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Journal

Journal of Proteome Research, 20(11)

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Publication Date

2021-11-05

DOI

10.1021/acs.jproteome.1c00603

Peer reviewed

HHS Public Access

Author manuscript

J Proteome Res. Author manuscript; available in PMC 2022 November 05.

Published in final edited form as:

J Proteome Res. 2021 November 05; 20(11): 4919–4924. doi:10.1021/acs.jproteome.1c00603.

A Quantitative Proteomic Approach for the Identification of DNA Guanine Quadruplex-Binding Proteins

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Abstract

DNA sequences of high guanine (G) content have the potential to form G quadruplex (G4) structures. A more complete understanding about the biological functions of G4 DNA requires the investigation about how these structures are recognized by proteins. Here, we conducted exhaustive quantitative proteomic experiments to profile the interaction proteomes of G4 structures by employing different sequences of G4 DNA derived from the human telomere and the promoters of c-*MYC* and c-*KIT* genes. Our results led to the identification of a number of candidate G4-interacting proteins, many of which were discovered here for the first time. These included three proteins that can bind to all three DNA G4 structures and 78 other proteins that can bind selectively to one or two of the three DNA G4 structure(s). We also validated that GRSF1 can bind directly and selectively toward G4 structure derived from the c-*MYC* promoter. Our quantitative proteomic screening also led to the identification of a number of candidate "antireader" proteins of G4 DNA. Together, we uncovered a number of cellular proteins that

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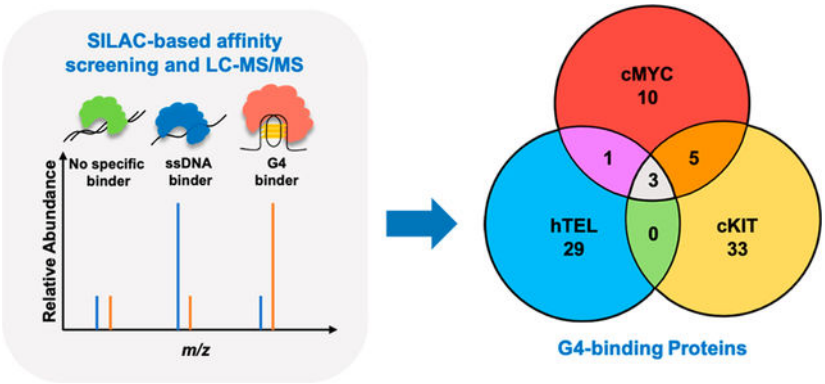
Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jproteome.1c00603.
Methods. Table S1: The DNA sequences employed for the affinity pull-down of cellular proteins that can bind to G4 DNA. Table S2: A complete list of G4-binding proteins identified from the SILAC-based interaction screening and their G4/M4 ratios. Table S3: A list of peptide ratios of c-*KIT*, c-*MYC*, and hTEL G4-binding proteins. Table S4: A complete list of G4-antireader proteins identified from the SILAC-based interaction screening and their G4/M4 ratios. Table S5: A list of peptide ratios of c-*KIT*, c-MYC and hTEL G4-antireader proteins. The mass spectrometry proteomic data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD026794 (PDF)

The authors declare no competing financial interest.

exhibit general and selective recognitions of G4 folding patterns, which underscore the complexity of G4 DNA in biology and the importance of understanding fully the G4-interaction proteome.

Graphical Abstract



Keywords

G quadruplex; quantitative proteomics; SILAC; nucleic acid-binding protein; gene regulation

INTRODUCTION

Regions of genomic DNA with contiguous runs of guanines exhibit the ability to fold into non-B-form secondary structures known as guanine quadruplexes (G4).[1] The G4 structures are assembled from multiple G-tetrads stacked upon one another, where a monovalent cation, primarily K^+ or Na^+, further stabilizes the G tetrad structure.[2]

Bioinformatic and experimental studies have revealed the widespread occurrence of G4 structures in the human genome. In this vein, computational analyses uncovered more than 300,000 putative G4-forming motifs in the human genome.[3–5] With the use of a G4 structure-specific antibody (BG4) and fluorescence microscopy analysis, Biffi et al.[6] revealed the presence of G4 structures in chromosomal DNA of human cells. Moreover, chromatin immunoprecipitation using BG4 followed by next-generation sequencing (ChIP-Seq) analyses led to the discovery of approximately 10,000 G4 structure sites in chromatin of cultured human cells.[6–8] These G4 structure sites are enriched at loci of important biological relevance and regulatory functions, including more than 2000 gene promoters and telomeric regions.[6,7]

