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G-Quadruplex Hinders Translocation of BLM Helicase on DNA: A Real-Time Fluorescence Spectroscopic Unwinding Study and Comparison with Duplex Substrates

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Abstract: Sequences with the potential to form G-quadruplex structures are spread throughout genomic DNA. G-quadruplexes in promoter regions can play regulatory roles in gene expression. Expression of protein-encoding genes involves processing of DNA and RNA molecules at the level of transcription and translation, respectively. In order to examine how the G-quadruplex affects processing of nucleic acids, we established a real-time fluorescent assay and studied the unwinding of intramolecular G-quadruplex formed by the human telomere, ILPR and PSMA4 sequences by the BLM helicase. Through comparison with their corresponding duplex substrates, we found that the unwinding of intramolecular G-quadruplex structures was much less efficient than that of the duplexes. This result is in contrast to previous reports that multistranded intermolecular G-quadruplexes are far better substrates for the BLM and other RecQ family helicases. In addition, the unwinding efficiency varied significantly among the G-quadruplex structures, which correlated with the stability of the structures. These facts suggest that G-quadruplex has the capability to modulate the processing of DNA and RNA molecules in a stability-dependent manner and, as a consequence, may provide a mechanism to play regulatory roles in events such as gene expression.

Introduction

In addition to the canonical double helix structure, guanine-rich (G-rich) nucleic acids can fold into a four-stranded G-quadruplex structure in the presence of K^+ or Na^+.[1,2] So far large numbers of such sequences have been found to be present in the genomes of humans and other species.[3−12] In human and chicken genomes, putative quadruplex sequences may total up to several hundred thousands copies.[11−16] Many of these sequences have been found to exist in essential regions of chromosomes,[8−12] for example, telomeres,[17] promoter of oncogenes,[18] and immunoglobulin switch[19] and the insulin regulatory[20] regions. G-quadruplex structures are believed to play roles in important biological processes, such as regulation of gene expression and maintenance of genome stability.[21] They are also considered as potential therapeutic targets for many diseases,[22,23] for instance, cancer.[24−27] Currently studies on quadruplexes have been mostly focused on the characterization of the structures and their interaction with small molecules. How G-quadruplexes affect gene expression, or in a more general sense, the processing of DNA and RNA in various physiological events, such as transcription and translation, is an important question deserving exploration.

In vivo, G-quadruplex structures are dynamic and their participation in biological processes involves structural assembly and disassembly. G-quadruplex has to be resolved or unwound

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in several essential events, such as DNA replication,[28] transcription,[29] and extension of telomere ends.[30] Unwinding of G-quadruplex can be achieved with a number of proteins, for example, the RecQ family of DNA helicases,[31−36] the FANCJ helicases,[37] the lagging strand DNA replication protein Dna2,[28] and the viral SV40 large T-antigen helicases.[38−40] Defect and dysfunction in these enzymes result in genome instability and are associated with many genetic diseases including cancer, neurodegenerative syndromes, and a myriad of other disorders.[41] Therefore, the processing of G-quadruplexes is of physiological importance to the cellular processes and therapeutic applications in which G-quadruplexes are involved.

So far there have rarely been studies on the unwinding of intramolecular G-quadruplex structures. Previous studies on G-quadruplex unwinding have been carried out almost exclusively with intermolecular G-quadruplexes, most often referred to as G4 DNA, in which multiple G-rich strands are hold together by stacked G-quartets.[32−39] Unwinding activity in these studies was determined by physical separation of radioisotope-labeled substrates with gel electrophoresis. The intermolecular structures and their unwindings may not be representative of the gene-regulating G-quadruplex structures because the latter are present as single copies in the middle of double-stranded genomic DNA and are likely to form intramolecular structures. Only until recently has an unwinding assay for the intramolecular G-quadruplex been presented using an affinity biosensor.[40] This method only accesses the amount of unwound substrate at the end of the unwinding reaction by using single-stranded DNA binding protein. To facilitate the study of intramolecular G-quadruplex unwinding, we established a fluorescent method to monitor the kinetics of intramolecular G-quadruplex unwinding in real-time. Using this method we studied the unwinding of intramolecular G-quadruplex from the human telomere, ILPR and PSMA4 sequences by the BLM helicase and compared it with the unwinding of their corresponding duplex substrates. Our study revealed that the unwinding of the G-quadruplex structures is significantly less efficient than that of the duplexes. These results are in contrast to previous reports on intermolecular G-quadruplexes that were shown to be far better substrates to the RecQ family helicases, BLM and Sgs1, than duplexes with at least 10 times higher unwinding efficiency.[32−34] In addition, our data show that the efficiency of G-quadruplex unwinding reversibly correlates with quadruplex stability. These facts imply that G-quadruplex can create a barrier to processes such as protein translocation on nucleic acids and can modulate the opening/annealing of genomic DNA. Since these processes are essential in many physiological events, for example, DNA replication, transcription and repair, nucleoprotein displacement, and protein synthesis, we suggest that the barrier created by G-quadruplex may provide a way to regulate such processes.

