Peptides tailored to interfere with protein interaction and function

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ABSTRACT

We have employed semi-rational design in combination with selection systems to generate peptides interfering with a pathogenic protein-protein interaction: the transcriptional regulator Activator Protein-1 (AP-1). Peptide inhibitors with high interaction stability were screened and selected using either phage display or protein-fragment complementation assays (PCA). The specificity of interaction was further enhanced by combining PCA with a “Competitive and Negative Design Initiative” (CANDI). Selected peptides binding their target revealed an impressive 70,000-fold increase in $K_d$ compared to the wild-type interaction, and major energetic differences of up to 5.6 kcal/mol between desired and non-desired interactions were achieved. Different strategies are discussed for targeting oncoproteins such as Jun and Fos using tailored peptides.

INTRODUCTION

Can one design stable and specific protein-protein interactions? Doing so at will should enable devising interfering peptides and peptidomimetic antagonists able to mimic natural proteins by binding and sequestering interaction partners, or conversely by increasing the activity of the natural protein. It will also help to understand how protein networks function, and will generate sequences capable of capturing proteins that behave abnormally and give rise to a pathogenic phenotype. In the following sections, and as a result of principle, we have focused our efforts on the coiled-coil region of the Activator Protein-1 (AP-1) transcription factor that is implicated in a variety of diseases.

COILED COILS

The coiled coil is an attractive protein model since it contains a simplistic quaternary structure but it is specific and diversely distributed (1-4). Indeed, it is found in 3-5 percent of the entire coding sequence (5) where it serves in transcriptional control (6), muscle contraction (7), viral infection (8, 9), cell signalling (10), molecular chaperones (11), and fertilisation (12), and is therefore an ideal test-bed for specificity. It contains a regular repeating unit of seven amino acids (the heptad repeat) which contains distinctive hydrophobic/hydrophilic repeats between interacting helices. The patterning of these charged and hydrophobic residues influences stability, specificity, orientation and oligomeric state of resultant interactions (13-15).

THE JUN/FOS ACTIVATOR PROTEIN-1 SYSTEM

The transcription factor Activator Protein-1 (AP-1) is composed of Jun (cJun, JunB, JunD) and Fos (cFos, FosB, Fra1, Fra2) proteins, with DNA binding occurring at a specific consensus sequence (TGAGTCA) known as the “TPA Response Element”, or TRE. Jun and Fos members contain a transactivation domain, a basic DNA binding region and a leucine zipper (coiled coil) domain that mediates homo- (for Jun members) or hetero-dimerisation. This basic-zipper or bZIP protein is found within several cell signalling cascades. Implicated in numerous cancers, AP-1 is considered a legitimate therapeutic target. Depending on the subunit composition, AP-1 can also have antiproliferative properties as is clear from the abundance of different AP-1 family members in different types of cancer. For example, c-Jun is central in skin and liver tumours, while JunB and JunD have very poor transactivation domains, weak transforming activities, and may have antagonistic function. Fra1 and Fra2 have weak transactivation domains, and are found in lung and epithelial tumours, possibly functioning by dimerising with other family members possessing intact transactivation domains (16).