DNA G4 structures have been shown to assume important roles in many biological processes, including DNA replication, transcription, alternative polyadenylation, and maintenance of genomic stability.[9–14] In this vein, promoter sequences with the ability to fold into G4 structures are of particular importance owing to the potential roles of these G4s in gene regulation. For instance, the nuclease hypersensitivity element III_1, which is found within the promoter of c-*MYC* oncogene and regulates 85–90% of its transcriptional activity, harbors a G4 motif.[15] Likewise, the c-*KIT* proto-oncogene contains two different G4 sequence motifs upstream to its core promoter, and these G4 structures are involved in

regulating the expression of the c-*KIT* gene.[16,17] Moreover, a recent study showed that G4 structure can remotely regulate gene expression by enabling DNA looping.[13] Apart from gene promoters, the human telomere is known to fold readily into G4 structure,[18–20] which modulates telomere integrity.[21]

Many proteins, including nucleolin, Pif1, PARP1, SLIRP, SUB1, Rif1, VEZF1, WRN, and YY1, were found to interact with G4 structures.[13,14,22–30] We reason that a better understanding about how DNA G4 structures function in gene regulation and human diseases entails a systematic investigation about how these structures are recognized by cellular proteins. Additionally, since the loop sizes and primary DNA sequence for each G4 are unique, we reason that cells may also be equipped with proteins that interact selectively with only certain G4-folding pattern(s).

In this study, we conducted an exhaustive quantitative proteomics-based interaction screening using three pairs of DNA probes that are capable or incapable of folding into G4 structures (Figure 1 and Table S1). Through these experiments, we identified more than 80 candidate G4-binding proteins (Table S2 and Figure 2). Interestingly, some of these proteins display preferential binding to all three G4 structures than their corresponding mutated sequences, whereas others interact uniquely with certain G4 structures.

RESULTS

To discover systematically novel G4-interacting proteins and to assess their binding specificities, we employed three G4 DNA probes derived from the G-rich sequences of the human telomere and the promoters of c-*KIT* and c-*MYC* genes, and these sequences were previously characterized by solution-phase NMR studies to adopt well-defined G4 foldings *in vitro*.[31–33] We also obtained the corresponding mutated probes incompetent in G4 folding.[30] The proper folding of the G4-containing probes and the inabilities of the mutant probes in G4 folding were confirmed by circular dichroism (CD) measurements, as described elsewhere.[30] In this vein, the sequences derived from the c-*KIT* and c-*MYC* promoters form parallel G4 folding topology, whereas that from the human telomere exhibits a G4 folding pattern with both parallel and antiparallel strands.[30]

Equal amounts of the heavy- and light-labeled nuclear proteomes, which were obtained from stable isotope labeling by amino acids in cell culture (SILAC),[34] were passed through streptavidin columns immobilized with biotin-conjugated G4 DNA and the corresponding mutated sequence (M4), respectively, which we designate as the reverse experiment (Figure 1). In this vein, by culturing cells in a medium in which arginine and lysine are replaced with their stable isotope-labeled counterparts, i.e., [$^{13}C_6$]-L-arginine and [$^{13}C_6$,$^{15}N_2$]-L-lysine, SILAC facilitates the labeling of the proteome with these heavy-labeled amino acids during protein synthesis. To remove potential experimental bias arising from incomplete SILAC labeling, we also conducted the forward experiment, where the light- and heavy-labeled nuclear proteomes were passed through streptavidin columns immobilized with biotin-conjugated G4 and M4 DNA probes, respectively.[35] In total, we conducted a minimum of 4 independent (2 forward and 2 reverse) SILAC-based interaction screening experiments for each pair of G4/M4 probes (Table S1).

After incubation with the nuclear protein lysate, the DNA-bound avidin beads were washed, and the proteins captured on the beads were eluted, combined, trypsin-digested, and subjected to LC-MS/MS analysis, as detailed in the Supporting Information. By performing this experiment on multiple G4 folding patterns, we could achieve a quantitative comparison about the binding selectivities of candidate proteins toward the three G4 structures.