Materials and Methods

Oligonucleotides. Oligonucleotides used in unwinding assays are given in Table 1. Unlabeled oligonucleotides were purchased from Invitrogen (Beijing, China). Tetramethylrhodamine (TMR)-labeled oligonucleotides were purchased from Invitrogen (Beijing, China) or TaKaRa Biotechnology (Dalian, China). Oligonucleotide concentration was determined by the absorbance at 260 nm using the OligoAnalyzer 3.1 from IDT (http://www.idtdna.com).

BLM Helicase. A truncated BLM variant, $BLM^{642-1296}$, was constructed from the plasmid pJK1 (gift from Dr. Ian Hickson at the University of Oxford, UK). Briefly, the region of the pJK1 plasmid encoding residues 642−1296 was amplified by PCR with primers GGAGGATCCGAGCGTTTCCAAAGTCTTAGTT and GTGCTCGAGGGAACTGTCTTCAGCTGGCGATGT, digested with BamH I and Xho I, and inserted into the pET28a plasmid. The resulting plasmid was amplified in *E. coli* strain DH5α, verified by sequencing, and expressed in *E. coli* strain BL21. The recombinant His-tagged fusion protein $BLM^{642-1296}$ was purified by nickel chelate chromatography. The purified protein was concentrated by ultrafiltration and stored in buffer of 50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 50% (v/v) glycerol at −80 °C until use.

Unwinding Assay. DNA substrate was made in buffer of 20 mM Tris-HCl (pH 7.4), 150 mM KCl and 3 mM $MgCl_2$, denatured at 95 °C for 5 min, then cooled down slowly to room temperature. G-quadruplex substrate was made at 5 nM (or otherwise indicated) and assayed directly. Duplex substrate was made at 2.5 μM and diluted to 5 nM right before assay. Unwinding was carried out on a Spex Fluorolog-3 spectrofluorometer (HORIBA Jobin Yvon, France) at 20 °C in a 2 mL volume by addition of 0.5 mM ATP followed by 50 nM BLM helicase or vice versa under constant stirring, unless otherwise indicated. Fluorescence change was monitored over time with excitation wavelength set at 555 nm and emission at 575 nm. For single-turnover kinetics analysis, the rate constant k_{obs} and amplitude of maximal unwinding U_{max} was derived by fitting experimental data to the single-exponential equation

$$U = U_{max}(1 - e^{-k_{obs}t})$$

where U is the percent unwinding at time t. In case the above equation could not be reasonably fitted, the following dual-exponential equation was used

$$U = U_{max}[1 - C \cdot e^{-k_{obs1}t} - (1 - C) \cdot e^{-k_{obs2}t}]$$

with C being the fractional constant. The mean rate constant, k_{obs}, was then calculated as

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$$k_{obs} = C \cdot k_{obs1} + (1 - C) \cdot k_{obs2}$$

Oligonucleotide Digestion by T4 DNA Polymerase. 5′-P^{32}-labeled oligonucleotides were mixed at equimolar in buffer of 20 mM Tris-HCl (pH 7.4), 0.05 mg/mL BSA, 10 mM KCl and 3 mM $MgCl_2$, heated at 95 °C for 5 min, then slowly cooled down to 37 °C and maintained for 30 min. Hydrolysis was initiated by addition of T4 DNA polymerase, stopped by addition of 10 μL of formamide solution containing 10 mM EDTA, 50 mM NaOH, and 0.1% bromophenol blue. Samples were electrophoresed on 19% denaturing polyacrylamide gel containing 7 M urea for 70 min, autoradiographed on a FLA 7000 biomolecular imager (Fujifilm, Japan), and quantified with the software ImageQuant 5.2.

