomeric overhang and tethers the telomeres via three SNS proteins (I, II, III) to a subnuclear compartment. TEBPα recruits TEBPβ (β) to the telomeric overhang and they form a heterodimer. (2) TEBPβ promotes the formation of telomeric G4 structure. (3) With the beginning of S phase the three SNS proteins and TEBPβ are phosphorylated (grey circles) by a cdc2 kinase (cdc2). Consequently the telomeric complex is no longer attached to the subnuclear structure. (4) The phosphorylation of TEBPβ leads to telomerase (TERT) recruitment with its associated helicase (Hel). Telomerase and the associated RecQ like helicase act together displacing TEBPβ from the heterodimer by unwinding G4 structures. One TEBPα stays bound at the replicating telomeres and traps the telomeric overhang in its linear form. (5) Following replication TEBPβ and the three SNS proteins are dephosphorylated probably by a phosphatase (Phosphatase) and TEBPα is again attached to the subnuclear structure components and recruits TEBPβ to the telomere/TEBPα complex.

mologous recombination. Sgs1, like human RecQ helicases can unwind G4 structures *in vitro* [68, 69]. A possible role for Sgs1 at telomeres might be to help promoting the formation of the telomeric G-tails by unwinding G4 structures [69]. Also the helicase Pif1 function, which binds to telomeres in late S phase and negatively regulates telomerase activity, has recently been connected to G4 structure unwinding and stability [69, 70]. So far, it is difficult to image how Pif1's ability to unwind G4 structures and regulation of telomerase could co-function, but future work will elucidate this question.

G4 STRUCTURE REGULATION AT TELOMERES IN HUMANS

Human telomeric DNA sequence (d(GGGTTA)$_n$) is also capable to form spontaneously into intermolecular G4 structures [30, 70]. Similar to all other tested telomeric G4 structures human telomeric G4 structures are stable under physiological conditions, with a thermal melting temperature of ~65°C. Other telomeric sequences studied to date can also form G4 structures with comparable thermal stability. At vertebrate telomeres, G4 structures cannot only form at the single-stranded 3' overhang but also within the double-stranded telomeric region during transcription or replication.

Several studies using natural or synthetic G4 structure-specific ligands, which bind to G4 DNA and RNA structures tightly (at best 1-5 nM) and stabilize them (reviewed in [71,72]), support the presence of G4 structures at vertebrate telomeres. The advantage of utilizing these ligands is that they stabilize or even promote the formation of G4 structures and by this enhance the inhibitory effect of G4 structures on telomerase [32]. These ligands are used in clinical trials for cancer treatment, where by stabilizing G4 structures they enhance the inhibition of telomerase action, which is active in 85% of all human cancers. Indeed, long-term applications of such

ligands to telomerase-positive cell lines led to telomere-shortening and apoptosis [73], but there are still many open question about their specificity *in vivo* and their targets. So far there are several different G4 stabilization ligands that claim to act specific on telomeric DNA and cause telomere dysfunction by altering the integrity of the shelterin complex (reviewed in [74]).

Furthermore, telomeric G4 structures in human can be unfolded by many telomere-binding proteins [3, 75] or destabilized by bordering proteins [76]. For example human POT1, which binds single-stranded telomeric overhangs, inhibits G4 structure formation *in vitro* [77]. Detailed work on telomere-associated helicases, like Werner, Bloom, RTEL, or Pif1 helicases, which can unwind G4 structures *in vitro* [78-81], support the model that G4 form at telomeres. In fact the loss of these helicases results in telomere defects that might be related to G4 structures (reviewed in [22]).

Due to the repetitive G-rich nature of telomeric DNA, the telomeric overhangs can form G4 at any position and leave an uncomplexed 3'distal tail ranging in length from 0-23 nt. Although telomerase activity is inhibited by G4 structures *in vitro* [32, 82], a 8-12 nt tail behind the G4 was shown to help telomerase processivity [82]. A recent study supports the presence of G4 at human telomeres by showing the localization of highly fluorescent compounds that are specific for G4 structures [83]. Deeper analysis of these compounds will help to shed light on G4 occurrence and regulation during the cell cycle.

CONCLUSION

Accumulating results from biophysical, bioinformatic, genetic, and cell biological approaches have yielded a remarkable series of findings that argues for the presence of G4 structures at telomeres. However, additional work is required to firmly establish the role of G4 structures for telomere maintenance. From published data there are some hypotheses what role G4 structures perform at telomeres. G4 could contribute to telomere end protection, by preventing end-to-end fusion and degradation of telomeres. A second possibility could be that G4 formation results in the recruitment of proteins that are important for telomere metabolism (e.g. length regulation, protection, replication). Thirdly, it is possible that G4 structures play an important step in telomere length regulation. Meaning that shorter telomeres, which have a different protein composition, will also have a different G4 conformation that results in activation of events like telomerase activation, DNA damage activation, or increased protection of the telomere. Fourthly, G4 formation at the single-stranded telomeric overhangs may itself be a DNA damage signal that triggers responses analogous to those of other mediators of telomere damage [21, 84]. There are also indications of functional interactions between G4 and poly(ADP-ribose) polymerase-1 [85]. Lastly, in ciliates, and maybe in yeasts, G4 structures are involved in telomerase recruitment [13, 41, 65]. The biological function of telomeric quadruplexes remains to be fully clarified and future experiments will shed light on G4 function and distribution at telomeres.

Future research will elucidate several different questions in the field of G4 structure research: Do G4 structures form during semi-conservative telomere replication? Which proteins are involved in G4 formation and unwinding during the cell cycle in eukaryotes other than ciliates? The most ambitious goal is to show direct evidence of G4 structures at human telomeres. Considerable effort is currently being undertaken to raise antibodies against human and yeast telomeric G4 structures. These experiments will enable the G4 research to go to a next level, although it is questionable whether the low concentration of telomeres in yeast and human will allow straightforward *in situ* analyses using current microscopic techniques. Another interesting question is whether G4 structures regulate telomerase and how telomerase function is affected by G4 ligands. In the last years a lot of effort was given to the improvement and development of G4 specific ligands. In the next couple of years *in vivo* experiments using these ligands will enhance not only the knowledge about G4 structures but also will help to improve anti proliferation drugs for cancer treatment.

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