Peptide Aptamers as Genetic Agents

Most cellular processes are regulated by networks of interactions among proteins. Classic "forward" transmission genetic approaches have succeeded in identifying proteins involved in such networks and provide information on the relationships between them. Approaches such as (1) isolation of mutants and mapping the genes responsible for mutant phenotypes, (2) epistasis analysis to determine the order in which gene products might act on each other, (3) dependency analysis to determine the relationship between gene products with respect to the completion of a cellular process, and (4) allele-specific suppression analysis to give clues as to the possible physical interaction between two proteins provide detailed information about genes and their positions and interactions within networks. Although these approaches are often highly informative, they are often difficult to perform, especially in diploid organisms. Their full application is thus generally limited to a few tractable organisms such as phage, bacteria, yeast, worms, and flies.

The ability to manipulate individual genes by recombinant DNA approaches allowed the development of "reverse" genetic techniques, in which investigators mutate the function of individual genes and monitor the resulting phenotypes. Reverse genetic approaches that mutate or knock out the function of genes at the DNA level suffer from the following limitations: (1) if the gene under study is essential, then mutations in it will yield nonviable organisms, but often no other information; (2) gene knockouts provide information about the phenotypic consequences of abolishing all the protein interactions in which the knockout gene product participates; (3) gene knockouts typically provide information related only to those phenotypes caused by the initial function of the gene in the development of the organism; and (4) in diploid organisms, as for forward genetic analysis, the observation of recessive phenotypes requires the generation of organisms homozygous for particular mutations, typically in the second generation.

More recently, dominant reverse approaches have been devised that affect gene products rather than genes. These dominant reverse approaches rely on generating "mutagenic" agents that inactivate gene products in trans without affecting their coding DNA. Examples of dominant genetic agents include small molecule inhibitors,\textsuperscript{4} dominant negative proteins,\textsuperscript{5} injection of antibodies,\textsuperscript{6} antisense RNA,\textsuperscript{7} ribozymes,\textsuperscript{8} and nucleic acid aptamers.\textsuperscript{9} These methods are particularly useful for studying gene function in diploids; however, they too have various weaknesses. Dominant negative proteins and small molecule inhibitors may not exist for all gene products, thus restricting their universality. Antibodies, antisense RNA, ribozymes, and nucleic acid aptamers can in principle be generated to inactivate almost any gene product. Antibodies, however, are not cell permeable and their injection into cells is time consuming and not practicable in all organisms. RNA-based agents are generally not stable in intracellular environments. Although RNA stability can be partly overcome by using RNAs with different chemistry, it remains difficult to predict the sites on the target RNA that are exposed for antisense inhibition. Also, the stability of the protein product of the antisense and ribozyme target gene affects the timing (perdurance) and extent (penetrance) of the mutant phenotype. Moreover, like DNA mutagenesis, mutagenesis at the RNA level results in phenotypes that are caused by the abolishment of all protein interactions in which the target gene product is involved.

A new class of dominant agents has been developed to facilitate the analysis of processes in diploid and genetically intractable organisms. We termed these molecules "peptide aptamers," because of their similarity to nucleic acid aptamers.\textsuperscript{10} We define peptide aptamers as antibody-like recognition agents that consist of conformationally constrained peptides displayed on the surface of scaffold proteins, and distinguish such molecules from peptides of variable sequence displayed that are not constrained at both ends by the scaffold. Peptide aptamers are designed to inhibit cellular processes by interacting with proteins and disrupting their biological functions. Combinatorial libraries of peptide aptamers in principle contain aptamers that bind almost any protein target. Peptide aptamers specific for numerous proteins have been isolated by using the yeast two-hybrid system.

Using this strategy, we and others have isolated peptide aptamers against Cdk2, Ras, human immunodeficiency virus type 1 (HIV-1) Rev, E2F as well as linear peptides against Rb, and mutagenized PDZ domains against C-terminal peptides. Peptide aptamers recognize their protein targets with \( K_d \) values and half-inhibitory concentrations between \( 10^{-6} \) and \( 5 \times 10^{-11} \) M. The fact that peptide aptamers are typically selected \textit{in vivo} by two-hybrid methods increases the likelihood that the molecules will function \textit{in vivo}. In support of this notion, peptide aptamers function as reverse dominant agents in both mammalian cells and \textit{Drosophila melanogaster}.

We and others have also used peptide aptamers as dominant agents for the forward "genetic" analysis of cellular processes. In this approach, combinatorial libraries of peptide aptamers are used as random "mutagens" that interfere with cellular processes. To make this work, the investigator (1) introduces combinatorial expression libraries of peptide aptamers into cells, (2) selects cells that carry peptide aptamers that cause a desired phenotype, and (3) identifies the targets of the peptide aptamers. Target identification is performed by screening the aptamers for interactions against panels of proteins or products of cDNA libraries, using the two-hybrid system. This combination of aptamer-mediated "mutagenesis" with the two-hybrid system to identify aptamer targets provides a general method for characterizing processes in a variety of hitherto genetically intractable organisms.

Here we describe how to obtain and characterize peptide aptamers for both reverse and forward analysis of cellular processes. We describe methods to isolate aptamers against specific proteins with the yeast two-hybrid system as well as methods to identify the targets of aptamers isolated from

\begin{enumerate}
  \item B. Cohen, Ph.D. thesis. Harvard University, Boston, Massachusetts, 1998.
  \item Xu \textit{et al.}, submitted (2000).
\end{enumerate}
forward selections. We also illustrate how to use the two-hybrid system to define the specificity of aptamers, to isolate mutant aptamers with enhanced affinity for their targets, and to obtain aptamers specific for allelic variants of proteins. We provide protocols that detail the necessary reagents and techniques to perform the above-described work. While a basic knowledge of yeast and molecular biology techniques is required, descriptions of reagents and techniques not described in this review can be obtained from basic molecular biology manuals.\textsuperscript{23,24}

Design of Intracellular Peptide Aptamers

\textit{In Vitro Selection Methods}

A number of \textit{in vitro} selection methods have been developed to express randomly encoded peptides (and peptide aptamers) from combinatorial libraries in order to isolate peptides that bind specific targets. These methods range from displaying peptides on the surface of phage,\textsuperscript{25} yeast,\textsuperscript{26} DNA-binding proteins,\textsuperscript{27} polysomes,\textsuperscript{28} and covalently attached RNA\textsuperscript{29} to displaying bona fide peptide aptamers on the main flagellar protein of \textit{Escherichia coli}.\textsuperscript{30} These methods allow isolation of peptides that bind extracellular proteins and proteins immobilized on solid supports. The main advantage of using \textit{in vitro} selection methods to isolate such peptides is the size of the random libraries that can be generated. Currently, libraries containing $10^{10}$ members are readily obtainable.\textsuperscript{31}

The tactics for displaying peptides on the surface of phages, bacteria, or yeast, or in association with the coding RNA, however, all suffer from the following limitations. First, with the exception of the flagellar display of peptide aptamers, the displayed peptides are typically unconstrained

and of low affinity. Second, none of these selection methods guarantee that the peptide or peptide aptamer will be functional in the cellular environment. For example, peptides may be toxic, they may not bind their target under cellular conditions, and the target may not have the proper conformation or display the same surfaces available for binding. Finally, most in vitro selections take place in oxidizing environments, in which disulfide bonds, which are sometimes used to constrain peptides in vitro, can form. The cytoplasm and the nucleus are reducing environments, in which disulfide bonds do not typically form.

In Vivo Selection Methods

Peptide aptamers can be directly selected to interact with proteins within cells by the two-hybrid system\textsuperscript{11,32} or by genetically screening for aptamers that cause particular phenotypes.\textsuperscript{20-22} Because these selections take place inside the cell, the aptamers are by definition active and nontoxic. However, when the selection is performed in yeast or in mammalian cells, the number of aptamer-expressing cells that can be easily surveyed ($\sim 10^6$--$10^7$) is typically smaller than the number of peptides that can be surveyed by in vitro methods. Libraries of this lower complexity still possess significant biochemical diversity\textsuperscript{33} and have been successfully used in both phage display\textsuperscript{34,35} and in vivo selections.\textsuperscript{11,15}

Properties of Intracellular Aptamers

The selection of peptide aptamers within cells restricts the types of scaffold proteins and variable region constraints that can be used. Constrained peptide libraries generally produce peptide aptamers with higher binding affinities and greater specificity relative to unconstrained peptide libraries.\textsuperscript{11,36} For in vitro selections, the variable region peptide is often constrained either by cyclization through the formation of disulfide bonds\textsuperscript{37} or by its immobilization on a protein scaffold.\textsuperscript{38} For selections in the reducing environment of a cell, peptides must either be unconstrained or constrained by means other than disulfide bonds. At least in \textit{E. coli}, unconstrained peptides tend to be unstable. For example, only 5% of the members of a random unconstrained peptide library are able to direct the synthesis

\textsuperscript{32} Deleted in proof.
of observable protein in *E. coli*. Moreover, higher proteolytic stability is expected for peptides that are displayed from a folded structure relative to a linear or unordered peptide. For these reasons, we prefer to use constrained peptide libraries for intracellular selections.

