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Engineered Protein Scaffolds as Leads for Synthetic Inhibitors of Protein-Protein Interactions

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Abstract

Rationally designed protein-protein interaction inhibitors mimic interfacial binding epitopes, specifically residues that contribute significantly to binding. However, direct mimicry often does not lead to high affinity ligands because the natural complexes themselves are functionally transient and of low affinity. The mimics typically need to be optimized for potency. Engineered proteins displaying conformationally-defined epitopes may serve as attractive alternatives to natural protein partners as they can be strictly screened for tight binding. The advantage of focused screens with conformationally-defined protein scaffolds is that conservation of the geometry of the natural binding epitopes may preserve binding site specificity while allowing direct mimicry by various synthetic secondary structure scaffolds. Here we review different classes of engineered proteins for their binding epitope geometry and as leads for synthetic secondary and tertiary structure mimics.

Introduction

Designed protein-protein interaction (PPI) inhibitors often mimic structural features present at natural protein-protein interfaces. However, despite preliminary success in inhibitor design for PPIs, it has proven difficult to develop high affinity ligands for extended protein surfaces [1–3]. Typically, it is common to quickly obtain peptides or small molecules that bind with millimolar to micromolar affinity. Translating these weak inhibitors to potent, specific modulators of intracellular interactions remains a significant challenge. The challenge in isolation of weak binders is often propagated from natural PPIs, which are functionally transient and thus of low affinity.

The goal in inhibitor design is to reduce the salient features of the natural binding interface to a cell-permeable small molecule or peptidomimetic while preserving the binding affinity and specificity for the chosen target. Based on this premise, we and others have surveyed high resolution complexes in the Protein Data Bank for helix- [4], strand- [5], loop- [6], and coiled-coil [7] -mediated protein complexes to define starting points for macrocycles [8], secondary [9], and tertiary structure mimics [10–12]. However, these mimics of natural

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interfaces are often weak binders because (i) by design the mimics do not capture all contacts available to the natural proteins, and (ii) the natural proteins themselves are often evolved for biological function rather than binding affinity. The leads require further optimization from analyses of structure-activity relationships, library screening, computational modeling, or non-natural residue incorporation [13–16]. These optimization steps are routinely employed and have been reviewed [17]. Direct screening of some conformationally constrained peptide motifs such as helices [16,18] and macrocycles [19,20] has also been described. However, a comprehensive approach that allows mimicry of various different binding epitopes may enhance success in developing specific inhibitors. Typically, it is difficult to access secondary structures in isolated peptide sequences for selection assays because short peptides do not readily adopt a single, predominant conformation in the absence of the native protein context. An alternative to screens with random peptides or macrocycles would be to utilize high-affinity, engineered protein scaffolds that preserve the conformation found in the native protein complex [21,22]; sequences from these protein scaffolds could then be transformed to synthetic motifs [23]. This approach assumes that specific backbone geometries have been evolved in nature because they may be the best geometrical matches for the crevices and curvatures found on the binding partner [24,25]. However, this may not always be the case as sheet epitopes can be mimicked by helices and vice versa [26]. We posit that while it may be possible to obtain conformationally distinct tertiary structures that bind a given protein surface with high affinity, it would be difficult to readily swap secondary structures because of their more focused binding epitopes. Access to conformationally-defined epitopes from engineered proteins would streamline design of small molecule and peptidomimetic scaffolds.

Several classes of engineered proteins have been described, making it rather straightforward to create high affinity proteins for a given target using various display technologies [21,22,27,28]. We evaluated the suitability of non-antibody protein scaffolds as conformation-specific leads for the generation of peptidomimetics or small molecules. The geometry of the binding epitope offered by different engineered proteins suggests that they can be readily transformed into synthetic secondary or tertiary structure motifs (Figure 1). (In this review, we do not distinguish between miniproteins, which are generally classified as proteins of less than 40–60 residues,[21] and engineered protein scaffolds, and focus solely on the geometry of engineered proteins available for combinatorial screens.)