Results

The BLM helicase is a member of the RecQ family of DNA helicases that can unwind duplex DNA and other secondary structures, including intermolecular G-quadruplex structures,[31−33] in a 3′−5′ direction driven by consumption of ATP as an energy source.[42] The interaction of BLM helicase with substrate and the unwinding of intramolecular G-quadruplex was first analyzed using a substrate carrying the human telomere G-rich core sequence $G_3(T_2AG_3)_3$ (Figure 1). The substrate can form either G-quadruplex or hairpin duplex. The base-pairing size of the hairpin was chosen such that in 150 mM K^+, which is the physiological concentration of this cation inside animal cells, the substrate preferentially folded into G-quadruplex leaving a 10 nt 3′ single-stranded tail as loading strand for the helicase. The fluorescent dye TMR covalently attached to the 3′ end of the loading strand served as a reporter to indicate the structure of the substrate, which was monitored simultaneously in real-time by fluorescence intensity and polarization. When helicase was added, it bound to the loading strand, resulting in a reduction in the rotational freedom of the dye, which was reflected by an increase in fluorescence polarization. Addition of ATP initiated G-quadruplex unwinding, during which the helicase translocated to the 5′ end of the substrate and allowed the loading region to fold back to form a hairpin structure by hybridizing with the G-rich region. Upon hybridization, the fluorescent dye was quenched by the guanines on the opposite strand[43,44] and further restrained in its rotational freedom. Thus, the unwinding process

Table 1. Sequence of DNA Substrates and 100% Unwound Controls Used in the Hairpin Format Unwinding Assay[a]

Substrate	Sequences
qTEL	5′-GGGTTAGGGTTAGGGTTAGGGTTTTCCTAAC-TMR-3′
Control	5′-GGGTTAGGGTTAGGGTTAGGGTTTTCCTAAC-TMR-3′ 3′-CCAAAAGGATTGGGA-5′
dTEL	5′-GTGTGAGTGGTAGGGTTAGCGTTTTGCTAAC-TMR-3′ 3′-CACACTCACCATCCCAATCGC-5′
Control	5′-GTGTGAGTGGTAGGGTTAGCGTTTTGCTAAC-TMR-3′
qTEL′	5′-GTTCCGTTGAGCAGAGTTAGGGTTAGGGTTAGGGTTAGGGTTATTGTTAG-3′ 3′-TMR-CAAGGCAACTCGTCTC-5′
dTEL′	5′-GTTCCGTTGAGCAGAGTTAGTGTGAGTGGAGGTGTGAGGTTTATTGTTAG-3′ 3′-TMR-CAAGGCAACTCGTCTC CACACTCACCTCCACACTCCA
Control	3′-TMR-CAAGGCAACTCGTCTC-5′
qILPR	5′-GGGGTGTGGGGACAGGGGTGTGGGGTTTTTACCCC-TMR-3′
Control	5′-GGGGTGTGGGGACAGGGGTGTGGGGTTTTTACCCC-TMR-3′ 3′-CCAAAAATGGGGTGT-5′
dILPR	5′-GCCGTGTGCCGACAGCGGTGTGGGGTTTTTACCCC-TMR-3′ 3′-CGGCACACGGCTGTCGCCACACCCC-5′
Control	5′-GCCGTGTGCCGACAGCGGTGTGGGGTTTTTACCCC-TMR-3′
qPSMA4	5′-GGGTTGGGGCGGGGGGAGCGGGTTTTTAACCC-TMR-3′
Control	5′-GGGTTGGGGCGGGGGGAGCGGGTTTTTAACCC-TMR-3′ 3′-CCAAAAATTGGGCGA-5′
dPSMA4	5′-GCGTTGCGGCGCGCGCAGCGGGTTTTTAACCC-TMR-3′ 3′-CGCAACGCCGCGCGCGTCGCCC-5′
Control	5′-GCGTTGCGGCGCGCGCAGCGGGTTTTTAACCC-TMR-3′

[a] Underlined nucleotide forms a hairpin stem when substrate is unwound.

