Unique motifs and hydrophobic interactions shape the binding of modified DNA ligands to protein targets

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Selection of aptamers from nucleic acid libraries by in vitro evolution represents a powerful method of identifying high-affinity ligands for a broad range of molecular targets. Nevertheless, a sizeable fraction of proteins remain difficult targets due to inherently limited chemical diversity of nucleic acids. We have exploited synthetic nucleotide modifications that confer protein-like diversity on a nucleic acid scaffold, resulting in a new generation of binding reagents called SOMAmers (Slow Off-rate Modified Aptamers). Here we report a unique crystal structure of a SOMAmer bound to its target, platelet-derived growth factor B (PDGF-BB). The SOMAmer folds into a compact structure and exhibits a hydrophobic binding surface that mimics the interface between PDGF-BB and its receptor, contrasting sharply with mainly polar interactions seen in traditional protein-binding aptamers. The modified nucleotides circumvent the intrinsic diversity constraints of natural nucleic acids, thereby greatly expanding the structural vocabulary of nucleic acid ligands and considerably broadening the range of accessible protein targets.

SELEX | pseudoknot | modified nucleotide

Since the advent of SELEX (Systematic Evolution of Ligands by EXponential enrichment) 22 years ago (1, 2), aptamers have been described that bind specifically and with high affinity to many different types of targets, including proteins, peptides, and small molecules (3). Binding interactions between aptamers and their targets are characterized by shape complementarity, polar contacts, hydrogen bonding interactions, and charge-charge interactions (3–5). Other than base stacking interactions, hydrophobic contacts, which are known to make key contributions to protein–protein interactions (6–8), have been notably limited, reflecting the lack of such moieties in nucleic acid libraries typically used in SELEX.

We have recently shown that augmenting the diversity of randomized libraries with functional groups absent in natural nucleic acids can dramatically improve the success rate of SELEX, especially against difficult protein targets (9, 10). We have named this unique class of binding reagents SOMAmers (Slow Off-rate Modified Aptamers), to account for their distinct composition and binding properties. Among the different types of modifications we have tested, functional groups with hydrophobic character have typically yielded SOMAmers with the highest binding affinity. Although the contribution of such functional groups to the outcome of SELEX experiments has been quite apparent (9), the structural basis for the effect of these “side chains” on folding and binding has been unclear. Here, we report two cocrystal structures of related SOMAmers bound to a protein target, platelet-derived growth factor B (PDGF-BB), solved at a resolution of 2.2 Å and 2.3 Å. The structures elucidate the striking impact of the hydrophobic aromatic functional groups in creating novel intramolecular motifs and their extensive participation in shaping the contact surface with the native protein. By combining nucleic acid secondary structure elements (hairpins, pseudoknots) with protein-like features (hydrophobic patches), SOMAmers enhance the vocabulary of structural biology and suggest the next frontier in the area of directed evolution to create unique high-affinity reagents.

Results

Selection and Optimization of PDGF-BB SOMAmers. Platelet-derived growth factors (PDGF-A, -B, -C, and -D) are ubiquitous mitogens and chemotactic factors for many connective tissue cells (11). PDGFs occur as disulfide-linked dimers that exert their biological effects by dimerizing their cognate tyrosine kinase receptors, leading to autophosphorylation and signal transduction (12). SOMAmers were selected to bind PDGF-BB using SELEX conditions that favor slow dissociation rates, starting from a DNA library composed of 40 randomized positions containing benzyl-dU (Bn-dU), -dA, -dC, and -dG. Following eight rounds of selection, the affinity-enriched pool contained multiple copies of a high-affinity clone and its variants. Systematic truncation led to a 29-nucleotide core sequence (SL1) that retained full binding activity (Fig. 1A). SL1 exhibited high-affinity binding to PDGF-BB ($K_d = 20$ pM) and PDGF-AB ($K_d = 60$ –80 pM) but ~100-fold weaker binding to PDGF-AA (Fig. 1A and Fig. S1A and D). Binding to the more distantly related PDGF-CC and PDGF-DD isoforms was essentially undetectable (Fig. S1D). Reducing the disulfide bonds of PDGF-BB with DTT diminished binding affinity by >100-fold, showing that SL1 selectively recognizes the native conformation of its target. Overall, SL1 binds to PDGF-BB with affinity that compares favorably to previously reported PDGF aptamers (13, 14). SL1 potently inhibits PDGF-BB-induced phosphorylation of PDGF β -receptors (PDGFR β) in cultured human fibroblasts (Fig. 1A and Fig. S1B).

