Display cloning: functional identification of natural product receptors using cDNA-phage display

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Background: The identification of cellular targets has traditionally been the starting point for natural product mode of action studies and has led to the understanding of many biological processes. Conventional experimental approaches have depended on cell-based screening and/or affinity chromatography. Although both of these techniques aid in the discovery of protein cellular targets, a method that couples protein identification with gene isolation would be extremely valuable.

Results: A procedure for the direct cloning of cellular proteins, based on their affinity for natural products, using cDNA phage display has been developed. The technique is referred to as display cloning because it involves the cloning of proteins displayed on the surface of a bacteriophage particle. The approach has been established by isolating a full-length gene clone of FKBP12 (FK506-binding protein) from a human brain cDNA library using a biotinylated FK506 probe molecule. During the affinity selection, the FKBP12 gene emerged as the dominant library member and was the only sequence identified after the second round of selection.

Conclusions: The development of display cloning greatly facilitates the investigation of ligand–receptor interaction biology and natural product mode of action studies. This procedure utilizes heterologous protein display on infectious phage, which allows the amplification and repeated selection of putative sequences, leading to unambiguous target identification. In addition, the direct connection of a functional protein to its gene sequence eliminates the subsequent cloning step required with tissue homogenate or cell lysate affinity methods, allowing direct isolation of an expressible gene sequence.

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Introduction

Natural products have historically played an important role in the elucidation of biological mechanisms [1–4]. The identification of a natural product's receptor provides an important link between its phenotype and cellular component [5–7]. The combination of *in vitro* genetic expression techniques with traditional biochemical approaches, such as affinity chromatography, offers the possibility of functional gene selection by bridging natural product affinity directly with gene structure. Phage display is one such *in vitro* technique that allows the expression, selection and subsequent amplification of proteins on the surface of bacteriophage viral particles, effectively linking a phenotype to its genotype [8–10]. Complementary DNA (cDNA) is the reverse transcriptase product of mRNA and represents the coding sequence of all transcribed genes at the time of mRNA isolation. By incorporating cDNA into phage display, it is possible to obtain an expressible transcript of every gene from any desired cell type. Many techniques for the construction of double-stranded cDNA from mRNA are available [11,12], and these procedures are so advanced that it is possible to extract and construct a cDNA library from a single cell [13]. Although several cDNA phage display systems have been described, including M13 pIII display [14–24], M13 pVI display [25] and tailed bacteriophage display [26–29], these systems have only been used to elucidate protein–protein interactions. The use of nonpeptidic small molecule probes to identify receptor proteins has remained elusive. In fact, it has been postulated that the small molecule epitope may be too small for use in phage display screening [30].

Display cloning is a method for the concomitant identification and isolation of natural product protein cellular targets, using a T7 cDNA phage display library and a biotinylated natural product probe. Validation of this procedure was obtained by using a T7 cDNA phage display library and biotinylated FK506 as a natural product affinity probe. The FK506–FKBP (FKBP; FK506 binding protein) interaction is a challenging proof of principle for two reasons. First, the carboxyl terminus of the protein is directly adjacent to the middle strand of a five-strand β sheet [31,32], making it difficult to remove the STOP codon in the cDNA transcript while maintaining an active fold. Second, FKBP12 is

a cytosolic protein and therefore not efficiently secreted through the periplasm of the bacterial cell wall. Both of these criteria are required for M13 display [10]. Display cloning utilizes T7 phage display, which overcomes these difficulties. With biotinylated FK506 as a natural product probe this procedure provided a single cDNA clone containing the entire FKBP12 gene sequence after only two rounds of affinity selection. An optimized protocol is presented, as well a discussion of the potential benefits of combining *in vitro* genetic techniques with affinity chromatography as a general approach for the investigation of small molecule ligand–receptor interactions.