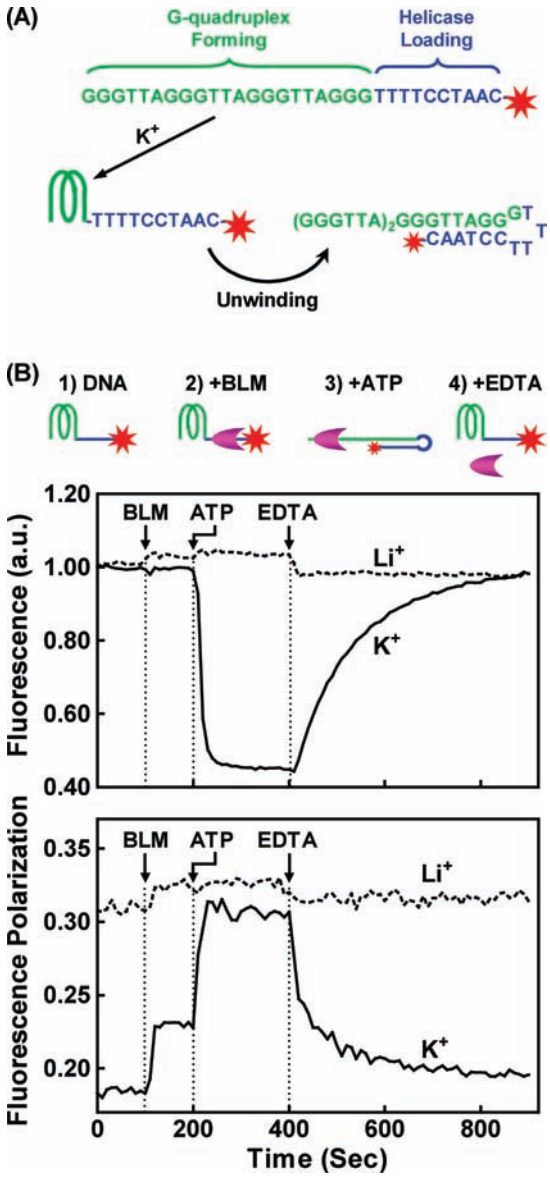

Figure 1. Real-time monitoring of intramolecular G-quadruplex unwinding by fluorescence quenching via G-quadruplex/hairpin conversion. (A) Illustration of G-quadruplex formation in qTEL and its structural conversion upon G-quadruplex unwinding. (B) Fluorescence monitoring of G-quadruplex unwinding. (1) In K^+ solution, qTEL initially formed G-quadruplex carrying a 10-nt 3′ tail. The fluorescent dye tetramethylrhodamine (TMR) at the end of the tail had high fluorescence emission and low fluorescence polarization (solid lines). (2) Loading of BLM helicase to the 3′ tail resulted in increase in fluorescence polarization caused by reduction in the rotational freedom of the dye. (3) Addition of ATP drove the helicase to unwind the G-quadruplex, allowing the 3′ tail to fold back into hairpin. Hybridization of the hairpin stem quenched the fluorescence of TMR and further increased its fluorescence polarization. The binding of helicase at the 5′ end of DNA substrate and formation of hairpin at the 3′ end prevented G-quadruplex formation. (4) Addition of EDTA depleted the Mg^{2+}, causing the helicase to release from the DNA. Thus the DNA refolded into G-quadruplex, liberating the 3′ tail to restore its fluorescence emission and polarization. Dotted line shows a control experiment carried out in 150 mM Li^+ solution in which G-quadruplex did not form. DNA (50 nM) was used to compensate the decrease in sensitivity caused by the polarizing filters.

was reflected concomitantly by a decrease in fluorescence emission and further increase in fluorescence polarization. The formation of hairpin and the binding of helicase at the 5′ end of the substrate prevented G-quadruplex formation after its unwinding. The binding of the BLM helicase with DNA requires

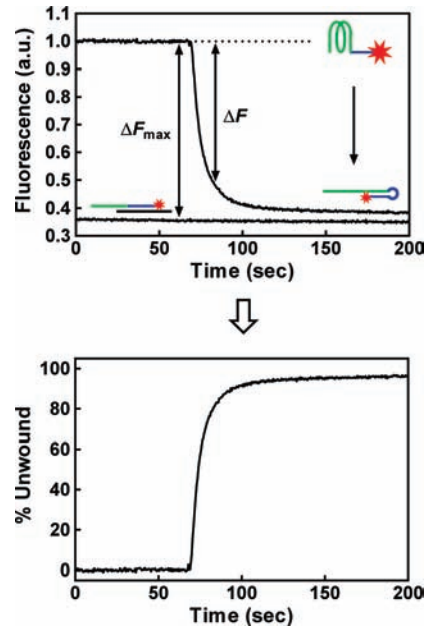

Figure 2. Quantification of unwinding in the fluorescence quenching assay. Maximal fluorescence quenching (ΔF_{max}) was obtained by addition of excess complementary DNA to fully hybridize the substrate DNA and create a local sequence environment for the fluorescent dye the same as that in the hairpin structure (Table 1). Percent unwinding at a given time was calculated as $100 \times \Delta F/\Delta F_{max}$.