We were able to identify many proteins exhibiting preferential binding toward G4 probes over the mutated single-stranded DNA probes (Figure 2, Tables S2–S3). We employed a stringent criterion for considering a protein to be a G4-binding protein, where the protein needs to be enriched on the G4 over the corresponding M4 probes in both forward and reverse SILAC experiments with an average G4/M4 ratio being greater than 1.5. With this criterion, we identified 41, 19, and 33 proteins that can bind preferentially to the G4 sequences derived from the promoters of c-*KIT* and c-*MYC* genes and the human telomere, respectively, over their mutant counterparts (Figure 2 and Tables S2–S3). In this context, it is worth noting that the SILAC-based proteomic approach provides a quantitative measure about relative, but not absolute, binding affinities of proteins toward G4 over the corresponding M4 probes. The method, therefore, does not offer insights into the relative binding affinities of different G4-binding proteins toward any specific G4 probe employed in this study.

Among these proteins, 11 were previously described to interact directly with G4 DNA structures, including DDX5, MAZ, NPM1, etc., where known DNA G4-binding proteins are highlighted in red in Figure 2.[36–46] Interestingly, we also observed several proteins (e.g., FUS and SRSF1) that were previously characterized as RNA G4-binding proteins.[47,48] Aside from proteins that bind specifically to all three G4 structures, i.e., SLIRP, YY1, and YY2 (Figure 2 and Table S2), we identified a number of proteins that bind exclusively to one or two of the G4 structures, e.g., NSUN2 (to c-*MYC* and c-*KIT* G4 structures) and GRSF1 (to c-*MYC* G4 structure) (Figures 2–3 and Table S2). In this vein, it is worth noting that more comprehensive proteomic data sets are employed in the present study; hence, the SILAC ratios for YY1 and SLIRP proteins differ slightly from our previously published results.[13,30]

The results from our quantitative proteomics-based interaction screening showed that GRSF1 binds selectively to the G4 structure derived from the c-*MYC* promoter, but not that from the human telomere or c-*KIT* promoter (Figures 2–3 and Table S2). Thus, we next asked whether this protein can bind directly and selectively to the G4 structure derived from the promoter of the c-*MYC* gene by using fluorescence anisotropy measurements. It turned out that GRSF1 indeed displays strong and selective binding toward the c-*MYC* G4 probe over the corresponding mutated probe, as manifested by the K_d values of 59 nM and 1.28 μM for the G4 and M4 probes, respectively (Figure 4). On the other hand, we did not detect obvious binding of the protein toward G4 structure derived from the human telomere or c-*KIT* promoter (Data not shown).

Our proteomic data also led to the discovery of a number of proteins that bind more strongly to M4 over the corresponding G4 DNA probes (Figure 5 and Table S3). Interestingly, several of these proteins (BLM, nucleolin, HNRNPA2, and SUB1) were previously characterized as

DNA G4-binding proteins, and TARDBP was characterized as a RNA G4-binding protein (Figure 5).[23,24,49–52] Along this line, BLM was shown to unfold G4 DNA, which may explain its preferential binding toward M4 over G4 DNA probes.[50] While these proteins were shown to bind to G4 DNA, it will be important to examine their relative affinities in binding to G4 DNA vs mutated single-stranded DNA that cannot fold into G4 structure. It will also be important to assess the functions of these proteins in modulating the biology of G4 DNA.

Our proteomic data revealed that CNBP binds preferentially with M4 over G4 probe derived from the c-*MYC* promoter, whereas it binds more strongly to G4 over M4 probe derived from the c-*KIT* promoter. The exact reason for the different selectivities of this protein in binding with G4 vs M4 probes derived from these two promoter sequences is not clear, though we reason that the nucleobases not involved in G tetrad formation and G4 folding (i.e., those residing in the loop regions of the G4 structure) may also modulate the differential interactions between CNBP and G4/M4 probes. In this respect, it is worth noting that purified CNBP protein was found to be capable of unfolding G4 structures, where the protein binds more strongly to unfolded than folded G4 DNA derived from the c-*MYC* promoter, but exhibits similar affinities to unfolded and folded G4 DNA derived from the c-*KIT* promoter.[45]

Our proteomic data showed that XRCC5 and XRCC6, a.k.a. Ku86 and Ku70, respectively, display stronger binding to M4 over G4 probes derived from all three sequences. These two proteins form a heterodimer and function in the repair of DNA double-strand breaks through the nonhomologous end-joining pathway.[53] At first glance, this seems to be incongruent with the heterodimer's ability in binding with and sliding along broken ends of DNA at the DSB sites.[53] However, Yuan et al.[54] showed that the Ku complex can also bind directly with single-stranded DNA, albeit at a lower affinity than that with the corresponding double-stranded DNA. In addition, Shao et al.[55] revealed that the Ku heterodimer can bind to RNA and assume an important function in 18S rRNA processing. Thus, the Ku complex's ability in binding with single-stranded nucleic acids may contribute to its preferential interaction with single-stranded M4 DNA over the corresponding folded G4 DNA.

