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Iodine-125 Radioprobng of Intramolecular Quadruplex Conformation of Human Telomeric DNA in the Presence of Cationic Porphyrin TMPyP4

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Abstract

Purpose—A repeated, non-coding, DNA sequence d(TTAGGG)_n is present in the telomeric ends of all human chromosomes. These repeats can adopt multiple inter- and intra-molecular non-B-DNA conformations that may play an important role in biological processes. We applied ¹²⁵I-radioprobng to assess the conformation of the human telomeric DNA fragment in a complex with the quadruplex-specific drug - cationic porphyrin TMPyP4.

Material and methods—Synthetic DNA oligonucleotides containing the telomeric sequence were labeled with ¹²⁵I. The probability of DNA breaks caused by decay of ¹²⁵I is inversely related to the distance between the radionuclide and the sugar unit of the DNA backbone; hence, the conformation of the DNA backbone can be deduced from the distribution of breaks.

Results—The obtained data indicate that the telomeric oligonucleotides predominantly fold into an intramolecular quadruplex conformation in the presence of TMPyP4. We propose a mixed-type (3+1) conformation of telomeric quadruplex in a complex with the cationic porphyrin TMPyP4 in solution. Binding of the porphyrin overrides the counterion effect on quadruplex conformation.

Conclusions—We have demonstrated that ¹²⁵I radioprobng can be successfully applied not only to determine folding in G-quadruplexes, but also to reveal the mode of quadruplex interaction with small ligands.

Keywords

radioprobng; quadruplex; cationic porphyrin TMPyP4; telomeric DNA

Introduction

Telomeric ends of human chromosomes contain long (2–3 kb) stretches of a double-stranded repeated, non-coding, DNA sequence d(TTAGGG)_n and 35–600 nt, long single-stranded 3' overhangs of the same sequence (Makarov et al., 1997, Wright et al., 1997, Sfeir et al., 2005). These single-stranded guanine-rich repeats can fold into multiple inter- and intra-molecular tetra-stranded DNA structures (quadruplexes) stabilized by the Hoogsteen hydrogen-bonded guanine-tetrads (G-tetrads) under physiological conditions (Sen et al., 1988, Panyutin et al., 1989, Williamson et al., 1989). Formation of the quadruplex structures may play an important role in regulation of different biological processes (Hurley, 2001, Neidle et al., 2003, Cech, 2004, Burge et al., 2006).

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Small-molecule ligands capable of binding to the quadruplex structures can potentially interfere with telomere structure maintenance, and thus, are considered as potential anti-cancer agents. Cationic porphyrin 5,10,15,20-tetrakis(1-methyl-4-pyridyl)-porphyrin (TMPyP4) was reported as an effective telomerase inhibitor by an *in vitro* assay (Izbicka et al., 1999). Despite extensive studies, there are still controversies about structure and stoichiometry of quadruplex/porphyrin complexes and different models have been proposed. These models describe TMPyP4 binding as intercalation between adjacent G-tetrads (Keating et al., 2004, Wei et al., 2006), intercalation into the ApG step of d(TTAGGG) (Mita et al., 2006), by stacking on the external ends of quadruplex G-tetrad (Han et al., 2001, Wei et al., 2006) or by stacking onto TTA loop nucleotides (Parkinson et al., 2007). The porphyrin was reported to catalyze the conversion of both chair and basket type structures into parallel strand quadruplex DNA (Arthanari et al., 1999), TMPyP4 was shown to bind to the anti-parallel intramolecular (Wei et al., 2006), and to the parallel intermolecular (Parkinson et al., 2007) telomeric DNA conformations.

The knowledge of the structure of different conformations of the telomeric DNA quadruplexes is important for the rational design of small-molecule ligands that are able to bind and stabilize them. Several quadruplex conformations that can be adopted *in vitro* by telomeric oligonucleotides are shown in Figure 1. In all these structures, the telomeric sequence adopts an intra-molecular quadruplex structure stabilized by G-tetrads but the ways the sequence folds into the quadruplex are different. The folds were ascribed as anti-parallel (Na^+ form, resolved by nuclear magnetic resonance spectroscopy (NMR)) (Wang et al., 1993) and parallel (K^+ form, X-Ray) (Parkinson et al., 2002). The most recent NMR studies indicate that in solution in presence of K^+ cations telomeric DNA oligonucleotides formed predominantly mixed-type 3+1 structure (Ambrus et al., 2006, Luu et al., 2006, Phan et al., 2006, Dai et al., 2007). In general, the conformation of the telomeric DNA quadruplex is affected by the type of counterions and by the presence of an additional sequence flanking dGGG(TTAGGG)₃ core repeat. High structural polymorphism of telomeric quadruplexes makes it difficult to resolve the fine structure in the complex with TMPyP4 in solution.