Mg^{2+}. When Mg^{2+} was depleted by addition of EDTA, the bound helicase released from the substrate, and the substrate refolded back into G-quadruplex. As a result, the fluorescence intensity and polarization restored to their original values. G-quadruplex does not form in Li^+ solution.[45,46] In this case, the fluorescence showed little or only minor changes in both intensity and polarization. This further confirmed that the change in fluorescence intensity and polarization was an indication of G-quadruplex unwinding activity.

In the above analysis, it can be seen that the fluorescence can be used to monitor the unwinding process. To quantitate the G-quadruplex unwinding, one has to know the fluorescence when all the substrates are in the hairpin form. To obtain this value, the substrate was hybridized with an excess of complementary DNA to create the same local sequence environment for the fluorescent dye as in the hairpin structure (Figure 2, Table 1). Therefore, the percentage unwinding of the available substrate can be calculated as $100 \times \Delta F/\Delta F_{max}$, where ΔF is the decrease in fluorescence at a given time after initiation of unwinding and ΔF_{max} is the maximal decrease in fluorescence when all the fluorescent dye is quenched. Using this definition, the unwinding of G-quadruplex under various concentrations of ATP and helicase was carried out, and the results are given in Figure 3. As the concentration of ATP and helicase increased, both the rate and extent of G-quadruplex unwinding increased. Maximal unwinding was achieved at 500 μM ATP and 50 nM BLM helicase. The unwinding assay was also expanded to

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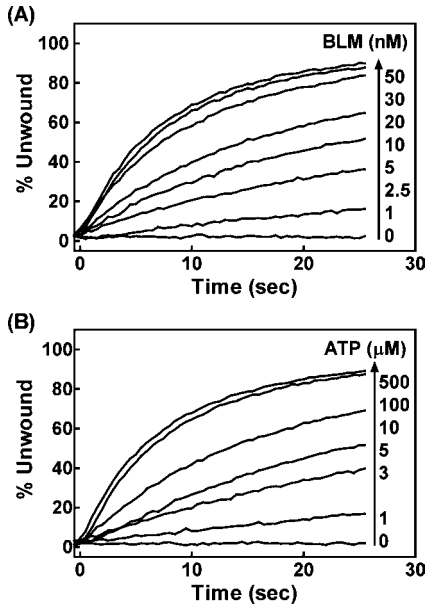

Figure 3. Unwinding of human telomere intramolecular G-quadruplex qTEL by BLM helicase at various concentrations of (A) BLM helicase and (B) ATP, monitored by fluorescence quenching.

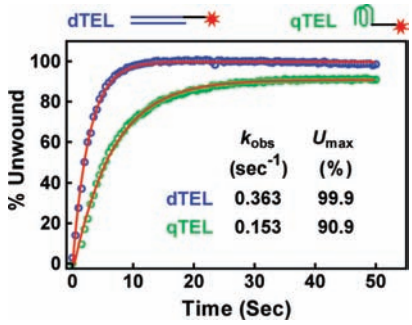

Figure 4. Unwinding of human telomere intramolecular G-quadruplex qTEL and duplex dTEL by BLM helicase measured by fluorescence quenching. The fluorescence was fitted to the single-exponential equation (red lines) to obtain the rate constant k_{obs} and maximal unwinding U_{max}. Structures of the substrates are shown above the graph.

analyze duplex unwinding by replacing the G-quadruplex with a duplex structure, which displayed similar changes in fluorescence intensity and polarization during BLM loading, substrate unwinding, and BLM releasing as in the G-quadruplex unwinding (Figure S1, Supporting Information). In Figure 4, the unwinding of the intramolecular G-quadruplex of the human telomere sequence $G_3(T_2AG_3)_3$ was compared with a duplex substrate in which the G-rich region was randomized from the $G_3(T_2AG_3)_3$ sequence. By fitting into a single-turnover kinetics, it can be seen that the unwinding of the G-quadruplex featured a significantly slower rate and a slightly lower magnitude than those of the corresponding duplex substrate.