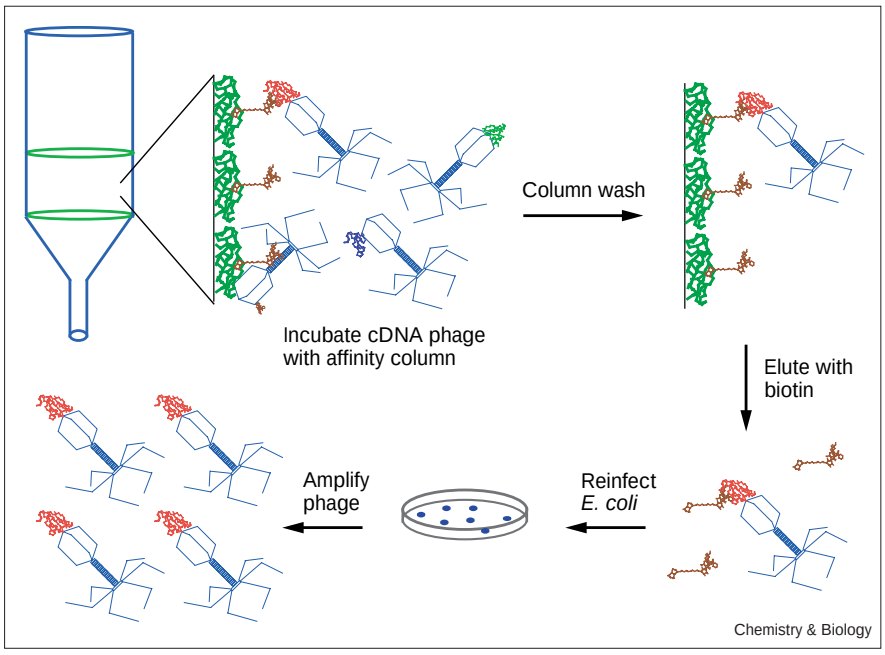
Results and discussion

The display cloning procedure is shown schematically in Figure 1. The protocol begins with the immobilization of the biotinylated natural product onto a monomeric-avidin agarose gel. The gel is utilized in a column and the cDNA phage are allowed a single pass over the column. This is followed by extensive washing to minimize any nonspecific binding interactions between the phage coat proteins and the agarose column. Phage that display proteins with specific affinity for the natural product probe will remain on the column during the wash step. These phage are then selectively eluted with excess biotin. After elution, the phage are rescued by immediate re-infection into *Escherichia coli* and amplified. This cycle represents one round of affinity selection and takes approximately eight hours. The amplified phage can either be used in an additional round of selection or the selected protein(s) can be analyzed by isolating and sequencing the phage DNA. The procedure requires either a biotinylated natural product or natural product affinity matrix and a cDNA phage display library.

The most useful option for probe derivatization for FK506 was biotinylation, as opposed to the construction of a covalent affinity matrix. After affinity purification, this same probe could be used for the quantitative analysis of affinity (K_d) and/or protein detection (western blot). Biotinylated natural product also offers the feature of a reversible solid-support reagent when combined with monomeric-avidin sepharose. The synthesis of FK506–biotin (**4**) is outlined in Figure 2 and starts with bis-silylated N-Boc-hexanediamine-FK506 (**1**) [33–35]. Simultaneous deprotection of the silyl groups and the Boc function of **1** with hydrofluoric acid in 7:1 acetonitrile:water gave FK506-amine **2**. Immediate treatment of this nucleophilic amine with N-hydroxysuccinimide–long-chain–biotin (NHS–LC–biotin) led to formation of FK506–biotin (**4**). The biotin component of compound **4** will serve as an anchor to the column resin, whereas the FK506 component is free to interact with the bacteriophage cDNA library.