We applied ^{125}I -radioprobng to assess the conformation of the human telomeric DNA fragments in the presence of different ions (sodium and potassium), and in complex with the cationic porphyrin TMPyP4. Iodine-125 radioprobng is based on the analysis of nucleic acid strand breaks produced in the close vicinity of the site of decay of an ^{125}I atom. The majority of local breaks produced by ^{125}I decay result from the direct action of radiation. The probability of breaks formation is inversely related to the distance from the radionuclide decay site to the corresponding nucleotides (Martin et al., 1981, Panyutin et al., 1997, Lobachevsky et al., 2000), and to a lesser extent on their accessibility to solution, like in other probing methods that rely on diffusible chemical species.

Iodine-125 can be easily incorporated into DNA as [^{125}I]-iododeoxycytosine (^{125}I -dC) using a DNA polymerase and commercially available ^{125}I -dCTP as a precursor (Panyutin et al., 1994). Most of the breaks (90%) produced by decay of ^{125}I are located within approximately one helical turn from the ^{125}I -dC incorporation site (Lobachevsky et al., 2000). Analysis of strand breaks in DNA and DNA-protein complexes with known 3D structure have confirmed experimentally that the probability of breaks depends primarily on the distance from ^{125}I to the sugar of a target nucleotide (Panyutin et al., 1997, Lobachevsky et al., 2000, He et al., 2004a). The strand breaks are due to the energy deposition or radical attack in the sugar-phosphate moiety because damage to the DNA bases generally does not lead to strand scission. Therefore, radioprobng provides information on the interatomic distances; and, in principle, on the 3D-structure of nucleic acids (Panyutin et al., 1998).

Materials and Methods

Oligodeoxyribonucleotides

All oligodeoxyribonucleotides (ODN, Table 1) were synthesized on an ABI394 DNA synthesizer (PE Applied Biosystems, Foster City, CA, USA), and purified by denaturing polyacrilamide gel electrophoresis (PAGE) as described in detail in (He et al., 2004b). The concentration of single-stranded ODN was measured at 260 nm on a HP 8452A Diode Array Spectrophotometer (Agilent Technologies, Inc., Santa Clara, CA, USA) and was calculated with the extinction coefficient calculator software - <http://www.basic.northwestern.edu/biotools/oligocalc.html> (Kibbe, 2007). The template ODN **VII** was synthesized with biotin groups so that it can be removed with magnetic Streptavidin Dynabeads (Invitrogen Corporation, Carlsbad, CA, USA) as described (Panyutin et al., 2001).

Labeling and purification of oligodeoxyribonucleotides

The telomeric ODN were labeled with ^{125}I using [^{125}I]-IdCTP and Klenow fragment of DNA polymerase I by primer extension reaction (Panyutin et al., 1994). The detailed protocol for ^{125}I labeling was as follows: 2 μl of 10x Klenow fragment of DNA polymerase I buffer [500 mM Tris-HCl (pH 8.0), 50 mM MgCl_2 and 10 mM dithiothreitol (DTT)], 1 μl of 10 μM duplexes (primers **V** and **VI** and template **VII** (Table 1) were annealed in equimolar amounts) and 16 μl of H_2O were added to freshly-dried 120 μCi [^{125}I]-IdCTP (Perkin Elmer, Waltham, MA, USA). After 15 min at RT, 1 μl of 10 U/ μl Klenow fragment of DNA polymerase I (Fermentas, Hanover, MD, USA) was added. After 15 min, 2 μl of 100 mM dNTP was added, and after 15 min incubation, the reactions were stopped with 1.5 μl of 0.5 M ethylene diamine tetraacetic acid (EDTA). The reaction mixtures were purified by MicroSpin G-25 columns (GE Healthcare, Piscataway, NJ, USA) to remove free [^{125}I]-IdCTP. The labeled ODN were separated and further purified from the template using Streptavidin Dynabeads (Invitrogen Corporation) (Panyutin et al., 2001) followed by purification with denaturing PAGE. The ODN were ^{32}P 5' end labeled using T4 Polynucleotide Kinase (Fermentas) following the standard protocol.