In the assays shown in Figures 1−4, there was a possibility that the hairpin structure could form before the G-quadruplex or duplex region was fully unwound once the hybridization region became available. Therefore, they might only reflect the initiation of unwinding. This situation is very unlikely because a BLM helicase (amino acids 642−1290) occupies a ssDNA stretch of 14 nucleotides;[47] thus, it must move to the far 5′ end

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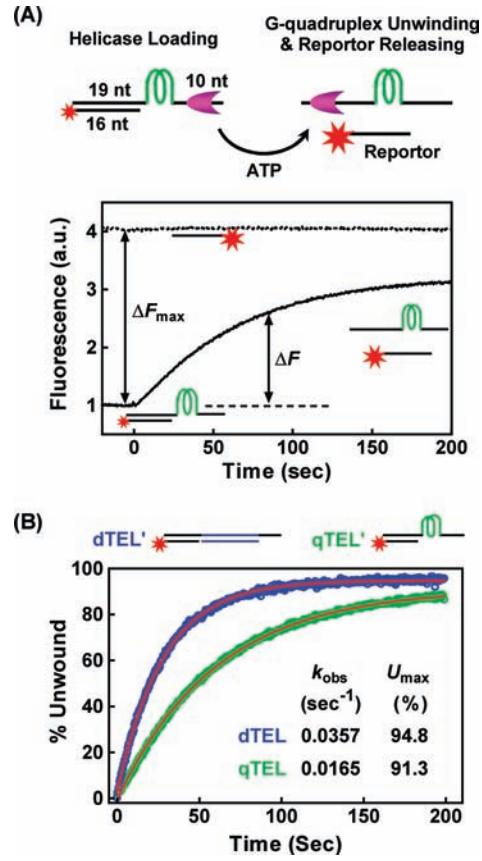

Figure 5. Real-time monitoring of G-quadruplex unwinding by fluorescence unquenching. (A) Fluorescence change during unwinding. Substrate DNA consisted of three sections, in which a G-quadruplex was flanked by a duplex at the 5′ side carrying a TMR-labeled oligonucleotide as reporter and a 10-nt single-stranded tail at the 3′ side as loading sequence for BLM helicase. The fluorescence of TMR was quenched in the duplex. Unwinding of the G-quadruplex followed by that of the duplex resulted in an increase in fluorescence of the reporter. Translocation of helicase to the 5′ end of DNA substrate sheltered off the displaced reporter. Maximal fluorescence that could be restored (ΔF_{max}) was obtained from the free reporter without hybridization. Percent unwinding at a given time was calculated as $100 \times \Delta F/\Delta F_{max}$. (B) Unwinding of intramolecular telomere G-quadruplex qTEL and duplex dTEL by BLM helicase measured by fluorescence unquenching. The fluorescence was fitted to the single-exponential equation (red lines) to obtain the rate constant k_{obs} and maximal unwinding U_{max}. Structures of the substrates are shown above the graph.

to unwind the whole substrate before the hairpin can form. To verify the observation we made, further experiments were carried out, using a different substrate format illustrated in Figure 5A. Here, the G-quadruplex was flanked by a reporter-binding sequence at its 5′ end and a 10-nt helicase-loading sequence at its 3′ end. The 5′ reporter-binding sequence was bound by a complementary 16-nt strand labeled at the 3′ end with a fluorescent dye TMR. The release of the reporter from the substrate as a result of unwinding activity led to an increase in fluorescence of the reporter, which only occurred after successful unwinding of the entire G-quadruplex and duplex regions. Similar to the previous assay, the percentage unwinding of the available substrate can be calculated as $100 \times \Delta F/\Delta F_{max}$, where ΔF is the increase in fluorescence at a given time and ΔF_{max} the difference of fluorescence between the reporter in the bound and fully released forms. Duplex unwinding was also analyzed by replacing the G-quadruplex with a duplex structure. The unwinding of telomere G-quadruplex monitored by this unquenching assay also exhibited similar dependence on ATP and helicase concentration as that in Figure 3 (data not shown). In