Other desirable properties for aptamer scaffolds include the following: (1) The scaffold should possess good solubility properties, in order to help prevent aggregation of aptamers that contain hydrophobic variable regions; (2) the scaffold should be small, stable, easily purified, and expressed at high levels without toxicity; and (3) the scaffold should be tolerant of modifications such as localization sequences, epitope tags, purification tags, and other protein moieties that allow the aptamer valency to be increased [e.g., aptamers can be dimerized by fusing them to glutathione S-transferase (GST)]

In the future, we imagine that individual selections will benefit from the use of promiscuous protein scaffolds that can provide additional functional groups for enhanced binding interactions, and through the development of scaffolds carefully designed to display variable regions of different lengths, with different distances between the constrained ends of the variable region, and with different distances between the variable region and the body of the scaffold protein.

**Scaffolds Used for Intracellular Aptamers**

To date, only a few different scaffolds have been used to display constrained or unconstrained variable regions inside cells. In two-hybrid selections, *E. coli* thioredoxin and PDZ domains have been used as scaffolds, and the Gal4 activation domain has been used to display linear peptides. A comparison of the binding constants shows that the aptamers, with their constrained variable regions, bind their targets between 100- and 10,000-fold better than linear peptides (see Table I). For direct phenotypic selec-

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tions, thioredoxin, green fluorescent protein (GFP), and staphylococcal nuclease have been used as aptamer scaffolds. Because the dissociation constants for these aptamers were not measured, it is difficult to compare them. However, the fact that the selected aptamers function in two-hybrid experiments suggests that binding constants for all these aptamers are likely to be tighter than the $10^{-6}$ M threshold for these reporters.

Properties of Thioredoxin Aptamer Proteins

In this review we focus on the properties and uses of *E. coli* thioredoxin (TrxA) as a scaffold protein for constrained variable regions. TrxA was originally used as a scaffold by J. McCoy and co-workers to display peptides on the surface of *E. coli* as a fusion to flagellin. Using this approach, TrxA aptamers were obtained against a number of monoclonal antibodies and against protein phosphatase.  

TrxA possesses many desirable properties that make it an effective intracellular scaffold protein. TrxA is a small (12-kDa) stable cytoplasmic protein that can be expressed at high levels in cells without toxicity. Structural studies have shown that the TrxA active site consists of a short disulfide-constrained peptide loop (–CGPC–). This loop is tolerant to insertion of peptides and thus provides a site to introduce peptides of random sequence. TrxA enhances the solubility of proteins fused to it, suggesting that it will enhance the solubility of peptides that would normally aggregate. TrxA can interact with many different protein substrates, so long as those contain disulfide bonds. The ability of TrxA to interact with many different proteins suggests that it may bear functional groups that make some contacts with the proteins recognized by different aptamer variable regions.

**Method 1: Construction of a Thioredoxin Peptide Aptamer Library**

**Reagents**

**Vectors**

- pJM-1: Peptide aptamer vector for use with the interaction trap
- pJM-2: Peptide aptamer vector for use in yeast genetic selections

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41 Lu *et al.*, 1995.
pJM-3: Peptide aptamer vector containing a nuclear localization signal for use in yeast genetic selections

See Fig. 1 for a detailed description of the above-described vectors.

Construction of a Random Thioredoxin Peptide Aptamer Library. Thioredoxin peptide aptamer libraries are constructed by inserting an oligonucleotide encoding a random 20-amino acid peptide into an *RsrII* restriction site that occurs naturally in the DNA encoding the TrxA active site loop. This generates a solvent-exposed random 20-amino acid peptide constrained by the active site of TrxA.

**Fig. 1.** Expression vectors used for interaction trap and forward genetic selection of peptide aptamers. The yeast–*E. coli* shuttle vectors are derived from pJG4-4. They contain an *E. coli* origin of replication (pMB1 ori), an ampicillin resistance gene (Amp<sup>R</sup>), a *TRP1* marker gene, a yeast 2 micron origin of replication, and one of the following expression cassettes: pJG4-5, yeast GAL1 promoter (P<sub>GAL1</sub>), SV40 nuclear localization signal, B42 activation domain and hemagglutinin epitope tag, *EcoRI* and *XhoI* cloning sites, and yeast ADH1 transcription terminator (T<sub>ADH1</sub>); pJM1, yeast GAL1 promoter (P<sub>GAL1</sub>), SV40 nuclear localization signal, B42 activation domain and hemagglutinin epitope tag, TrxA, and yeast ADH1 transcription terminator (T<sub>ADH1</sub>); pJM2, yeast GAL1 promoter (P<sub>GAL1</sub>) and hemagglutinin epitope tag, TrxA, and yeast ADH1 transcription terminator (T<sub>ADH1</sub>); pJM3, yeast GAL1 promoter (P<sub>GAL1</sub>) and hemagglutinin epitope tag, TrxA, and yeast ADH1 transcription terminator (T<sub>ADH1</sub>) (J. McCoy, personal communication, 1999; and Ref. 21).
Protocol 1-1: Construction of Random Peptide Aptamer Library

1. Choose one of the three TrxA peptide expression vectors (pJM-1, pJM-2, or pJM-3) shown in Fig. 1. pJM-1 is designed for use in the yeast interaction trap two-hybrid system.\textsuperscript{11} pJM-2 and pJM-3 are, respectively, designed for genetic selections of nuclear-localized and nonlocalized peptide aptamers in yeast.\textsuperscript{21}

2. Digest the appropriate pJM vector (Fig. 1) with RsrlI and dephosphorylate with calf intestine phosphatase.

3. Prepare the following oligonucleotide on an automated DNA synthesizer: 5'-GACTGACTGGTGCCG (NNG/C)\textsubscript{20}GGTCCTCAGTCAGTCAGT-CAG, where N represents G, C, A, or T.

4. Add 200 μg (a 10-fold excess) of the primer 5'-CTGACTGACT-GAGGACC to 100 μg of the above-described random oligonucleotide to give a total volume of 890 μl in 1× Klenow polymerase buffer. Anneal the primer by heating the sample to 94 ° for 5 min and slowly cool to room temperature.

5. Add 90 μl of a 5 mM mixture of the four dNTPs and 20 μl of Klenow polymerase (5 U/μl) and incubate the reaction for 3 hr at 37 °.

6. Phenol–chloroform extract the mixture. Follow this with a chloroform extraction.

7. Ethanol precipitate and wash the pellet with 80% (v/v) ethanol. Dissolve the pellet in 0.9 ml of H\textsubscript{2}O.

8. Add 0.1 ml of AvaiI buffer and digest the DNA with 1000 units of AvaII for 4 hr. Optional: Digest the DNA from step 7 with 1000 units of BfaI overnight. BfaI cleaves DNA containing CTAG and eliminates TAG stop codons following C.

9. Phenol–chloroform extract the mixture, followed by a chloroform extraction.

10. Ethanol precipitate the restriction digest and dissolve the DNA in 10 mM Tris (pH 8) and a 1/5 volume of nondenaturing loading buffer.

11. Purify the DNA on a 10% non-denaturing polyacrylamide gel. Expose the DNA by UV shadowing. Cut out the DNA band and elute in 10 mM Tris, pH 8, overnight. Ethanol precipitate the supernatant and dissolve in 10 mM Tris, pH 8.

12. Ligate 8 μg of the random sequence DNA insert into 12 μg of the RsrlI-cut dephosphorylated pJM vector from step 1 with 80,000 units [New England BioLab (Beverly, MA) units] of ligase in a 1-ml reaction.

13. Purify the DNA with a QIAquick gel extraction kit from Qiagen (Chatsworth, CA) and elute with 30 μl of ultrapure H\textsubscript{2}O.

14. Add 30 μl of DNA to 350 μl of competent \textit{E. coli} (e.g., MC1061; Bio-Rad, Hercules, CA) in 0.2-cm gap electroporation cuvettes. Electroporate,
using the following settings: 2.5 kV, 200 Ω, 25 μF. Recover the cells in 25 ml of SOC for 1.5 hr.

15. Take a small sample to determine the transformation efficiency and transfer the cells to 1 liter of LB medium with ampicillin (50 μg/ml). Incubate overnight at 37°C.

16. Purify the plasmid DNA with a plasmid megakit from Qiagen, or with successive ethidium bromide–CsCl gradients.

Reverse Genetics with Peptide Aptamers

Selection of Aptamers to Specific Proteins

Two-hybrid systems have been used to detect protein–protein interactions\(^{46,47}\) and are effective for screening proteins for interactions with cDNA and genomic libraries.\(^{48,49}\) The two-hybrid system has been used to screen libraries of peptide aptamers\(^{11}\) and linear peptides\(^{15}\) for those that interact with given baits. A number of working two-hybrid systems have been developed,\(^{46,47,50–52}\) all of which have the following features: (1) a target protein of interest fused to a DNA-binding domain referred to as the "bait," (2) a library of proteins or peptide aptamers fused to a transcription activation domain, sometimes referred to as the "prey," and (3) one or more reporter genes that detect interactions between the bait and prey proteins.

In this review we describe the use of the interaction trap two-hybrid system\(^{47}\) to isolate peptide aptamers (illustrated in Fig. 2). In the interaction trap, the protein of interest is fused to the LexA DNA-binding domain (bait). The bait protein is expressed constitutively from the yeast ADH1 promoter. The bait protein binds to the LexA operators upstream of the two reporter genes, an integrated LexA operator–LEU2 gene and a LexA operator–lacZ gene, but does not activate transcription of the reporters. The peptide aptamer library is fused to the amino-terminal moiety consisting of three domains: nuclear localization signal, transcriptional activation domain, and an epitope tag. The peptide aptamer library is expressed from the GAL1 promoter, allowing high levels of expression in the presence of

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\(^{49}\) Finley and Brent, 1996.


