A biochemical and biophysical model of G-quadruplex DNA recognition by positive coactivator of transcription 4

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DNA sequences that are guanine-rich have received considerable attention because of their potential to fold into a secondary, four-stranded DNA structure termed G-quadruplex (G4), which has been implicated in genomic instability and some human diseases. We have previously identified positive coactivator of transcription (PC4), a single-stranded DNA (ssDNA)-binding protein, as a novel G4 interactor. Here, to expand on these previous observations, we biochemically and biophysically characterized the interaction between PC4 and G4DNA. PC4 can bind alternative G4DNA topologies with a low nanomolar Kd value of ∼2 nM, similar to that observed for ssDNA. In consideration of the different structural features between G4DNA and ssDNA, these binding data indicated that PC4 can interact with G4DNA in a manner distinct from ssDNA. The stoichiometry of the PC4-G4 complex was 1:1 for PC4 dimer:G4 substrate. PC4 did not enhance the rate of folding of G4DNA, and formation of the PC4-G4DNA complex did not result in unfolding of the G4DNA structure. We assembled a G4DNA structure flanked by duplex DNA. We find that PC4 can interact with this G4DNA, as well as the complementary C-rich strand. Molecular docking simulations and DNA footprinting experiments suggest a model where a PC4 dimer accommodates the DNA with one monomer on the G4 strand and the second monomer bound to the C-rich strand. Collectively, these data provide a novel mode of PC4 binding to a DNA secondary structure that remains within the framework of the model for binding to ssDNA. Additionally, consideration of the PC4-G4DNA interaction could provide insight into the biological functions of PC4, which remain incompletely understood.

Cells are confronted with a barrage of diverse genotoxic insults that can threaten cellular subsistence and organismal fitness (1). Distinct from the covalent modifications that can develop from chemical bombardment of genomic DNA, the inherent structure of the DNA can function as a source of genomic instability once the protein constituents that recognize or resolve DNA secondary structures become compromised. Among the variety of secondary structures that DNA is able to adopt, the G-quadruplex (G4)2 structure is one that has received a generous amount of interest to discern the biological consequence of these structures in cellular regulation and disease (2).

G-quadruplexes are four-stranded DNA or RNA structures that form from appropriately spaced di- or tri-guanine nucleotide repeats. Four guanine nucleotides are arranged in a cyclic conformation through Hoogsteen hydrogen bonds to form a G-quartet subunit (Fig. 1A). Multiple G-quartets stack, driven by the stabilization of π-π interactions between the planar face of adjacent G-quartets and the monovalent cations (K+ or Na+) to form the complete G4 structure (Fig. 1B) (3, 4). The topological arrangement of G4 structures can be highly heterogeneous based on the number of strands involved and the strand directionality to adopt parallel, anti-parallel, or hybrid type G4 conformations (5, 6). G4-forming sequences are located in distinct, non-random regions of the genome, which include telomeres, DNA origins of replication, ribosomal DNA, and the promoters of proto-oncogenes (7–10). G4 sequences have also been mapped to the 5'- and 3'-untranslated regions of mRNA (11, 12). This non-random distribution of G4 sequences has led to the suggestion that G4 structures are involved in the regulation of genomic processes, such as transcription, translation, and DNA replication (13, 14). The development of effective ligands that can stabilize or destabilize specific G4 structures is being pursued to probe G4 function and to exploit their potential as chemotherapeutic targets (10, 15).

The transcriptional coactivator (PC4) is a highly conserved, small, homodimeric single-stranded DNA (ssDNA)-binding protein that was first discovered as an enhancer of activator-dependent transcription in HeLa cells (16). Multiple groups have since reported the involvement of PC4 in a variety of genomic activities including transcription (16–27), DNA replication (28), DNA repair (29–31), chromatin condensation (32, 33), oxidative damage suppression (34), and potential tumor suppressor activity by direct and indirect activation of p53 (22, 35, 36). PC4 has also been implicated in the progression and metastasis of several cancer types and might serve as a potential chemotherapeutic target (37–42). Interestingly, one report has

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2 The abbreviations used are: G4, G-quadruplex; PC4, positive coactivator 4; ssDNA, single-strand DNA; RMSD, root mean square deviation; HADDOCK, High Ambiguity Driven Docking; hTelo, human telomeric; DMS, dimethyl sulfate; RPA, replication protein A.
suggested PC4 associates with Aurora kinases to enhance their kinase activity during cell cycle progression (43). Reports have also provided evidence for the involvement of a PC4 homolog in cellular migration and virulence of the parasitic amoeba *Entamoeba histolytica*, which is a common cause of amoebic colitis, dysentery, and liver abscesses (44, 45).

PC4 has a bimodal domain architecture with an unstructured, lysine- and serine-rich regulatory domain that comprises the N-terminal half of the protein. The remaining C-terminal half is composed of the ssDNA-binding domain (Fig. 1C). PC4 consists of two shallow, anti-parallel DNA-binding grooves separated by a β-ridge region that is located at the interface between the two monomers (Fig. 1D). This anti-parallel DNA-binding site orientation is thought to allow for accommodation of the anti-parallel strands of the DNA upon strand separation (46–48). Indeed, biochemical evidence for this binding mode was supported when PC4-mediated duplex destabilization occurred on DNA substrates that contained 8 nucleotides of unpaired DNA (49).

Recently, PC4 and the yeast homolog suppressor of TFIIB (Sub1) were identified as novel G4DNA interactors through pulldown assays and fluorescence equilibrium-binding analysis (50). Reported here is an expanded analysis of G4DNA recognition by PC4. Fluorescence equilibrium binding analysis of PC4 with G4DNA sequences that adopt multiple conformations resulted in similar $K_d$ values to that observed for ssDNA substrates. Additional biophysical, molecular docking simulations, and biochemical footprinting experiments support a model whereby PC4 can stabilize G4DNA.