To assess the importance of individual nucleotides in SL1, we systematically replaced each nucleotide with a flexible linker (C3 spacer) that lacks both the sugar ring and the base but maintains the same number of carbons between phosphates (Fig. 1A). At most positions, even a single substitution was extremely deleterious to PDGF-BB binding. This linker-scan also revealed several nonessential positions, including the 3' terminal dG residue, a six-nucleotide stretch d(GAC[Bn-U]AC) near the 5' end, and a d(AC) dinucleotide in the center. This internal hexanucleotide could be replaced with a single hexaethylene glycol (HEG) linker (SL2, Fig. 1A). We also identified four positions that could accommodate 2'-O-methyl substitutions, resulting in the considerably shorter and more nuclease-resistant variant SL3 (Fig. 1A).

Modified Nucleotide Structure Activity Relationship and Affinity Maturation. To examine the contribution of each of the eight benzyl side chains to binding, we performed another series of

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 4HQX and 4HQU).

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Fig. 3. Modified nucleotides facilitate unique structural features of the PDGF SOMAmer. (A) S1 and L2 side view. Color scheme for bases and backbone is as follows. Miniknot stem 1: Watson–Crick base pairs, orange; noncanonical base pair, cyan; loop 2, white; stem 2, blue; 5' stem loop: Watson–Crick base pairs, gray; noncanonical base pair, pink. (B) Detail of noncanonical base pair between Pe-dU17 and Bn-dU20. (C) Axial view of S2. (D) Axial view of the 5' stem domain highlights deviation from B-form DNA (Table S2). (E) Noncanonical base pair between Bn-dU2 and Bn-dU8. (F) Bn8 makes edge-to-face π - π interactions with Bn16 and Bn20 that define the topology of the interdomain junction. The effect of a single modified nucleotide substitution at the junction is evident in the space-filling images of SOMAmer SL5 (G) versus SL4, where iB8 group is shown in red (H).

rest of SL5 and with PDGF; Bn-dU8 base stacks with Bn20, whereas Bn8 lies perpendicularly between the rings of Bn16 and Bn20 in consecutive π - π edge-to-face interactions. These long-range tertiary interactions define a precise hinge between the miniknot and the stem-loop domains (Fig. 3F and Fig. S5D). The lack of curvature between the first two nucleotides prevents clashing of Bn-dU1 base with Bn2, augmenting stacking of the rings (Fig. 3D). Bn2 sits in the middle of a hydrophobic cluster created by Bn7 and Bn8 (from the 5' stem) and Bn16, Pe17, and Bn20 (from the miniknot) (Figs. 2B and 3D and Fig. S5D). This hydrophobic cluster contributes to stabilization of the SOMAmer, supported by the observation that SL5 exhibits a melting temperature (T_m) of 64 °C, which is >30 °C higher than its analog that lacks the modified nucleotides (Fig. S7).

