Technique review

Peptide aptamers: Tools for biology and drug discovery

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Abstract

Peptide aptamer technology is relatively youthful. It has the advantage over existing techniques that the reagents identified are designed for expression in eukaryotic cells. This allows the construction of molecular tools that allow the logic of genetics, from knockouts to extragenic suppressors, to be applied to studies of proteins in tissue culture cells. Until recently, the available tools have limited our understanding of cell biology. The same limitation restricts our ability to validate the numerous candidate drug targets emerging from genome-wide approaches to cellular biology. Peptide aptamers represent a stride forwards in the evolution of a modular, molecular tool kit for cell biology and for drug target validation. The authors predict that they will also play a role in the transition from genomic to proteomic microarray technology.

Keywords: constrained peptides; nanotechnology; protein engineering; protein genetics; proteolysis; cellular targeting signals

PEPTIDE APTAMERS: PRINCIPLES AND ADVANTAGES

Background

The use of peptide aptamer technology has been adopted by a number of groups.1–8 The terms peptide aptamers (our term of choice),1 paptamers,4 thioredoxin-insert proteins (TIPs)2 or perturbagens9 are used to describe artificial proteins where inserted peptides are expressed as part of the primary sequence of a structurally stable protein, called the scaffold. This is achieved by the insertion of oligonucleotides encoding the peptide into existing or engineered restriction sites in the open reading frame encoding the scaffold. The peptides displayed can be isolated from random libraries, or can be amino acid sequences taken from previously identified proteins whose biology requires further characterisation. The three-dimensional folding of the scaffold is predicted to force the peptide to adopt a discrete shape from the range of conformational space that is available to it.10 The final shape of the presented peptide will be determined both by the amino acid composition and sequence of the peptide and by the primary sequence and tertiary structure of the scaffold protein.

The site of insertion is chosen such that the peptide will disrupt a region of the scaffold that would otherwise mediate a protein–protein interaction. This ensures both that the peptide will be displayed on the solvent-exposed surface of the scaffold, and that at least one important and potentially confounding activity of the scaffold is lost. An ideal scaffold will be biologically neutral: it will not interact with any cellular molecule or organelle and will possess no enzymatic activity.

Functionally, peptide aptamers can be compared to antibodies. The heavy and light chains of an antibody combine to form a stable scaffold whose role is to present peptide sequences that can recognise epitopes. In fact, peptide aptamers have dissociation constants that are comparable to, or sometimes better than, antibodies and can be used in the same way as antibodies in many
applications. The authors have used them as probes of proteins immobilised on nitrocellulose membranes, similar to the way that immunoblots are probed with a primary antibody, and as affinity reagents in pull-down experiments, where the peptide aptamer replaces the antibody in an immunoprecipitation experiment. Visintin et al.\textsuperscript{11,12} have taken the converse approach — to simplify the antibody scaffold so as to make it suitable for intracellular expression.

Peptide aptamers are most commonly used as disrupters of protein–protein interactions in vivo, but the flexibility of protein engineering means that peptide aptamers can be turned into tools for virtually any type of biological study (Figure 1). The following section describes these applications in more detail. Future fusions of GFP to peptide aptamers (or peptide aptamers constrained in an auto-fluorescent protein scaffold) will be expressed from titratable promoters to serve as biological tracers of their target proteins, allowing the visualisation of protein localisation in living cells — a marked improvement over immunocytochemistry performed on dead cells. It will also be interesting to determine whether peptide aptamers can be used to probe proteins in paraffin-embedded sections or in tissue microarrays.

Peptide aptamer libraries have taken four forms to date: yeast two-hybrid libraries, yeast expression libraries, bacterial expression libraries and retroviral libraries for expression in mammalian cells. Historically, peptide aptamers have been used in two approaches to the study of protein biology.\textsuperscript{5,6,9,13-15} In so-called ‘reverse genetics’, a peptide aptamer found to bind to a particular protein by yeast two-hybrid screening is used to study that protein in vivo. By contrast, ‘forward genetics’ or ‘dominant effector genetics’ is the term used where peptide aptamer libraries are screened for the ability to produce a particular phenotype in a given cell, and the targets of active peptide aptamers are subsequently identified. Examples that illustrate these themes are given later in this paper.

