G4 Resolvase 1 Binds Both DNA and RNA Tetramolecular Quadruplex with High Affinity and Is the Major Source of Tetramolecular Quadruplex G4-DNA and G4-RNA Resolving Activity in HeLa Cell Lysates*

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Quadruplex structures that result from stacking of guanine quartets in nucleic acids possess such thermodynamic stability that their resolution in vivo is likely to require specific recognition by specialized enzymes. We previously identified the major tetramolecular quadruplex DNA resolving activity in HeLa cell lysates as the gene product of DHX36 (Vaughn, J. P., Creacy, S. D., Routh, E. D., Joyner-Butt, C., Jenkins, G. S., Pauli, S., Nagamine, Y., and Akman, S. A. (2005) J. Biol Chem. 280, 38117–38120), naming the enzyme G4 Resolvase 1 (G4R1). G4R1 is also known as RHAU, an RNA helicase associated with the AU-rich sequence of mRNAs. We now show that G4R1/RHAU binds to and resolves tetramolecular RNA quadruplex as well as tetramolecular DNA quadruplex structures. The apparent Kd values of G4R1/RHAU for tetramolecular RNA quadruplex and tetramolecular DNA quadruplex were exceptionally low: 39 ± 6 and 77 ± 6 pm, respectively, as measured by gel mobility shift assay. In competition studies tetramolecular RNA quadruplex structures inhibited tetramolecular DNA quadruplex structure resolution by G4R1/RHAU more efficiently than tetramolecular DNA quadruplex structures inhibited tetramolecular RNA quadruplex structure resolution. Down-regulation of G4R1/RHAU in HeLa T-REx cells by doxycycline-inducible short hairpin RNA caused an 8-fold loss of RNA and DNA tetramolecular quadruplex resolution, consistent with G4R1/RHAU representing the major tetramolecular quadruplex helicase activity for both RNA and DNA structures in HeLa cells. This study demonstrates for the first time the RNA quadruplex resolving enzymatic activity associated with G4R1/RHAU and its exceptional binding affinity, suggesting a potential novel role for G4R1/RHAU in targeting in vivo RNA quadruplex structures.

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moter regions of the human genome are enriched in quadruplex forming sequences (8) as well as the first intron region of many genes, especially at a region about 200 bases downstream of the start of transcription (9). Interestingly, sequence analysis has also shown that within the human genome the potential of quadruplex formation is significantly higher in oncogenes and lower in tumor suppressor genes (10).

In addition to these theoretical studies that highlight the prevalence of sequences with potential to form quadruplex structures, a number of empiric studies directly show long lived quadruplex structures in vivo. Some of this body of work includes: development of quadruplex specific antibodies that have detected quadruplex structures in the telomeres of the ciliate macronucleus of *Stylonychia lemnæ* (11, 12), the demonstration of a quadruplex binding-dependent fluorescence emission wavelength shift of a carbazole derivative in the telomeres of human chromosomes (13), and the construction of an inducible transcript in *Escherichia coli* incorporating the mammalian immunoglobin Sµ and Sγ3 switch regions that forms a cleavable quadruplex structure in the G-rich nontemplate strand of the gene upon induction of transcription (14). More indirect empiric evidence for the existence of quadruplex structures in control regions is found in a number of specific genetic control elements that readily form quadruplex structures in *vitro* under physiological salt conditions including: the aforementioned telomeres (15, 16), the immunoglobulin heavy chain switch region (3), guanine-rich regions of ribosomal DNA (17), the d(pCGG) repeats of the fragile-X mental retardation gene (18), and the promoters of a number of proliferation-associated genes such as c-MYC (19, 20), PDGF-A (21, 22), *RET* (22), and the diabetes susceptibility locus (23). In addition, a promoter region in the human insulin gene can form a quadruplex structure in *vitro* capable of specifically binding insulin suggesting a quadruplex based feedback expression loop (24).

In RNA the 5’ untranslated region of a number of proto-oncogenes contain quadruplex forming motifs including *NRAS*, *BCL2*, *JUN*, and *FGR* (25). In the case of *NRAS*, 5’ untranslated region mutations that disrupt the ability of the transcript to form quadruplex structures appear to increase translation over 3-fold, suggesting that the 5’ untranslated region quadruplex has a function in inhibiting translation of certain mRNAs (25). There is also evidence that preRNA termination regions have G-rich regions capable of forming quadruplex structures and possibly effect usage of alternative polyadenylation sites (26).