Bn-dU8 Is Critical for Junction Topology and Functional Activity. In addition to SL5, we also solved the structure of SL4, which is identical to SL5 except for the replacement of Bn-dU8 with isobutyl-dU (iB-dU). When iB-dU8 was combined with Pe-dU17 and Th-dU18 in variant SL4, the SOMamer showed substantially weaker binding (~20- to 50-fold vs. SL5) and a 75-fold lower in vitro inhibitory activity (Fig. 1*A* and Fig. S1*B*). The smaller non-aromatic isobutyl side chain cannot form the energetically favorable π - π edge-to-face stacking seen with the benzyl side chains of Bn-dU20, Bn-dU8, and Bn-dU16 in SL5 (Fig. 3*G* and *H*). This creates a hole in the center of the hydrophobic cluster at the protein interface, effectively unlocking the hinge between the 5' stem and the miniknot domains. The structural effect of this substitution is directly analogous to a Phe to Leu mutation in the hydrophobic core of a protein. Such protein mutations are well described (26–28) and usually have a significant destabilizing effect. Junctions between secondary structure motifs are known to play a critical role in determining nucleic acid tertiary structure (29).

Despite markedly weaker target-binding affinity, SL4 exhibits a thermal melting profile similar to that of SL5 in the absence of ligand (T_m values of 62 °C and 64 °C, respectively; [Fig. S7A](#)). This is consistent with the notion that the cavity and altered junction topology created by iB-dU8 substitution in SL4 destabilizes the protein-binding interface, while leaving the intradomain structures of the SOMAmer intact. The conformations of free SOMAmers in solution may well be very different from those in the complex with the protein, which could also diminish the relationship between T_m and binding affinity. In fact, because the energetic cost of solvating a large hydrophobic surface of the SOMAmer is likely to be substantial, we expect the uncomplexed SOMAmer to collapse around the hydrophobic side chains and adopt a conformation in which they are partially protected from the solvent (30).

Extensive Hydrophobic Interactions Define the SOMamer:Protein Interface. In contrast to previously described protein:aptamer complexes, hydrophobic interactions dominate the interface between SL5 and PDGF (Figs. S3 and S6). Binding to PDGF-BB creates a buried surface area of $\sim 1,225 \text{ \AA}^2$ per SOMamer. The

eight modified nucleotides of SL5 create an extensive hydrophobic interface that interacts with 13 nonpolar amino acids of PDGF (Ala35, Phe37, Leu38, Val39, Trp40, Pro42, Cys52, Cys53, Ile75, Ile77, Pro82, Ile83, and Phe84), which account for approximately half of the total nonpolar contacts, with the remainder composed of aliphatic regions of polar or charged amino acids such as Glu24, Arg27, Asn36, Asn54, Asn55, Arg56, Arg73, Lys74, Lys80, Lys85, and Lys86 (Fig. S6). Similar interactions between completely nonpolar residues and nonpolar moieties of charged amino acids are often observed in proteins. Thus, the structural diversity afforded by the modified nucleotides in SOMamers enables them to mimic the rich repertoire of interactions accessible to proteins. The striking difference in the extent of hydrophobic contacts made by the SOMamer compared with traditional aptamers is evident when the interface atoms are displayed on the surfaces of the target proteins (Fig. 4A). SL5 exhibits remarkably few polar interactions, having just six H-bonds and one charge-charge interaction with PDGF, despite close proximity to basic amino acids. Relative to the contact surface area, this is significantly lower than what is typical for aptamers. The total number of H-bonds and charge-charge interactions (that is, polar contacts) for six traditional aptamers increases approximately linearly in direct proportion to the interface area (Fig. 4B and Fig. S84) with a correlation coefficient of 0.91 and an average of 1.9 ± 0.4 polar contacts per $100\text{-}\text{\AA}^2$ interface area. SL5, as well as two additional SOMamers in other cocrystal structures, clearly fall outside of the 99% confidence intervals of this trend, with less than half the number of polar contacts per interface area (average of 0.7 ± 0.2 per $100\text{-}\text{\AA}^2$ interface area), while exhibiting a trend toward higher binding affinities for their targets (Fig. 4B and Fig. S8C). In terms of ligand efficiency (free energy of binding per nonhydrogen contact atom) (31), aptamers and SOMamers do not appear to be different (Fig. S8C), encompassing a range of values observed with protein-based and small-molecule-based ligands (32). Free energies of binding per interface area are also similar (Fig. S8C). What is different, however, is the value of free energy of binding per polar contact, which is about twice as large for SOMamers as for aptamers (Fig. S8B and C), consistent with the notion that SOMamers derive a larger contribution to binding from hydrophobic interactions.