Preparation of G-4 quadruplexes

The telomeric ODNs were incubated in presence or absence of the TMPyP4 in radioprobable buffer (RB) solution (20 mM Tris-HCl (pH 7.4), 10% dimethyl sulfoxide (DMSO) and 1 mM EDTA) with addition of 100 mM NaCl or 100 mM KCl at 37 °C for 1 hour. Then samples were quickly frozen in liquid nitrogen and stored at -80 °C for 2 weeks to accumulate ^{125}I -induced DNA strand breaks.

DNA strand break analysis

After 2 weeks, the samples were thawed and the strand breaks were analyzed by 12% denaturing PAGE. The DNA strand breaks were quantified using a BAS-2500 Bioimager (FUJI Medical Systems USA, Stamford, CT, USA). To measure the intensity of the individual bands, the intensity profile of each lane was generated from the digitized gel image using semi-automated footprinting analysis (SAFA) software (Das et al., 2005). The probability of breaks were calculated from the areas of the individual peaks using a recursive formula and assuming that probability of breaks at [^{125}I]-IdC equals 1 as described in detail in (Lobachevsky et al., 2000). Briefly, if there is >1 break/decay then only the break closest to the ^{32}P -labeled 5'-end of the oligonucleotide will be detected. To obtain the probabilities of breaks in i^{th} position (p_i) we used next recursive expression: $p_i = F_i / (1 - p_i - 1)(1 - p_i - 2) \dots (1 - p_1)$, where is F_i is the observed frequencies of breaks at nucleotide in position i .

Results

Telomeric oligonucleotides adopt intramolecular quadruplex conformation in the presence of Na⁺ and K⁺

The ODN containing G-rich telomeric sequences are able to adopt different intramolecular as well as intermolecular conformations. Thus, it is important to confirm under the experimental conditions used, that the telomeric oligonucleotides adopt only intramolecular quadruplex conformations. In our previous work (He et al., 2004a), a band-shift assay and chemical probing with dimethyl sulfide (DMS) and diethylpyrocarbonate (DEPC) methods were applied to address this issue. Here, we used a radioprobng-based approach to test if the used ODN were present exclusively in an intramolecular conformation. An equimolar mixture of fluorescein-labeled telomeric ODN **III** and ¹²⁵I-labeled ODN **I** were used. If intermolecular quadruplex conformations were present in the solution, then ¹²⁵I-induced cleavage of **III** should be observed. ¹²⁵I labeled fluorescent ODN **II** was used as a control.

Primers **V** and **VI** were labeled by primer extension reactions as described in “Materials and Methods” to yield ODN **I** and **II** and then purified by denaturing PAGE. The samples of **II** or **I+III** were preincubated in RB buffer solution with addition of 100 mM NaCl or 100 mM KCl at 37° C for 1 hour. Control duplex of **II** with a complementary strand **VII** and control ODN **III** were preincubated in RB solution with addition of 100 mM NaCl at 37° C for 1 hour. Then, samples were quickly frozen in liquid nitrogen and stored at –80° C to accumulate ¹²⁵I-induced DNA strand breaks. After 14 days, samples were analyzed in the sequencing PAGE. The results of radioprobng of fluorescein-labeled telomeric oligonucleotides **V** and **VI** are shown in Figure 2. When ODN **II** was stretched in the duplex with the complementary oligonucleotide **VII** (lane 1) the maximum of ¹²⁵I-induced DNA strand breaks was observed at the position C18 with a rapid decrease of band intensities (which corresponds to breaks at the residues between the C18 position and the fluorescein-labeled 5' end), reflecting the increase of the distances from these nucleotides to the C18 residue in this B-DNA duplex. In samples of ODN **II**, double labeled with ¹²⁵I and fluorescein, in the presence either of Na⁺ (lane 2) or K⁺ (lane 3) a compact quadruplex structure was formed and an additional maximum of strand breaks was observed at the loop T5-A7. In contrast, ¹²⁵I decays produced no breaks in conditions when radioisotope and fluorescein were attached to two different oligonucleotides – mixtures of **I+III** (lanes 5 and 6). A minor band located at the A19 position is also presented in the sample of ODN **III** incubated in the absence of ¹²⁵I (lane 4) and therefore, is not the result of radioisotope decay but probably due to some impurities in the oligonucleotide sample. An equimolar mixture of **I+III** was incubated in the same conditions described for **II**. Formation of an intermolecular quadruplex would have brought the separate strands labeled with fluorescein (ODN **III**) and with ¹²⁵I (ODN **I**) into close proximity, and radioisotope decay would have produced DNA strand breaks in both oligonucleotides. Therefore, the absence of detectable breaks in lanes 5 and 6 proves that the tested telomeric oligonucleotides predominantly fold into intramolecular quadruplex conformations in the specified experimental conditions.