Fig. 2. Isolation of peptide aptamers with the interaction trap. The yeast strain (EGY48) used in the interaction trap contains an integrated LexAop–LEU2 reporter (required for growth on Leu− medium) and a plasmid (pSH18-34) containing a LexAop–lacZ reporter (required for blue color on X-Gal medium). The bait proteins are constitutively expressed from a plasmid (pEG202) as fusions to LexA (represented by black circles). The library of random peptide aptamer preys is expressed as fusions to an activation domain from a plasmid (pJM-1) containing a galactose-inducible promoter (Pgal1). In the presence of glucose the peptide aptamer preys are not expressed and no interactions occur. In the presence of galactose the peptide aptamer preys are expressed. Interactions between the bait protein and peptide aptamer prey activate the LexAop–LEU2 and LexAop–lacZ reporters, allowing the yeast to grow on Leu− medium and turn blue on X-Gal.

Peptide aptamers have been isolated against Cdk2,11 Ras,12 E2F,14 and HIV-1 Rev13 with the interaction trap. These aptamers bind their targets with $K_d$ values and half-inhibitory concentrations ranging from $10^{-8}$ to $5 \times 10^{-11} \text{M}$. In general, approximately 1 of every $10^5$ aptamers interacts with the target protein.11,14,18 For the most part, the variable regions of peptide aptamers isolated against target proteins with the interaction trap (Cdk2, Ras, and HIV-1 Rev) show no resemblance to any known proteins. However, in one case, the variable region of an anti-E2F aptamer resembled DP, a natural protein that interacts with E2F. The anti-E2F aptamer contains a −WIGL− motif, which is present in the DP family of proteins and is responsible for the heterodimerization of E2F–DP.14

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The majority of peptide aptamers isolated with the interaction trap function effectively in vivo. The anti-Cdk2 aptamer inhibits the progression of human cells through the G₁ phase of the cell cycle. The anti-Cdk2 aptamer also functions in Drosophila melanogaster by inhibiting the cell cycle during organogenesis, resulting in eye defects. Anti-Ras aptamers function effectively in mammalian cells by blocking EGF-stimulated Raf kinase activation. The anti-E2F aptamers function in mammalian cells as growth inhibitors by impeding the entry of cells into S phase. In summary, the interaction trap provides an effective method for obtaining high-affinity peptide aptamers that bind specific protein targets. These peptide aptamers function effectively under a variety of in vivo conditions.

**Method 2: Isolation of Peptide Aptamers with the Interaction Trap**

**Reagents**

**Strain**
EGY48: * Mata his3 trp1 ura3-52 leu2::LexA6op-LEU2*

**Vectors**
pEG202: LexA fusion plasmid for the construction of bait proteins (pBait) (see Fig. 4 for details)

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FIG. 4. Bait protein expression plasmid, pEG20247 is a yeast shuttle vector consisting of an E. coli origin of replication (pMB1 ori), an ampicillin resistance gene (AmpR), a HIS3 marker gene, a yeast 2 μm origin of replication, and an expression cassette that contains a yeast ADH1 promoter (P_{ADH1}), DNA encoding amino acids 1–202 of the bacterial repressor protein LexA, a polylinker for creating in-frame fusions to LexA, and a yeast ADH1 transcription terminator (T_{ADH1}).

pJM-1 peptide aptamer library: Peptide aptamer prey library for use in the interaction trap (see Fig. 1 for details)
pSH18-34: lacZ reporter plasmid (see Fig. 554a–54d for details)
pJK101: Repression assay plasmid (see Fig. 5 for details)

Media

YPD plates and liquid media

1. Combine the following:
   - Yeast extract 10 g
   - Peptone 20 g
   - Agar (only for plates) 20 g
   - H2O 950 ml

2. Autoclave and add 50 ml of 40% (w/v) glucose

Dropout plates and liquid media

1. Combine the following:

FIG. 5. lacZ reporter plasmids. The lacZ reporters are derived from a plasmid containing an E. coli origin of replication (pMB1 ori), an ampicillin resistance gene (AmpR), URA3 marker gene, yeast 2μm origin of replication, and a GAL1 promoter fused to a lacZ reporter.\textsuperscript{54a} pJK101 is used for the repression assay and contains two LexA operators between the UAS\textsubscript{G} and the GAL1 TATA.\textsuperscript{54b} lacZ reporter plasmids are derived from pLR1Δ1, in which the UAS\textsubscript{G} is deleted.\textsuperscript{54c} pSH18-34, pJK103,\textsuperscript{54d} and pRB1840\textsuperscript{54} contain binding sites for eight, two, and one LexA dimers, respectively.

Yeast nitrogen base without amino acids 6.7 g
Dropout mixture minus appropriate amino acids 2 g
(see below)
Agar (plates only) 20 g
H\textsubscript{2}O 950 ml (glucose plates or media)
or
925 ml (galactose/raffinose plates or media)

2. Autoclave and add either 50 ml of 40\% (w/v) glucose or 50 ml of 40\% (w/v) galactose and 25 ml of 40\% (w/v) raffinose

Dropout mixture: Combine the required nutrients listed in the tabulation to make the appropriate selection plates. Grind the components of the mixture into a fine powder, using a mortar and pestle
<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Amount (g)</th>
<th>Concentration in medium (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>2.5</td>
<td>40</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>1.2</td>
<td>20</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>6.0</td>
<td>100</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>6.0</td>
<td>100</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>1.2</td>
<td>20</td>
</tr>
<tr>
<td>L-Isoleucine</td>
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<td>30</td>
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<td>L-Leucine</td>
<td>3.6</td>
<td>60</td>
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<tr>
<td>L-Lysine</td>
<td>1.8</td>
<td>30</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>1.2</td>
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<tr>
<td>L-Phenylalanine</td>
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<td>50</td>
</tr>
<tr>
<td>L-Serine</td>
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</tr>
<tr>
<td>L-Threonine</td>
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<td>200</td>
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<tr>
<td>L-Tryptophan</td>
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<tr>
<td>L-Tyrosine</td>
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<td>30</td>
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<tr>
<td>L-Valine</td>
<td>9.0</td>
<td>150</td>
</tr>
<tr>
<td>Uracil</td>
<td>1.2</td>
<td>20</td>
</tr>
</tbody>
</table>

X-Gal plates

1. Combine the following:
   - Yeast nitrogen base without amino acids 6.7 g
   - Dropout mixture 1.5 g
   - Agar 20 g
   - H₂O 850 ml (glucose plates or media)
   - or 825 ml (galactose/raffinose plates or media)

2. Autoclave and add either 50 ml of 40% (w/v) glucose or 50 ml of 40% (w/v) galactose and 25 ml of 40% (w/v) raffinose.
3. Cool to 55°.
4. Add 100 ml of 10× BU salts (see below).
5. Add 4 ml of 20 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) dissolved in dimethylformamide.

BU salts (10×):

1. Combine the following:
   - Na₂PO₄·7H₂O 70 g
   - NaH₂PO₄ 30 g
2. Adjust to pH 7 and autoclave.
High-Efficiency Lithium Acetate Yeast Transformation. (After Geitz and Schiestl.\textsuperscript{55}) Transformations with lithium salts typically result in approximately $10^5$ transformants per microgram of plasmid DNA. The protocol described below may need to be optimized for individual yeast strains. Factors such as the final concentration of the cells prior to transformation and the heat shock time should be optimized. In general, the highest transformation efficiencies per microgram of plasmid are obtained when 1 µg of plasmid is used per 50-µl aliquot of competent yeast cells.

**PROTOCOL 2-1: YEAST TRANSFORMATION**

1. Inoculate a 10-ml culture with the yeast to be transformed in appropriate media and incubate overnight at 30° with shaking.
2. Determine the OD\textsubscript{600} and dilute colonies in 50 ml of appropriate medium to a concentration of 5 × 10\textsuperscript{6} cells/ml of culture.
3. Continue to grow the cells at 30° with shaking until an OD\textsubscript{600} of 0.6–0.8 is reached. This will provide enough cells for ten 50-µl transformations.
4. Centrifuge the yeast cells at 4000 rpm for 5 min at room temperature.
5. Remove the supernatant and resuspend the yeast pellet in 25 ml of H\textsubscript{2}O. Centrifuge again as in step 4.
6. Remove the supernatant and resuspend the yeast in 1 ml of 100 mM lithium acetate. Centrifuge at 13,000 rpm for 15 sec.
7. Remove the supernatant and resuspend the yeast pellet in 100 mM lithium acetate to give a final volume of ~500 µl.
8. Split the samples in 50-µl aliquots. Centrifuge at 13,000 rpm for 15 sec.
9. Remove the supernatant and add the following ingredients in the order listed to one yeast aliquot from step 8.
   - Polyethylene glycol (50%, w/v) 240 µl
   - Lithium acetate (1 M) 36 µl
   - Single-stranded DNA (ssDNA, 2 mg/ml) 25 µl
   - Plasmid DNA (1.0 µg) 50 µl

*Note:* Single-stranded carrier DNA (ssDNA) must be boiled for 5 min and cooled rapidly on ice prior to use.
10. Vortex the mixture vigorously until the pellet is completely resuspended.
11. Incubate the sample at 30° for 30 min.
12. Heat shock the sample at 42° for 20 min.
13. Centrifuge the sample at 8000 rpm for 15 sec and remove the supernatant.