The predicted biological problems of blockage of replication, translation, and perhaps RNA processing through the formation of highly stable quadruplex structures led us and others to search for enzymatic quadruplex resolvases. RecQ family proteins BLM and WRN have been shown to possess DNA quadruplex resolving activity (18, 27) as has recently the *dog-J* homolog DEXH helicase FANCJ (28) whose loss is associated with familial breast cancer and Fanconi anemia. We took a biochemical approach to detect and fractionate quadruplex G4-DNA resolving activity, and initially we characterized a detectable human G4-DNA resolvase activity that was NTP-dependent and free of nuclease activity (29). Subsequently, through using an affinity chromatography/proteomics approach, we identified the major tetramolecular DNA resolvase in HeLa cells as the DHX36 gene product, naming the protein G4R1* for G4 Resolvase 1 (30). This protein was previously isolated for its affinity to AU-rich mRNA, possibly through interaction with the protein PARN, and termed RHAU (RNA helicase associated with AU-rich elements (31)). Therefore, we now refer to this protein by the double acronym G4R1/RHAU. Further work has shown that G4R1/RHAU can co-localize in nuclei with and co-immunoprecipitate with the RNA processing helicases p68 and p72 (32).

These observations led us to consider that the G4-RNA quadruplex may be a target of G4R1/RHAU activity. Logically, there should be great opportunity for quadruplex structures to form in RNA. RNA quadruplexes have been found to be more stable than their cognate DNA quadruplexes of the same sequence and structure under physiological salt conditions (33). RNA molecules are largely single-stranded for extended periods of time, allowing the kinetic potential for the formation of bimolecular and tetramolecular quadruplex structures. In addition, complementary strand hybridization does not compete for single-stranded RNA as it does for DNA quadruplex structures formed from duplex DNA.

In this study we demonstrate and characterize a tetramolecular RNA quadruplex resolvase activity associated with G4R1/RHAU for the first time. In addition, we determine that G4R1/RHAU has a very tight binding constant for tetramolecular quadruplex structures of both RNA and DNA. The robust resolution of RNA quadruplex by G4R1/RHAU may suggest that removal of quadruplex in RNA is an important part of RNA processing in cells.

**EXPERIMENTAL PROCEDURES**

**G4-Nucleic Acid Formation.—**Tetramolecular G4-quadruplex DNA and RNA were formed using an identical procedure and are herein referred to as “dAGA” or “rAGA.” The oligonucleotides used were of the identical base sequence whether of DNA or RNA: AAAAA AAAAA AAAAA AAAAA AAAAA AAAAA. Oligoribonucleotides (unlabeled or 5’-TAMRA*-labeled) were purchased from Dharmacon Research and oligodeoxyribonucleotides (unlabeled or 5’-TAMRA*-labeled) were purchased from either Oligos Etc. or Integrated DNA Technologies. Oligoribonucleotides were dissolved at a concentration of 0.5 mM in RNase-free 10 mM Tris-HCl, 1 mM EDTA, pH 7.5. Oligoribonucleotides were deprotected according to the manufacturer’s instructions and dissolved in RNase-free 10 mM Tris-HCl, 1 mM EDTA, pH 7.5, at a concentration of 0.5 mM. Oligonucleotide solutions were aliquoted into PCR tubes and incubated in a thermocycler (Eppendorf epGradient S) at 98 °C for 10 min, then held at 80 °C. Immediately the tubes were opened and EDTA, pH 8, was added to a final concentration of 25 mM. The tubes were reclosed and allowed to come slowly to room temperature. Aliquots were combined and stored at 4 °C for 2–3 days.

4 The abbreviations used are: G4R1, G4 resolvase 1; GSPB, G4-DNA streptavidin paramagnetic beads; RHAU, DHX36 gene product from RNA helicase associated with AU-rich element of urokine mRNA (H≥N), 11 repeats of a nucleotide sequence, where H = C, A, or T/U, and N = A, C, G, or T/U; TAMRA, 5,6-carboxytetramethylrhodamine; shRNA, short hairpin RNA; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.
days. PAGE analysis of the annealed oligonucleotides indicated that this annealing procedure resulted in over 99% conversion of monomer to tetramolecular quadruplex (data not shown). G4 nucleic acids formed from 5′-TAMRA-labeled oligonucleotides were further purified by electrophoresis on a 10% polyacrylamide gel and band excision. Electrophoretic bands containing G4 nucleic acids were recovered by electroelution in a Schleicher and Schuell Elutrap with 1× TBE buffer additionally containing 3 mM KCl. The Elutrap was run with buffer recirculation. 5′-TAMRA-labeled G4 nucleic acids were aliquoted and stored at −20 °C or lower.

5′-[32P]-End Labeling of G4 Nucleic Acids—5′-[32P]-End-labeled G4 oligonucleotides were obtained by incubating unlabeled G4 oligonucleotides annealed as described above with T4 polynucleotide kinase (Promega Corp.) and [γ-32P]ATP for 0.5 h at 37 °C, according to the manufacturer’s instructions. 5′-[32P]-Labeled G4 oligonucleotides were purified with a MicroSpin G25 column (GE Healthcare) equilibrated with TEK buffer (10 mM Tris, 1 mM EDTA, and 50 mM KCl) and stored at 20 °C.

