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Characterization of the intramolecular G-quadruplex promoting activity of Est1

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ABSTRACT

In the budding yeast *Saccharomyces cerevisiae*, telomeric DNA includes $TG_{1-3}/C_{1-3}A$ double-stranded DNA and a protruding G-rich overhang. Our previous studies revealed that the telomerase regulatory subunit Est1 promotes telomeric single-stranded DNA to form intermolecular G-quadruplex in vitro, and this activity is required for telomere replication and protection in vivo. In this study, we further characterized the G-quadruplex promoting activity of Est1. Here we report that Est1 is able to promote the single-stranded oligonucleotide of $(TGTGTGGG)_4$, which mimics the natural telomeric DNA, to form intramolecular G-quadruplex. Therefore, it remains possible that the intramolecular G-quadruplex promoting activity of Est1 is biologically relevant in telomere replication in vivo.

1. Introduction

Telomeres are the protein–DNA complexes at the ends of linear chromosomes in eukaryotic cells, and they play critical roles in maintenance of genome integrity and chromosome stability [1–2]. The telomeric DNA is composed of guanine (G)-rich double-stranded DNA as well as a protruding single-stranded overhang [1,3]. The G-rich single-stranded DNA has the ability to form a higher order DNA structure, known as G-quadruplex structure in vitro and in vivo [4–5]. The G-quadruplex structure is composed of several layers of G-quartets. Each G-quartet contains four guanines arranged in a square-planar unit by hydrogen-bonding and each guanine serves as both the donor and acceptor in a Hoogsten base pair. The G-quadruplex has a parallel or antiparallel configuration formed within one (intramolecular) or several (intermolecular) single-stranded DNA molecules [6–7]. G-quadruplex structure can be regulated by *trans* and *cis* elements [8–9]. The only evidence to support the existence of in vivo G-quadruplex came from the studies in ciliate, in which Paeschke et al. developed an antibody that specifically recognizes the antiparallel G-quadruplex in vivo, and they found that telomere binding factors TEBPα and TEBPβ are both required for in vivo G-quadruplex formation [10–11].

In the budding yeast *Saccharomyces cerevisiae*, each telomere contains ~350 bp of irregular $TG_{1-3}/C_{1-3}A$ repeats [1]. Telomeric DNA is elongated by the telomerase or homologous recombination pathway when telomerase is absent [12–14]. Telomerase consists of the catalytic subunit Est2, RNA template TLC1, as well as the regulatory subunits Est1 and Est3 [15–19]. Est2 and TLC1 form the telomerase core enzyme, which can exert the nucleotide addition activity in vitro [20]. Est1 and Est3 are also required for the telomerase activity in vivo, and deletion of any of the four components causes telomere shortening and cellular senescence [18]. Est1 is expressed and binds to telomeres in S phase [21–23], and recruits telomerase through interactions with TLC1 and single-stranded DNA binding protein Cdc13 [24–27]. Est1 is able to promote telomeric single-stranded DNA to form tetra-molecular parallel G-quadruplex structure in vitro. This activity has been shown to be required for telomere replication and protection in vivo [28–29]. Mutations in the conserved sites of the EF-hand motif in Est1 impair the G-quadruplex promoting activity resulting in telomere shortening and cellular senescence [28–29].

The tetra-molecular G-quadruplex DNA structures are very thermally stable; however, they have large kinetic formation barriers, requiring high concentrations of monovalent cations and long periods of incubation [30]. Because of this, it has been proposed that the unimolecular (or intramolecular) G-quadruplex could be more biologically relevant in vivo [31–33]. In order to extend the studies of Est1 on G-quadruplex formation, we examined the intramolecular G-quadruplex promoting activity of Est1 by using several single-stranded telomeric DNA oligonucleotides of 32- to 38-nucleotides containing either three or four repeats of three consecutive

Abbreviations: DMS, dimethyl sulfide; CD, circular dichroism

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guanines. Our results showed that Est1 could promote the oligonucleotide of $(TGTGTGGG)_4$ to form intramolecular G-quadruplex.