**Results**

**PC4 recognizes multiple G4DNA topologies**

The G4DNA substrates that have been used previously to investigate recognition by PC4 have largely consisted of a G4 sequence modified from the c-MYC gene promoter (50, 51). This modified c-MYC promoter sequence (Table 1, c-MYC) predominantly adopts a parallel G4 structure indicated by a characteristic CD spectral profile with a global maximum and minimum at ~265 and ~245 nm, respectively (Fig. 2, A and B). Several reports have indicated that human telomeric (hTelo) DNA can adopt multiple topologies with respect to the strand directionality between adjacent loops within the G4 structure and these different topologies are dependent on the identity of the monovalent cation (Na$^+$ or K$^+$) present (6, 7). To confirm that hTelo can adopt multiple G4 topologies, hTelo was analyzed by CD in 100 mM KCl or NaCl. In the presence of 100 mM NaCl (Fig. 2C), hTelo spectra possess a global maximum and minimum near 295 and 260 nm, respectively. This spectral profile corresponds to the hybrid G4 conformations (Fig. 2A, bottom left structure). However, in the presence of 100 mM KCl (Fig. 2D), hTelo spectra possess a global maximum and minimum near 290 and 235 nm, respectively. This spectral profile corresponds to a predominantly anti-parallel G4 conformation (Fig. 2A, bottom left structure). These CD data are consistent with previous observations that the identity of the monovalent cation present in solution has an important impact on the overall G-quadruplex conformation. To determine whether PC4 can interact with alternative G4DNA structures, equilibrium binding assays were conducted.
Table 1
Sequences of oligonucleotide substrates

| DNA            | Sequence (5' → 3')                              |
|----------------|-----------------------------------------------|
| c-MYC F        | TGGGTGGGTAGGGTGGGTTT FAM                      |
| hTelo F        | AGGGTAGGGTTAGGGTTAGGGTT FAM                   |
| ssDNA F        | FAM TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT |
| Cy5-Cy3 G4     | Cy5 TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT |
| c-MYC          | TGGGTGGGTAGGGTGGGTTT                        |
| T30            | TGGGTGGGTAGGGTGGGTTT                        |
| T50            | TGGGTGGGTAGGGTGGGTTT                        |
| G4-multiplex   | GCGTTGTAGAACTCAG Cy3 ATATGGGTGGTGGGTTAGGGT |
| G4-multiplex complement | CGCGTAGCTAGCAC Cy5 TAAATCCACCTACCCACCCCTATCGAGTTTCGAGACCC |
| c-MYC reporter | TGGGTGGGTAGGGTGGGTAACGCTGATGTCGC            |
| c-MYC reporter complement | GCCGAATCAACCC |

PC4 and G-quadruplex DNA

Figure 2. A, CD spectra of G-rich oligonucleotides indicate formation of various G-quadruplex topologies dependent on sequence and conditions. B–E, CD spectra of 10 μM each of c-MYC (B), hTelo (C and D), and ssDNA (E) with 100 mM of the indicated salt. Each CD spectrum is color-coded to the name of the G-quadruplex topology represented in A.

with fluorescently labeled c-MYC, hTelo, and ssDNA sequences (Table 1) in the presence of 100 mM KCl (Fig. 3A) or NaCl (Fig. 3B). In the presence of 100 mM KCl, PC4 bound equally well to hTelo, c-MYC, and ssDNA substrates ($K_d = 3.0 \pm 0.5, 3.1 \pm 0.3,$ and $3.5 \pm 1.5$ nM, respectively). The strength of this interaction was not drastically altered in the presence of 100 mM NaCl ($K_d = 2.6 \pm 0.7, 4.9 \pm 1.1,$ and $2.8 \pm 0.6$ nM, respectively). The $K_d$ values that are reported here differ somewhat from those reported previously in which PC4 bound more tightly to G4DNA ($K_d = 1.4 \pm 0.3$ nM) compared with...
ssDNA ($K_d = 12.3 \pm 1.7$) (50). However, multiple preparations of PC4 displayed binding isotherms that are consistent with that reported here, so we conclude that the differences observed are likely preparation-dependent.

Mutation of the aromatic amino acid residues Phe77 and Trp89, which are reported to be essential for ssDNA recognition, to alanine residues increased the $K_d$ value of the PC4-G4DNA complex (Fig. 3C) ~7–10-fold ($K_d = 12.1 \pm 2.7$ and 16.7 ± 3.2 nM for W89A and F77A). A similar fold increase in the $K_d$ value was also observed for the PC4-ssDNA complex (Fig. 3D) with $K_d = 15.0 \pm 2.3$ and 17.5 ± 2.4 nM for W89A and F77A, respectively. Increasing the oligonucleotide concentration to 2 nM in the presence of 100 mM KCl resulted in $K_d$ values similar to that observed in Fig. 3, which indicates that equilibrium binding was observed (supplemental Fig. S1A). The fluorescence intensity values were also not affected during the course of the titrations (supplemental Fig. S1B).

Collectively, the CD and binding data indicate that PC4 recognition of G4DNA is not dependent on the strand directionality of the G4 structure because PC4 does not discriminate between different G4 topologies. Mutational analysis revealed the aromatic amino acids (Phe77 and Trp89) had similar deleterious effects on binding to both G4 and ssDNA substrates, which indicates that the DNA-binding domain is important for G4-binding activity. Thus, PC4 can accommodate alternative G4DNA structures with similar affinity to that observed for ssDNA, and the alternative topological G4 arrangements have no apparent effect on binding activity in vitro.

PC4 does not unfold G4DNA but slows the rate of G4 folding

It has been reported previously that PC4 can destabilize duplex DNA, which ultimately leads to complete strand separation (49). To examine the possibility that PC4 binding to G4DNA results in unfolding of the G4DNA structure, a FRET-based assay that has been described previously was used (52–54). In this assay, 25 nM of c-MYC G4DNA with Cy5 and Cy3 labels on the 5’ and 3’ ends, respectively (Cy5-Cy3 c-MYC G4; Table 1) is mixed with varying concentrations of PC4 or $0.2 \mu M$ Pif1 helicase. When the G4DNA is folded, the fluorophors are in close proximity, which gives a high fluorescence signal. Upon unfolding of the G4 structure, the distance between the Cy5 and Cy3 fluorophors is increased and results in a decrease in the fluorescence signal (Fig. 4A). Upon rapid mixing of the folded c-MYC G4 sequence with 0.2, 1, and $3 \mu M$ PC4, there was no observable decrease in the fluorescence signal up to 500 s. However, when rapidly mixed with $0.2 \mu M$ of the Pif1 helicase with ATP, which unfolds many different G4DNA structures (54–57), there was a robust decrease in the fluorescence signal (Fig. 4B). These data indicate that PC4 alone is unable to induce G4DNA unfolding.