As biological probes, conformationally constrained peptides offer significant advantages over free peptides. They possess greater specificity, as their constraint makes them likely to recognise cognate surfaces, rather than simply being able to mould around surfaces they encounter. Because they are already folded, the conformational entropy lost upon binding to a target surface is decreased compared with free peptides, increasing the affinities of peptide aptamers for target surfaces in vivo. In one experiment, the measured affinity of a free peptide was 1,000-fold weaker than its constrained counterpart.\textsuperscript{16} This study, along with many others, has indicated that the constrained nature of peptide aptamers contributes to their high specificities and affinities and allows them to compete, in living cells, for endogenous protein–protein interactions.

**Protein genetics?**

To date, peptide aptamers have usually been employed to disrupt intracellular protein–protein interactions. One outstanding goal is to use peptide aptamers to manipulate proteins at will in diploid eukaryotes, so as to apply the logic of genetics to diploid tissue culture cells.

Genetic experiments are founded on four common types of mutation. Point mutations (which are sometimes conditional) often lead to the loss of a specific protein–protein interaction. Deletion mutations, by preventing the expression of an entire gene, lead to a loss of all the protein–protein interactions the lost protein would normally make. Suppressor mutations can be intragenic (mutation of a second residue in a protein compensates for the deleterious effects of the first mutation) or extragenic, where loss of an inhibitor or gain of a novel interactor compensates for the loss caused by the first mutation. Finally, synthetic lethal mutations occur when the combination of two non-lethal mutations leads to the death of the host cell. A fifth
class of mutations is the gain-of-function mutant, which is not so commonly sought but is worth mentioning here.

Placing peptide aptamers within this genetic framework is very straightforward (Figure 1). Peptide aptamers are already most commonly used as inhibitors of specific protein–protein interactions in eukaryotic cells, mimicking the effects of point mutations in model genetic systems. Peptide aptamers have the benefit in biological studies that they recognise individual domains of a pleiotropic protein and may thus inhibit just a subset of its activities. Using inducible or repressible promoters will allow the creation of conditions that parallel the very useful conditional alleles of classical genetics. In the future, protein engineering of peptide aptamers will allow the creation of deletion mutants at the level of the protein (protein knockouts) and gain-of-function or suppressor mutations. Because peptide aptamers can be thought of as artificial protein domains, they can be fused to other domains to create designer proteins that can be used to accomplish these goals (Figure 1). These ideas are discussed below.

In order to make targeted protein knockouts, the authors are seeking to construct artificial E3 ubiquitin ligases. Ubiquitin ligases are comprised of a substrate recognition domain and a catalytic domain, and they represent both the ultimate step and the specificity determinant in the conjugation of ubiquitin to specific proteins subsequently targeted for destruction by the proteasome (reviewed in ref. 17). By replacing the substrate recognition domain of an ubiquitin ligase with a peptide aptamer, one creates an ubiquitin ligase whose sole substrate is the protein to which the peptide aptamer binds. When applied to the study of proteins within the cellular context, these reagents should allow us to create protein knockouts in tissue culture cells. This idea may seem reminiscent of anti-sense or RNAi technologies; however, these techniques are used to eliminate or at least reduce the translation of a target protein by depleting the pool of messenger RNA. There is a major drawback to this approach: if the anti-sense targeted transcript encodes a stable protein, reducing the level of the RNA will not reduce the level of the protein unless the host cell undergoes sufficient rounds of cell division to dilute the original pool of protein.