2. Materials and methods

2.1. Oligonucleotides

The oligonucleotides were synthesized by Sangon Biotech (Shanghai, China). In most of the experiments, the DNA samples were prepared by mixing 80 pM ^{32}P-labeled probes with 4 μM unlabeled DNA. Because the amount of ^{32}P-labeled probes was small, the DNA concentration was considered to be approximately 4 μM. For the intramolecular G-quadruplex formation induced by KCl, the DNA samples were incubated in a 10 mM Tris–HCl (pH 7.5) buffer, heated at 95 °C for 5 min and slowly cooled down (over 2 h) to room temperature, then supplemented with 100 mM KCl and incubated at 25 °C for different time as indicated. For Est1-promoted intramolecular G-quadruplex formation, if no specific indication, 4 μM DNA, 200 nM ^{GST}-Est1 protein were incubated in the buffer containing 25 mM Tris–HCl, pH 8.0, 10% (v/v) glycerol, 1 mM DTT, 50 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA and 0.1 μg/μl BSA at 25 °C for 15 min.

2.2. Protein purification

The expression plasmids of pEGKT-*EST1* and pEGKT-*est1-D514A* were introduced into yeast strain BCY123, respectively. The protein overexpression and purification were performed as described previously [28].

2.3. DMS interfering assay

Before the induction of G-quadruplex, 1% DMS in ethanol (v/v) was added to the ^{32}P-labeled probes and incubated at 25 °C for 20 min. The DMS-methylated probes were mixed with the unlabeled DNA, and the assay for G-quadruplex formation induced by KCl or Est1 was then performed as above.

2.4. Gel electrophoresis

The G-quadruplex DNA was analyzed by native or denaturing polyacrylamide gel electrophoresis (PAGE). The native gel electrophoresis was performed on a 15% polyacrylamide gel containing 20 mM KCl, 1 × TBE and 2.5% glycerol in 0.5 × TBE running buffer containing 20 mM KCl at 4 °C. Denaturing gel electrophoresis was performed on 15% polyacrylamide gel containing 7 M urea, 1 × TBE and 2.5% glycerol. The gels were dried, and signals were detected with FLA-9000 (Fuji, Japan) and quantified with Fuji Multi Gauge software.

2.5. Circular dichroism spectroscopy

The products induced by KCl or Est1 were subjected to circular dichroism (CD) analysis. The CD spectra were recorded on a JASCO-715 spectropolarimeter using a 1-cm pathlength quartz cuvette with a reaction volume of 300 μl. For each sample, an average of two scans was taken, and the spectrum of the buffer was subtracted.

3. Results

3.1. Potassium induces guanine-rich telomeric single-stranded DNA oligonucleotide $4G_3$ to form parallel intramolecular G-quadruplex

The G-quadruplex DNA structures are highly polymorphic. According to the numbers of DNA strands folded in, the G-quadruplex can be divided into intermolecular and intramolecular structures. Different types of G-quadruplex may play distinct roles in telomere replication, recombination and protection [34]. The telomeric repeats in yeast are much more heterogeneous than that in ciliate and metazoan, and it is often denoted as TG_{1-3} [1]. However, the sequence containing four GGG-tracts exists naturally within telomeric DNA of different chromosomes (Table 1) [35]. Previous study showed that the $4G_3$ oligonucleotide (Table 1), which is derived from *S. cerevisiae* telomeric DNA and contains regular four G-tracts of three consecutive guanines, forms intramolecular G-quadruplex in the presence of monovalent cations, such as potassium and sodium [36]. We performed the G-quadruplex-formation assay as previously reported [37], and found that in the presence of 100 mM KCl, 1 M KCl or 1 M NaCl, $4G_3$ can form a structure with increased mobility (Fig. 1A). The intramolecular G-quadruplex is thought to fold into a more compact structure than the loose single-stranded DNA, and it is predicted to have a faster migration rate than the single-stranded DNA in a native gel [38]. Interestingly, the products induced by sodium migrated slightly faster than those induced by potassium (Fig. 1A). The reason for this is not clear but most likely because the G-quadruplex induced by sodium has a different configuration from that induced by potassium. In a denaturing gel, both the induced and uninduced DNA oligonucleotides behaved the same (Fig. 1B). This result is consistent with previous reports, and indicates that potassium is a better inducer for the formation of intramolecular configuration of G-quadruplex [36]. Thereby, we used 100 mM KCl in the following experiments.