The absence of any observable G4 unfolding activity by PC4 presents the possibility that PC4 could stabilize or even enhance the folding of the G4DNA structure. To address this potential activity, 25 nM of the unfolded Cy5-Cy3 c-MYC G4 sequence was rapidly mixed with 100 mM KCl alone or with varying concentrations of PC4 (Fig. 4C). In the absence and at lower concentrations of PC4, the data were best fit to a double
exponential equation, which is consistent with a multistep G4 folding process (5, 6, 58). Rate constant values obtained from the fitting analysis are presented in Table 2. Upon addition of 0.2 μM PC4 (Fig. 4C, green versus gray traces), there was a \( \sim 4 \)-fold reduction in the rate constant for the initial fast folding step and a \( \sim 2 \)-fold reduction in the rate constant for the slower step of the Cy3-Cy5 c-MYC G4 (0.56 ± 0.07 s\(^{-1}\) versus 0.15 ± 0.02 s\(^{-1}\) and 0.037 ± 0.001 s\(^{-1}\) versus 0.018 ± 0.001 s\(^{-1}\)) for the fast and slow rate constants, respectively, in Fig. 4C. Upon addition of 1 or 3 μM (Fig. 4C, red and blue, respectively), there was a greater reduction in the rate constant for folding, and the data fit best to a single exponential equation with rate constants of 0.011 ± 0.004 and 0.009 ± 0.001 in the presence of a large excess of PC4 relative to DNA. Collectively, the data presented indicate that PC4 binding to a prefolded G4DNA structure does not induce G4DNA unfolding even in high excess of PC4. However, folding of G4DNA from an unfolded state is slowed in slight excess of PC4 and is severely perturbed at higher concentrations of PC4 that are in great excess of the DNA concentration.

### G4DNA scaffold can accommodate a single dimer of PC4

G4DNA adopts a more rigid, compact structure compared with an ssDNA counterpart of the same sequence. Despite this difference, the fluorescence equilibrium binding data combined with the absence of G4DNA unfolding activity by PC4 suggest that PC4 recognizes the folded G4DNA scaffold. However, the stoichiometry of the PC4-G4DNA interaction is a question that has not been addressed. It is known that PC4 possesses oligomeric activity on unpaired ssDNA strands of an open duplex conformation if enough unpaired nucleotides are available (34). To appraise the stoichiometry of the binding interaction between PC4 and G4DNA, intrinsic protein fluorescence quenching experiments were conducted with 1 μM PC4 titrated with the indicated DNA substrates in the presence of 100 mM KCl or LiCl (Fig. 5). The data were plotted as the ratio of [DNA]/[PC4] versus 

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F_{\text{bound}} / F_{\text{PC4-DNA}}.
\]

The data display a characteristic linear increase that reaches a point of saturation that is represented as a plateau in the binding isotherm. The value of the [DNA]/[PC4] ratio at the point of saturation is the stoichiometry of the interaction (59). Titration of 1 μM PC4 with the folded c-MYC (Fig. 5, purple) resulted in a DNA/PC4 = 0.49 ± 0.03, which corresponds to 1 dimer of PC4 bound per folded G4DNA substrate. When titration of 1 μM PC4 was performed in the presence of 100 mM LiCl (which does not support G4DNA formation), there was an observed decreases in the DNA/PC4 = 0.34 ± 0.11, which corresponds to 1–2 dimers of PC4 per unfolded G4DNA substrate. These data are consistent with the hypothesis when the G4DNA scaffold is unfolded, additional binding sites are available to be bound by PC4. The same trend was observed for the \( T_{30} \) and \( T_{50} \) ssDNA substrates. DNA/PC4 = 0.15 ± 0.02 and 0.08 ± 0.002, which correspond to three dimers bound to the \( T_{30} \) substrate and six dimers bound to the \( T_{50} \) substrate.

### G4DNA structure is nested into the binding site of a single PC4 monomer

In lieu of a crystal structure of the PC4-G4DNA complex, \textit{in silico} molecular docking simulations were conducted with the previously solved structures of PC4 and c-MYC G4DNA (46, 51). Atomic structures were downloaded from the Protein Data Bank, and simulations were carried out with the High Ambiguity Driven DOCKing (HADDOCK) program (60, 61). The simulation analysis resulted in 51 structures that were grouped into 9 clusters that represented 25.5% of the water-refined models.
The statistics of these 9 clusters are tabulated in Table 3. The top-scored cluster based on the HADDOCK score, which is a weighted sum of the electrostatic, van der Waals, desolvation, and restraint violation energies, was cluster 3 with a value of $-147.5 \pm 7.0$. However, upon a more detailed analysis of the interface and ligand RMSD values in addition to the fraction of native contacts ($F_{\text{nat}}$) with respect to the Critical Assessment of PRedicted Interactions (CAPRI) criteria (62), cluster 4 resulted in the only favorable values ($\text{HADDOCK} = -133.5 \pm 16.3$, interface RMSD = 0.88 $\pm$ 0.5, ligand RMSD = 1.7 $\pm$ 0.9, and $F_{\text{nat}} = 0.79 \pm 0.12$).