Figure 1. Schematic representation of the iPEP strategy. A peptide interfering with coiled-coil mediated interaction of cellular protein of interest, e.g. a transcription factor is selected from designed semi-rational libraries (18, 19, 24, 29-31). The iPEP (red) captures its target (blue) and prevents the natural interaction partner (green) from binding, resulting in a loss of DNA-binding capability (19).
Specific inhibition of inappropriate AP-1 formation is likely to be better served via interference with the dimerisation driving coiled-coil propensity, and solubility, and contribute to improved overall stability. Libraries were screened in vivo for an interaction with a partner helix using a Protein-fragment Complementation Assay (PCA) (14, 17) (Figure 2). In PCA, one half of murine dihydrofolate reductase (mDHFR) is genetically fused to the target, and the second half of mDHFR is fused to the protein library. Library members that bind to the target will recombine the two halves of DHFR, render the enzyme active, and generate a bacterial colony under selective conditions. Growth competition under selective conditions enriches for fastest bacterial growth, which correlates in most cases very well with coiled-coil interaction strength (14, 17). Selected winning sequences were termed JunW and JunW. These 37-mer peptides targeted cJun and cFos, respectively (18), and were analysed for both stability and specificity together with wild-type Jun and Fos family members (cJun, JunB, JunD and cFos, FosB, Fra1, Fra2) (Figure 3). Electrostatic e/g pairings, core residues pairings, and helical propensity were dominant factors in determining coiled coil stability (see below and also Mason et al. 2006 for further details) (18). Resulting dimers, cJun/FosW and cFos/cJunW, displayed significant stabilities with $T_m$ values of 63°C and 44°C, relative to 16°C for the wild-type cJun/cFos coiled-coil interaction. This first criterion of higher stability for cJun/FosW represented an impressive 70,000-fold $K_D$ improvement and was due to optimised hydrophobic packing, a-helical propensity, and electrostatics. PCA is carried out entirely in vivo such that only soluble, non-aggregating, protease-resistant, stable inhibitors that bind their targets with high efficacy are selected. Sequencing one half of AP-1 with such potency should strongly and indefinitely inhibit transcription of the target gene.

**Peptide Selection Using Phage Display As A Comparison**

Selecting peptides from the same parent library as the PCA-selected JunW peptide, we used the phage display technique to permit a direct comparison of selected sequences and their ability to disrupt the cFos/cJun interaction (19). Explicitly, a cJun-based library was screened to enrich for peptides that disrupt the AP-1 complex by binding to the cFos coiled-coil domain. Phage display identified one helix, JunW$_{ph}$, (phage display-selected interfering peptide targeting cFos), which differed by only two of ten randomised positions relative to JunW (PCA-selected interfering peptide targeting cFos). These two positions, Q21R and E23K were located at an edge e and g position respectively. Phage-selected peptides revealed higher affinity to cFos than wild-type cJun, harbouring a $T_m$ of 53°C compared to 16°C for cJun/cFos or 44°C for cFos/JunW. Importantly, all inhibitory peptides were able to interfere successfully with DNA binding as demonstrated in gel shift assays.

**The ‘Basic Coiled Coil Interaction Prediction Algorithm’ (bCIPA)**

Despite not being a factor in the initial library design criteria, selected sequences displayed much improved a-helical propensity. Thermal melting data for every possible dimeric interaction between homologues and interfering peptides (as shown in Figure 3) generated quantitative rankings of bZIP stability. Based on these data, the ‘basic coiled coil interaction prediction algorithm’ (bCIPA) was developed, predicting...
A ‘COMPETITIVE AND NEGATIVE DESIGN INITIATIVE’ (CANDI) GENERATES STABLE AND SPECIFIC PROTEIN-PROTEIN INTERACTIONS.

The previous study demonstrated that, although increased stability is relatively straightforward to design and select for, it is much harder to confer a specific interaction while maintaining this high interaction stability. Designing peptide-based inhibitors to bind with both high affinity and specificity to pathogenic proteins implicated in disease is of utmost importance, and promises to yield important rules. Designed inhibitors arising from “single-state” approaches may bind their target, but in vivo derived inhibitors can do so with increased target specificity. This so-called negative or “multi-state” design can derive specific high affinity peptide-based drugs. An in vivo selection of specific and stable interactions was achieved by expressing homologous peptides lacking DHFR-fragment fusions in the PCA assay. These homologues are then forced to compete with protein libraries for an interaction with a target molecule during PCA (24). Library members must bind their target, promote cell growth, while out-competing competitor-target interactions (i.e., competition) and evade binding to the competitors (i.e., negative design). Thus, library members with high interaction stability and specificity will confer DHFR activity and result in the fastest bacterial growth rates (see Mason et al, 2007 for further details) (24). This is termed a Competitive And Negative Design Initiative (CANDI). By combining CANDI with PCA we observed major specificity improvements, by driving selection of interfering library members that bind their target with maximum efficacy, ensuring that otherwise energetically accessible alternatives are inaccessible (24). CANDI-PCA has been used with libraries targeted at coiled-coil regions of oncopgenic AP-1 components c-Jun and c-Fos. We demonstrated that comparable hydrophobic and electrostatic contributions in desired species are compromised in non-desired species when CANDI is executed, demonstrating that both, core and electrostatic residues are required to direct specific interactions. Major energetic differences (5.6 kcal/mol) are observed between desired and non-desired interaction stabilities for a CANDI-PCA derived peptide relative to a conventional PCA derived helix, with significantly higher stability (3.2 kcal/mol) than the wild-type c-Jun/c-Fos complex. As a negative control, a library lacking a residue repertoire able to generate a specific and stable helix was also tested. In this case a specific interaction was not capable of being generated, and therefore implementation of CANDI had no effect.