14. Resuspend the pellet in 500 μl of H₂O and plate 200 μl onto a 10-
cm-diameter plate containing the appropriate dropout medium.

Construction and Characterization of Bait Plasmid (pBait). (After Ausubel et al.23) Insert DNA encoding protein that the peptide aptamers are
to target into the bait plasmid pEG202 (Fig. 4). Using standard subcloning
techniques, insert the coding region for the protein of interest into the
polylinker of pEG202 in frame with LexA to create pBait—from plasmid that
directs the synthesis of a chimeric protein LexA fusion protein that contains
the bait moiety. Introduce pBait with pSH18-34 and pEG202 with pSH18-
34 (control) into the yeast selection strain EGY48, using the lithium acetate
method (protocol 2-1). Select transformants on Glu His⁻ Ura⁻ plates. Test
the baits for their ability to self-activate the LEU2 and lacZ reporters.

Protocol 2-2: Testing Baits for Self-Activation of Reporters

1. Take four colonies from both pBait with pSH18-34 and pEG202
with pSH18-34 transformations and inoculate 5 ml of Glu Ura⁻ His⁻ cul-
tures. Grow the cultures overnight at 30° with shaking. Dilute cultures to
an OD₆₀₀ of 0.2 and continue to grow the cultures at 30° with shaking until
they are in midlog phase (OD₆₀₀ ~0.6).
2. Make 100- and 1000-fold dilutions of the culture in H₂O.
3. Spot 10 μl of the stock culture and each dilution onto the following
plates and incubate at 30°: Gal/Raf Ura⁻ His⁻, Gal/Raf Ura⁻ His⁻ Leu⁻,
and Gal/Raf Ura⁻ His⁻ X-Gal.
4. Check the growth of the yeast after 3 days. The yeast should all grow
on Gal/Raf Ura⁻ His⁻ plates, indicating that the baits are not toxic to the
yeast. Yeast should not grow on Gal/Raf Ura⁻ His⁻ Leu⁻ plates or have
blue color on Gal/Raf Ura⁻ His⁻ X-Gal plates, indicating that the baits do
not activate the LEU2 or lacZ reporters.

After confirming that the bait(s) do not activate the reporter genes, it
is useful to determine whether the baits enter the nucleus and bind the
LexA operators. This is accomplished with a repression assay that tests
whether the baits can repress transcription of a lacZ reporter on plasmid
pJK101 (Fig. 5), which contains LexA operators between the TATA and
the GAL1 upstream activating sequence. lacZ expression is induced in the
presence of galactose. Baits that enter the nucleus and do not activate
transcription bind to the lexA operators and repress the transcription of
lacZ.

Protocol 2-3: Repression Assay

1. Transform pBait with pJK101 and pEG202 with pJK101 into EGY48
as described in protocol 2-1. Select transformants on Glu Ura⁻ His⁻ plates.
2. Take four transformants from each transformation and streak them onto Glu Ura\(^{-}\) His\(^{-}\) X-Gal and Gal/Raf Ura\(^{-}\) His\(^{-}\) X-Gal plates. Incubate the plates at 30\(^{\circ}\).

3. Monitor the plates for 3 days. Yeast with pEG202 and pJK101 should turn blue on Gal/Raf Ura\(^{-}\) His\(^{-}\) X-Gal plates after 1 day and should be light blue on Glu Ura\(^{-}\) His\(^{-}\) X-Gal after 2–3 days. Yeast expressing baits that enter the nucleus should turn blue more slowly than the controls.

4. Baits that do not cause a conspicuous diminution in blue color on X-Gal plates relative to the controls should be measured by liquid \(\beta\)-galactosidase assays (see protocol 2-4).

5. If no differences are detected by either plate or liquid \(\beta\)-galactosidase assay, then the baits may not be expressed correctly, or they may be unable to enter the nucleus and bind the operator. Expression of the full-length protein can be verified by standard Western blot methods.\(^{23}\) If the protein is expressed, a nuclear localization signal may need to be added to the bait to aid its entry into the nucleus (Breitwieser and Ephrussi, unpublished, 1999).

**Protocol 2-4: \(\beta\)-Galactosidase Assay.** (After Stern et al.\(^{56}\) and Miller.\(^{57}\))

1. Isolate three individual colonies from each sample and grow cells to log phase in liquid culture (\(OD_{600}\) ~0.6).

2. Centrifuge 1 ml of culture (in triplicate) at 13,000 rpm for 5 min at room temperature.

3. Remove the supernatant and resuspend pellet in 1 ml of Z buffer\(^{*}\) without 2-mercaptoethanol.

4. Centrifuge again as in step 2 and resuspend the pellet in 150 \(\mu l\) of Z buffer with 2-mercaptoethanol (27 \(\mu l/10\) ml), 50 \(\mu l\) of chloroform, and 20 \(\mu l\) of 0.1\% (w/v) sodium dodecyl sulfate (SDS) in the order listed.

5. Vortex the mixture vigorously for 15 sec.

6. Start the reaction by adding 700 \(\mu l\) of \(o\)-nitrophenyl-\(\beta\)-galactopyranoside (ONPG) prewarmed to 30\(^{\circ}\) (1 mg/ml in Z buffer plus 2-mercaptoethanol).

7. Incubate the reaction at 30\(^{\circ}\) until the reaction turns a medium yellow color (20 min to 3 hr).

8. Quench the reaction with 0.5 ml of 1 \(M\) NaCO\(_3\).

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\(*\) Z buffer (1 liter): \(Na_2HPO_4\cdot7H_2O\), 16.1 g (8.5 g anhydrous); \(NaH_2PO_4\cdotH_2O\), 5.5 g; KCl, 0.75 g; \(MgSO_4\cdot7H_2O\), 0.246 g.
9. Centrifuge the reaction mixture at 13,000 rpm for 10 min at room temperature.

10. Measure the OD$_{420}$ of the supernatant and calculate the $\beta$-galactosidase activity in Miller units.

$$\text{Miller unit} = \left(\frac{A_{420}}{A_{600}}\right) \left(\frac{1000}{(\text{time})(\text{volume})}\right)$$

where time is reaction time (minutes) and volume is reaction volume (milliliters).

**Selection of Aptamers that Bind the Bait Protein.** The pJM-1-derived peptide aptamer library expresses proteins that contain a nuclear localization signal and a transcription activation domain. This library is used in the interaction trap to select aptamers that bind a specific protein target. The library is introduced into a selection strain that contains the bait plasmid and LexA fusion-responsive $\text{LEU2}$ and $\text{lacZ}$ reporters and transformants are selected on dropout plates. Transformants are collected by scraping the plates, and stored as frozen stocks. In this procedure, each yeast cell containing an aptamer is allowed to grow as a colony and the colonies are pooled when they are approximately the same size. Selection of transformants on plates prior to selection for expression of the reporter genes results in a more uniform representation of yeast that contain unique peptide aptamers. Aliquots of the frozen stock are then plated onto galactose Leu$^-$ plates. Galactose induces the expression of the peptide aptamer preys. The absence of leucine selects for peptide aptamer preys that interact with baits and activate the $\text{LEU2}$ reporter. Colonies that grow on the galactose Leu$^-$ plates are subsequently tested for galactose-dependent growth and blue color on Leu$^-$ and X-Gal plates, respectively.

**Protocol 2-5: Selection of Peptide Aptamers**

1. Introduce 50–100 $\mu$g of pJM-1 aptamer library into EGY48 containing pBait and pSH18-34 to give between $10^6$ and $10^7$ transformants (protocol 2-1). Plate the transformation on Glu Ura$^-$ His$^-$ Trp$^-$ plates and incubate at 30°C for 2–3 days (colonies should be ~1 mm in diameter).

2. Scrape the yeast cells from the plate by adding 1–2 ml of H$_2$O to a 24 × 24 cm plate, collect the yeast with a glass spreader, and transfer them to a 50-ml Falcon tube. Pool the transformants from all plates and centrifuge at 3500 rpm for 4 min at room temperature. Remove the supernatant and resuspend the pellet in 25 ml of H$_2$O. Centrifuge again and wash the pellet once more in H$_2$O.

3. Resuspend the pellet in H$_2$O. Add an equal volume of 65% (v/v) glycerol, 0.1 $M$ MgSO$_4$, 25 mM Tris-HCl, pH 7.4. Divide into 1-ml aliquots and freeze at ~70°C.
4. Determine the plating efficiency of the frozen aliquot. Inoculate 10 library equivalents in 1 ml of Gal/Raf Ura⁻ His⁻ Trp⁻ liquid medium. Incubate at 30° for 4 hr with shaking.

5. Pellet the yeast cells by centrifuging at 3500 rpm for 4 min at room temperature. Remove the supernatant and resuspend the pellet in H₂O.


7. Streak colonies onto a Glu Ura⁻ His⁻ Trp⁻ master plate and incubate at 30° for 1 day.

8. Replica plate the master plate on the following four plates: Glu Ura⁻ His⁻ Trp⁻ X-Gal, Gal/Raf Ura⁻ His⁻ Trp⁻ X-Gal, Glu Ura⁻ His⁻ Trp⁻ Leu⁻, and Gal/Raf Ura⁻ His⁻ Trp⁻ Leu⁻ and incubate at 30°. Monitor the plates for blue color on X-Gal plates and growth on Leu⁻ plates.

9. Select colonies that show galactose-dependent growth and blue color on Leu⁻ and X-Gal plates, respectively, for further characterization.