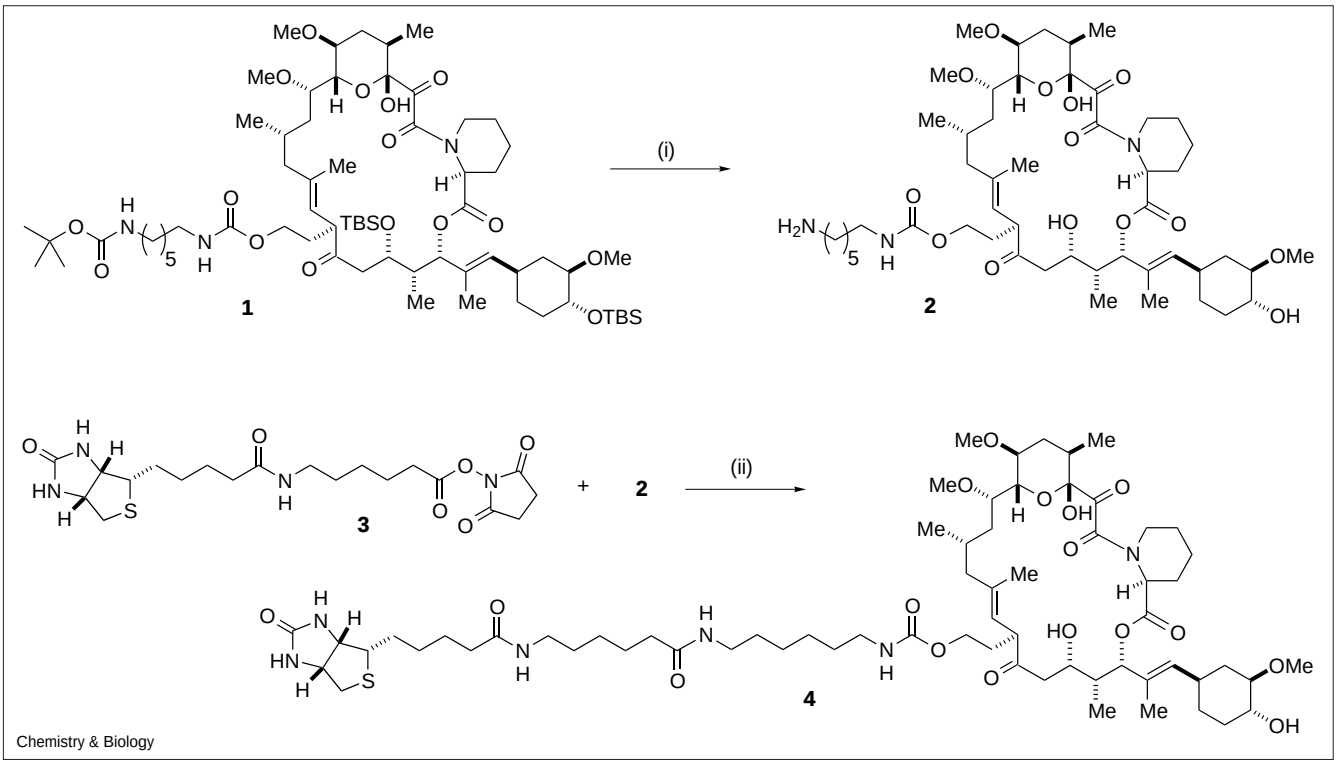
Monomeric avidin bound to agarose was chosen as the optimal solid-phase resin. It has a significantly lower affinity for biotin (K_d ~10^{-7} M) enabling the selective release of any biotin-bound species [36]. The elution is specific for phage bound to the column through the FK506–biotin interaction and is independent of the natural product–target interaction because the entire complex is eluted as a whole. This anchor-specific elution

Figure 1



Display cloning procedure for one round of affinity selection using a biotin-probe-treated monomeric avidin column and cDNA phage. At the end of the first round of selection, the amplified phage can either be analyzed by DNA sequencing or directly subjected to additional round(s) of selection.

Figure 2



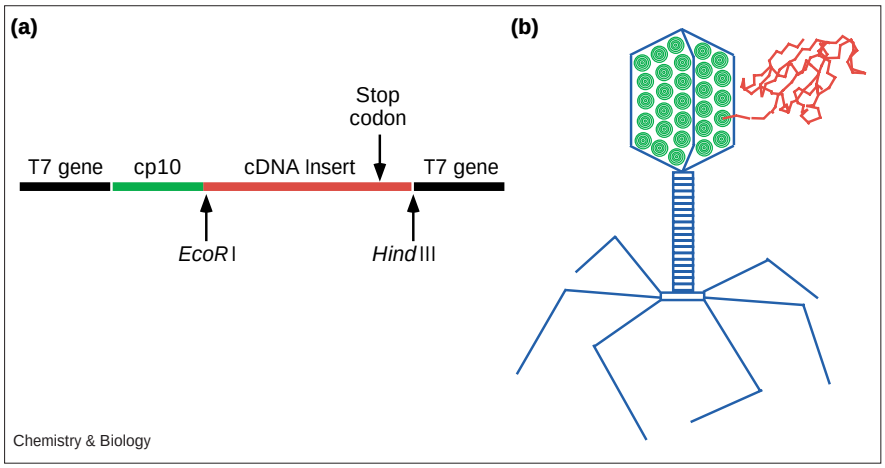
Synthesis of the biotinylated FK506 (4) probe molecule. (i) HF, CH_3CN, H_2O, rt (ii) pyridine, rt.