Radioprobng of telomeric DNA in presence of cationic porphyrin TMPyP4

We applied radioprobng to examine the influence of cationic porphyrin TMPyP4 on the intramolecular quadruplex structure of telomeric oligonucleotide **I**-³²P-5'. ODN **V** was labeled with ¹²⁵I and ³²P to yield **I**-³²P-5', which was purified and allowed to fold in the following conditions. The samples were incubated in the presence or absence of TMPyP4 in RB solution containing 100 mM NaCl or 100 mM KCl at room temperature for 1 hour. Then, samples were quickly frozen in liquid nitrogen and stored at –80° C to accumulate ¹²⁵I-induced DNA strand breaks. After 14 days, the samples were analyzed by the sequencing PAGE. For further analysis, the probabilities of breaks were calculated from the intensities of the bands by applying the recursive formula described previously (Panyutin et al., 1997, Lobachevsky et

al., 2000). The resulting probabilities of breaks are shown in Figure 3. The values were normalized such that probability of break at the [^{125}I]-C18 position equals 1. Only a part of the nucleotides of $\text{I-}^{32}\text{P-5'}$ are shown to highlight the fine structure of the breaks distribution. The data in the Figure 3 show that the probability of ^{125}I induced strand breaks rapidly decreases with distance from the [^{125}I]-C18 position in the duplex. The higher probability of breaks at the nucleotides from A1 to T12 in the single-stranded samples reflect folding of telomeric ODN $\text{I-}^{32}\text{P-5'}$ into a compact structure. When $\text{I-}^{32}\text{P-5'}$ was folded in the presence of Na^+ or K^+ it shows an increased probability of breaks with a maximum in the region of the loop T5-A7. This fold is characteristic for the basket and chair type conformations when the $^{125}\text{I-dC}$ containing loop is in the proximity to the T5-A7 loop (see Figure 1). In addition, in the presence of K^+ ions, there is a breaks probability maximum in the tail region A1-G2. These results are similar to those described in our previous work (He et al., 2004a).

Samples of $\text{I-}^{32}\text{P-5'}$ folded in presence of TMPyP4 either in the presence of K^+ or Na^+ , show a different DNA breaks probability distribution pattern, reflecting rearrangement of the telomeric quadruplex conformation. In the presence of TMPyP4, the maximum probability of breaks in $\text{I-}^{32}\text{P-5'}$ shifts to the G8-G9 region. Despite the difference in absolute values of breaks probability (0.112 at G8 in the presence of Na^+ and 0.075 at G8 in the presence K^+) samples of $\text{I-}^{32}\text{P-5'}$ folded in the presence of TMPyP4 with the addition of either Na^+ or K^+ , show a very similar breaks probability distribution pattern, indicating the similarity of telomeric quadruplex conformations under these conditions. In both cases, the probability of breaks in the T5-A7 loop is lower than at the G8-G9 nucleotides in the core, that is consistent with the mixed-type 3+1 quadruplex conformation (Figure 1). Therefore, we conclude that binding of TMPyP4 promotes folding of telomeric ODN $\text{I-}^{32}\text{P-5'}$ into the mixed-type 3+1 quadruplex structure, thus overriding the effect of counterions.

Discussion

The structure of the TMPyP4 complex with a bimolecular human telomeric quadruplex was recently solved by X-ray crystallography (Parkinson et al., 2007). In this structure, the quadruplex is in the parallel propeller conformation (Figure 1). TMPyP4 molecules are bound to the TA nucleotides both in the loops and in the 5' region of the stacked quadruplex. No interactions of the porphyrin with the G-tetrads were observed. Although the X-ray crystallography provides superior resolution and invaluable structural insights, the results could be affected by the selectivity of the crystallization. For example, until now the parallel propeller conformation of the human telomeric quadruplex was observed only in the crystal, most likely because it is the most compact of the quadruplex conformations and therefore, is the best fit for crystallization.