Production and Purification of Recombinant G4R1/RHAU—G4-DNA bound streptavidin paramagnetic beads (GSPB) were prepared as described previously (30). E. coli strain Rosetta 2 (Novagen) was transformed with TriEx-4 DHX36 (30) expression plasmids. Cultures were grown to a density of A600 = 0.4 – 0.6 at 37 °C in a Brunswick shaker incubator. Cultures of 500 ml were induced with 1 mM isopropyl β-D-galactopyranoside at 4 °C and grown overnight at 14 °C with gentle agitation. Recombinant G4R1/RHAU protein was initially purified by means of a His6 tag by utilizing the TALON cobalt beads and xTractor kit according to manufacturer’s (Clontech) instructions with 2× Sigma protease inhibitor mixture and 15 μg/ml leupeptin added. Rosetta 2 cell lysates were isolated and bound to TALON cobalt (0.5 ml bead volume per 500 ml of E. coli culture) resin as recommended by the manufacturer. Cobalt resin was washed three times with ice-cold SSC (4×) with β-mercaptoethanol (0.5 μl/ml). Recombinant protein was eluted from resin with three washes of 0.5 ml of histidine elution buffer (0.7 M histidine, pH 6.0, 8.6 mM β-mercaptoethanol, 1× Sigma protease inhibitor mixture), followed by one 0.5-ml wash of 200 mM EDTA, pH 6.0. For the second phase of purification, the four eluates were combined with 1 ml (3 ml total) of 3× Res buffer (1×, 50 mM Tris acetate, pH 7.8, 50 mM NaCl, 70 mM glycine, 0.5 mM MgCl2, 0.012% bovine α-lactalbumin, 1× Sigma protease inhibitor mixture, 10% glycerol) and bound to GSPB at 37 °C for 15 min. Bound GSPB were washed twice in ice-cold SSC (4×) with 0.1% α-lactalbumin and 0.5 μl/ml β-mercaptoethanol. High purity recombinant His-tagged DHX36 protein (10,000 units/μg, units defined previously (29)) was obtained by ATP-dependent elution of GSPB as described previously (30), except bovine α-lactalbumin and Sigma protease inhibitor mixture were added to the elution buffer. Purified enzyme was stored at −80 °C.

Electrophoretic Mobility Shift (Bandshift) Assays and Apparent Kd Determination—The apparent Kd of G4R1/RHAU bound to tetramolecular G4-DNA or -RNA was estimated by bandshift assay. Estimation of recombinant G4R1/RHAU concentration was performed on 4–12% SDS-PAGE (Invitrogen) and Coomassie Blue stain (ProtoBlue Safe, National Diagnostics). A dilution series of recombinant G4R1/RHAU was loaded next to a dilution series of protein standards (Promega, V849A). Gels were scanned on an Epson 2450 flatbed scanner using transmissive mode and Silverfast (version 6) TWAIN. Band densities were analyzed using Multi Gauge (Fuji) software. Recombinant G4R1/RHAU at concentrations of 10–300 pM was incubated with 1 pM 5′-32P-labeled G4 nucleic acid in K-Res buffer with 10 mM EDTA at 37 °C for 30 min. Binding mixtures were then loaded as is (monitored visually by Schlieren lines) and analyzed by 10% non-denaturing PAGE. Electrophoresis was performed at 70 volts for 10 h in a cold room (7 °C). Gels were imaged on a Typhoon 9210 Imager (GE Healthcare). Band densities were analyzed using Multi Gauge (Fuji) software and statistical analysis with MS Excel. Linear regression models were fit to the data points corresponding to at least 50 and 50 pm for percent bound substrate. The apparent Kd value was estimated from each model at 50% bound substrate for four experiments, separately for RNA and DNA. A two-sided t test was used to compare the average apparent Kd values for RNA and DNA. A two-sided p value <0.05 was considered to be statistically significant.

G4-DNA and G4-RNA Competition Assay—Resolvase activity was determined as previously described (30) but in a modified RES buffer (“K-Res,” 100 mM KCl, 10 mM NaCl, 3 mM MgCl2, 50 mM Tris acetate, pH 7.8, 70 mM glycine, 0.012% bovine α-lactalbumin, 10% glycerol). 0.2 pmol of 5′-end-labeled quadruplex (32P or TAMRA) were included per 50-μl reaction. Reactions were allowed to proceed at 37 °C for 30 min, stopped by addition of 5 μl of 200 mM EDTA, and analyzed by electrophoresis through a 10% non-denaturing polyacrylamide TBE gel with 10% glycerol. G4 nucleic acids used in competition experiments included tetramolecular dAGA and rAGA quadruplexes and E. coli tRNA (Sigma). Competitor nucleic acid concentrations ranged from a 128-fold molar excess of competitor to labeled tetramolecular quadruplex DNA substrate down to an equimolar competitor to substrate ratio. In a half-volume reaction mixture (25 μl), 2× competitor nucleic acids were mixed with 0.2 pmol of TAMRA-labeled G4 nucleic acid substrate on ice. Next, 25 μl of reaction mixture containing 1 unit of recombinant G4R1/RHAU was added (50 μl total reaction volume) at 4 °C, then reaction mixtures were incubated at 37 °C for 30 min, dropped to 4 °C, and stopped with 5 μl of 200 mM EDTA. Gels were scanned on a Typhoon 9210 Imager (GE Healthcare) and images were analyzed using FUJI Multi Gauge version 3.0 imaging software. Statistical analysis was done in Microsoft Excel 2003 and standard deviations were calculated by the STDEV function.