DISCUSSION

Although *in vitro* formation of G4 structure has been known for decades, only recently has the widespread presence of these DNA structures in human cells come to light. [6,7] Previous studies also suggested the functions of G4 structures in many different biological processes. In this vein, G4 sequence motifs are highly enriched in genomic regions of biological importance, e.g., promoters of genes.[7] Hence, it is important to have a more complete understanding about how G4s are sensed by cellular proteins and how these proteins modulate the biological functions of G4 DNA. Many studies have attempted to address this question using diverse approaches and have led to the discovery of a number of proteins that bind directly and strongly with G4 DNA. High-resolution mass spectrometry-based techniques are particularly well suited to explore the interaction proteomes of G4 DNAs, and they were previously employed for the identification of a diverse set of G4 DNA-binding proteins.[23,51] Different folding patterns have been described for G4 DNA;

nevertheless, many of these prior interactome studies only employed one G4 folding pattern. Given the high structural diversity of G4 folding patterns, we aimed to expand this knowledge by comparing directly the interaction proteomes of three unique G4 folding patterns. Interestingly, we discovered three proteins that can bind specifically and recognize G4 structures derived from all three G-rich sequences (Figure 2).

Among the identified G4-binding proteins, we recently validated that SLIRP and YY1 can bind directly to all three G4 structures with low-nanomolar binding affinity *in vitro*, and ChIP-Seq results showed that SLIRP and YY1 can also bind with G4 structures in chromatin.[13,30] It will be important to assess whether YY2, a closely related protein of YY1, can bind directly with G4 DNA structures and to explore the biological functions of such interactions.

Aside from the generic G4-binding proteins, our method allowed for the discovery of proteins that specifically recognize selected G4 structure(s). We also revealed that purified GRSF1 protein can bind strongly and selectively to G4 structures derived from the G4 sequence contained in the c-*MYC* promoter, but not to that derived from the human telomere or c-*KIT* promoter (Figure 4).

In summary, we identified, from exhaustive SILAC-based quantitative proteomic experiments, many novel putative G4-binding proteins that recognize all G4 folding patterns and/or selected G4 folding patterns. We further demonstrated that GRSF1 can interact directly and selectively with G4 DNA derived from c-*MYC* promoter region with high affinity *in vitro*. Hence, our study revealed that G4-binding proteins hold the ability to differentiate and selectively bind only certain G4-folding patterns, which may bear a significant impact on understanding the biological functions of G4 DNA. Moreover, the large number (~80) of candidate G4 DNA-binding proteins identified in the present study constitute an important resource for the research community to assess the functions of these proteins in the biology of G4 DNA. Our work also revealed a number of putative “antireader” proteins for G4 DNA, which calls for the assessment of the functions of these proteins in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health (R35 ES031707).

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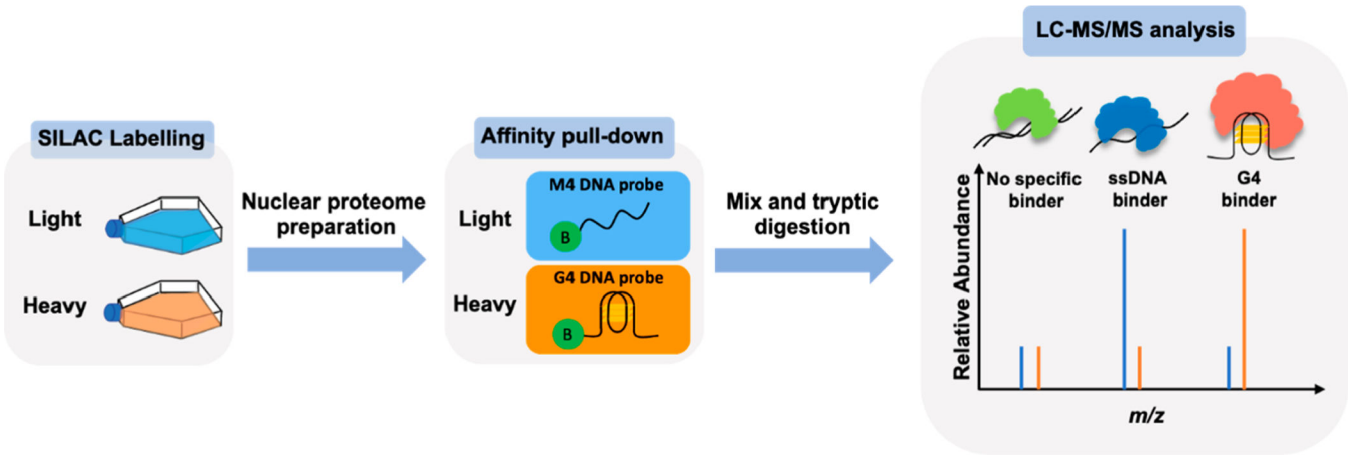