In an alternative strategy, a functional knockout may be created by re-localising a protein within the cell, which may, for example, have the effect of removing a cytoplasmic protein from its usual interactors. This may of course have the side-effect of creating a novel (gain-of-function) phenotype, by allowing the protein to interact with proteins it may normally never encounter, or may only encounter in a regulated or pathological condition. To these ends, the authors have already shown that fusion to a nuclear localisation sequence (NLS) of a peptide aptamer that binds human CDK2 will cause the re-localisation of a cytoplasmic pool of this protein to the nucleus in yeast cells. This approach will be readily extended using other intracellular targeting signals (see Figure 1) that may be effective in trans, such as lysosomal, mitochondrial or peroxisomal targeting sequences, prenylation domains, etc.

Finally, one may wish to suppress the effects of a loss-of-function point mutation, such as might occur in a disease protein. In principle, the binding of tightly interacting peptide aptamers to a misfolded protein should be capable of driving the correct folding of the distant domain in the same way that some anti-p53 antibodies are able to restore function to mutant p53. The authors are testing this idea with the goal of restoring function to defective tumour suppressor proteins. For the sake of completeness, it should be pointed out that synthetic lethal combinations of peptide aptamers with each other or with defects in human or mouse cells represent a straightforward extension of the ideas described in this section.
**Peptide aptamers compared with existing technologies**

In addition to improving on antibody and RNA-based technologies, peptide aptamers compare favourably to a number of other techniques currently used in many laboratories. For example, a frequently published experiment involves the overexpression in cells of truncated forms of proteins, yielding dominant—negative, loss-of-interaction phenotypes. It is only in a minority of cases that proteins are sufficiently well characterised, or a mutant form has been fortuitously identified, that dominant negative proteins can be employed to induce and characterise a non-functional phenotype. Peptide aptamer technology allows one to screen for peptide aptamers that both bind to a protein of interest and produce a measurable phenotype.

Some of the applications where peptide aptamers compare well with antibodies have already been mentioned. Of course, although well suited to extracellular recognition, antibodies are not optimal for intracellular applications. In addition to being relatively large, multichain proteins, antibodies are usually stabilised by disulphide bonds, which will not always form in the cytoplasm of eukaryotic cells. There are exceptions to these problems, and single-chain antibodies are also being developed as scaffolds.\(^1\)\(^2\)

Given a source of the target protein, it is relatively trivial to identify ligands (peptides, nucleotide aptamers or even small molecules) that bind to it *in vitro*; however, *in vitro* selection techniques, powerful as they are, suffer from a large number of drawbacks, not the least of which are the problems associated with the large-scale purification of the target protein, even using recombinant protein expression technologies. In addition, recombinant proteins expressed in prokaryotes for screening may not be correctly folded, whereas peptide aptamer screens take place in eukaryotic cells, circumventing this problem. It is probably worth emphasising that a source of pure protein is neither necessary nor even advantageous in reverse peptide aptamer screens — for a yeast two-hybrid screen, all that one needs is a clone.

Once a source of the target protein has been identified, the *in vitro* screening methodology will bring its own problems. In phage display or in other *in vitro* selections, inter-molecular competition between many potential ligands for a limited number of binding sites on the target can potentially reduce the effective size of the library. In typical screens of peptide aptamer libraries, each peptide aptamer is expressed within a single cell so that there is no competition for binding to a given target. Another drawback to all *in vitro* selections is they do not immediately provide information regarding the stability and efficacy of a given ligand, whereas *in vivo* selection does. In addition, the *in vitro* behaviour and folding of both the target protein and the ligand (peptide or antibody) may not reflect the situation inside the target cell. In the worst case, a peptide identified in an *in vitro* screen will fold so differently in a eukaryotic cell that it will completely fail to recognise its target, requiring a wasteful secondary screen of many false positives. Another advantage of peptide aptamer screens is that they take place within a rich environment, where a degree of specificity is granted by the presence of competing proteins. For example, in the commonly used yeast two-hybrid screen of peptide aptamer libraries, more than 6,000 competing yeast proteins are present.

**PEPTIDE APTAMERS: APPLICATIONS**

**Reverse genetics**

Peptide aptamers identified in yeast two-hybrid screens against a variety of wild-type and mutant protein targets have been used for biological studies both in tissue culture and *in vivo*.