Development of Tetracycline Inducible shRNA for G4R1/RHAU Down-regulation—The T-REx™ HeLa cell line (pTER-HAUG25) that expresses G4R1/RHAU-targeting shRNA molecules under doxycycline control was described previously (32). Cells were grown in Dulbecco’s modified Eagle’s medium + 10% fetal bovine serum, 1% penicillin/streptomycin, 2 mM L-glutamine, 450 μg/ml Zeocin, and 3 μg/ml blasticidin in T-75 filter-cap culture flasks. At ~60–70% confluency, doxycycline (1 μg/ml final) was added daily with fresh media for 5
days. Cells were scraped in cold phosphate-buffered saline, collected in 1.5-ml microtubes, and centrifuged into pellets. The pellets were frozen by storage at −80 °C.

Assay of G4 Nucleic Acid Resolving Activity from HeLa Cells Expressing G4R1/RHAU shRNA—T-REX™ HeLa cell extracts were prepared as reported previously (30). Briefly, cell pellets were thawed and manually homogenized on ice in an equal volume of cold 2× Lysis buffer (100 mM Tris acetate, pH 7.8, 0.4 mM EDTA, 40 mM β-mercaptoethanol, 0.02% Triton X-100, 20% glycerol, 50 μg/ml leupeptin, 1× Sigma protease inhibitor mixture) with a microtube pestle (USA Scientific, 1415–5390). Cell particulates were removed by centrifugation (Eppendorf microcentrifuge, 14 krpm for 5 min). Whole cell extracts were aliquoted and stored at −80 °C. Extracts were assayed for G4 nucleic acid resolving activity as described above in K-Res buffer supplemented with 1/100th reaction volume of nuclease resolving activity as described above in K-Res Multi Gauge (Fuji) software and data analyzed with MS Excel.

Western Blotting of HeLa Cell Extracts—Western blotting was done by standard methods. Briefly, 10 μg each of HeLa pTER-RHAU25 whole cell extracts were resolved by SDS-PAGE on 4–12% BisTris Invitrogen gradient gels and transferred to Hybond P (polyvinylidene difluoride, Amersham Biosciences). Blots were blocked with 5% (Carnation Instant Nonfat Dry) milk, TBST (0.1% Tween) and probed with RHAU monoclonal antibody 12F33 (30) at 1:15,000 dilution in 2% milk TBST and β-actin monoclonal antibody at 1:10,000 dilution (Sigma A5441) in 5% milk TBST. Bio-Rad goat anti-mouse IgG (H+L)-horseradish peroxidase conjugate was used as the secondary antibody at concentrations ranging from 1:7,500 to 1:10,000 in 2–5% milk in TBST. Membranes were activated for chemiluminescence by ECL-plus (Amersham Biosciences) and exposed to Kodak Biomax Light film. Films were scanned on an Epson (2450) flatbed scanner using transmissive mode and Silsoft (version 6) twain. Band densities were measured using Multi Gauge (Fuji) software and data analyzed with MS Excel.

RESULTS

G4R1/RHAU Displays Robust Resolving Activity on Both RNA and DNA Tetramolecular Quadruplex Substrates—Our previous work showed that recombinant G4R1/RHAU protein resolves tetramolecular DNA quadruplex efficiently and specifically (30). We have previously defined 1 unit of G4R1 resolvase activity as that amount that resolves 50% of 0.2 pmol of tetramolecular DNA quadruplex DNA in 30 min at 37 °C in 30 μl of reaction buffer (29). Fig. 1 compares resolution of both RNA and DNA tetramolecular quadruplex substrates for a range of G4R1/RHAU concentrations under standard reaction conditions. Nondenaturing gel electrophoresis of reaction contents demonstrated G4R1/RHAU concentration-dependent resolution of both RNA and DNA quadruplex structures into single strands. At concentrations of greater than 1 unit/reaction, G4R1/RHAU catalyzed complete resolution of both RNA and DNA tetramolecular quadruplex substrates 5’-end-labeled with either [32P] (Fig. 1, A and C) or the fluorescent molecule TAMRA (Fig. 1, B and D) into single strands. One unit of G4R1/RHAU resolved slightly more 32P-labeled quadruplex DNA than quadruplex RNA substrate (compare Fig. 1A, lane 4 with C, lane 4), whereas 1 unit of G4R1/RHAU resolved a similar amount of TAMRA-labeled quadruplex DNA and RNA (see Fig. 1, B lane 3 and D, lane 3). G4R1/RHAU concentrations less than 1 unit/reaction catalyzed less than 50% resolution of tetramolecular DNA or RNA quadruplex substrates as expected. These data demonstrate that G4R1/RHAU has robust resolving activity upon RNA quadruplex substrates in addition to DNA quadruplex substrates. Moreover, these data indicate that there is little difference in G4R1/RHAU-catalyzed resolving activity on tetramolecular quadruplex RNA and DNA substrates under these experimental conditions.