will allow the procedure to be generalized for any receptor–ligand complex, regardless of off rate, allowing even a covalent interaction to be efficiently eluted. More importantly, the elution conditions are mild, thus lowering the possibility that nonspecifically bound phage will also be removed. This is important because nonspecific interactions in bacteriophage selections are well documented [10]. The agarose column does provide an increase in surface area, which increases the number of phage that can be evaluated, but this can also lead to high background phage titers and a loss of signal amplification with increasing selection rounds. The anchor-specific elution reduces the number of nonspecific phage eluted, and as long as the background interactions remain constant and weak, even low abundance clones with high natural product affinity will be selected for and become the dominant members of the population after affinity maturation.

The choice of phage is crucial for cDNA incorporation. Tailed phage alleviate two of the major problems encountered with M13 expression of cDNA libraries, namely the STOP codon problem and protein secretion. The T7 bacteriophage display system is readily available, exhibits rapid growth and was therefore selected for this study [26]. Incorporation of cDNA into the traditional M13 phagemid is problematic because of the presence of a STOP codon in the cDNA insert, which is amino-terminal in this phage display fusion. This prevents the expression of a complete insert-pIII fusion protein that is required for display on the surface of the phage particles. Solutions for the incorporation of cDNA into an M13 system have been described [14,15,17,25,37], but even proteins that remain functional must be secreted through the periplasmic space of the bacterial cell wall in order to be properly displayed on M13 phage [10]. Although this is likely to benefit proteins that are normally secreted, such as antibodies and membrane-bound receptors, it will provide a selective bias against most cytosolic and nuclear localized proteins, and will hamper affinity selection. Several examples of cDNA libraries in M13 display systems are known [19–21,23,24], but at this point none has been described for the study of small molecule ligand–receptor interactions. In fact, these reasons are probably why our attempts to utilize M13 cDNA display systems [25,37] did not lead to identification of the target gene when probed with FK506.

A human brain cDNA library was cloned into bacteriophage T7 [26]. Construction of the directionally cloned, randomly primed cDNA library was performed with polyA$^+$ purified mRNA from human brain. The new cDNA library was directionally cloned using *Eco*RI and *Hind*III restriction sites, then packaged *in vitro* and

Figure 3

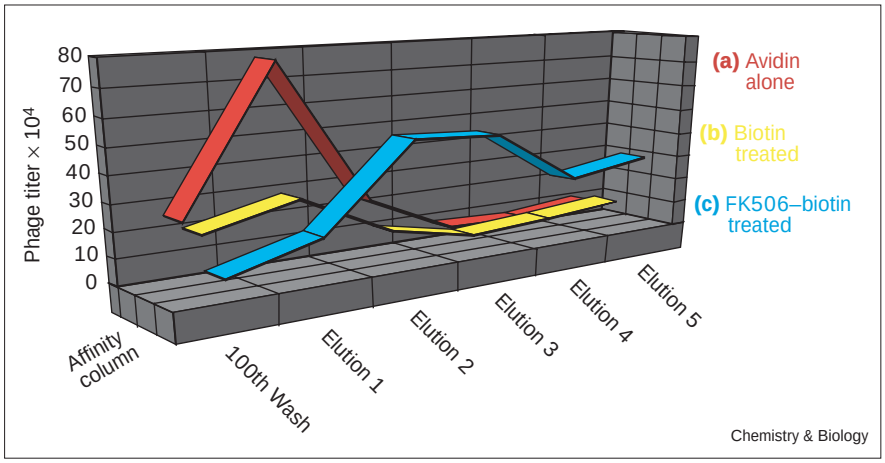


Schematic representation of the T7 display vector and graphic diagram of coat protein fusion to the expressed cDNA insert. **(a)** The cDNA library is directionally cloned using *EcoR* I and *Hind* III restriction sites. Efficient surface expression of all clones is achieved, regardless of the presence of a STOP codon in the cDNA transcript, because the cDNA insert follows the coat protein (cp10). **(b)** Graphic representation of bacteriophage T7 and the cp10 coat fusion with cDNA protein product. The fusion takes place on the phage head, leaving the neck and tail free for *E. coli* infectivity.