**Anti-viral agents**

Hepatitis B virus (HBV) is strongly linked to liver cancers. During chronic HBV
infection, pre-genomic RNA is packaged together with viral polymerases into capsids, which require viral core proteins for formation. Butz et al. identified several peptide aptamers in a yeast two-hybrid screen that could bind to the HBV core capsid protein. One of these was capable of inhibiting viral capsid formation, DNA replication and virion production in mammalian cells.

**Cell cycle analysis**

In mammalian cells, CDK2 is required for progression from G1 into S phase of the cell cycle. Colas et al. isolated peptide aptamers that bind tightly to CDK2, with in vitro affinities in the nM range. When expressed in mammalian cells, such peptide aptamers cause an increase in the number of cells in the G1 phase of the cell cycle. Interestingly, one of the peptide aptamers differentially inhibited the kinase activity of CDK2 by blocking the phosphorylation of histone H1, but not pRb. This highlights the usefulness of artificial proteins to elucidate the functional significance of specific protein–protein interactions. This was taken a step further in later studies by Kolonin and Finley, who demonstrated that some of the CDK2-interacting peptide aptamers also bound specifically and avidly to Drosophila CDK1 and CDK2. Temporal expression of the

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**Figure 1:** Applications of peptide aptamers to biological studies. Peptide aptamers can be used in ‘forward studies’, such as phenotypic screens or microarrays, where their target proteins are initially unknown. They also represent modular tools for a range of ‘reverse’ studies, where a peptide aptamer that binds to a protein of interest can be used to probe all aspects of its biology, from intracellular function (dominant-negative inhibition of protein–protein interactions, target protein knockouts, cellular targeting studies) and localisation (GFP fusions expressed in living cells) to biochemical interactions (complex purification, ligand overlays). See text for details.
peptide aptamers during development resulted in eye defects characteristic of abnormal cell cycle regulation. Kolonin and Finley\textsuperscript{21} also showed that the effect was dosage dependent and could be rescued by the overexpression of wild-type CDKs, demonstrating \textit{in vivo} specificity.

The activity of the E2F family of transcription factors is a key target of cell cycle regulation by the Rb pathway and appears to play a role in the switch between cell division and differentiation (reviewed in ref. \textsuperscript{22}). Fabbrizio \textit{et al.}\textsuperscript{23} generated peptide aptamers capable of preventing E2F binding to DNA \textit{in vitro} and showed that they could inhibit the proliferation of fibroblasts in culture. The peptide aptamer may be competing for the binding of DP1 (a natural E2F-binding protein) as its random region contains a WIGL motif, which is evolutionarily conserved in DP1 and is required for heterodimerisation with E2F. The identification of sequence motifs in peptide aptamers identified by the yeast two-hybrid method that recall sequences selected during natural evolution is infrequent, as would be expected given the much larger number of 20-mer peptides present in the Brent library ($2.7 \times 10^9$) than in the human proteome ($3.5 \times 10^9$). If there were as many as ten different spliced forms of each of 35,000 proteins with an average length of 1,000 amino acids. A rigorous attempt to use the sequences identified in peptide aptamer screens to predict unidentified protein–protein interactions has yet to be performed.

More recently, peptide aptamers have begun to be applied to the dissection of the signalling pathways regulated by small GTP-binding proteins such as Ras\textsuperscript{24} and Rho.\textsuperscript{25} Studies such as these will be the first extensive test of the power of peptide aptamer technologies. Finally, peptide aptamers (to use the authors’ definition of them) that use a selected antibody chain as a scaffold have been used to target the BCR–ABL oncogenic fusion protein.\textsuperscript{26}

**Forward genetics**

**Yeast pheromone signalling pathway**

When \textit{Saccharomyces cerevisiae} cells are exposed to \textalpha-factor, they arrest in mid-G1 and transcribe genes required for mating. Several groups\textsuperscript{4,13,27} have screened for peptide aptamers that prevent cell cycle arrest associated with the mating response to pheromones. Many of the resultant peptide aptamers interacted with known components of the pheromone pathway. Interestingly, a novel role of a known protein was identified, and many peptide aptamer targets remain to be identified. Norman \textit{et al.}\textsuperscript{4} extended their study to investigate essential components of transcriptional silencing and the spindle checkpoint, further highlighting the potential use of peptide aptamers in dissecting biological pathways.