NMR and UV-spectroscopy studies (Mita et al., 2006; Wei et al., 2006) revealed that TMPyP4 could intercalate between G-tetrads. The human telomeric quadruplex used in (Mita et al., 2006) consisted of four parallel single telomeric repeat sequences. It is not clear if TMPyP4 can intercalate in intramolecular human telomeric quadruplexes; the presence of the TTA loops may preclude this mode of binding. Although, we cannot rule out TMPyP4 intercalation in our results based on radioprobings experiments.

Our findings are consistent with the mode of binding of TMPyP4 to a quadruplex formed by a G-rich sequence located upstream of the c-MYC oncogene proposed in (Seenisamy et al., 2004). Similar to our results, the Seenisamy et al. described transition of the quadruplex to the mixed-type (3+1) conformation upon binding with TMPyP4. We believe that the mixed-type (3+1) conformation, with two lateral and one diagonal loops, ideally fits TMPyP4, providing two flat surfaces for stacking at the top and bottom of the quadruplex, as well as a possibility for interaction of the porphyrin with the lateral loops.

In conclusion, we have demonstrated that ^{125}I radioprobng can be successfully applied not only to determine folding in G-quadruplexes, but also to reveal the mode of quadruplex interaction with small ligands. Although radioprobng cannot compete with X-ray crystallography and NMR in resolution of structural details, it could be very helpful in distinguishing between different conformations of the quadruplexes in solution. In addition, radioprobng studies can be carried out in a wide variety conditions in solution, including physiological, and, possibly, *in vivo* conditions.

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Figure 1.
The schematic diagram of possible intramolecular conformations of human telomeric quadruplexes.

Figure 2.

Analysis of DNA breaks in the G4 one tail telomeric oligonucleotides by 12% denaturing PAGE. 1. Duplex of **II** with complementary strand **VII**; 2. **II** folded in RB+100 mM NaCl; 3. **II** folded in RB+ 100 mM KCl; 4. Fluorescein-labeled telomeric ODN **III**; 5. Telomeric ODNs **III** + **I** folded in RB+100 mM NaCl; 6. Telomeric ODNs **III** + **I** folded in RB+100 mM KCl; 7. G sequencing ladder.

Figure 3.

Distribution of ^{125}I induced strand breakage probability in the telomeric ODN **I**- ^{32}P -5'. The samples were incubated (1 hour at room temperature) in RB containing: 100 mM of KCl (closed triangles); in the presence of 100 mM NaCl (closed squares); in the presence of 100 mM of KCl and 5 μM TMPyP4 (open triangles); in the presence of 100 mM of NaCl and 5 μM TMPyP4 (open squares); and a duplex of ODN **I**- ^{32}P -5' and complementary template **VII** (open circles). Then the samples were quickly frozen in liquid nitrogen to preserve the structure. After 14 days the breaks in the samples were analyzed with denaturing PAGE and the resulting frequencies of breaks are shown.

Table 1

Oligonucleotides used in this study

G4 one tail series		
I	5'-A ₋₁₀ C ₋₉ G ₋₈ T ₋₇ T ₋₆ A ₋₅ T ₋₄ A ₋₃ T ₋₂ G ₋₁ A ₁ G ₂ G ₃ G ₄ T ₅ T ₆ A ₇ G ₈ G ₉ G ₁₀ T ₁₁ T ₁₂ A ₁₃ G ₁₄ G ₁₅ G ₁₆ T ₁₇ C* ₁₈ A ₁₉ G ₂₀ G ₂₁ G ₂₂	
II	5'-Fluor- ACGTTATATGAGGGTTAGGGTTAGGGTC*AGGG	Same as I, fluorescein-labeled
III	5'-ACGTTATATGAGGGTTAGGGTTAGGGTCAGGG	Same as I, no ¹²⁵ I
IV	5'-Fluor-ACGTTATATGAGGGTTAGGGTTAGGGTCAGGG	Same as II, no ¹²⁵ I
Oligonucleotides used for ¹²⁵ I-labeling of V and VI		
V	5'-ACGTTATATGAGGGTTAGGGTTAGGGT	Primer
VI	5'-Fluor-ACGTTATATGAGGGTTAGGGTTAGGGT	Fluorescein-labeled primer
VII	3'-BCGATCBCTATACAATATACTCCCAATCCCAATCCCAGTCCC	Complementary template

B, Biotin; Fluor, Fluorescein; C*, [¹²⁵I]-dC