infected into *E. coli* to yield 3.3×10^6 transformants, based on phage titer. The fusion of the gene encoding coat protein 10 (cp10) and the cDNA insert allows one cDNA protein product to be presented on the surface of each phage particle (Figure 3a). As the cDNA insert is carboxy-terminal to the coat protein (cp10), expression of the intact fusion is not affected by the presence of a STOP codon. Figure 3b shows a schematic of the T7-phage particle with the displayed protein fused to the phage head, leaving the tail free for *E. coli* infectivity. A small aliquot of the stored human brain cDNA library was used to generate the phage employed in the FK506 selection by inoculating a log phase culture of *E. coli*. On average, T7 bacteriophage production yields a titer of 10^{10} pfu/ml. By inoculating one liter of culture with approximately 10^7 phage, a total of 10^{13} phagemids are formed. As the cDNA library contains 10^6 distinct transformants, each clone is represented by approximately 10^7 phage particles.

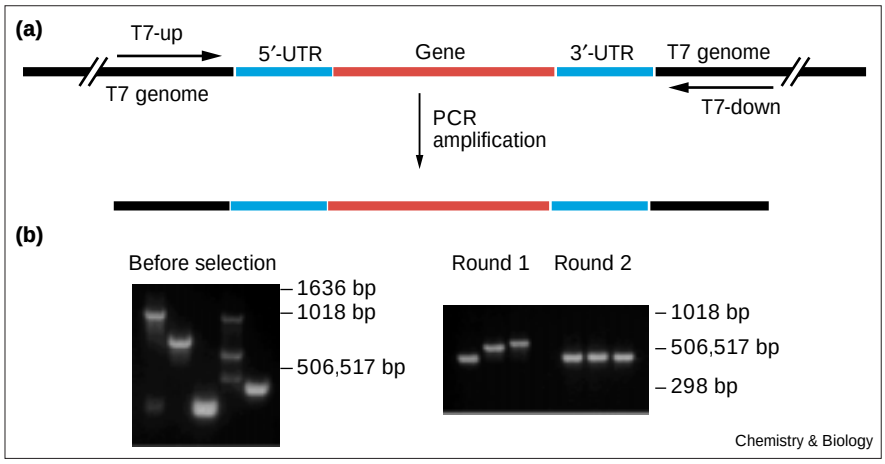
Three 1 ml affinity columns were prepared: the monomeric avidin column alone, a biotin pretreated column and a column treated with FK506–biotin. In the first round of selection phage supernatant (100 ml; ~10^{12} phage) was poured over each 1 ml column. The columns were subjected to washing with 100 ml phage wash buffer (PWB: 1 × phosphate-buffered saline (PBS), 450 mM NaCl, 0.05% Tween20) followed by 10 ml dH_2O. The bound phage were eluted with a biotin solution (5 mM in PWB) and collected in 0.5 ml aliquots. Figure 4 shows a plot of phage titer as a function of elution. As evidenced by the elution titer (Figure 4a), quite a few cDNA proteins have inherent affinity for the avidin binding sites on the column. This is not unexpected because certain peptides have been shown to bind the avidin protein [38]. These interactions can, however, be suppressed by biotin pretreatment of the column (Figure 4b). It is useful to evaluate the phage titer for at least the first selection because

Figure 4



Elution titers used for the evaluation of the FK506/cDNA phage affinity selection. Phage titers are evaluated as the last wash (typically 100× column volume) and the first few biotin elutions. **(a)** Monomeric avidin alone (red) shows the ability to bind cDNA-phage, as is seen by the spike in titer with the biotin-containing elution fractions. **(b)** When the column is preincubated with biotin (yellow), the column does not show appreciable titer with specific elution. **(c)** Incubation with FK506–biotin (4) restores retention of titer, which extends over many more elutions than the avidin alone. The elution is anchor specific, suggesting that a specific interaction is taking place between the biotin–FK506 and members of the cDNA phage library.

Figure 5



PCR analysis of amplified phagemids after each selection round. (a) Primers annealing to the vector sequence allow the amplification of a band corresponding to the size of the insert +118 bp of vector sequence. In addition to size analysis, this amplified sequence can be also used for DNA sequencing and/or subcloning. (b) PCR band-size analysis of selected clones. The clones analyzed prior to the affinity selection show a wide range of sizes (five out of 20 shown). After the first round of selection, the evaluated clones and appeared to have a diverse representation. The second-round phage all gave the same band size and were found to be identical by DNA sequencing. It should be noted that no FKBP gene was isolated prior to selection. During the course of library construction and evaluation, 35 independent clones were isolated and sequenced without the identification of the FKBP gene.

this can be an indication of both the level of background phage–column interaction and of an affinity interaction. The FK506–biotin treated column showed an elevated titer, relative to the biotin control, suggesting a strong interaction between FK506 and members of the cDNA library (Figure 4c).