Figure 1.

Experimental workflow for the identification of novel DNA G4-binding proteins. Shown in the scheme is a reverse SILAC-labeling experiment, where the heavy- and light-labeled nuclear protein lysates are incubated with 5′-biotinylated G4 DNA probe and the corresponding single-stranded DNA probe (M4), respectively. The “B” in the green circle denotes 5′-biotin labeling.

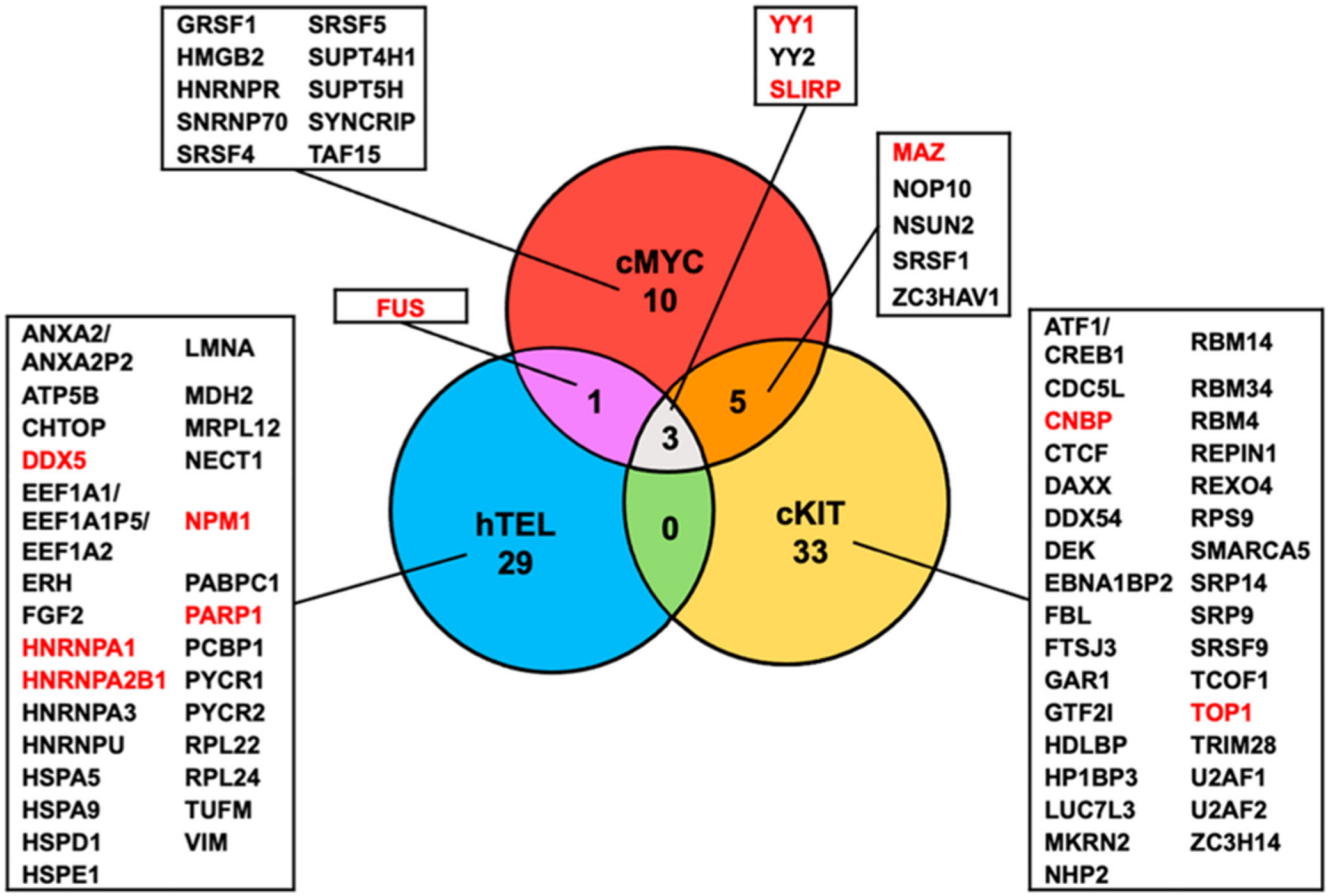

Figure 2.

Venn diagram displaying the overlap in interacting proteins among the three G4 folding patterns studied. Candidate G4-binding proteins identified from SILAC-based affinity screening are listed. Among the identified candidate G4-binding proteins, unique peptides were detected for YY1; the peptides detected for YY2 are shared with YY1. Common peptides were detected for ATF1 and CREB1; for ANXA2 and ANXA2P2; and for EEF1A1, EEF1A1P5, and EEF1A2. Proteins highlighted in red are known to bind to DNA G4 structures.

