Complementary-DNA Phage Display

Simultaneous Identification of Multiple Protein Targets by Using Complementary-DNA Phage Display and a Natural-Product-Mimetic Probe**

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Both natural-product and cofactor affinity chromatography have long played a crucial role in the elucidation of biological mechanisms. More recent advances have shown that small-

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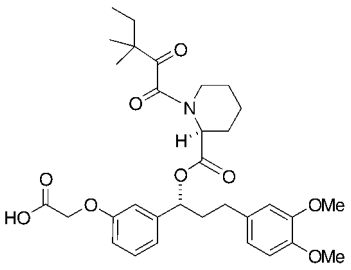
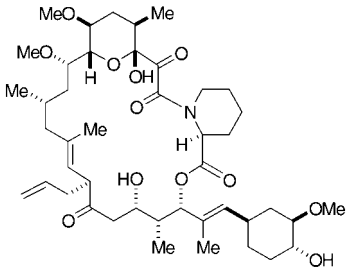
molecule affinity-based techniques have the potential to play a major role in functional proteomics.[1] Toward this end we, and others, have employed T7 complementary-DNA (cDNA) phage display with affinity chromatography in order to clone genes encoding protein cellular targets (display cloning).[2] It remains a challenge, however, to develop a selection protocol that is capable of isolating more than one protein homologue from a native cDNA library, especially when there is a disparity in the relative abundance and affinity among family members. In this paper we describe the simultaneous cloning of three homologues of the FK506 binding protein (FKBP) from a T7 phage-display cDNA library by using novel elution conditions and a biotinylated form of AP1497, a structural mimetic of the natural product FK506.[3] Furthermore, we describe an on-phage titer binding assay, which can be used to rapidly determine relative binding affinities of isolated cDNA phage clones.



AP1497



FK506

Display cloning is a technique that involves the capture of bacteriophage, displaying cDNA-derived proteins on their outer coat, by using biotinylated molecules on an avidin-coated surface. After elution of bound phage, clones are amplified and either resubjected to selection conditions or analyzed. During optimization of the experimental protocol, we discovered that FK506 affinity selections, when performed using *Escherichia coli* to rescue phage between selection rounds, provided a homologue of FKBP (FKBP13) that was different from the FKBP12 homologue isolated in a previous study.[2a] This finding was intriguing, since FKBP13 has a markedly lower-affinity than FKBP12 for FK506,[4] a fact making it an unlikely victor in an affinity-based selection process. Furthermore, it has been reported that *E. coli* infection is a less efficient method for phage rescue than specific elution conditions.[5] We postulated that the appearance of the lower-affinity homologue, observed with *E. coli* elution, may be due to the reinfection process itself. At equilibrium, a higher proportion of a lower-affinity phage would be expected to be in solution at any given time. If the disparity between phage-binding affinities is large enough, the lower-affinity phage would have a larger representation in solution and, therefore, a distinct advantage for reinfection.

We therefore sought to develop a selection protocol that takes advantage of this observation. By using an *E. coli* elution, lower-affinity homologues may be amplified in greater ratios, relative to higher-affinity homologues. Combined with clone screening at an earlier stage in the selection process, before the population reaches homogeneity, we may also increase the number of different genes that can be isolated from a single cDNA library. In addition to improving the number of homologues we can isolate, we were also interested in evaluating molecules with lower affinity for their target proteins as potential probes in this assay. The FKBP-binding pipecolyl α-keto amides[6] were viewed as optimal for this endeavor, since their synthesis has been described[3] and these molecules have a lower affinity[1b,7] for the FKBP family of proteins than FK506.[3]

Functionalization of AP1497 was achieved by activation of the carboxylic acid with *N*-hydroxysuccinimide, followed by coupling with a polyethylene-linked amine containing biotin (biotin-PEO-LC-NH_2) in dimethyl sulfoxide to provide biotinylated AP1497 (bio-AP1497). A single well of an avidin-coated 96-well plate was treated with 10 μM bio-AP1497, and this was followed by blocking with 1 mM biotin. This secondary biotin incubation greatly reduces the amount of background binding by blocking unoccupied binding sites on the avidin plate that would otherwise provide a pocket for peptide affinity.[8] A 100-μL aliquot of a human-brain T7 cDNA phage library[2a] was precleared by incubation with an untreated avidin plate, in order to remove any avidin-binding proteins in the cDNA library, before it was transfered to the plate treated with bio-AP1497. After incubation and removal of the unbound phage, the remaining phage were amplified by addition of a log-phase culture of *E. coli* (100 μL). The resulting phage lysate was used directly in the next selection round, and this cycle was repeated for a total of three rounds. After the third round, the bound phage were eluted by using 1% sodium dodecylsulfate (SDS; in tris-buffered saline), diluted, and applied to Luria–Bertani top agarose plates in order to obtain spatially separated phage plaques.