The structure that represents cluster 4 is presented in Fig. 6. This structure has the c-MYC G4DNA cradled within one of the PC4 monomers (Fig. 6, A–C) with one planar face of the G4 structure placed against the DNA-binding groove of PC4 (Fig. 6B). This “face-down” mode of interaction places the free adenine nucleotides within congruent loops of the G4DNA (Fig. 6C, circled) in close ($\sim$6 Å) proximity to the aromatic amino acids Trp$^{89}$ and Phe$^{77}$. A more detailed view of the interaction surface illustrates the positioning of the key residues Trp$^{89}$ (Fig. 7A) and Phe$^{77}$ (Fig. 7B), which are suggested to be essential for DNA recognition (18). The distance between Trp$^{89}$ and Phe$^{77}$ and their respective base stacking partners was calculated to be $\sim$6 Å, which is on the upper limit of the maximum distance allowed for a stable $\pi-\pi$ stacking interaction to form. A small ($\sim$2 Å) contraction in the PC4 structure could bring these residues into closer proximity to allow for more favorable interactions.

One interesting feature predicted by this docking simulation was the formation of a molecular “pocket” formed around the Phe$^{77}$ residue by the phosphate back bone and the adenine residue predicted as a base stacking partner (Fig. 7C). In addition to the “poised” stacking interactions, several hydrogen bond interactions were predicted between the phosphate backbone and residues Arg$^{75}$, Lys$^{78}$, Lys$^{80}$, and Arg$^{100}$ (Fig. 7D). These hydrogen-bonding interactions are analogous to the interactions that have been observed within the crystal structure of the PC4-ssDNA complex (47). Collectively, the data from the in silico molecular docking simulation and biophysical stoichiometric experiments suggest that the G4DNA structure is recognized by one monomeric unit of the PC4 dimer.

**Expanded model of DNA multiplex recognition by PC4**

The current model to suggest how PC4 might interact with DNA was first presented by Brandsen et al. (46) upon solving the crystal structure of PC4. This model suggests the anti-parallel DNA-binding grooves of PC4 are positioned to accommodate the anti-parallel strands of the DNA duplex, which are separated by the $\beta$-ridge region of PC4 (Fig. 8, scheme 1). This model was supported further by biochemical data suggesting that PC4 interacts with unpaired strands of the DNA to destabilize the duplex and also inhibit transcription (18, 49).

In consideration of the data in the literature and the data presented here, an expanded model of DNA recognition by PC4

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**Table 3**

| Cluster | i-RMSD | l-RMSD | $F_{\text{nat}}$ | z score |
|---------|--------|--------|-----------------|---------|
| 1       | $-95.7 \pm 4.3$ | 9.7 $\pm$ 0.3 | 18.1 $\pm$ 0.7 | 0.11 $\pm$ 0.01 | 0.9 |
| 2       | $-107.9 \pm 5.8$ | 11.9 $\pm$ 0.1 | 19.6 $\pm$ 0.2 | 0.13 $\pm$ 0.007 | 0.2 |
| 3       | $-147.5 \pm 7.0$ | 10.9 $\pm$ 0.1 | 16.7 $\pm$ 0.1 | -2 |
| 4       | $-133.5 \pm 16.3$ | 0.88 $\pm$ 0.5 | 1.7 $\pm$ 0.9 | 0.79 $\pm$ 0.12 | -1.2 |
| 5       | $-94.7 \pm 14.7$ | 11.1 $\pm$ 0.1 | 18.1 $\pm$ 0.1 | 1 |
| 6       | $-96.0 \pm 9.7$ | 12.1 $\pm$ 0.04 | 27.5 $\pm$ 0.2 | 0.11 $\pm$ 0.01 | 0.9 |
| 7       | $-101.6 \pm 11.4$ | 9.4 $\pm$ 0.2 | 15.6 $\pm$ 0.3 | 0.02 $\pm$ 0.02 | 0.6 |
| 8       | $-107.3 \pm 5.5$ | 9.5 $\pm$ 0.3 | 16.6 $\pm$ 0.6 | 0.09 $\pm$ 0.001 | 0.3 |
| 9       | $-126.9 \pm 4.7$ | 9.4 $\pm$ 0.1 | 14.7 $\pm$ 0.2 | 0.03 $\pm$ 0.02 | -0.8 |
It has been difficult to study G4DNA-associated activities in the context of duplex DNA because the stability of the GC-rich duplex out-competes the stability of the folded G4 structure under typical buffer conditions in vitro. However, if the two strands are annealed in the presence of a molecular crowding agent (PEG 200, glycerol, etc.), the structure of the G4DNA can persist in the presence of the complementary C-rich strand (63). In efforts to ascertain whether the model for PC4 binding to G4-multiplex is consistent with the model of duplex binding by PC4, a FRET-based assay that has been previously described was utilized (64). Briefly, a Cy3-labeled c-MYC G4DNA sequence flanked by 18 nucleotides on the 5’ and 3’ ends is annealed to a Cy5-labeled complementary C-rich strand. Annealing in the absence of PEG results in formation of the complete duplex structure, which should result in a low FRET signal. However, annealing in the presence of a molecular crowding agent, in this case 40% PEG 200, would result in persistence of the G4 structure and an increase in FRET signal even in the presence of adjacent duplex DNA (Fig. 9A).

In the absence of PEG 200, the Cy5 fluor is distanced from the Cy3 fluor because of complete duplex formation, which resulted in a relatively low fluorescence value of ~0.25 V (Fig. 9B). Neither the addition of PEG after the annealing reaction (+PEG PA) nor 3 μM PC4 post-annealing had any effect on the fluorescence of this complete duplex substrate (Fig. 9B and C). However, when the annealing reaction was conducted in the presence of PEG 200, the G4DNA structure persisted, which positions the Cy5 and Cy3 flours in relatively closer proximity to one another, resulting in a ~2-fold increase in fluorescence signal from 0.25 to 0.5 V (Fig. 9B, +PEG). The DNA substrate, which consists of G4DNA, duplex, and single-stranded DNA, will be referred to as a G4-multiplex. However, when the G4-multiplex substrate was mixed with PC4 (1.5 and 3 μM) there was a further enhancement in the fluorescence signal from ~0.5 to ~0.7~0.8 V (Fig. 9B). Additionally, the formation of the PC4-G4-multiplex complex did not result in an extensive loss of fluorescence over time, which indicated that the integrity of the duplex was maintained up to 500 s (Fig. 9D).