G4R1/RHAU Displays a Tight Binding Affinity for Both DNA Quadruplex and RNA Quadruplex—The ability of G4R1/RHAU to bind quadruplex RNA and DNA structures was measured by gel mobility shift assays. In previous studies using tetramolecular G4-DNA affinity beads to isolate G4R1/RHAU, we found that addition of EDTA inhibited the resolution, but not the binding of enzyme to quadruplex nucleic acids (32). Therefore, binding could be studied in the presence of EDTA without resolution of substrate. Under these conditions, binding reactions were performed with increasing concentrations of G4R1/RHAU protein against a fixed concentration of 32P-labeled tetramolecular DNA quadruplex or tetramolecular RNA quadruplex. Fig. 2A shows the ability of G4R1/RHAU to bind
**Fig. 2C** represents a similar titration of a range of G4R1/RHAU protein concentrations versus a fixed concentration of $^{32}$P-labeled RNA quadruplex. There are striking differences in the binding of G4R1/RHAU to RNA quadruplex substrate compared with DNA quadruplex substrate of the identical sequence. At 50 pm G4R1/RHAU (Fig. 2C, lane 3) three distinct shifted bands appear, likely representing a monomer and two multimers of G4R1/RHAU protein molecules bound to the RNA quadruplex. However, only a single major binding species of G4R1/RHAU-nucleic acid complex was observed upon binding to DNA quadruplex of the identical sequence (compare Fig. 2, A, lanes 4–7 with C, lanes 3–6). This observation may suggest that, upon binding to RNA quadruplex, binding of G4R1/RHAU to itself is induced or stabilized. Alternatively, there may be three separate recognizable binding sites of similar binding affinities for G4R1/RHAU on RNA quadruplex but only one on DNA quadruplex structures. Assuming that multiple binding species of G4R1/RHAU-nucleic acid complexes did not affect the binding affinity, apparent $K_a$ was determined as the concentration of G4R1/RHAU protein at which 50% of the RNA was bound (Fig. 2E). The apparent $K_a$ of G4R1/RHAU binding to quadruplex RNA is 39 ± 6 pm ($n = 4$ independent experiments), significantly lower than the apparent $K_a$ of G4R1/RHAU bound to quadruplex DNA ($p = 0.0001$). There was little detectable binding of G4R1/RHAU to the single-stranded 33-base random sequence oligoribonucleotide r(HHN)$_{11}$ at G4R1/RHAU protein concentrations over 75-fold greater than the apparent $K_a$ for quadruplex RNA binding (Fig. 2D). In summary, these gel mobility shift studies show that G4R1/RHAU binds both DNA and RNA quadruplex with high affinity; however, G4R1/RHAU binds tetramolecular RNA quadruplex with a somewhat greater affinity than DNA quadruplex. Furthermore, multiple species of G4R1/RHAU and nucleic acids form upon binding to RNA quadruplex.

**G4R1/RHAU Is Selective for Quadruplex Structures as Substrates and Has a Preference of RNA Quadruplex Over DNA Quadruplex in Competition Studies**—Fig. 3 demonstrates inhibition of DNA quadruplex resolution in the presence of increasing molar excess of potential substrates that are unlabeled. We have previously shown that 300-fold excesses of Y form DNA, 5’-duplex DNA, or 3’ duplex DNA were required to inhibit resolution of 5’-$^{32}$P-labeled tetramolecular G4-DNA quadruplex as effectively as equimolar unlabeled G4-DNA (30). To further characterize the ability of unlabeled substrates to inhibit 5’-$^{32}$P-labeled tetramolecular G4-DNA resolution catalyzed by G4R1/RHAU, G4-DNA resolution reactions were titrated with various molar excesses of unlabeled G4-DNA, G4-RNA, or tRNA. Resolution of 5’-$^{32}$P-labeled tetramolecular G4-DNA in the presence of increasing molar concentrations of these competitors was monitored by nonnucleating gel electrophoresis (Fig. 3A). Fig. 3B shows the graphical representation of the mean values of three separate experiments. Addition of equimolar unlabeled G4-DNA inhibited resolution of 5’-$^{32}$P-labeled tetramolecular G4 DNA quadruplex by a lower range value of 60%, near the 50% inhibition expected. This “competition” is due to a dilution of the labeled substrate by equimolar unlabeled identical substrate. Interestingly, tetramolecular G4-RNA of the identical sequence and structure as that of sub-