Recover peptide aptamer plasmids from the two-hybrid selection strain by a yeast minipreparation (protocol 2-6). Use the plasmid DNA from the yeast minipreparation to transform *E. coli* so that the aptamer plasmids can be separated from the pBait and pSH18-34. Plasmids isolated with this yeast minipreparation can also be used as templates for sequencing.

**Protocol 2-6: Yeast Minipreparation.** (After the “smash and grab” procedure of Hoffman and Winston.⁵⁸)

1. Inoculate 2 ml of Glu Trp⁻ medium with a single colony from the two-hybrid selection and grow overnight at 30° with shaking.

2. Centrifuge 1.5 ml of the saturated overnight culture at 13,000 rpm for 15 sec. Discard the supernatant.

3. Resuspend the yeast pellet in 200 μl of breaking buffer [2% (v/v) Triton X-100, 1% (v/v) SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8), 1 mM EDTA]. Add ~0.3 g of glass beads and 200 μl of phenol–chloroform–isoamyl alcohol (25:24:1, v/v/v). Vortex the mixture vigorously for 2 min.

4. Centrifuge at 13,000 rpm for 5 min at room temperature. Remove 50 μl of the aqueous layer. The aqueous layer can be stored at −20° or 1–5 μl can be used directly for transforming *E. coli*.

5. *Escherichia coli* transformants that contain the peptide aptamer plasmid can be identified by performing the polymerase chain reaction (PCR) directly from the colonies, using primers that flank the thioredoxin insert. Perform a PCR for 20 cycles. Colonies that contain the aptamer will give

a bright band on an ethidium bromide–agarose gel. Colonies that do not contain the thioredoxin plasmid will not give a band. Colonies that contain a library plasmid that does not contain a variable region insert will give a band that runs 60 bp slower than the thioredoxin aptamer.

Determining Peptide Aptamer Specificity

The specificity of peptide aptamers isolated with the interaction trap can be evaluated by reintroducing the peptide aptamers into yeast (EGY48) that contain the original target protein bait as well as related proteins. The procedure for determining the specificity of the aptamers is identical to that used in the peptide aptamer library screen (protocol 2-5) except that single peptide aptamers, rather than libraries of aptamers, are screened for interactions with chosen baits. Variations of this two-hybrid method are also used to identify regions or specific amino acids of the target protein that are involved in the aptamer–protein interaction (see below).

**Determining Peptide Aptamer Specificity Using Interaction Mating**

An alternative method for determining peptide aptamer specificity against a large panel of proteins is to use interaction mating. Haploid yeast exist in one of two mating types (α or α) and opposite mating types mate to form diploids (reviewed in Herskowitz). Haploid yeast that contain the bait or prey in strains of the opposite mating types can be mated to generate diploids that carry both the bait and prey. Use of interaction mating to test specificity is outlined in Fig. 6.

For example, Colas et al. used interaction mating to study the specificity of anti-Cdk2 peptide aptamers (Fig. 7). All the aptamers isolated against Cdk2 interact with Cdk2 and not with unrelated proteins such as Max and Rb. Some of the aptamers isolated against Cdk2 interacted with related cyclin-dependent kinases, whereas other aptamers were specific for Cdk2. The results suggest that aptamers are capable of recognizing different epitopes on Cdk2, some of which are conserved among different subsets of the cyclin-dependent kinases.

Interaction mating assays can also determine the amino acids and/or regions of the target protein that are required for the interaction. For example, Cohen et al. showed that one anti-Cdk2 aptamer is a competitive inhibitor of the Cdk2–cyclin E phosphorylation of histone H1. They used interaction mating against a panel of mutant Cdk2 proteins to show that

Fig. 6. Interaction mating assay. Yeast strain EGY48 (Mata) containing the individual peptide aptamer preys is streaked horizontally on Glu Trp\textsuperscript{+} plates. Yeast strain EGY42 (Mata) containing individual baits and a lacZ reporter is streaked vertically on Glu His\textsuperscript{+} Ura\textsuperscript{−} plates. The two strains are mated by replica plating on YPD plates. The strains mate at the intersections, forming a/a diploids that contain the peptide aptamer prey, bait, and the lacZ reporter. The YPD plate is then replica plated onto the mating interaction scoring plates: Glu Ura\textsuperscript{−} His\textsuperscript{−} Trp\textsuperscript{−} Leu\textsuperscript{−}, Gal/Raf Ura\textsuperscript{−} His\textsuperscript{−} Trp\textsuperscript{−} Leu\textsuperscript{−}, Glu Ura\textsuperscript{−} His\textsuperscript{−} Trp\textsuperscript{−} X-Gal, Gal/Raf Ura\textsuperscript{−} His\textsuperscript{−} Trp\textsuperscript{−} X-Gal. Peptide aptamer preys and baits that interact grow on Gal/Raf Leu\textsuperscript{−} plates and turn blue on Gal/Raf X-Gal plates.
Fig. 7. Mating interaction specificity test (from Ref. 11). Specificity of anti-Cdk2 peptide aptamers against a panel of cyclin-dependent kinases and two unrelated proteins (Max and Rb). The specificity is assessed on galactose X-Gal plates. Interactions between the peptide aptamer preys and the baits result in the activation of the lacZ reporter, giving blue color on galactose X-Gal plates.

residues in the active site of Cdk2 are required for the aptamer–Cdk2 interaction (Fig. 8). Specifically, two point mutations within the active site (Cdk2-145: D145N and Cdk2-30: V30A) and two point mutations outside the active site (Cdk2-38: D38A, E40A and Cdk2-150: R150A, A151F, F152A) diminished the interaction between the peptide aptamer and Cdk2. Interestingly, these results showed that the Cdk2 residues recognized by this anti-Cdk2 aptamer differed from those recognized by other Cdk2-binding proteins tested (Cdi1, Cks1, p27, and p21). For example, the Cdk2-30 V30A mutation affects the binding of both the aptamer and p21 but not p27. This aptamer also inhibits the ability of Cdk2 to phosphorylate one substrate but not another, suggesting that it can block specific protein–protein interactions while leaving other interactions uninterrupted. Similarly, this strategy can also be expanded to determine the domains or regions of protein targets where the aptamers interact.21

In summary, use of interaction mating together with appropriately chosen panels of wild-type and mutant proteins can give insight into the details of aptamer binding. The assay is able to discriminate between aptamers that recognize different but related proteins, as well as variants of individual proteins. This two-step approach to isolating peptide aptamers with the
interaction trap and their subsequent characterization by the mating assay provides a simple and effective method for obtaining aptamers with different specificities toward the same target.

**Method 3: Defining Recognition Specificity with Interaction Mating**

**Reagents**

**Strains**
- EGY48: *Mata his3 trp1 ura3-52 leu2::LexA6op-LEU2*
- EGY42: *Mata his3 trp1 ura3 leu2*

**Vectors**
- pEG202: LexA fusion plasmid for the construction of bait proteins (pBait) (see Fig. 4 for details)
- pBait: pEG202 containing bait protein of interest inserted into the polylinker in frame with LexA
- pJG4-5: Nuclear localization, activation domain, and hemagglutinin (HA) epitope tag fusion for the construction of prey proteins (see Fig. 1 for details)
- Peptide aptamer prey: pJM-1 containing peptide aptamer (see Fig. 1 for details)
- pSH18-34: lacZ reporter plasmid (see Fig. 5 for details)

**Media:** See method 2 for recipes
**Mating Interaction Assay.** Here, the peptide aptamer prey is introduced into yeast of the α-mating type and the bait protein is transformed into yeast of the α-mating type. The two yeast strains are mated by replica plating streaks of yeast of opposite mating types on top of each other (see Fig. 6). The resulting diploid yeast contain both the bait and peptide aptamer prey. Interaction between the bait and prey is detected by the activation of LexAop-LEU2 and LexAop-lacZ reporters. This assay allows peptide aptamers to be simultaneously screened for interactions against panels of bait proteins.

**Protocol 3-1: Interaction Mating Assay**

1. Introduce the peptide aptamer prey plasmid into EGY48 (Mata) (protocol 2-1) and select for transformants on Glu Trp- plates. At the same time, perform an identical transformation with pJG4-5, a control plasmid with no peptide aptamer.

2. Introduce the bait plasmid (pBait) that expresses the protein target, together with pSH18-34, a lacZ reporter, into the yeast strain EGY42 (Mata) (protocol 2-1). Select transformants on Glu His- Ura- plates. At the same time, perform an identical transformation with pEG202, a control plasmid with no bait protein with pSH18-34.

3. Streak individual peptide aptamer strains and pJG4-5 control strain (from step 1) in parallel lines on Glu Trp- plates. Streak individual bait strains and control strain (from step 2) in parallel lines on Glu His- Ura- plates. Incubate the plates at 30° overnight or until the streaks show heavy growth.

4. Replica plate the bait and prey stains on the same replica velvet by pressing the replica block onto the bait plate, rotating the block by 90°, and pressing it again onto the prey plate. Transfer the imprint on the replica velvet to a YPD plate and incubate overnight at 30°. Mating occurs at patches where the two strains intersect.