Dimethyl sulfate (DMS) footprinting of the folded G4-multiplex substrate validated the presence of the G4DNA structure (Fig. 10, A and B). The N7 position of guanines involved in G4DNA formation exhibit a greater degree of protection (~3.4-fold) from reaction with DMS. This protection was not observed in the duplex or the duplex treated with 4% PEG 200, which suggests that PEG alone does not induce G4DNA formation and is consistent with the formation of the G4DNA structure when annealing is performed in the presence of PEG (64, 65). Additionally, CD analysis of the complete duplex and the folded G4-multiplex substrates (Fig. 10C) suggests that the formation of the G4 structure did not perturb the flanking duplex regions because there was no discernible shift in the spectra minimum and maximum at ~245 and ~280 nm, respectively (66). The data in Figs. 9 and 10 support the conclusion that the structure adopted by the DNA substrate is the G4-multiplex.
Furthermore, PC4 can bind to this structure in a manner that maintains the G4DNA structure itself.

**G4 structure within the G4-multiplex is stabilized in the presence of PC4**

To address the G4-multiplex further, an experiment was conducted in which PEG 200 was dialyzed away from the folded G4-multiplex substrate in the absence or presence of PC4 prior to examination by DMS footprinting. It was hypothesized that if the G4DNA structure is stabilized by PC4, the guanine nucleotides involved in G4DNA formation would exhibit a greater degree of protection from DMS in the presence of PC4 compared with the G4-multiplex in the absence of PC4.

DMS footprinting gels for the G4-multiplex are shown in Fig. 11A in the presence or absence of PC4 after dialysis to remove PEG. The bands corresponding to the guanine residues are visibly darker in the absence of PC4. Quantitation of the band intensity reveals a significant increase in guanine reactivity in the absence of PC4 (Fig. 11B). Hence, PC4 does appear to remain bound to the G4DNA structure in the context of the duplex substrate, even after the removal of PEG.

The current binding model of PC4 suggests that the anti-parallel strands of an open DNA duplex are bound by the anti-parallel DNA-binding sites of the PC4 homodimer (Fig. 8). This binding model predicts that the complementary C-rich strand opposite the folded G4DNA would be protected against chemical attack in the presence of PC4. Cytosine-specific bromine footprinting of the complementary C-rich strand of the G4-multiplex was carried out in the absence and presence of 500 nM PC4 (Fig. 11, C and D). In the presence of PC4, there was a 2-fold reduction in reactivity of the cytosine residues opposite the G4DNA structure within the G4-multiplex. The protection provided by PC4 in the guanine and cytosine footprinting data (summarized in Fig. 12, A and B, respectively) suggests that the recognition of G4DNA by PC4 results in stabilization of the G4 structure and complementary C-rich strand.

To address the stabilization of G4DNA by PC4 further, a G4DNA reporter substrate that has been used previously to investigate Pif1-mediated G4 unfolding was used (54). In this assay, the c-MYC G4DNA sequence is flanked on the 5’ and 3’ ends by ssDNA and 12-bp duplex, respectively (Table 1, c-MYC reporter). Pif1-mediated unfolding of the c-MYC reporter is monitored by unwinding of the 12-bp duplex on the 3’ end. There was a large (~5-fold) decrease in the rate constant of Pif1 unwinding in the presence of PC4 compared with Pif1 alone (Fig. 13). The difference in Pif1 unwinding was not dependent on the order of addition as preincubating Pif1 with the DNA prior to addition of PC4 followed by ATP did not change the difference in rate constants. This unwinding activity was not observed for PC4 alone. The footprinting and Pif1-mediated G4

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Figure 7. Highlighted interactions of the PC4-c-MYC G4 complex from molecular docking simulations. Close-up view of Trp89 (red) and Phe77 (blue) to their respective adenine (ADE) base stacking partners (A and B, respectively). C, the Phe77 molecular “pocket” created by the phosphate backbone and the adjacent adenine residue is shown as a space-filling model. D, additional interactions between Arg75, Lys78, Lys80, and Arg100 and the phosphate backbone.
G-quadruplex binding (50, 67). The transcriptional coactivator PC4 is a ssDNA-binding protein that has multiple reported functions including transcription, DNA replication, telomere maintenance, and recombination (14). Functional characterization of the proteins and G4DNA-forming sequences as regulatory components in transcription, replication, and disease (2, 13, 14). Presently, there is some controversy over the exact number of G4DNA-forming sequences within the mammalian genome (2). The values of potential G4DNA-forming sequences range from 300,000 to 750,000, and the broad range in number is largely due to the degree of stringency in loop length allowed within the search algorithm (2). Regardless, the non-random distribution within or near functional regions of the genome largely lends the role of G4DNA-forming sequences as regulatory components in transcription, DNA replication, telomere maintenance, and recombination (14). Functional characterization of the proteins and cofactors that recognize these structures is essential if a comprehensive physiological model of G-quadruplex structure is to be attained. The transcriptional coactivator PC4 is a ssDNA-binding protein that has multiple reported functions including G-quadruplex binding (50, 67).

Here it was shown that PC4 can recognize alternative G4DNA conformations with similar $K_d$ values to that observed for ssDNA (Fig. 3). No unfolding of the G4DNA structure was observed upon binding by PC4 (Fig. 4B). This is in contrast to RPA, another abundant SSB, which has been shown to readily unfold G4DNA (68). However, PC4 significantly perturbed the folding rate of G4DNA (Fig. 4C).

PC4 binds the folded c-MYC G4 substrate with a stoichiometric ratio of 1:1, PC4 dimer:c-MYC G4. This stoichiometric ratio increases ϒ2-fold to ϒ2:1 upon unfolding of the G4DNA structure (Fig. 5B). There are two important conclusions that can be drawn from this data: 1) a reduction in the number of binding sites available through folding of G4DNA (Fig. 5) could prevent the oligomerization of PC4 onto the DNA and potentially serve as a mechanism of regulation, and 2) the occlusion of ϒ17–23 nucleotides into a compact folded G4DNA structure artificially increases the effective binding site size of PC4 (minimum of ϒ8 nucleotides) ϒ2–3-fold depending on the loop lengths of the G4DNA.