STUDYING THE FOLDING MECHANISM OF JUN-FOS AP-1 COILED COIL MOTIFS

We further probed the structural determinants of stability by dissecting the folding pathway of four c-Jun leucine zipper variants that bind with high affinity to c-Fos (25). These encompassed our previous PCA-selected interfering peptide (J unW) and the phage-display selected interfering peptide (J unW\textsubscript{M}). These peptides differed in only two of ten semi-randomised positions with ΔT\textsubscript{m} values of 28°C and 37°C over wild-type. To separate the effect of these two mutations, we also looked at the two intermediate mutants (J unW\textsubscript{E23K} and J unW\textsubscript{Q21R}), c-Fos/J unW, c-Fos/J unW\textsubscript{M}, and intermediate mutants (c-Fos/c-JunW\textsubscript{Q21R} and c-Fos/c-JunW\textsubscript{E23K}) displayed biphasic kinetics in the folding direction, indicating the existence of a folding intermediate. The first reaction phase is fast and concentration dependent, showing that the intermediate is readily populated and dimeric. The second is independent of concentration and is exponential. In contrast, in the unfolding direction, all molecules displayed two-state kinetics (25). Collectively, this implies a transition state between unfolded helices and a dimeric intermediate that is readily traversed in both directions. We have demonstrated that the added stability of c-Fos/J unW\textsubscript{M} relative to c-Fos/J unW is achieved via a combination of kinetic rate changes; c-Fos/J unW\textsubscript{Q21R} exhibited an increased initial dimerisation rate, prior to the major transition state barrier while c-Fos/J unW\textsubscript{E23K} displayed a decreased unfolding rate. The former implies that improved hydrophobic burial and helix-stabilising mutations exert their effect on the initial, rapid, monomer-collision event. In contrast, electrostatic interactions exert their effect late in the folding pathway. Although our focus was on the leucine zipper region of the oncopgenic transcription factor AP-1, coiled coils are ubiquitous and highly specific in their recognition of partners. Consequently, generating kinetic-based rules to predict and engineer their stability is of major significance in peptide-based drug design and nanobiotechnology.

CONCLUSIONS

The importance of AP-1 and the targeting of its coiled coil and/or DNA binding regions in antagonist design and development have been highlighted previously (26-29). Peptides that bind and sequester coiled-coil interactions have been generated using PCA and phage display combined with semi-rational design. CANDI-PCA was used to confer specificity and stability on resulting protein-protein interactions. Inspection of the folding pathway of J un-Fos variants has given new rules to aid in the design of stable and specific future antagonists, including hydrophobic and/or high helical propensity residues to increase folding rates, and intermolecular electrostatic interactions to decelerate unfolding rates. PCA has been applied to therapeutically relevant systems to generate interfering peptides that free DNA from J un-Fos bZIP protein and the Myc-Max bHLHZip protein (26-29). Collating techniques and rules, and demonstrating in vivo efficacy, we will harness negative protein design to generate specific and therapeutically relevant peptides, as well as peptides suited to a range of other nanobiological functions.
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REFERENCES AND NOTES