**Increasing amounts of labeled tetramolecular quadruplex DNA with increasing concentrations of protein. In the presence of 70 pm G4R1/RHAU protein (lane 4) a clear mobility shift occurs, shifting about 50% of the quadruplex DNA.**

**GRAPHIC REPRESENTATION OF DATA FROM FOUR INDEPENDENT GEL MOBILITY SHIFT EXPERIMENTS SHOWING AN ORBIT OF HN MODELS OF CENTRAL SUBSTRATE AND AN ABSCISSA OF pm CONCENTRATION OF G4R1/RHAU.**

**Error bars represent S.D.**

**FIGURE 2. Equilibrium binding gel mobility shift assay of purified recombinant G4R1/RHAU incubated with tetramolecular quadruplex DNA and tetramolecular quadruplex RNA substrates yields apparent $K_a$ values of 77 ± 6 and 39 ± 6 pm, respectively. A, lanes 1–7 show a gel mobility shift assay with increasing concentrations of G4R1/RHAU, 0, 30, 50, 75, 100, and 150 pm incubated with 1 pm 5’-$^{32}$P-labeled DNA quadruplex. Lanes 3–7 show one major protein-quadruplex DNA species shifted to a higher molecular weight. B, lanes 1–7 show the mobility shift assay for single-stranded DNA of the randomer d(HHN)$_{11}$, at concentrations of G4R1/RHAU of 0, 100, 150, 300, 500, 1000, and 3000 pm, respectively. C, lanes 1–7 show a gel mobility shift assay of 1 pm 5’-$^{32}$P-labeled tetramolecular RNA incubated with increasing concentrations of G4R1/RHAU, 0, 30, 50, 75, 100, and 150 pm. Three different protein-quadruplex RNA species are seen shifted to higher molecular weights. D, lanes 1–7 show the mobility shift assay for single-stranded RNA of the randomer r(HHN)$_{11}$, at concentrations of G4R1/RHAU of 0, 100, 150, 300, 500, 1000, and 3000 pm, respectively. E, graphic representation of data from four independent gel mobility shift experiments showing an ordinate of percent bound substrate and an abscissa of pm concentration of G4R1/RHAU. Error bars represent S.D.**
strate G4-DNA inhibited G4R1/RHAU-catalyzed resolution of 5'-32P-labeled tetramolecular G4-DNA more effectively than did unlabeled tetramolecular G4-DNA over a large range of competitor molar excess. However, 100-fold molar excess of tRNA did not inhibit the reaction by more than 20%. tRNA was chosen as a nonspecific control inhibitor because it contains double-stranded, single-stranded, and Y-form fork structures.

Considering that the experimental results illustrated in Fig. 3 suggested that G4-RNA is a better substrate for G4R1/RHAU, inhibition of G4R1/RHAU-catalyzed resolution of 5'-32P-labeled tetramolecular G4-RNA by G4-DNA or tRNA was monitored by nondenaturing gel electrophoresis (Fig. 4A). One representative titration of each competitor is shown in Fig. 4A. Fig. 4B shows a graphical representation of mean values of three separate experiments. As expected, equimolar unlabeled G4-RNA substrate inhibited G4R1/RHAU-catalyzed resolution of 5'-32P-labeled tetramolecular G4-RNA by about 50%. This competition can also be viewed as a dilution of the labeled substrate by equimolar unlabeled substrate. However, 64-fold molar excess of tRNA caused only a modest inhibition of the resolution of the G4-RNA. Unlabeled tetramolecular G4-DNA was consistently less effective at inhibiting G4-RNA resolution than was unlabeled G4-RNA itself. These inhibition experiments suggest that tetramolecular G4-RNA is a preferred substrate of G4R1/RHAU as compared with tetramolecular G4-DNA of an identical sequence. However, both G4-DNA and G4-RNA are readily resolved by G4R1/RHAU.

Down-regulation of G4R1/RHAU by Doxycycline-inducible shRNA Significantly Reduces the G4 Nucleic Acid Resolving Activity of HeLa Cell Lysates on Tetramolecular DNA Quadruplex and Tetramolecular RNA Quadruplex Substrates. Consistent with G4R1/RHAU being the Major Tetramolecular Quadruplex Resolvase Activity—Previous work had indicated that immunodepletion of G4R1/RHAU from HeLa cell lysates significantly reduced detectable tetramolecular G4-DNA resolving activity, suggesting that G4R1/RHAU is the major tetramolecular G4-DNA resolvase in HeLa cells (30). The ability of RNA interference technology to specifically inhibit protein expression suggests a more direct way to test how much of the total G4-DNA resolvase activity in cell lysates may be attributable to G4R1/RHAU activity. Furthermore, considering that the data presented above indicate that tetramolecular G4-RNA is also a substrate of G4R1/RHAU, the question arose regarding how much of the cellular tetramolecular G4-RNA resolving activity present in HeLa cell lysates is attributable to G4R1/RHAU enzymatic activity.