Ninety-six randomly selected clones from the third round were amplified and tested individually for their ability to bind a 96-well plate treated with bio-AP1497. The phage clones were then ranked, based on elution titer, from highest to lowest, and the cDNA inserts from the top 30 bio-AP1497-binding clones were sequenced. Each of the top 22 clones were found to contain one of three different genes, which encode either FKBP12,[9] FKBP12.6,[10] or FKBP13,[11] all of which are known binders of the natural product FK506. In these clones, all three genes are in-frame with the T7 coat protein (cp10). The FKBP12 and FKBP12.6 clones contain the entire coding portion of the gene, while for FKBP13, only the FK506-binding region of the gene was isolated; this result suggests the potential of this affinity-based cloning method to

Figure 1. Protein sequence alignment of translated FKBP12 (top), FKBP 12.6 (middle), and FKBP13 (bottom) isolated cDNA phage clones.

identify not only a target protein but also the minimal binding domain. The alignment of the translated peptide sequences for the three isolated genes is shown in Figure 1.[12]

In order to establish the probe dependence of phage binding, and to develop a quantitative binding assay that can be performed directly by using the protein expressed on the phage particle, the FKBP phage were subjected to affinity analysis in the presence of varying concentrations of the probe. Figure 2 shows the rescued phage titer (1% SDS

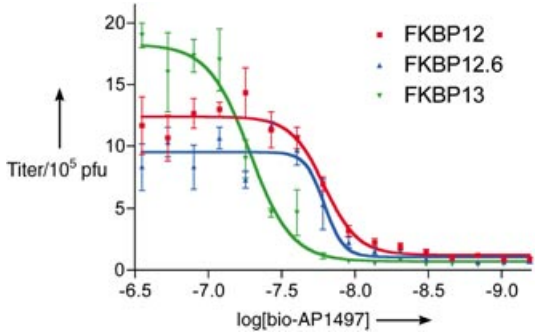


Figure 2. Titer binding assays at multiple probe concentrations for FKBP12-, FKBP12.6-, and FKBP13-displaying phage. pfu = plaque-forming units.

elution), as a function of log[bio-AP1497], for FKBP12, FKBP12.6, and FKBP13 phage. In each case, phage binding is probe dependent and can be fitted to a sigmoidal dose–response curve by using nonlinear regression analysis.[13] The calculated phage-binding EC_{50} values of bio-AP1497 for FKBP12 and FKBP12.6 are essentially identical at 16 nM (14–19 nM), while the EC_{50} value for FKBP13 is somewhat higher, 52 nM (44–62 nM), which is consistent with literature values.[3,4]

This on-phage binding assay is useful since phage clones can be analyzed within hours of affinity selection without the need for subcloning or additional instrumentation. Methods for quantitative phage-binding analysis at single probe concentrations have been described;[14] however, multiple-concentration titer data are a more accurate measure of relative protein affinity than single-concentration titer data, unless the relative protein display on the phage particle is taken into account.[15] As seen in Figure 2, the EC_{50} values observed for phage binding more accurately reflect the affinity of these phage-displayed proteins than the absolute titer observed at maximal probe concentrations does.

The utilization of small molecules in order to affinity purify phage-displayed proteins from cDNA libraries has great potential for the rapid identification of cellular protein targets; however, several factors must be taken into account when the appropriateness of this approach is evaluated for a particular molecule. A major factor that must be considered is whether a cDNA library actually contains the gene of interest. Protein expression can vary from cell to cell, and library quality can also vary, based on the mRNA source and the method used to construct the library. By their very nature, cDNA phage libraries are an enormous mixture of genes and gene fragments, some in-frame and some out-of-frame with the phage coat proteins. Therefore, particular care must also be taken in choosing the conditions employed for affinity selection, as evidenced by the results described above. Another consideration is the type of protein that can be isolated with this technique. To date, reports utilizing this protocol have primarily described the isolation of cytosolic single-domain proteins or single domains from large multi-domain proteins.[2] It is not clear at this time whether this trend suggests a limitation of the overall approach or is a result of the tendency for genetic-based selection techniques to provide the most efficient member of a given population. Rather conspicuously missing from the array of successes are integral membrane proteins or proteins that bind as either homo- or heterodimers. However, a recent paper describing the use of T7 cDNA phage display for the isolation of mRNA-binding proteins, which was not achievable without modification of the *E. coli* expression system,[2h] suggests that attention to details concerning protein expression may facilitate the isolation of more varied protein families.

In conclusion, the *E. coli* reinfection of affinity-captured cDNA phage provided selection conditions that allowed the isolation of a low-affinity FKBP homologue (FKBP13). A 96-well phage-based affinity binding screen, performed before the phage population reached homogeneity, allowed for the identification of a low-copy, yet high-affinity FKBP homologue (FKBP12.6). Neither of these modifications obviated the isolation of FKBP12, a high-copy, high-affinity FKBP homologue, and they resulted in a direct and rapidly verifiable method for the simultaneous isolation and identification of target genes. Development of a concentration-dependent on-phage binding assay allowed for the rapid assessment of relative binding affinities for each of the isolated clones. Furthermore, we have shown that a natural-product structural mimetic can be used as an effective affinity probe, with a T7 cDNA phage-display library, for the cloning of multiple homologues of human genes corresponding to the natural-product protein target. Efforts to expand this methodology for general use in small-molecule-based proteomic studies are currently underway.

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