Charge–charge interactions often contribute less than 0.2 kcal/mol to the stability of a folded protein (33). In contrast, burying just a single methylene group is estimated to contribute ~1–1.5 kcal/mol to globular protein stability and/or binding interactions (6, 7). SOMAMer structures reveal a strong reliance on hydrophobic interactions, and in this sense their binding to proteins more closely resembles typical protein–protein interactions. Consistent with this observation, the affinity of SL5 for PDGF shows virtually no decrease across a broad range of salt concentrations (0.1–1.0 M NaCl) or pH values (5.0–8.8), in contrast to the effects seen with traditional aptamers (14, 34).

Post-SELEX optimization facilitates fine tuning of shape complementary and hydrophobic packing interactions. For example, the

Fig. 4. Hydrophobic interactions dominate SOMAmer-target binding interface. (A) Interface contact atoms of PDGF in the SOMAmer cocrystal are compared with six currently available cocrystal structures for aptamer:target complexes with K_d values <500 nM (Fig. S8C). Noncontact atoms are colored gray, and atoms that make ≤ 4 Å contact with the nucleic acid ligand are colored yellow (nonpolar contacts), red (oxygen participating in a H-bond or salt bridge), or blue (nitrogen participating in a H-bond or salt bridge). (B) Plot of the number of polar contacts (hydrogen bonds plus charge-charge interactions) vs. contact surface area vs. reported binding affinities (also Fig. S8) for the six aptamer-target complexes (blue bars) and for three SOMAmers (yellow bars) including the PDGF:SL5 complex and two other SOMAmer:target structures. The area outside the 99% confidence interval for the polar contacts vs. contact surface area trendline defined by the six aptamers is indicated by gray shading on the floor of the figure. (C) Detailed view of shape complementarity of the PDGF:SOMAmer complex as exhibited by Pe-dU17 and Th-dU18 residues in space-filling representations (Left) or Pe-dU17 in stick representation revealing the contours of the thiophene ring of Th-dU18 (Right). PDGF chains 1 and 2 are shown as dark and light green surfaces, respectively. (D) Detail of Bn-dU1 interaction with PDGF.

exceptional shape complementarity of Pe-dU17 and Th-dU18 at the protein interface (Fig. 4C) is corroborated with the structure-activity relationships (Fig. 1B). Bn-dU1 also forms a unique interaction with PDGF-BB, with the benzyl ring sitting in a tunnel formed by the Cys43–Cys52 disulfide bond and a salt bridge between Glu24 of PDGF chain1 and Arg56 of chain 2 (Fig. 4D). The crystal structure suggests that the binding pocket can accommodate a variety of side chains, including larger bicyclic substituents, thereby enhancing this point of contact with the protein. Indeed, we have identified several modified nucleotide substitutions at this position that confer 5- to 10-fold enhancement in binding affinity (Fig. 1B).

SOMAmer Binding to PDGF Mimics Receptor Binding. A notable feature of the PDGF-BB:SL5 structure is the degree to which the SOMAmer mimics PDGFR β . The receptor binds to PDGF primarily through hydrophobic interactions, including seven hydrophobic amino acids at the PDGF interface (35). SL5 binding site largely overlaps that of the receptor with the Bn-dU aromatic rings occupying the same hydrophobic groove on the protein (Fig. 5). PDGF contacts both the receptor and SL5 with 24 residues, of

which 10 are shared. These shared, or “promiscuous,” residues likely represent a hot spot of binding energy on the surface of PDGF (32, 36). However, compared with PDGFR β , SL5 exhibits a 10-fold higher affinity for PDGF-BB (37). Consistent with these observations, SL5 is a potent inhibitor of PDGF-BB (Fig. 1A and Fig. S1).