Molecular docking simulations support a binding mode whereby one monomer of a PC4 dimer cradles the G4DNA (Fig. 6), which is consistent with the observed stoichiometry of the complex (Fig. 5). Docking studies revealed that the close proximity of the aromatic amino acid residues Trp89 and Phe77 to nucleobases within the loops of the G4DNA (Fig. 7, A and B) is consistent with the binding data (Fig. 2), which supports the role of these amino acids in G4DNA binding. Collectively, the data presented here suggest a structural model of G4DNA recognition by PC4 that builds on the existing structural and biochemical data for binding of ssDNA.

A G4DNA structure was formed within the context of duplex DNA by using a molecular crowding agent, PEG 200 (64). The resulting structure is referred to as a G4-multiplex. Evidence for direct binding of PC4 to the G4-multiplex was observed by an increase in Cy5 fluorescence of the folded G4-multiplex substrate that was not observed in the completely annealed duplex (Fig. 9, compare C and D). The observed PC4-mediated FRET enhancement of the folded G4-multiplex substrate in 4% PEG 200 would suggest that PC4 shifts the equilibrium of the complex from a partially annealed state to a stabilized G4-multiplex complex. Stabilization of the G4-multiplex is proposed to occur through either: 1) sequestration of the single-stranded C-rich strand, which would then allow the complementary G-rich strand to adopt a folded G4 structure, 2) recognition of a partially folded G4 structure that is then stabilized upon PC4 binding, and/or 3) the simultaneous recognition of both the G4 and C-rich strands by PC4 to enhance the stability of the open G4-multiplex.

Additionally, others have reported that PC4 can denature a canonical open duplex through an oligomerization-dependent mechanism (49). However, the modest decrease in Cy5 fluorescence upon PC4 binding to the G4-multiplex (Fig. 9D) indicates that the open G4-multiplex is not denatured by PC4. If PC4 were to unwind or unfold the complete G4-multiplex, a greater reduction in fluorescence would be expected.

The ssDNA-binding domain of PC4 was initially thought to possess a unique structural arrangement, but upon superimposition of PC4 and replication protein A (RPA) crystal structures, a similar positional arrangement of amino acid residues between the two proteins was observed (46–48). Indeed, sev-
Figure 9. Formation of the G4-multiplex in the presence of the crowding agent PEG and stabilization of the structure by PC4. A illustrates the annealing of the duplex resulting in relatively low FRET in the absence of PEG or formation of the G4-multiplex structure in the presence of PEG resulting in relatively high FRET. The change in Cy5 fluorescence was monitored in the presence of 25 nM duplex or G4-multiplex substrates as a function of PC4 and PEG. B, (+)PEG PA indicates fluorescence of the duplex substrate with the addition of PEG post-annealing. C and D, fluorescence change over time for the duplex (C) and G4-multiplex (D) substrates up to 500 s.

Figure 10. Biochemical evaluation of the formation of the G4-multiplex. A, DMS footprinting reactions were performed with duplex or G4-multiplex substrates in the presence or absence of 4% PEG. M denotes the mock reacted substrates. B, quantification of guanine reactivity of duplex (blue circles), duplex (+) 4% PEG 200 (red squares), and the folded G4-multiplex (green diamonds) from the DMS footprinting reaction. C, CD spectra of duplex (blue) and multiplex (green) substrates compared with ssDNA, T15.
eral reports suggest that there is compensational or complementary activity between both PC4 and RPA because both proteins are simultaneously involved in replication and DNA damage repair and have been reported to directly associate (28). However, the evidence presented here suggests that there is a potential dichotomy of function between PC4 and RPA on G4DNA. Others have reported RPA can efficiently unfold G4DNA (68, 69). The evidence here suggests that, unlike RPA, PC4 does not possess the same G4DNA unfolding ability on the c-MYC G4DNA substrate. In fact the c-MYC G4DNA structure appears to be stabilized by the recognition of PC4 (Fig. 11).

Possible roles for the G4DNA-binding capacity of PC4 are increasingly being recognized. PC4 is involved in various stages of transcription. At the initiation stage of transcription and during the formation of the preinitiation complex, significant torsional strain on the DNA scaffold is produced to promote open complex formation and progression into transcription elongation (70). The torsional strain that is invoked on the DNA can cause negative superhelical stress that can be transmitted along the DNA scaffold and away from the preinitiation complex. Formation of G4DNA is proposed to relieve the superhelical stress and serve as a temporary means of relieving the strain on the DNA and to serve as a sensor that can be recognized by regulatory factors (71). Apart from a regulatory function, formation of G4DNA downstream of an elongating RNA polymerase could provide the transcriptional machinery with a physical obstacle that must be resolved or bypassed before transcription is allowed to proceed (13). This could provide additional down-

Figure 11. The folded G4 structure in the G4-multiplex is stabilized by PC4. A, DMS footprinting reactions were performed on the G4-multiplex after removal of the PEG crowding agent in the absence or presence of PC4. B, quantification of guanine reactivity in the G4-multiplex alone (red squares) or in the presence of 500 nM PC4 (green diamonds). C, bromine footprinting of the C-rich complementary strand indicates some protection by PC4. D, quantification of cytosine reactivity of the C-rich strand in the absence (red squares) or in the presence of 500 nM PC4 (green diamonds).
stream “sensors” that relay the DNA damage status to the active transcription complex to abort transcription in times of cellular stress (72).

PC4 has several reported functions throughout the process of transcription including transcriptional repression (71). PC4-mediated transcriptional repression is dependent on the ssDNA-binding domain, although the precise mechanism for how PC4 binds to the DNA and inhibits transcription has been largely unexplored. Given the overlapping roles of PC4 and G4DNA in transcriptional regulation, it is possible that formation of G4DNA during the transcriptional process could provide a structural “signal” that is then recognized by PC4 to regulate transcription.