**Anti-bacterial agents**

Bacterial cell wall impermeability poses a problem in conventional screens for new drugs. Blum \textit{et al.}\textsuperscript{5} circumvented this problem by utilising the intracellular nature of peptide aptamer screens. Several peptide aptamers were isolated in a screen for resistance to trimethoprim in cells requiring thymidine for growth — ie an indirect screen against thymidylate synthase, an important target in cancer chemotherapy. The specificity of the peptide aptamer–protein interaction was confirmed using yeast two-hybrid analysis. In addition, Blum \textit{et al.}\textsuperscript{5} also performed a non-specific screen for peptide aptamers toxic to \textit{Escherichia coli}. This produced five peptide aptamers: four bacteriostatic and one bacteriocidal. Unfortunately, the targets of these peptide aptamers were not described.

**Peptide aptamers in proteome studies**

The specificity of peptide aptamers for their targets is highlighted by the work of Colas \textit{et al.}\textsuperscript{18} who showed that individual peptide aptamers that bind to wild-type CDK2 showed different affinities for single point mutants of that target. One such peptide aptamer (Apt12) barely
recognises wild-type CDK2 but binds very strongly to only one of a panel of point mutants (cdk2-217). This raises the possibility that peptide aptamers can be identified that distinguish between different activation states (post-translational modifications) of any protein, or between the wild-type and pathological forms of the many proteins implicated in human disease. Indeed, Xu and Luo\(^{24}\) have reported peptide aptamers that can distinguish between inactive and active forms of Ras. Although the dual bait system\(^{28}\) is not yet amenable to high throughput, this system would allow the creation of arrays of peptide aptamers that can distinguish between the various activation states of proteins inside living cells, in a ‘non-living’ peptide array, as discussed by Emili and Cagney.\(^{29}\) The authors believe that the advent of microarrayed peptide aptamer chips will play a key role in ushering in the next revolution in ‘omic’ studies.

The term ‘peptide aptamer’ encompasses proteins whose interaction surfaces are randomised — that is to say where a surface normally used for protein–protein interactions is replaced by random (or, sometimes, designed) peptide sequences, so that the rest of the protein becomes a scaffold that is used to present peptide sequences that will interact with targets other than that originally recognised by the interaction surface. Some of the ways that peptide aptamers have been used have been discussed and, more importantly, some of the future applications of the technology to biological studies have been considered. Four major challenges lie ahead: translating peptide aptamers to microarrays; using GFP fusions expressed from titratable promoters as intracellular tracers in living cells; creating a truly modular tool kit that will allow rapid and effortless application of peptide aptamers to biological studies; and, finally, translating peptide aptamers to drug discovery. The validation of candidate drug targets that are being identified daily is the rate-limiting step in the design of therapeutics. Peptide aptamers can clearly be used for open-ended and hypothesis-driven experiments that can be used to determine the role (if any) that each candidate plays in a given pathology. Once a peptide aptamer that reverses the pathology has been identified, there are four ways that the peptide aptamer can be used in therapy. One is simply to use peptides, or peptidomimetics, as therapies. An alternative would be to co-crystallise the peptide aptamer and its target, and use the information for \textit{in silico} screening or for the rational chemical synthesis of small molecule drugs. A third, more long-term, goal would be to develop gene therapy vectors for the delivery of peptide aptamers or of engineered modular enzymes with peptide aptamers as their substrate-targeting domain. But perhaps the simplest way forwards would be to perform drug displacement screens of small molecule libraries for those that can displace the peptide aptamer from the target proteins. Because the peptide aptamer would already have been used to confirm the importance of its binding site for the pathological mechanism implicated in cell-based models of the disease, any small molecules that bind to that site should represent relatively high-affinity lead compounds for drug development.

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