The eluted phage aliquots from the first FK506–biotin column selection round were combined and infected into log phase *E. coli*. The resulting amplified phage were then subjected to a second round of selection. After each round, five random phage plaques were selected for analysis. As T7 genomic DNA is quite large (39 kb), analysis of insert size was performed by polymerase chain reaction (PCR) amplification of the insert region using primers designed to anneal to the vector sequence flanking the cloning site (Figure 5a). Although this procedure results in a band with 118 base pairs (bp) more than the gene insert, it does allow rapid monitoring of the progress of the selection at each round. By comparing the relative size of each band, the number of identical clones may be inferred. This is useful information because an affinity selection should produce one or more clones that dominate the phage population after several rounds of selection.

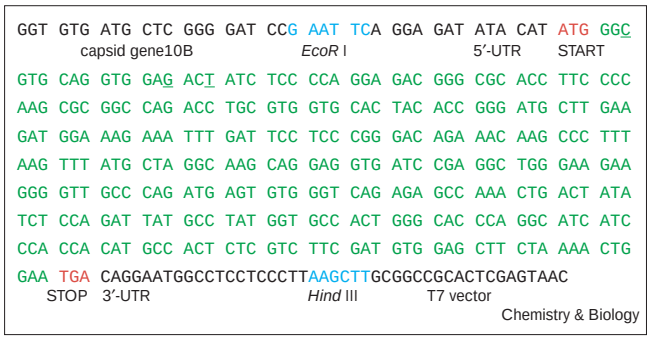
One of the most remarkable features of display cloning with the natural product FK506 was the rapidity with which target selection took place. Random analysis of the phage library prior to the first selection showed no redundant clones in the library (Figure 5b). In addition, no duplicate sequences were noted after the first round of selection. However, one of the clones identified in the first round became the dominant clone in the second round, giving a band size of 450 bp. By the second round of selection this was the only clone detected in the phage population. A BLAST [39] search of the DNA sequence obtained for this clone revealed sequence identity to the FKBP12 gene, the known cellular target of FK506 [40–42]. Figure 6 shows the gene sequence for the isolated clone. The gene was obtained in its fully expressible form, as observed by the presence of 5′- and 3′-untranslated region (UTR) as well as START and STOP codons, flanked by the *Eco*RI and *Hind*III cloning sites. The efficiency of this selection is probably related to the high affinity that FK506 has for FKBP12 (K_d = 0.4 nM) [43]. It remains to be seen what level of affinity is required for target identification. In principle, a small molecule with micromolar affinity would also be amenable to this technique, but may require more than two rounds of selection.

Several FKBP homologs have been reported [1–2]. As display cloning is selective in nature, it provided the highest affinity and smallest protein in the FKBP family. Modification of this procedure to screen for additional binding proteins might include DNA sequencing of clones from earlier selections and/or additional affinity rounds after DNA-level subtraction of the dominant clone, in this case FKBP12.

Advantages of affinity chromatography with cDNA phage display

One of the major benefits of display cloning is the ease with which consecutive rounds of selection can be employed, because the phage retain their infectivity. This allows for an iterative protocol, leading to an amplification of signal, which is important when probing cDNA libraries for low abundance genes. In addition to signal amplification, the competition for the natural product, in this case

Figure 6



Sequence analysis of the isolated phage clone. Sequence analysis reveals that the FKBP12 gene is in frame with the capsid protein cp10, as required for expression. A segment of 5′-UTR (untranslated region) is observed, followed by an open reading frame, the FKBP12 gene sequence and finally a short 3′-UTR segment. No poly A tail is present because the library was constructed using random primers. Three silent mutations were noted in the sequence (underlined bases) which is the only difference between the sequence we identified and those previously described [41,42,95].

FK506, provided a selective pressure between the clones in the cDNA phage library and allowed the unambiguous assignment of FKBP12 as the affinity target of FK506, because it was the only phage in the population after affinity maturation. When display cloning is used for natural products for which the cellular target is unknown, the evaluation of earlier selection rounds may prove useful for the identification of additional binding proteins and/or smaller binding domains within the target protein.