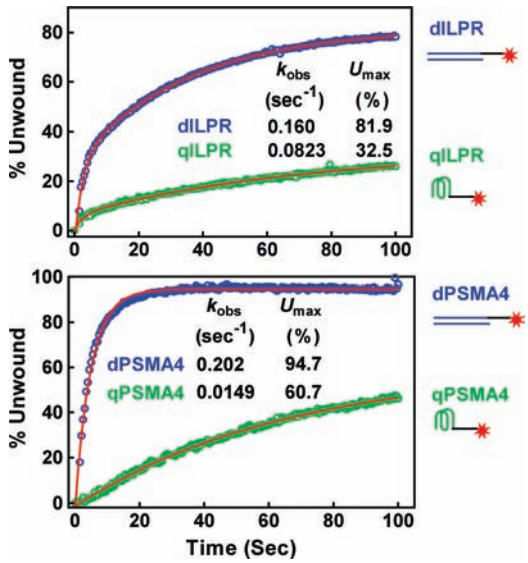

Figure 6. Unwinding of intramolecular G-quadruplex and duplex from ILPR and PSMA4 sequence by BLM helicase monitored by fluorescence quenching. Fluorescence was fitted (red lines) to the single-exponential equation for PSMA4 or the double-exponential equation for ILPR to obtain the rate constant k_{obs} and maximal unwinding U_{max}. Structures of the substrates are shown at the right side.

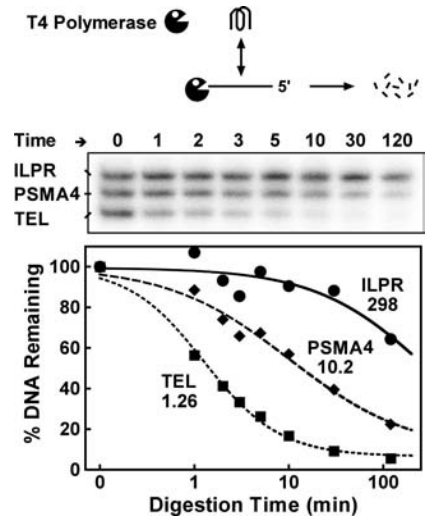

Figure 7. Digestion of TEL, PSMA4, and ILPR G-quadruplex structures by T4 DNA polymerase. A T_6, T_{11}, or T_{15} was added to the 5′ end of a TEL, PSMA4, or ILPR sequence, respectively, to separate the sequence on the gel. The number by the curve gives the time ($T_{1/2}$, min) required to hydrolyze half of the substrate, which was derived by fitting the experimental data to the sigmoidal function. If assuming that only the intramolecular G-quadruplex (~82%, Figure S1 in Supporting Information) in the PSMA4 could be digested; a slightly smaller $T_{1/2}$ of 7 min was obtained.

Figure 5B, the unwinding of telomere G-quadruplex was compared with its corresponding duplex substrate. With an additional duplex to unwind, the unwinding rate was significantly reduced for both the G-quadruplex and duplex, but the influence on the maximum unwinding was marginal. The results confirmed that the unwinding of the G-quadruplex was significantly slower in rate and lower in magnitude than that of the duplex substrate.

Additional unwinding assays using the quenching format shown in Figure 2 were carried out for two other sequences, ILPR and PSMA4, which are known to form G-quadruplex.[8,48−50] The unwinding of qPSMA4 was analyzed, assuming it occurred only to intramolecular structure (82%, Figure S2, Supporting Information), which would only overestimate the unwinding if intermolecular structures (18%, Figure S2, Supporting Information) could be unwound. Similar to the telomere sequence, the two G-quadruplex structures all showed a lower unwinding efficiency compared to that of their equivalent duplex substrates with respect to the rate and magnitude (Figure 6). The unwinding of a G-quadruplex breaks the Hoogsteen hydrogen bonds among the guanines in the stacked G-quartets that stabilize G-quadruplex. We next examined the stability of the three G-quadruplex structures. To avoid the formation of intermolecular structures, the concentration of ILPR and PSMA4 had to be kept low. This prevented us from using the conventional thermodynamic techniques such as UV or fluorescent melting. Thus, we adopted the T4 polymerase digestion assay. The T4 polymerase has been shown to only cleave DNA in the relaxed state from the 3′ end, but not those folded into G-quadruplex.[51] As a result, more stable G-quadruplex will be more resistant to T4 polymerase digestion. As is shown in Figure 7 and Table 2,

Table 2. Kinetic Parameters of the Unwinding by BLM Helicase and Digestion by T4 Polymerase of G-Quadruplex Structures

substrate	U_{max} (%)	k_{obs} (s^{-1})	$T_{1/2}$ (min)
qTEL	90.9	0.153	1.26
qPSMA4	60.7	0.0149	10.2
qILPR	32.5	0.0823	298

the difference in unwinding among the three G-quadruplexes inversely correlated with the stability of the G-quadruplex structures.