Notable examples of engineered protein mimicry by synthetic secondary and tertiary structure mimics have been reported [29,30]. Ghosh and coworkers reported a functional small molecule mimetic that reproduces the binding epitope of an evolved protein scaffold [23]. Phage display on a β -sheet-rich engineered protein, HTB1, was used to identify selective binders of thrombin that utilized a dityrosine motif. The authors demonstrated that the engineered protein could be utilized as a platform for generating secondary structure mimetics via a functional epitope grafting strategy. Gellman and coworkers developed α/β -peptide foldamers to target vascular endothelial growth factor (VEGF) by mimicking a potent phage-display selected Affibody construct [31]. The group showed that α/β -foldamers can structurally and functionally mimic the binding surface of Affibody but offer significant improvement in proteolytic stability [10].

The binding epitopes of non-immunoglobulin proteins

Several classes of engineered proteins that can engage their biomolecular targets with high affinity and specificity have been described [32–34]. Although the binding epitope of these tertiary-structure-mediated contacts is more complex than those made by secondary structures and small molecules, their high stability and tolerance to directed evolution techniques have led to their success as alternatives to traditional antibodies and small molecules. We analyzed the binding epitopes within several classes of engineered protein complexes and compared them to secondary structure-mediated binding epitopes found in PPIs [4–6,35,36].

Engineered proteins have shown particular success in modulating *extracellular* PPIs, and hold numerous advantages over traditional antibodies such as their small size (4–20 kDa), favorable expression profile and stability, long half-lives, and amenability to directed evolution techniques. The protein scaffolds themselves are most commonly derived from human parent proteins, however, some scaffolds possess eukaryotic, prokaryotic or synthetic origin. Roughly twenty engineered proteins have been reported to date [32–34]; the physical and structural characteristics of several proteins in clinical development are described in Figure 2. We focused on non-antibody protein scaffolds (NAPS) as they contain conformationally-defined epitopes that can be used as defined starting points for secondary structure mimics.

We systematically analyzed the binding epitopes of the protein crystal structures of non-redundant, pharmacologically-relevant protein complexes (n=51) using Rosetta-based computational alanine mutagenesis scanning [37,38] and the 2P2I database [39]. The results of this analysis are summarized in Figure 2. The critical binding residues, or hot spot residues, are defined as residues whose mutation to alanine results in an estimated ΔG of binding >1 kcal/mol. Qualitative assessment revealed that the number of secondary structure contacts ranged from 2–16 α -helices, β -strands or loops. Although the average size of analyzed engineered proteins spans 105 ± 63 residues, the contact surface is much smaller with roughly seven amino acid residues contributing significantly to binding. This is surprising considering libraries generally randomize 10–20 surface mutations to generate high affinity and specificity; however, the results give credence to the hypothesis that these engineered proteins can serve as leads for peptidomimetics and small molecules because clustered hot spots can be readily displayed on small molecular weight scaffolds[40].

Non-immunoglobulin proteins engage their targets with more ionic contacts than natural interfacial protein secondary structures

While the interaction between two proteins often occurs with shape and electrostatic complementarity, the residual detail in which they interact gives high resolution insight into their complex binding epitopes. It is well appreciated that proteins utilize surface tryptophan, tyrosine, and arginine residues to engage their protein partners [41,42]. Engineered proteins engage their targets in a similar manner (Figure 3A); tryptophan and tyrosine residues are highly enriched at target interfaces likely due to library bias in selection techniques. In earlier studies, we and others found that aromatic residues and arginine are most likely to be