Indeed, it has been previously shown that the helicase function of the XPB subunit within the TFIIH complex is required to alleviate PC4-mediated transcriptional repression that is not dependent on the XPD subunit (73). Additionally, it has been suggested that there is a differential activity between XPD/XPB subunits in G4DNA unfolding activity with XPB efficiently unfolding the G4DNA structure with no apparent G4DNA unfolding activity observed by XBP (58). It is not known whether PC4 stabilization of G4DNA would inhibit XPB-mediated G4DNA unfolding, but based on the G4 multiplex stabilization activity observed here by PC4, it is hypothesized that the PC4-G4DNA complex would serve as a physical block to XPB progression.

Experimental procedures

Oligonucleotides and purification

The DNA and oligonucleotides used in this study were purchased from Integrated DNA Technologies (Coralville, IA) and Dharmacon (Lafayette, CO), respectively. The sequences of the oligonucleotides used are presented in Table 1. Before use, the oligonucleotide precipitants were briefly centrifuged and resuspended in storage buffer containing 10 mM Tris-Cl, pH 7.5. A 1:1 mixture of oligonucleotide stock and loading buffer containing 1× TBE, and 90% formamide was purified on denaturing PAGE consisting of 20% acrylamide and 7 M urea for several hours at 15–22 mA. Separated oligonucleotide bands were excised from the gel and were electroeluted. Oligonucleotides were then desalted on a C18 Sep-Pak column purchased from Waters (Milford, MA) and were eluted with 60% methanol. DNA oligonucleotides were then dried via vacuum centrifugation and resuspended in 10 mM Tris-Cl, pH 7.5. Final concentrations of oligonucleotides were quantified by UV absorbance with calculated extinction coefficients.
Recombinant PC4 expression and purification

Full-length recombinant PC4 was expressed and purified from the pET11a expression vector as previously described but with slight modifications (18). Briefly, pET11a-PC4 was transformed into BL21 (DE3) pLysS-competent Escherichia coli cells (Agilent Technologies). Positive transformants were then selected with 100 μg/ml of ampicillin. Recombinant PC4 induction was initiated with 1 mM isopropyl β-D-thiogalactopyranoside and was allowed to continue for 4 h. Induced cultures were harvested via centrifugation at 3000 g for 30 min at 4 °C. Cell pellets were resuspended and processed in a Dounce homogenizer in lysis buffer (20 mM Tris-Cl, pH 7.3, 1 mM EDTA, 5 mM DTT, 10 mM Na₂S₂O₅, 10% glycerol) supplemented with 500 mM KCl, 1 mM PMSF, and 4 μg/ml pepstatin A, at a volume of 2 ml/gram of cell pellet. Suspended cells were flash frozen in liquid N₂, thawed on ice, and then sonicated. Lysis solution was then ultracentrifuged at 148,000 g for 90 min. The supernatant was diluted to 200 mM KCl with lysis buffer and loaded onto a HiTrap™ heparin-Sepharose column (GE Healthcare). Recombinant PC4 was eluted with lysis buffer supplemented with 500 mM KCl. The PC4 fractions that were the most pure by 15% SDS-PAGE analysis were pooled and diluted with lysis buffer again to 75 mM KCl and was loaded onto a ssDNA-cellulose column (Affymetrix). Full-length PC4 was eluted with a linear salt gradient from 75 to 1000 mM KCl. Purified PC4 was concentrated and buffer-exchanged into storage buffer (20 mM HEPES, pH 7.5, 150 mM KCl, 1 mM EDTA, 5 mM DTT, 20% glycerol) with Amicon® Ultra-4 centrifugal filter with a molecular weight cutoff of 3,000 (Millipore). Final concentration of purified protein was determined by UV absorbance at 280 nm with the calculated extinction coefficient, flash frozen in liquid N₂, and stored at −80 °C.

Circular dichroism analysis

CD analysis was performed on a Jasco J-715 spectropolarimeter. DNA substrates were heated to 95 °C for 10 min and slow cooled to room temperature before analysis. Spectra were recorded at 25 °C with 10 μM DNA in 10 mM Tris-Cl, pH 7.5, with 100 mM salt unless indicated otherwise. Five CD spectra were recorded, averaged, and normalized to the buffer sample without DNA.

Fluorescence equilibrium binding analysis

Equilibrium binding analysis was performed with a PerkinElmer Life Sciences Victor^™ V 1420 multilabel counter with filters set at 485 and 535 nm. Fluorescence polarization measurements were recorded at 25 °C in assay buffer (10 mM Tris-Cl, pH 7.5, 0.1 mM EDTA, 1 mM DTT, 100 mM KCl or 100 mM LiCl, and 0.1 mg/ml bovine serum albumin). 6-Carboxyfluorescein (FAM)-labeled substrates (1 nM final concentration) were titrated with increasing concentrations of protein to measure binding. The FAM-labeled substrate without protein was measured and used to normalize the change in fluorescence polarization observed in the protein-titrated samples. Fluorescence data were plotted as change in anisotropy against protein concentration using Kaleidagraph software (Synergy Software, Reading, PA) and fit to the binding quadratic equation to obtain dissociation constants (Kd).

Stopped-flow G4 unfolding analysis

All concentrations listed are final. Cy3-Cy5-c-MYC G4 in PC4 reaction buffer (25 mM Tris-Cl, pH 7.5, 1 mM DTT, and 100 mM KCl) was mixed with 0.2, 1, or 3 μM PC4 in an SX.18MV stopped-flow reaction analyzer (Applied Photophysics). The reaction assay was excited at 550 nm, and FRET signal was detected after a 665-nm cutoff filter (Newport Corp., catalog no. 51330). Pif1 catalyzed G4 unfolding served as the positive control. Cy3-Cy5 c-MYC G4 in Pif1 reaction buffer (25 mM...
**PC4 and G-quadruplex DNA**

HEPES, pH 7.5, 2 mM β-ME, 10 mM MgCl₂, 100 mM KCl, and 5 mM ATP) was mixed with 0.2 μM Pif1 to initiate the reaction.