Discussion

Hydrophobic interactions have long been recognized as a fundamental driver of macromolecular stability and complex formation (6, 36, 38). We have shown here that the hydrophobic functional groups of SOMAmers augment the contours of the binding surface and its physicochemical complexity beyond that which can be achieved with natural nucleic acids. This situation is similar to antibodies where relatively limited shape diversity of the canonical main-chain conformations in CDRs (39) is enhanced with amino acids that can sweep out large volumes of space, interspersed with smaller amino acids that allow such motions to take place (15, 16). Indeed, CDR libraries composed of only two amino acids, tyrosine and serine, which are overrepresented in CDRs, have been used to derive specific antibodies with relatively high affinity (40). However, such a minimalist approach is not always sufficient for certain antigens (analogous to difficult targets for aptamers) and additional diversity is needed for high-affinity binding to a larger fraction of epitopes (40).

With traditional aptamers, and for a subset of targets, good success has been achieved to date with relatively limited functional group diversity inherent to natural nucleic acids (compared with proteins), but compensated for by a much larger shape repertoire (compared with antibodies) provided by random libraries of enormous size ($\sim 10^{15}$ molecules). Conformational flexibility of nucleic acids is intrinsically far greater than that of proteins because the backbone of each monomer has seven rotatable bonds, compared with two in proteins (ϕ and ψ). The addition of novel functional groups to nucleic acid libraries

Fig. 5. Comparison of SL5 and PDGFR β binding to PDGF-BB. (A) Receptor cocrystal showing PDGF homodimer (chain 1, dark blue; chain 2, light blue) and receptor extracellular domain colored gray (35), with aromatic rings of hydrophobic amino acids highlighted red. (B) Complex of PDGF homodimer (chain 1, dark green; chain 2, light green) and SL5 (gray), with aromatic rings of modified nucleotides highlighted yellow. (C) Comparison of SL5 and PDGFR β binding footprints. PDGF is shown in three orientations as a surface representation, with residues that make 4 Å contact only with SL5 or PDGFR β or both (“overlap” residues) shown in yellow, red, or orange, respectively.

results in a unique type of ligand that incorporates the best attributes of two types of biopolymers: the huge conformational repertoire of nucleic acids combined with diversity-enhancing protein-like side chains. This has demonstrably translated into considerable improvements in target binding affinity as well as expansion of protein targets accessible to SELEX (9). The incorporation of various functional groups into nucleic acids has also been used to enhance nucleic acid-mediated catalysis (41, 42). The cocrystal structures described in this report provide a glimpse into the astonishing array of unique structural themes that can be achieved with modifications to standard nucleotide chemistry.

Many protein targets that were previously resistant to aptamer selection have proven amenable to SOMAmer selection (9), and this has allowed us to identify SOMAmers to over 1,000 human proteins (9). We are currently investigating the potential of this nascent technology for applications ranging from biomarker discovery and diagnostics (43, 44) to histochemistry and imaging (45). With the well-established role of PDGF-BB in proliferative disorders including atherosclerosis, fibrosis, macular degeneration, and cancer (11, 46, 47), the SOMAmers described here also have clear potential utility as therapeutic agents.

Materials and Methods

SOMAmers were selected and synthesized by methods described previously (9). PDGF-BB was obtained from Creative BioMart. Crystals of SL4:PDGF-BB and SL5:PDGF-BB diffracted to 2.3 and 2.2 Å resolution, respectively, and structures were solved by molecular replacement and refined to final $R_{\text{work}}/R_{\text{free}}$ values of 23.9/27.9% and 22.5/26.2%, respectively. Binding assays were performed using radiolabeled SOMAmer and capture of protein-bound SOMAmer by Zorbax beads. Cellular phosphorylation assays were performed in human fibroblasts cells. Results were quantified by phospho-specific ELISA. Sequence analysis of SELEX pools was performed using 454 pyrosequencing data. Detailed materials and methods can be found in *SI Materials and Methods*.

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