**One-Step Selection for Peptide Aptamers Specific for a Desired Protein**

A “two-bait” version of the interaction trap has been developed that enables a one-step selection of peptide aptamers that bind one protein but
FIG. 9. Two-bait interaction trap. The yeast strain (pWX200) contains two reporters, an integrated Tetop–URA3 reporter and a LexAop–lacZ reporter on a plasmid (pCWX24). The bait proteins (LexA and TetR fusions) are constitutively expressed from plasmids (pEG202 or pCWX200). The library of random peptide aptamer preys is expressed as fusions to an activation domain from a plasmid (pJM-1) containing a galactose-inducible promoter (Pgal). In the presence of glucose the peptide aptamer preys are not expressed and no interactions occur. In the presence of galactose the peptide aptamer preys are expressed. Interactions between the bait proteins and peptide aptamer prey activate the Tetop–URA3 and LexAop–lacZ reporters. The specificity of the peptide aptamer preys toward the two baits is observed by the differential activation of the reporters.

The two-bait interaction trap is outlined in Fig. 9 and uses two DNA-binding domains, Tet repressor (TetR) and LexA, to localize protein targets of interest upstream of two reporter genes. The Tet repressor binds to integrated Tet operator upstream of a URA3 reporter. The LexA DNA-binding domain binds to LexA operator upstream of the lacZ gene on a 2 μm plasmid. Peptide aptamers that interact with either of the proteins are detected by differential activation of the reporters (Fig. 9).

The two-bait interaction trap has been used successfully to isolate allele-specific Ras aptamers.12,18 Ras proteins cycle between two states, GTP bound (active) or GDP bound (inactive). This variable conformation allows Ras to function as a molecular switch in signal transduction pathways.61

Allelic variations of Ras exist that are either locked in the GTP-bound (RasV12) or GDP-bound (RasA15) state. Using the two-bait interaction trap, two classes of peptide aptamers were isolated that displayed different interactions with the allelic variants of Ras. The first class of aptamers interacted with RasV12 but not RasA15, and the second class interacted with both RasV12 and RasA15. In addition, some of the aptamers that interacted with RasV12 (GTP bound) preferentially bound this allelic variant compared with wild-type GTP-bound Ras (Ras\(^+\)). These results demonstrate that peptide aptamers are capable of discriminating between epitopes on GTP-bound active Ras (RasV12), GDP-bound inactive Ras (RasA15), and allelic variants of the GTP-bound active Ras (RasV12 and Ras\(^+\)).

The two-bait interaction trap provides a rapid and efficient method for obtaining aptamers that interact differently with two related proteins. This strategy should be especially useful for isolating peptide aptamers that are specific for polymorphic variants of proteins.

**Method 4: One-Step Determination of Peptide Aptamers Specific for One of Two Chosen Proteins**

**Reagents**

**Strain**

CWXY2: \textit{Mata ura3 his3 trp1 leu2 lys2::Tetop-URA3}

**Vectors**

pEG202: LexA fusion plasmid for the construction of bait proteins (pBait) (see Fig. 4 for details)

pCWX200: TetR fusion plasmid for the construction of bait proteins (pBait). The plasmid is similar to pEG202 except for a \textit{LEU2} marker and a TetR operator

pJM-1 peptide aptamer library: Peptide aptamer prey library for use in the interaction trap (see Fig. 1 for details)

pCWX24: \textit{lacZ} reporter plasmid similar to pSH18-34, except that it contains a LYS2 marker

**Media:** See method 2 for recipes

**Selection of Aptamers with the Two-Bait Interaction Trap.** The two-bait interaction trap can be used to select peptide aptamers specific for one of two chosen proteins. In this system, the target protein to which the peptide aptamer preys are selected to bind are constructed as TetR fusions (TetR bait). A second target protein that the peptide aptamers can be screened to bind or not to bind is constructed as LexA fusion (LexA bait). In one use of this method, peptide aptamers are first selected for interactions with the TetR bait. The TetR bait binds to the Tet operator upstream of the \textit{URA3} reporter. Peptide aptamer preys that interact with the bait are se-
lected on the basis of their ability to grow on Ura\textsuperscript{-} media. Second, the URA\textsuperscript{+} aptamers are screened for interactions with the LexA bait. The LexA bait binds to the LexA operator, which is upstream of the \textit{lacZ} reporter. Aptamers that interact with the LexA bait are blue on X-Gal plates. Aptamers that do not interact with the LexA bait are white.

**Protocol 4-1: Two-Bait Interaction Trap**

1. Create the desired target bait protein to isolate aptamers against in pCWX200. Create related target protein or allelic variant in pEG202. Confirm that the bait proteins (1) do not activate transcription and (2) are properly expressed, and (3) that the LexA fusions enter the nucleus and bind operator as described previously in protocols 2-2 and 2-3.

2. Transform the bait proteins (pCWX200 and pEG202 fusions) with pCWX24 (\textit{lacZ} reporter) into CWXY2 as described in protocol 2-1. Plate transformants on Glu Leu\textsuperscript{−} His\textsuperscript{−} Lys\textsuperscript{−} dropout plates.

3. Transform 50–100 µg of pJM-1 library into CWXY2 with baits and pCWX24 to give between $10^6$ and $10^7$ transformants (protocol 2-1). Plate transformation on Glu Leu\textsuperscript{−} His\textsuperscript{−} Lys\textsuperscript{−} Trp\textsuperscript{−} plates and incubate at 30 °C for 2–3 days (colonies should be ~1 mm in diameter).

4. Scrape the yeast cells with a glass spreader from the plate by adding 1–2 ml of H\textsubscript{2}O to a 24 × 24 cm plate and collect the yeast in a 50-ml Falcon tube. Pool the transformants from all plates and centrifuge at 3500 rpm for 4 min at room temperature. Remove the supernatant and resuspend the pellet in 25 ml of H\textsubscript{2}O. Centrifuge again and wash the pellet once more in H\textsubscript{2}O.

5. Resuspend the pellet in H\textsubscript{2}O and add an equal volume of 65% (v/v) glycerol, 0.1 M MgSO\textsubscript{4}, 25 mM Tris-HCl, pH 7.4. Divide into 1-ml aliquots and freeze at −70 °C.

6. Determine the plating efficiency of the frozen aliquot. Inoculate 10 library equivalents in 1 ml of Gal/Raf Leu\textsuperscript{−} His\textsuperscript{−} Lys\textsuperscript{−} Trp\textsuperscript{−} liquid medium. Incubate at 30 °C for 4 hr with shaking.

7. Pellet the yeast cells by centrifuging at 3500 rpm for 4 min at room temperature. Remove the supernatant and resuspend the pellet in H\textsubscript{2}O.

8. Spread yeast onto Gal/Raf Leu\textsuperscript{−} His\textsuperscript{−} Lys\textsuperscript{−} Trp\textsuperscript{−} Ura\textsuperscript{−} plates at a density of $10^6$ yeast per 10-cm-diameter plate and incubate at 30 °C. Colonies should begin to appear between 2 and 5 days.

9. Streak colonies onto Glu Leu\textsuperscript{−} His\textsuperscript{−} Lys\textsuperscript{−} Trp\textsuperscript{−} master plates and incubate at 30 °C for 1 day.

10. Replica plate the master plate on the following four plates: Glu Leu\textsuperscript{−} His\textsuperscript{−} Lys\textsuperscript{−} Trp\textsuperscript{−} X-Gal, Gal/Raf Leu\textsuperscript{−} His\textsuperscript{−} Lys\textsuperscript{−} Trp\textsuperscript{−} X-Gal, Glu Leu\textsuperscript{−} His\textsuperscript{−} Lys\textsuperscript{−} Trp\textsuperscript{−} Ura\textsuperscript{−}, and Gal/Raf Ura\textsuperscript{−} Leu\textsuperscript{−} His\textsuperscript{−} Lys\textsuperscript{−} Trp\textsuperscript{−} and
incubate at 30°C. Monitor the plates for blue color on X-Gal plates and growth on Ura\(^{-}\) plates.

11. Select colonies that are URA\(^{+}\) and LacZ\(^{+}\) or URA\(^{+}\) and LacZ\(^{-}\) on galactose for further characterization.

12. Isolate and sequence selected aptamer plasmids as described in protocol 2-6.

Affinity Maturation of Peptide Aptamers

Aptamer selections do not take advantage of the entire space of possible variable regions. There are \(20^{20}(10^{27})\) possible 20-mer variable regions. Libraries of aptamers contain from \(10^{9}\) to \(10^{10}\) members, of which \(\sim 10^{7}\) peptides can be looked at in any given selection. In addition, depending on the construction and the length of the random peptide insert, many of the aptamers will contain stop codons within the random region. Together, these limitations reduce the sequence space that can be sampled by any one interaction trap selection. In fact, for the 20-mer variable regions described above only \(\sim 9 \times 10^{-6}\%\) of the sequence space is represented.

As a result, peptide aptamers isolated with the interaction trap may not represent the highest affinity aptamers from all the possible sequences in a random 20-amino acid thioredoxin library.

However, in strict analogy to the recognition molecules of the immune systems, mutation of the aptamer variable regions, followed by selection for higher affinity aptamers, allows isolation of tighter binding aptamers\(^{13,62}\) (see Fig. 10). Mutations can be introduced into individual aptamer sequences by random PCR mutagenesis or by synthesizing degenerate oligonucleotides with varying degrees of randomness. The stringency of selection in the interaction trap is manipulated by the choice of reporter genes that have LexA operators in different numbers, affinity, and distance upstream of the reporter genes. The \(\text{LexA-lacZ (pSH18-34)}\) and \(\text{LexA-LEU2 (in EGY48)}\) reporters used in the "standard" interaction trap detect dissociation constants as weak as \(<1 \mu M\).\(^{63}\) A series of \(\text{lacZ}\) reporter genes\(^{64}\) that contain fewer and lower affinity LexA operators (Fig. 5) is used to allow selection of peptide aptamers that show increased affinity for their targets. Reporters containing one and two operators allow the detection of interactions from 20 nM to \(<1 \mu M\).\(^{63}\) Mutagenesis of DNA encoding the aptamer variable region, followed by selection of mutant aptamers in a strain with a low-sensitivity reporter, allows selection of peptide aptamers with higher affinity.