**Fluorescence quench titration**

Fluorescence quench titrations were performed on a Series 2 Luminescence spectrometer (AMINCO-Bowman). PC4 (1 μM) was titrated with increasing concentrations of c-MYC and T₅₀ and T₅₀ ssDNA substrates (Table 1). Three fluorescence values were recorded after each titration point and averaged. Fluorescence data from each titration point were plotted as a fluorescence ratio $F_{\text{buff}}/F_{\text{DNA}}$ (where $F_{\text{buff}}$ is the fluorescence from the buffer titrated sample, and $F_{\text{DNA}}$ is the fluorescence of the PC4-DNA complex) versus [DNA]/[PC4] ratio to obtain binding stoichiometry values. The data were fit to two linear equations, and the point of intersection of the two linear equations was calculated.

**G4 multiplex recognition by PC4**

All concentrations are final. Cy3-Cy5-c-MYC duplex and G4 multiplex substrates were annealed following protocols that have been previously reported with modifications (64). Briefly, Cy3-Cy5-c-MYC was annealed to the complementary strand in a 1:1 ratio in buffer (10 mM Tris-Cl, pH 7.5, 100 mM KCl) in the absence or presence of 40% PEG 200 to form the duplex or G4 multiplex structures, respectively. Duplex or G4 multiplex substrates (25 nM) in reaction buffer (20 mM Tris-Cl, pH 7.5, 100 mM KCl, and 4% PEG 200, if indicated) were mixed with 1.5 or 3 μM PC4 under instrumentation conditions that are described for measurements of G4 unfolding.

**Molecular docking simulations with HADDOCK**

Docking simulations were performed with the HADDOCK software package (60, 61). The crystal structure of the apo-DNA-binding domain of PC4 (PDB code 1PCF (46) and a single NMR model of the G-quadruplex structure from the c-MYC gene promotor were used to determine the ambiguous interaction restraints to drive the docking simulation. The residues in the PC4 DNA-binding domain Arg⁷⁵, Phe⁷⁷, Gly⁷⁹, Lys⁸⁰, and Trp⁸⁹ were set as “active” residues. Passive residues were allowed to be defined automatically around the active residues. The free base residues located in the loops of the G-quadruplex DNA structure were set as the active residues with the passive residues automatically defined around the active residues. Docking simulations were run using the default HADDOCK protocol of 1000 rigid body minimization docking simulations, followed by 200 semiflexible refinement simulations and 200 final explicit solvent refinement simulations. The RMSD cutoff for clustering was set at 0.75% of common contacts with a minimum cluster size of 4.

**DMS DNA footprinting**

DMS footprinting reactions were performed in reaction buffer containing 25 mM HEPES, pH 7.6, 100 mM KCl, 5 mM radiolabeled (79) G4-multiplex DNA that possessed a 3’ biotin label on the G-rich strand, and where applicable, 4% PEG 200 and 500 nM PC4. DMS was added to a final concentration of 5% to initiate the reaction and was allowed to proceed for 10 s. The reactions were quenched by the addition of a quench solution to a final concentration of 1.2 M β-mercaptoethanol and 166 mM EDTA. Biotinylated DNA samples were then conjugated to M-280 streptavidin-conjugated Dynabeads® (Life Technologies Inc.). Dynabeads were prepared following the protocol for nucleic acid coupling provided by the manufacturer. DNA coupling to the beads was performed at a final concentration of 0.04 mg/ml Dynabeads, 20 mM Tris, pH 7.5, 1 mM EDTA, and 150 mM KCl. Coupling reaction was allowed to proceed for 30 min at room temperature with vortexing. Dynabeads containing the coupled DNA was precipitated with a strong magnet, and the supernatant was aspirated off. The Dynabead-DNA samples were then resuspended in 100 μl of cleavage solution containing 1 m piperidine, 0.1 mM biotin and heated for 30 min at 90 °C. DNA samples were then aspirated from the Dynabeads and evaporated to dryness using a SpeedVac. DNA samples were then resuspended in denaturing loading buffer containing 95% formamide, 0.025% bromphenol blue, and 20 mM EDTA. DNA samples were heated to 90 °C for 10 min and separated on a denaturing 20% polyacrylamide gel with 7 M urea. The samples were visualized on a Typhoon Trio phosphorimaging system and ImageQuant software. The radioactivity of each band representing the guanines involved in G4 formation were corrected for the background. The average corrected radioactivity of triplicate experiments for each band was divided by the total radioactivity in the lane to determine the fractional reactivity.

**Bromine DNA footprinting**

Bromine footprinting reactions with 5 nM DNA, 100 mM KCl, 4% PEG 200 with and without 500 nM PC4 were carried out as previously described with slight modifications (80). Briefly, molecular bromine was generated in situ by the addition of 120 μM KBr followed by addition of 240 μM H₂SO₄ to initiate the reaction. The reaction was allowed to proceed for 10 min before the reaction was quenched by the addition of 25 mM HEPES, pH 7.5. DNA was precipitated, cleaved with piperidine, and visualized as described above for the DMS footprinting protocol.

**Pif1 unwinding of c-MYC reporter substrate**

All of the concentrations listed are final. 25 nM Pif1 and/or 50 nM PC4 were preincubated on ice with 2 nM radiolabeled c-MYC reporter in Pif1 reaction buffer containing 25 mM HEPES, pH 7.5, 100 mM KCl, 2 mM β-mercaptoethanol, 0.1 mM EDTA, 10 mM MgCl₂, and 0.1 mg/ml BSA. The reactions were initiated at 25 °C with 5 mM ATP. The reactions were terminated at various times in sample buffer containing 25 mM Tris-Cl, pH 7.5, 0.1 mM EDTA, and 10% glycerol with 60 nM of unlabeled c-MYC reporter complement and 100 μg/ml of proteinase K. Prior to loading, the quenched samples were spiked with proteinase K to ensure complete proteolysis of Pif1 and PC4. The samples were separated on 15% native PAGE, visualized using a Typhoon Trio phosphorimaging system, and quantified with ImageQuant software (GE Healthcare). The fraction of substrate that was converted to product was determined, and the data were fit to a single exponential equation.

**Author contributions**—All authors designed and conceptualized experiments. W. C. G performed experiments and prepared the manuscript. All authors edited the manuscript.
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