To validate that the DNA product of $4G_3$ induced by potassium is the G-quadruplex, we performed a DMS interfering assay. DMS can methylate the N7 position of guanine to inhibit the formation of G-quartet. When $4G_3$ was pretreated with DMS, the faster migrating band induced by potassium disappeared (Fig. 1C). To further confirm the band with slightly faster migration rate is the intramolecular G-quadruplex, we used the oligonucleotide of $4G_3M$, in which the middle G in the first GGG-tract of $4G_3$ was changed to T (Table 1), to induce intramolecular G-quadruplex formation. As expected, the $4G_3M$ oligonucleotide could not form a band with an increased mobility in the presence of 100 mM KCl (Fig. 1D). Taken together, these results support the idea that the faster migrating band of $4G_3$ products induced by KCl represents the G-quadruplex structure. Because the $4G_3$ oligonucleotide used in previous study [36] harbors several non-telomeric nucleotides at the 5′ end, we synthesized Telo-$4G_3$ (Table 1), an oligonucleotide with telomeric sequence, and performed the G-quadruplex formation assay (i.e., induce Telo-$4G_3$ with 100 mM KCl). The result showed that this oligonucleotide could fold to intramolecular G-quadruplex structure in the presence of potassium (Fig. 1D).

Table 1
List of oligonucleotides mentioned or used in this work.

Name	Sequence (5′ to 3′)
Chr IR	TGT**GGG**TGT**GGG**TGT**GGG**TGT**GGG**TGT**GGG**TGT**GGG**
Chr IIIL	**GGG**TGT**GGG**TGTGT**GGG**TGT**GGG**TGTGG
Chr IIIR	TGTGT**GGG**TGT**GGG**TGTGT**GGG**TGTGGTGTGT**GGG**TGTGG
Chr IIIR	TGTGT**GGG**TGT**GGG**TGTGT**GGG**TGTGGT**GGG**TG
Chr IVL	TGTGT**GGG**TGT**GGG**TGTGT**GGG**TGT**GGG**TGTGG
Chr XIR	**GGG**TTGT**GGG**TGTGT**GGG**TGTGGTGT**GGG**TGTGG
Chr XIIIL	GTGT**GGG**TGT**GGG**TGT**GGG**TGT**GGG**TGTGG
Chr XIVL	TGTGT**GGG**TGTGT**GGG**TGT**GGG**TGTGT**GGG**TGT**GGG**TGTGG
$4G_3$	AATTCT**GGG**TGTGT**GGG**TGTGT**GGG**TGTGT**GGG**TGTGG
$4G_3M$	AATTCT*G*TGTGT**GGG**TGTGT**GGG**TGTGT**GGG**TGTGG
Telo-4G3	TGT**GGG**TGTGT**GGG**TGTGT**GGG**TGTGT**GGG**TGTGG
TG32	GTGT**GGG**TGTGTGT**GGG**TGTGGTGTGTGT**GGG**
TG37	GTGTGGTGT**GGG**TGTGTGT**GGG**TGTGGTGTGTGT**GGG**

The telomeric oligonucleotides are named Chr XXX, such as Chr IR, which indicates the right arm telomere of the first chromosome. GGG-tracts are shown in bold. Mutation of $4G_3$ is shown in italic.

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