\(^{62}\) Colas \textit{et al.}, submitted (2000).
Fig. 10. Affinity maturation. Interaction mating assays comparing the affinities of Cdk2 bait for the original Cdk2 aptamer prey and the affinity-matured Cdk2 aptamer prey. Yeast strains (EGY48 Mata) containing either the original Cdk2 aptamer, affinity-matured Cdk2 aptamer, or a control aptamer were mated to yeast strains (EGY42 Mata) containing a Cdk2 bait with a lacZ reporter carrying either eight (pSH18-34), two (pJK103), or one (pRB1840) LexA operator. Interactions between the Cdk2 aptamer preys and the Cdk2 bait result in the activation of the lacZ reporter. The strength of the interaction is related to the intensity of the blue color on galactose X-Gal plates.

For example, the affinity of an anti-Cdk2 aptamer has been enhanced by using PCR to randomly mutate DNA encoding the variable region, followed by selecting those that bound Cdk2 in a bait strain with a more stringent reporter. In this work, a library of 15,000 mutant aptamers was screened for interactions with Cdk2, using a strain that contained a single LexA operator upstream of a lacZ reporter gene. A peptide aptamer was recovered with two amino acid changes that activated this lacZ reporter. Evanescent wave measurements revealed that the $K_d$ of the aptamer was reduced 20-fold from 10.5 $\mu$M to 5 nM. It should be possible to further increase the binding constants of peptide aptamers by using more stringent lacZ reporters. Moreover, it should be possible to isolate aptamers of higher affinity by screening larger numbers of mutagenized aptamers, by using more stringent LEU2 reporters, rather than lacZ reporters, as the primary screen, or by using URA3 reporters in the presence of increasing concen-
trations of the URA3 inhibitor 6-azauracil. Finally, it is possible to decrease bait and prey concentrations by changing the copy number of the plasmid encoding them [e.g., by changing the origin of replication from a 2 µm to a CEN autonomous replication sequence (ARS)]. We imagine that the use of such reduced copy bait and prey plasmids may facilitate the isolation of increased affinity aptamers.

**Method 5: Affinity Maturation**

**Reagents**

**Strain**
EGY48: Mata his3 trp1 ura3-52 leu2::LexA6op-LEU2

**Vectors**
pBait: pEG202 (Fig. 4) containing the bait protein of interest inserted into the polylinker in frame with LexA
pJG4-5: Nuclear localization, activation domain, and HA epitope tag fusion for the construction of prey proteins (see Fig. 1 for details)
Peptide aptamer prey: pJM-1 containing peptide aptamer (see Fig. 1 for details)
pRB1840: lacZ reporter plasmid containing one LexA operator (see Fig. 5 for details)

**Media:** See method 2 for recipes

**Mutagenic Polymerase Chain Reaction.** Amplify by PCR the variable region of the aptamer to be mutated in the presence of Mn²⁺, as described by Cadwell and Joyce.⁶⁵

**Protocol 5-1: Mutagenic Polymerase Chain Reaction**

1. Add 1 µl of Taq polymerase, 12 µl of H₂O, 1 µl of template DNA, and 10 µl of Mg/Mn mixture (45 mM MgCl₂ and 5 mM MnCl₂) to 76 µl of PCR premixture.

**PCR premixture**

- Taq polymerase buffer, 10× 500 µl
- MgCl₂, 1 M 5 µl
- dATP, 100 mM 10 µl
- dGTP, 100 mM 10 µl
- dCTP, 100 mM 50 µl
- dTTP, 100 mM 50 µl
- 5' Primer, 20 µM 125 µl
- 3' Primer, 20 µM 125 µl
- H₂O 2.9 ml

2. PCR amplify the reaction for 10 cycles. After one cycle remove 13 μl and add it to a solution containing 10 μl of Mg/Mn mixture, 1 μl of Taq polymerase, and 76 μl of PCR premixture. Perform the following PCR cycle: 95° for 30 sec, 55° for 1 min, and 72° for 1 min; repeat four times.

Selection of Higher Affinity Aptamers. The PCR-mutagenized variable region is used to construct a small library of mutagenized aptamers. Higher affinity aptamers are identified from this pool with selection strains that contain a low-sensitivity lacZ reporter (pRB1840).

Protocol 5-2: Selection of Mutagenized Aptamers

1. Introduce the mutated variable region from protocol 5-1 into RsrII-cut pJM-1, using standard subcloning techniques.
2. Transform the mutagenized peptide aptamer library into EGY48 (protocol 2-1) that contains the pBait of interest and pRB1840. Select transformants on Glu His- Ura- Trp- plates.
4. Incubate at 30° until galactose-dependent blue colonies are visible.
5. Rescue the plasmids from the galactose-dependent blue colonies (protocol 2-6) and reintroduce the plasmids into EGY48 containing the pBait of interest and pRB1840 to reconfirm the interaction phenotype.
6. Rescue the plasmid from the galactose-dependent blue colonies and sequence their variable regions.

Forward Genetics with Peptide Aptamers

Classic forward genetics involves isolating organisms displaying altered phenotypes and identifying the mutations responsible for the phenotypic changes. Forward genetic analysis is extremely powerful for characterizing cellular processes; however, it is limited by the ability to detect mutations causing the phenotypic change. Mutations causing phenotypic changes are readily identified in haploid organisms. In contrast, only dominant mutations can easily be detected in diploid organisms. Although in some diploid organisms recessive mutations are discernible, their detection requires the generation of organisms homozygous for the recessive mutation. Because creation of these homozygotes requires two generations of breeding, use of homozygous recessive mutations is generally confined to organisms with well-developed genetics.

To alleviate the problem of analyzing recessive mutations in diploid organisms, dominant agents have been developed that mutate gene function
without altering the genetic material. Combinatorial peptide aptamer libraries can function as dominant "mutagenic" that randomly inactivate gene function. Forward analysis of cellular processes with peptide aptamers involves expressing combinatorial peptide libraries in cells and screening or selection for aptamer-induced variations of cellular processes. The specific proteins and key points of disruption in the cellular process are subsequently identified by two-hybrid experiments. The advantage of using peptide aptamers for analyzing cellular processes is that they execute their "mutagenic" function at the protein level, eliminating problems associated with analyzing recessive mutations in diploid organisms. This strategy allows traits in diploid organisms and multicopy gene phenotypes to be examined.

For example, we and others have used aptamers in the forward analysis of a complex phenotype, the yeast pheromone response pathway, to identify genes and protein interactions involved in causing the phenotype.\textsuperscript{20-22} In the presence of mating pheromone, yeast arrest at the G\textsubscript{1} phase in the cell cycle.\textsuperscript{5} Peptide aptamers that inhibited the pheromone response pathway were selected on the basis of their ability to grow in the presence of mating pheromone. The protein targets of the selected peptide aptamers were identified with yeast two-hybrid systems.

Mating interaction assays successfully identified peptide aptamer targets from panels of proteins known to be involved in the pheromone response pathway\textsuperscript{20,21} as well as from a larger panel containing proteins from the entire yeast genome.\textsuperscript{22} We have also identified peptide aptamer targets by using them as baits in two-hybrid experiments to find interacting proteins in a yeast partial genome interaction library.\textsuperscript{21} Interestingly, this interactor hunt identified \textit{CBK1}, a gene not previously known to be involved in the pheromone response pathway.

This result demonstrated the benefits of combining library screening with mating interaction assay panels. However, due in part to the poor quality of the partial yeast genomic library, the library screens were not as effective as the interaction mating panels for identifying the aptamer targets. Many of the peptide aptamer targets that were identified with the mating interaction assay were not observed in the interaction trap hunt using the yeast genomic library.\textsuperscript{21} These results illustrate the utility of identifying targets by mating with ordered yeast arrays rather than by library screens. Mating interactions against ordered arrays of proteins have the advantage of (1) presenting the potential interactors as a fully normalized library, (2) easily detecting interactions that result in activation above the basal activation of the bait alone, and (3) detecting interactions that are independent of differences in the plating efficiency due to differences in reporter activation.\textsuperscript{63} However, until complete panels of proteins are available for
a variety of organisms, mating interactions with limited panels of known proteins will still need to be supplemented with two-hybrid genomic and cDNA library selections to identify peptide aptamer targets.