To address this we utilized T-REx-HeLa cells stably transfected with doxycycline-inducible shRNA against G4R1/RHAU to down-regulate endogenous G4R1/RHAU protein levels (32). Fig. 5A illustrates a Western blot of extracted proteins from a T-REx-HeLa cell line with and without doxycycline induction probed with monoclonal antibodies for G4R1/RHAU and actin. In the pres-
FIGURE 5. HeLa cells with a doxycycline (dox)-inducible shRNA to G4R1/RHAU show loss of the majority of quadruplex resolving activity for both DNA and RNA tetramolecular quadruplex upon down-regulation of G4R1/RHAU by shRNA. A. lanes 1–3 of a Western blot of identical loadings of 10 μg of lysate from HeLa cells not induced (−dox) for shRNA show high levels of G4R1/RHAU when probed with a G4R1/RHAU monoclonal antibody. Lanes 4–6 show identical loadings of 10 μg of protein after 5 days of induction for shRNA (+(+)dox); G4R1/RHAU is substantially down-regulated with dox treatment. Actin levels were also detected by monoclonal antibody and were used as a loading control. B, nondenaturing gel electrophoresis of tetramolecular quadruplex DNA incubated with increasing concentrations of HeLa lysates from (−)dox and (+(+)dox) lysates for 30 min at 37°C. Lane 1 shows the effect of no lysate. Lanes 2–5 show results for 1.5, 3.0, 6.0, and 12.0 μg of (−)dox lysate, respectively. Lanes 6–9 show results for (+(+)dox) lysates for 1.5, 3.0, 6.0, and 12.0 μg, respectively. C, nondenaturing gel electrophoresis of tetramolecular quadruplex RNA incubated with increasing concentrations of HeLa lysates from (−)dox and (+(+)dox) lysates for 30 min at 37°C. Lane 1 shows the effect of no lysate. Lanes 2–5 show results for 1.5, 3.0, 6.0, and 12.0 μg of (−)dox lysate, respectively. Lanes 6–9 show results for (+(+)dox) lysates for 1.5, 3.0, 6.0, and 12.0 μg of (−)dox lysate, respectively. (+(+)dox) lysates show diminished resolution activity compared with (−)dox lysates of the identical protein concentration. The reduction in tetramolecular quadruplex resolving activity was 8-fold for both RNA (S.D. = 0.30) and DNA (S.D. = 0.21) for (+(+)dox lysates measured at 1.5, 3.0, and 6.0 μg of protein.

dege of doxycycline induction, the endogenous G4R1/RHAU protein is diminished substantially.

Fig. 5B demonstrates total tetramolecular G4-DNA resolvasse activity with and without doxycycline induction for different amounts of HeLa cell extract. Tetramolecular G4-DNA resolvase activity in the G4R1/RHAU knockdown (+dox) is reduced 8-fold compared with the activity present in HeLa cells containing wild-type levels of G4R1/RHAU. This result is consistent with G4R1/RHAU being the major tetramolecular G4-DNA resolvase activity in HeLa cells. Fig. 5C illustrates tetramolecular G4-RNA resolving activity present in HeLa cell lysates from cells with wild-type and decreased levels of G4R1/RHAU.

DISCUSSION

Guanine is a uniquely problematic base due to its propensity to spontaneously self-assemble into Hoogsteen-bonded guanine quartets that are thermodynamically very stable. Yet the genome and transcriptome are replete with potential quadruplex forming sequences that contain runs of three or more guanines (5–7). These sequences form DNA or RNA quadruplexes under physiological salt conditions and have high melting temperatures that human cells could not endure. Quadruplex structures are expected to form in cells under physiological conditions at least in a stochastic manner and these bulky complexes have been shown in vitro to impede normal DNA and RNA metabolism (25, 34). Thus, an essential cellular problem emerges with quadruplex formation in vivo impeding normal metabolism and it invites an enzymatic solution by specific G4-nucleic acid resolvases.

In this regard, G4-DNA resolvase activity catalyzed by several human enzymes, e.g. BLM and WRN, and dog-1 human ortholog FANCJ helicase (18, 27, 28) has been demonstrated in vitro. An affinity chromatography/proteomics approach allowed us to identify the DHX36 gene product as a tetramolecular G4-DNA resolvase (30) designated as a G4 resolvase 1 (G4R1/RHAU). Sequence analysis showed that this enzyme is a DEHX/D box helicase family member whose closest BLAST homolog (that has a characterized function) is nuclear DNA helicase II (NDH II), also a known RNA helicase (RNA helicase A). NDH II has biological roles in both DNA and RNA metabolism (see Ref. 35 for review). Additionally, G4R1/RHAU had been previously identified in chromatographic fractions of HeLa nuclear extracts that associated with the AU-rich element derived from urokinase mRNA (31). These perspectives on G4R1/RHAU led us to consider that G4R1/RHAU might not only be capable of resolving tetramolecular G4-DNA, but may have a broader range of enzymatic activity including resolution of G4 structures occurring in both RNA and DNA. In this study, we asked the essential questions of whether and how well G4R1/RHAU recognizes tetramolecular quadruplex RNA as a enzymatic substrate and whether G4R1/RHAU has a low and specific equilibrium dissociation constant for both RNA and DNA quadruplex structures. Affirmative answers to these questions would suggest that G4R1/RHAU may have a pivotal role in the resolution of quadruplex structures formed in both DNA and RNA in vivo.