Many methods for identifying receptor–ligand complexes have been developed, including cell screening [44], biochemical elucidation [45,46], affinity chromatography [40,47–54], and more recently mRNA expression arrays [55–57]. All of these methods require that the gene sequence for the target protein must either be inferred from the screen or peptide sequenced from an isolated protein, with subsequent cloning of the identified gene. To circumvent the need for a subsequent cloning step, many gene-linked methods for analyzing small molecule–protein interactions have been described [58–63], and one has even been applied to natural product target elucidation [61]. These cell-based methods have inherent limitations such as requiring the spatial segregation of cells as well as the solubility and membrane permeability of the natural product probes. This can lead to either a low signal or the use of a large quantity of precious natural product. Indeed, even with the availability of these methods, affinity chromatography remains the predominant approach for natural product mode of action studies [52–54].

In vitro techniques that incorporate cDNA in an expressible format offer direct access to gene products. Expression cloning is one example that takes advantage of the link between gene and expression product, allowing the cloning of a desired gene directly from a cDNA library [64,65]. This technique is based on an antibody's affinity for the target protein or a nucleotide's affinity for its Watson–Crick complement. Although peptides [66], nucleoside derivatives [67] and a drug–BSA (bovine serum albumin) conjugate [68] have been used with expression cloning, a natural product interaction has not been reported. One potential explanation for this may be that, like the cell-based methods, expression cloning requires a direct signal readout, is not readily amenable to affinity amplification, and is therefore limited by probe solubility and availability. Alternate *in vitro* techniques, designed to link genotype with phenotype, have been described and include: phage display [8–10], plasmid display [69,70], polysome display [71,72], protein tagging [73], *in vitro* expression cloning (IVEC) [74], ribosome display [75–77], RNA-fusion display [78], baculoviral display [79], bacterial cell-surface display [80,81] and eukaryotic cell-surface display [82]. Although not all of these systems are amenable to the incorporation of cDNA libraries, they have the benefit of signal amplification and the ability to perform multiple selection rounds, bypassing the signal sensitivity problem. Affinity chromatography could, in principle, be combined with any of these methods to establish novel functional cloning procedures.

Tailed phage systems, such as T7, are emerging as a viable alternative to M13 bacteriophage display; several have recently been described [26,83–87] and a number of examples incorporate cDNA libraries [26–29]. The tailed phage are inherently superior to the M13 phage for cDNA expression because many involve expression at the carboxyl terminus of the surface fusion. Tailed phage are also lytic. As M13 phage are secretory, displayed proteins must be secreted through the periplasmic space of *E. coli*. Although this is possible, it does transfer a selective advantage to those proteins that are inherently membrane associated or endogenously expressed for extracellular localization. Tailed phage burst the cell membrane upon maturation and therefore no selective advantage is conferred through protein characteristics. Additionally, cell lysis is able to provide cell-based cofactors (i.e. DNA, RNA, additional proteins, and so on) that may also be involved in the affinity complex. This would be most useful in ligand–receptor systems where more than one cellular component may be required for activity [88].

Affinity chromatography offers the benefit of evaluating many potential protein interactions at once, because all available proteins can be applied to the solid support simultaneously [89]. Traditional approaches involve the use of either tissue homogenate or cell lysate for the production of the natural receptor. This requires the homogenization of many kilograms of animal tissue or, in the case of cell

lysates, culturing many liters of mammalian cells [90]. Once the receptor has been identified, the procedure is scaled up and the protein is isolated and purified to homogeneity in order to chemically determine its protein sequence. Subsequent database queries allow the identification of genes that encode known proteins or expressed sequence tags (ESTs) [39]. This sequence information is then used to probe cDNA libraries for the genetic clone [91].

Display cloning combines the benefit of gene-linked *in vitro* protein display with the power of affinity chromatography to provide direct access to the human genes of natural product receptors. The technique also has the potential to identify even the least abundant genes, because it employs a selective amplification, therefore bypassing the need for cDNA library normalization [92]. This is crucial because it is estimated that between 50,000 and 100,000 different genes are transcribed across all eukaryotic cell types and between 10,000 and 30,000 are expressed in any one cell type [93]. In addition, because the total number of phage that display cloning can evaluate extends beyond 10^{12} particles, it is possible to envision large libraries of cDNA gene fragments that would increase the potential of identifying natural product binding domains within a target protein.