**Method 6: Forward Analysis of Cellular Process in Yeast with Peptide Aptamer Mutagens**

**Reagents**

**Vectors**
- pJM-2: Peptide aptamer vector for use in yeast genetic selections (see Fig. 1 for details)
- pJM-3: Peptide aptamer vector containing a nuclear localization signal for use in yeast genetic selections (see Fig. 1 for details)
- pEG202: LexA fusion plasmid for the construction of bait proteins (pBait) (see Fig. 4 for details)
- pSH18-34: lacZ reporter plasmid (see Fig. 5 for details)
- pJG4-5: Nuclear localization, activation domain, and HA epitope tag fusion for the construction of prey proteins (see Fig. 1 for details)

**Media:** See method 2 for recipes

**Yeast Genetic Selection of Peptide Aptamers.** The design of yeast genetic selection strategies is beyond the scope of this review. A typical genetic selection of peptide aptamers involves transforming a peptide aptamer expression library consisting of at least $10^6-10^7$ members into the selection strain. On the basis of results obtained with the yeast pheromone response pathway, approximately 1 of every $10^5-10^6$ TrxA peptide aptamers with 20-mer variable regions is able to inhibit this process. Therefore, a collection of yeast containing approximately $10^6-10^7$ unique 20-mer aptamers should possess enough aptamer diversity to inhibit at least many cellular processes.

In this procedure, the peptide aptamer transformation is plated under nonselective conditions to allow uniform representation of colonies containing individual peptide aptamers. The transformants are then scraped from the plates and pooled as a frozen stock. An aliquot of the stock containing 10 equivalents of the library is subjected to the selection conditions. Plasmids encoding the peptide aptamer from the selected colonies are isolated and retransformed into the selection strain to reconfirm the aptamer phenotype. In the procedure given below, the peptide aptamers are conditionally expressed by the yeast GAL1 promoter. The inducible promoter allows the confirmation of aptamer-dependent phenotypes by comparing the phenotypes of the yeast with the aptamer in the presence and absence of the inducer.
**Protocol 6-1: Yeast Genetic Selection of Peptide Aptamers with a Galactose-Inducible Promoter**

1. Transform selection strain with 50–100 μg of pJM-2 or pJM-3 peptide aptamer library to give $10^6$–$10^7$ transformants (protocol 2-1). Plate transformation on Glu Trp- plates and incubate at 30° until colonies form.

2. Scrape the yeast cells with a glass spreader from the plate by adding 1–2 ml of H$_2$O to a 24 × 24 cm plate and collect the yeast in a 50-ml Falcon tube. Pool the transformants from all plates and centrifuge at 3500 rpm for 4 min at room temperature. Remove the supernatant and resuspend the pellet in 25 ml of H$_2$O. Centrifuge again and wash the pellet once more in H$_2$O.

3. Resuspend the pellet in H$_2$O and add an equal volume of 65% (v/v) glycerol, 0.1 M MgSO$_4$, 25 mM Tris-HCl, pH 7.4. Divide into 1-ml aliquots and freeze at −70°.


5. Pellet the yeast cells by centrifuging at 3500 rpm for 4 min at room temperature. Remove the supernatant and resuspend the pellet in H$_2$O.


7. Streak positive colonies onto Glu Trp- plates.

8. Replica plate the master plate onto Glu Trp- and Gal/Raf Trp- selections plates and incubate under selection conditions.

9. Isolate aptamer-encoding plasmids from colonies showing the galactose-dependent phenotype (protocol 2-6).

10. Retransform the peptide aptamers into the selection strain and reconfirm the galactose-dependent phenotype.

11. Isolate the galactose-dependent aptamer encoding plasmids from step 10 (protocol 2-6) for further characterization and sequencing.

**Identification of Peptide Aptamer Targets.** The targets of the peptide aptamers obtained from a genetic selection can be identified by interaction mating experiments or by interaction trap hunts against genomic or cDNA libraries. The peptide aptamer baits are constructed by transferring the aptamer encoding DNA from the expression vector used in the genetic selection to the bait vector, pEG202 (Fig. 4). The aptamer baits are then screened for interactions against panels of known prey proteins or genomic or cDNA libraries of prey proteins. Panels of prey proteins are constructed by inserting the coding region of a desired protein into pJG4-5 (Fig. 1).
The construction of genomic and cDNA libraries is beyond the scope of this review; however, details of their construction can be found elsewhere.\textsuperscript{47,66,67}

Identification of peptide aptamer targets by the mating interaction assay is performed as described in method 3, with the following alterations. To identify aptamer targets, aptamers are expressed as baits rather than preys as in method 3, because the same aptamers can also be screened for interactions against genomic and cDNA libraries. Genomic and cDNA libraries are constructed as preys because they contain many sequences capable of activating transcription in the bait configuration. The peptide aptamer targets are identified by transforming the aptamer baits and pSH18-34 (\textit{lacZ} reporter; Fig. 5) into EGY48 (\textit{Mata}) and the library of preys into EGY42 (\textit{Mata}). The strains are mated and interactions between baits and preys are scored on interaction detection plates.

Identification of the peptide aptamer targets by interaction trap library hunts is performed as described in method 2. However, instead of screening a library of peptide aptamers preys for interactions against a specific protein bait, a library of protein preys is screened for interactions against a specific peptide aptamer bait. The peptide aptamer targets are identified in the interaction hunt by transforming the peptide aptamer baits and pSH18-34 into EGY48. A genomic or cDNA library is then introduced into this strain. Cells that contain a prey protein that interacts with the aptamer bait are detected by the activation of their \textit{LexAop--LEU2} reporters, allowing them to form colonies on Leu\textsuperscript{−} medium. Aptamer--target protein interactions that activate the \textit{LexAop--LEU2} reporter are subsequently tested for their ability to activate a \textit{LexAop--lacZ} reporter.

Once putative peptide aptamer targets are identified with the two-hybrid system, their significance can be further corroborated by a variety of approaches. Immunoprecipitation can be used to ensure that the aptamer--target complex forms under \textit{in vivo} conditions. Epistasis analysis of the peptide aptamer can be used to confirm that it functions in the same "neighborhood" of a known pathway as the protein target. The peptide aptamer target can be deleted or overexpressed and the resulting phenotypic consequences compared with those caused by the peptide aptamer. Finally, we have extended the interaction trap to allow identification of specific protein interactions that the aptamer disrupts.\textsuperscript{21}

Concluding Statements

In this review, we have described properties of peptide aptamers and methods to isolate them. We have also provided examples describing the
use of this new class of dominant "genetic" agent for the analysis of cellular processes. Although peptide aptamers are still in their early stages of development, the pilot studies presented make evident a variety of applications for peptide aptamers in the analysis of cellular processes. Peptide aptamers are particularly well suited for this task, as they can (1) bind a variety of protein targets, (2) bind protein targets with high affinities, (3) specifically recognize their protein targets, and (4) function effectively within cells. Peptide aptamers can inhibit protein function by a variety of mechanisms. Peptide aptamers can disrupt protein interactions within cells\textsuperscript{14,18,54} and in two-hybrid assays.\textsuperscript{21} In addition, peptide aptamers can competitively compete with substrates for the active site of enzymes.\textsuperscript{54} Alternatively, peptide aptamers can be used to modify their protein targets. Peptide aptamers with nuclear localization signals can mislocalize their protein targets to the nucleus.\textsuperscript{62} Peptide aptamers have also been used to direct a ubiquitin ligase to a specific protein target.\textsuperscript{62}

Peptide aptamers targeted to specific proteins can be used in the reverse analysis of cellular processes. By disrupting the function of their protein targets, peptide aptamers can be used to analyze the phenotypic consequences of individual genes. The high specificity of peptide aptamers enables them to inhibit specific protein–protein interactions while leaving other interactions with the target protein intact.\textsuperscript{21} Unlike gene knockout phenotypes, peptide aptamers should enable the analysis of phenotypes caused by the disruption of individual protein interactions in genetic networks. The ability of peptide aptamers to recognize allelic variants of proteins will aid in the functional characterization of polymorphic protein variants now being rapidly identified by genome sequencing projects.\textsuperscript{68} In addition, peptide aptamer expression levels and timing can be controlled with inducible promoters. This fact will allow the penetrance and timing of the aptamer phenotype to be controlled.

Random peptide aptamer expression libraries can be used in the forward analysis of cellular processes. Peptide aptamer "mutagens" can be identified that disrupt a cellular process. These aptamers can be subsequently used to identify proteins and protein interactions required for the process. In addition to identifying protein targets, the location of the target protein in a cell can be determined. Peptide aptamer libraries with different localization signals can be used in genetic selections. Further, a comparison of the protein targets obtained from each selection would permit researchers to deconvolute the locations of individual proteins in a genetic pathway.

In higher eukaryotes, various processes such as senescence and metastasis are not well understood. The success of dominant peptide aptamers in

the reverse analysis of cellular processes in mammalian cell culture and in *Drosophila* indicates that peptide aptamer expression libraries will be useful for the forward analysis of cellular process in higher eukaryotes. Entire libraries of peptide aptamers have been utilized to "mutagenize" mammalian cells, to try to identify proteins that function in particular processes (A. Colman-Lerner, personal communication, 1999). Such forward "genetic" analysis with random peptide aptamer expression libraries should expand our ability to analyze processes in genetically intractable organisms.

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[14] Detection of Protein–Protein Interactions by Protein Fragment Complementation Strategies

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Introduction

Much of modern biological research is concerned with identifying proteins involved in cellular processes, determining their functions and how, when, and where they interact with other proteins involved in specific biochemical pathways. Advances in genome projects have led to rapid progress in the identification of novel genes. In applications to biochemical research, there is now the pressing need to determine the functions of novel gene products. It is in addressing questions of function that genomics-based research becomes bogged down and there is now the need for advances in the development of simple and automatable functional assays. Identifying proteins by functional cloning of novel genes remains a significant experimental challenge. Many ingenious strategies have been devised to simultaneously screen cDNA libraries in the context of assays that allow both