Our results suggest that G4R1/RHAU is efficient at binding and resolving both RNA and DNA tetramolecular quadruplex. In rapidly proliferating cells such as HeLa, the G4R1/RHAU protein is expressed abundantly (30), and it is found in both the cytoplasm and the nucleus (31). These qualities, along with a low and specific $K_d$ are those expected of a comprehensive quadruplex nucleic acid surveillance protein capable of quick
removal of quadruplex “knots” allowing smooth metabolic processing of G-rich RNAs and DNAs.

Of particular interest is the extraordinarily low apparent $K_d$ values of 39 ± 6 and 77 ± 6 pm of G4R1/RHAU binding for quadruplex RNA and DNA, respectively. To our knowledge, only one protein in the literature has been reported with a similarly low binding constant for quadruplex structures: the mouse ortholog mXRN1p of the yeast Xrn1 or Kem1 protein. mXRN1p is one of only two 5’ to 3’ exonuclease found in RNA turnover and is of key importance for RNA metabolism. mXRN1p has a reported apparent $K_d$ of 33.8 pm for RNA quadruplex and 79.4 pm for DNA quadruplex (36). Reported apparent $K_d$ values for other mammalian quadruplex binding proteins climb very quickly, with LR1, a B cell-specific duplex binding factor, having a reported apparent $K_d$ of 250 pm for quadruplex DNA (37), and with nucleolin having a reported apparent $K_d$ of 1 nm for quadruplex DNA (17).

The tetramolecular quadruplex RNA and DNA binding constants found for G4R1/RHAU of 39 and 77 pm, respectively, coupled with the high concentrations of G4R1/RHAU protein found in rapidly growing cells such as HeLa cells (30), suggests that quadruplex structures may be rapidly bound and resolved by G4R1/RHAU. If, however, G4R1/RHAU is sequestered or deprived of cofactors necessary for resolution, but not binding, of G4 nucleic acids (e.g. Mg$^{2+}$ or ATP) it is possible that G4R1/RHAU could bind G4 nucleic acids for extended time periods. This may be a useful function of G4R1/RHAU, binding and moving quadruplex RNA structures to cellular rendezvous points such as endosomes. G4R1/RHAU could also avidly bind to G4 sites in DNA and attract other binding partner proteins to the marked sites.

We have previously shown that a number of different tetramolecular DNA quadruplex structures compete effectively for G4R1/RHAU enzymatic activity (30). However, in this study we observed that not only does tetramolecular RNA quadruplex serve as an excellent substrate for G4R1/RHAU, but it can compete more effectively for enzymatic activity in competition assays than can tetramolecular DNA quadruplex of the same sequence. Furthermore, G4R1/RHAU uniquely forms multimers of the enzyme and nucleic acid upon binding RNA quadruplex structures. By this means, RNA quadruplex structures might deplete more G4R1/RHAU protein from a limited pool available for DNA quadruplex binding and resolution. Considering that cellular RNA is largely single-stranded, has selected sequences of greater concentration than DNA, and generally can form more stable quadruplex than DNA quadruplex of the same sequence, it is compelling to suggest that quadruplex RNA structures are likely to be more abundant in the cell than are DNA quadruplex structures. Therefore, the most common in vivo substrate of G4R1/RHAU might well be RNA quadruplex structures.

Although resolution of quadruplex DNA by G4R1/RHAU may be a less common event, this activity might also be of importance for preventing genetic alterations in replicating sequences that stochastically form quadruplex DNA. In this regard G4R1/RHAU may be a candidate tumor suppressor gene, as the FANCJ DEXH helicase has proven to be in breast cancer (38). It will be of great interest to follow the replication of G-rich regions in cells down-regulated and knocked out for G4R1/RHAU and determine whether such G-rich sequences are preferentially lost over cell divisions, as was found for the Caenorhabditis elegans protein dog-1 and its mouse homolog Rtel (39, 40). It is important to note that G4R1/RHAU is unique in being both an avid binder of quadruplex and a helicase without detectable nuclease activity. G4R1/RHAU therefore might be useful in removing quadruplex structures in DNA without cleavage, a role that nucleases such as mXRN1p and WRN could not perform without damaging DNA. It is possible that the context of G4R1/RHAU action is guided by protein binding partners, and it is likely that future genetic studies of G4R1/RHAU and candidate binding partners will help identify specific sequence substrates of G4R1/RHAU in vivo.

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