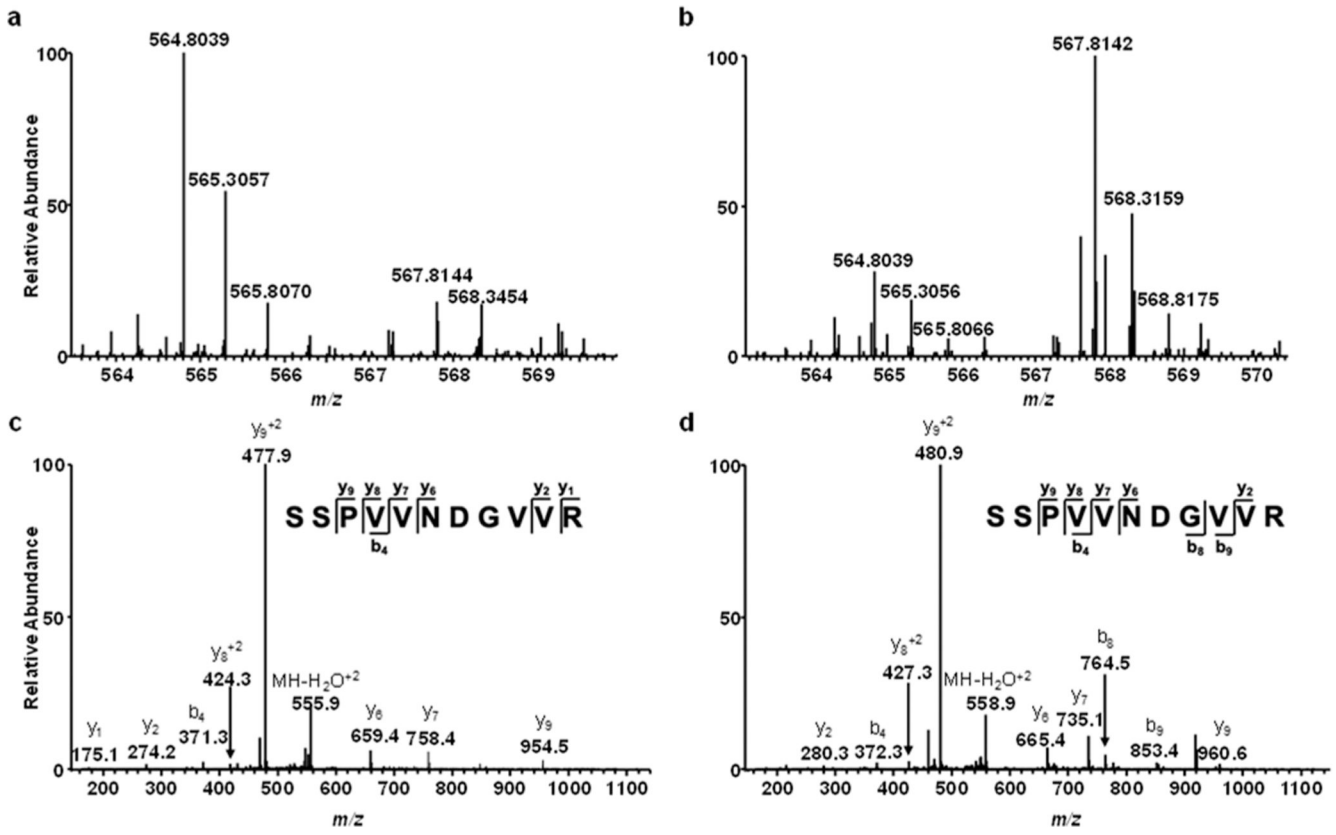

Figure 3.

GRSF1 binds preferentially to G4 structures derived from the promoter of the c-*MYC* gene. (a,b) ESI-MS showing the $[M + 2H]^{2+}$ ions of light and heavy arginine-containing peptide SSPVVNDGVVR with monoisotopic *m*/*z* values of ~564.8 and 567.8, respectively, obtained from forward (a) and reverse (b) SILAC-based interaction screening experiments. (c,d) MS/MS for the $[M+2H]^{2+}$ ions of the light (c) and heavy (d) arginine-containing peptide, SSPVVNDGVVR, derived from GRSF1.