It was noticed that the unwinding of both the G-quadruplex and duplex of ILPR could not be satisfactorily fitted to the single-exponential, but a double-exponential model. It has been reported that ILPR forms a mixture of antiparallel and parallel G-quadruplex structures in 100 mM K^+ solution with the former being more stable than the later.[48] The double-exponential behavior for the ILPR G-quadruplex may be explained by the unwinding of two structural species with different stabilities. However, we could not think of a reasonable explanation for the double-exponential behavior of the ILPR duplex, which might be a sequence-dependent characteristic for this specific substrate.

Discussion

In the past decade, genome-wide analysis has revealed several hundred thousand DNA sequences with the potential to form G-quadruplex structures in the genome of human and other species.[3−12] Many of such sequences have been shown to readily form stable G-quadruplex in double-stranded DNA under cell-mimicking condition.[52] Evidences for their presence *in vivo* and in regulatory roles are convincing.[13,53] It is therefore of interest to know how G-quadruplex structures affect physiological

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processes associated with DNA or RNA. G-rich nucleic acids can form intermolecular G-quadruplex via association of multiple strands or intramolecular G-quadruplex by folding of a single strand containing at least four G-tracts.[54,55] Many previous studies have reported that the eukaryotic RecQ family helicases, BLM and Sgs1, unwind intermolecular G-quadruplex structures with at least 10-fold higher efficiency than they unwind duplex substrates.[32−34] Our finding that intramolecular G-quadruplex structures are more difficult to unwind than the corresponding duplex substrates by the BLM helicase revealed a different property of G-quadruplex structures on the processing of nucleic acids.

The resistance of the G-quadruplex structures to the resolving activity of the BLM helicase may present an example of how G-quadruplex structures can affect the processing of nucleic acids. This property might be important to the biological function of G-quadruplex structures, such as the regulation of gene expression. Translocation activity is required for gene expression at both the DNA and RNA level where products are proteins. The formation of G-quadruplex may directly affect the rate of transcription and translation processes. G-quadruplex formation is also expected to affect the processing and structure of genomic DNA. Unwinding of genomic DNA is essential in many biological events where the DNA duplex has to be separated so that single-stranded DNA can be accessed, for example DNA replication, repair, and RNA transcription. In these events, motor proteins, such as DNA polymerase, RNA polymerase, and helicase, translocate along DNA tracks. The formation of G-quadruplex may create obstacles to the helicase and likely other translocation activities if it is present at the loading strand. This may lead to arrest or slowing down of translocation. On the other hand, the formation of G-quadruplex may facilitate the separation or inhibit the annealing of genomic DNA. For the above reasons, the formation of G-quadruplex structures can apparently provide an effective means to modulate the structure and function of genomic DNA. This also implies that failure to resolve G-quadruplex structures will lead to deleterious effects in the above-mentioned aspects.

Because of the dependence on DNA strand separation, the methods currently used for analyzing multimeric DNA unwinding are not applicable to the monomeric intramolecular G-quadruplex structures. Only recently, a method for unwinding the intramolecular G-quadruplex has been reported using an affinity biosensor.[40] In this method, G-quadruplex DNA was immobilized on a sensing chip, and the G-quadruplex was unwound by injection of the SV40 large T-antigen helicase. After the helicase was removed, a single-stranded DNA binding protein (SSB) was subsequently injected to detect the unwound DNA. Since the SSB only recognizes relaxed DNA, the amount of G-quadruplex unwound was reflected by the amount of SSB bound to the substrate. This method is not real-time, but it is an end-point detection. On the other hand, the unwound DNA may refold back into a G-quadruplex during the interval of the two injections, thus leading to underestimation of unwinding. The fluorescence method we adopted is dependent on a G-quadruplex−hairpin conversion induced by the unwinding of G-quadruplex and utilizes the phenomenon of fluorescence quenching that occurs upon DNA hybridization via the interaction between fluorescent dye and nucleobase.[43,44] The assay is simple, sensitive, quantitative, real-time, and fast, which can be done in around one minute. We expect that the method will provide a convenient tool for analyzing the unwinding of intramolecular G-quadruplex structures. Fluorescence resonance energy transfer (FRET) is another fluorescent method that has been used to analyze the folding/unfolding of the intramolecular G-quadruplex by proteins[56,57] that is analogous to the unwinding of the intramolecular G-quadruplex. This technique uses dual fluorescent labeling to monitor the change of the distance between the two fluorophores associated with DNA folding/unfolding. Therefore, it may also be adopted to analyze the unwinding of intramolecular G-quadruplex structures.

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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