hot spot residues on secondary-structure-mediated PPIs regardless of the type of secondary structure, i.e. α -helix, β -strand or loops [4,5,7,35]. The engineered proteins show a striking enrichment in both aromatic and ionic residues when compared to interfaces mediated by α -helices [4], β -strands [5], and loops [6] (Figure 3B). The enrichment in arginine, phenylalanine, and aspartic acid residues demonstrate that the protein scaffolds engage their targets with more electrostatic complementarity than secondary structures. Owing to their larger surface area, NAPS have access to a high number of surface contacts to engage the targets with high affinity and specificity. The diversity in NAPS tertiary folds allows for convex, concave, and planar binding epitopes for geometric matching, which cannot be easily reproduced with isolated secondary structures. Examining contact surface area ratios in our NAPS data set, revealed Darpins and Anticalins to be the most concave, Alphabodies and Affibodies are the most planar, and Kunitz domains are the most convex. Interestingly, the Monobody scaffold is able to span all different types of geometric interactions agreeing with previous reports [25,43].

Conclusion

Engineered proteins have vast potential as leads for synthetic scaffolds. Three features of how these proteins engage their partners stand out: (a) interfacial hot spot residues are more densely clustered in engineered protein complexes than natural protein hot spots. (b) Conformationally defined secondary and tertiary structures can be readily accessed for direct mimicry. (c) A higher proportion of arginine and aspartate hot spot residues are found in engineered protein complexes. The presence of critical ionic residues identified in our analysis beyond aromatic residues may aid in developing inhibitors with higher affinity and specificity as some of these critical features are not always available in natural protein complexes.

We anticipate that combining the recent progress in secondary and tertiary structure mimicry [9,12,17,48–55] with advances in conformationally-defined epitope display will lead to new avenues for protein-protein interaction inhibitor design (Figure 4). These efforts will complement existing strategies for rational design of ligands inspired by natural protein complexes. Engineered protein scaffolds are a highly successful class of molecules that are currently being evaluated clinically. Their success can be attributed to their ease of production, amenability towards directed evolution techniques, and small size relative to traditional monoclonal antibodies. However, their success as inhibitors of PPIs has largely been limited to extracellular protein targets as they have no intrinsic cell permeability; intracellular targeting currently requires exogenous delivery systems and transfection strategies [56–59]. The potency of these combinatorial randomized protein scaffolds can potentially be harnessed for intracellular targets, by miniaturizing their binding epitopes onto synthetic scaffolds.

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Figure 1. Conformation-specific engineered proteins as an alternative starting point for peptidomimetic inhibitor design

In this perspective, we explore the potential of engineered protein scaffolds as leads for synthetic secondary and tertiary structure mimics. Mimicry of engineered proteins, which can be selected for tight binding, provides an alternative to reproduction of the natural binding epitopes. Yellow and red spheres represent native and optimized contacts, respectively.

Figure 2.

Analysis of several classes of non-antibody protein scaffolds. The high-resolution crystal structures of protein complexes (n=51) were analyzed in Rosetta. REU = Rosetta Energy Unit.

Figure 3. Analysis of critical binding residues utilized by high affinity non-antibody protein scaffolds (NAPS)

Graphs compare hot spot enrichment in NAPS versus those found at (A) globular protein interfaces and (B) interfacial secondary structures (helices, strands and loops). The hot spot residue data for secondary structures (n=205,184) is obtained from reference [35] and for natural proteins (n=2,325) from reference [41]. We analyzed non-redundant NAPS-protein complexes whose high-resolution structures have been reported in the PDB (n=51). Omitted residues do not have statistically significant enrichment.

Figure 4. Miniaturization of combinatorially randomized engineered protein scaffolds to secondary and tertiary structure mimics

Several classes of secondary and tertiary structure mimics have been described and could be employed for miniaturizing proteins. Examples of hydrogen bond surrogate (HBS) helices and stapled helices as helix mimics [44], and crosslinked helix dimer as coiled coil mimics (top row) [12], triazolamers as strand mimics and diproline β -hairpin mimics (middle row) [45,46], and macrocycles and bicyclic peptides as loop mimics [6,47] are shown.