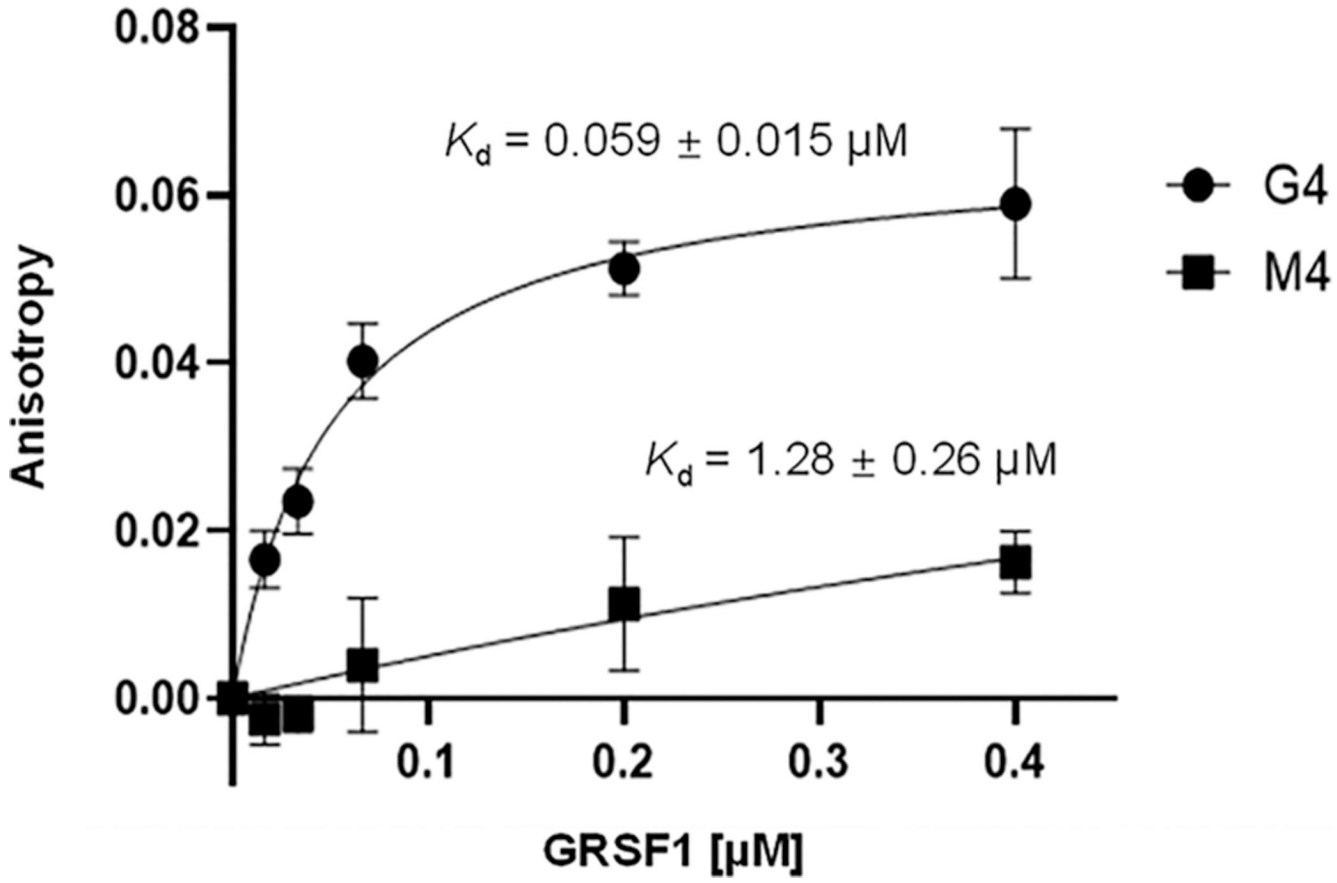

Figure 4.

Fluorescence anisotropy for measuring the K_d values for the binding of the GRSF1 protein toward G4 structures derived from the promoter of c-*MYC* gene.

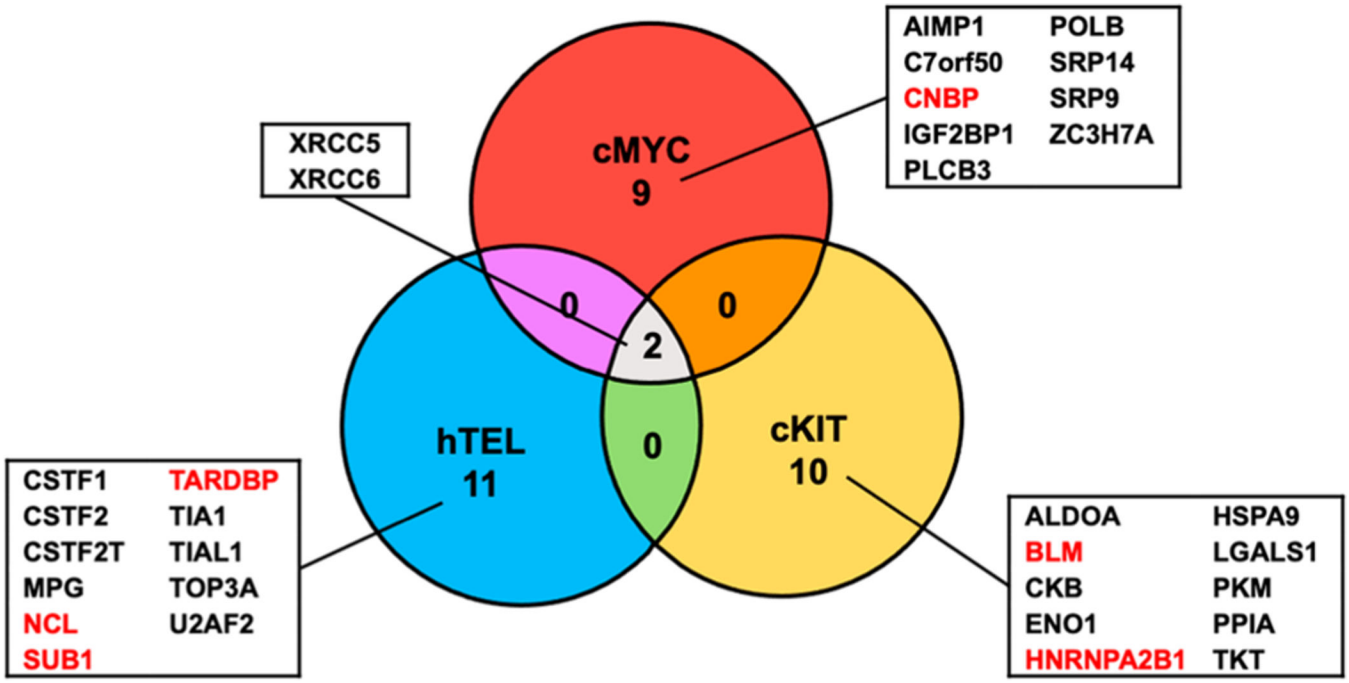

Figure 5.

Venn diagram displaying the overlap in proteins that bind more strongly to mutated single-stranded DNA probes (M4) over the corresponding G4 DNA probes derived from human telomere (hTEL) and the promoters of c-*MYC* and c-*KIT* genes. Putative antireader proteins for G4 DNA are listed. Proteins highlighted in red are known to bind to DNA G4 structures.