Conclusions and future work

We have described a cDNA-phage-based system for the elucidation of small molecule–receptor interactions, leading directly to the genetic element of the target protein. More complex display systems offer the hope of achieving expression conditions that will provide proper folding and post-translational modifications. It also remains to be shown whether membrane-associated proteins can be displayed in this manner. It is likely that these problems will require the use of eukaryotic display systems such as baculoviral [79] or mammalian [82] systems.

The link between nucleic acid sequence and protein function is one that nature has surely made from its beginning [94]. Our understanding of the relationship between genome structure and function will depend on our ability to rediscover this link and probe it with natural products. Future experiments will involve efforts to query these systems with protein display of cDNA libraries. Display cloning can be used in conjunction with natural products and combinatorial chemical libraries for the exploration of functional genomics.

Significance

Display cloning is a new method for the *in vitro* analysis of receptor–ligand interactions, directly from cDNA. The cellular target of FK506 is well defined. We have shown that this cytosolic target can be functionally displayed and affinity selected in an *in vitro* system. The validation of a small molecule epitope for the functional cloning of a gene target has been established and will serve as a starting point for additional efforts in natural product target elucidation. The ability to probe natural diversity with a natural product probe, leading to the direct identification of the target gene, will greatly enhance natural product mode of action studies. The power of combining affinity chromatography with a gene-linked system is that, in theory, any cell type in any phase of cell cycle or growth can be targeted. This will prove important in the exploration of teratogenic natural products to identify proteins important to organismal development, particularly because of the inherent limitation of human fetal tissue and the potential lack of developmentally important proteins in mature animal tissue.

Materials and methods

*Synthesis of FK506–biotin (**4**)*

To a solution of Boc-FK506-TBS_2 (7.5 mg, 5.8 μmol) [34] in 250 μl 7:1 CH_3CN:H_2O was added to 50 μl 48% HF in an Eppendorf tube. The solution was allowed to stir at room temperature until all of the starting material was consumed and ninhydrin staining revealed the presence of an amine with low R_f (0.1 in 31.1 ml *t*-butylacetate, 15.7 ml glacial acetic acid, 6.0 ml *n*-butanol and 14.4 ml water). Upon addition of NHS-LC-biotin (3.3 mg, 5.8 μmol; Pierce Chemical Company, Rockford, IL) the ninhydrin spot immediately disappeared and a new spot with a slightly higher R_f appeared. The reaction was quenched with saturated sodium bicarbonate (250 μl), extracted with methylene chloride (3×500 μl) and dried over sodium sulfate. The crude mixture was subjected to column chromatography (from 9:1 to 4:1, CH_2Cl_2:MeOH) and provided 1.7 mg (5.8 μmol) of FK506–biotin (4) (R_f 0.1 in 9:1 CH_2Cl_2:MeOH) in 35% yield.

Construction of human brain cDNA phage library

Bovine brain has traditionally been a good tissue source for the FKBP protein [40]. As display cloning only requires a small amount of mRNA for library construction, human brain was chosen as the mRNA source. Purified human brain poly A^+ mRNA (obtained from normal whole cerebral brain tissue of a 50 year old Caucasian male, Clontech, Inc., Palo Alto, CA) was randomly primed in the first-strand synthesis followed by second-strand synthesis to yield double-stranded complimentary DNA. The cDNA was doubly digested with *EcoR*I and *Hind*III endonucleases and size fractionated. The purified cDNA was then directionally ligated into T7Select1-1b vector arms and subsequently *in vitro* packaged into phagemids (Novagen, Madison, WI). Ligation efficiencies were evaluated with a small aliquot of the packaged phage and indicated a total of 3.3×10^6 plaque forming units. These initial transformants were subjected to one amplification step by infecting 50 ml log phase BLT5403 cells, providing a human brain library of cDNA phage with an overall titer of 3.6×10^{10} pfu/ml. The library was stored in aliquots (100 μl) at −78°C as 8% glycerol stocks.

Generation of cDNA phage for library selection

To one liter of $2 \times$ YT culture was added BLT5403 cells (from a 1 ml saturated culture) and incubated at 37°C until cell growth reached log phase (OD_{600} = 0.5–0.8). The culture was then inoculated with 10 μl (9×10^8 phage) of the human brain cDNA library stock described above. The number of phage used was 200-fold over the original library diversity in order to maintain an appropriate representation of all library members. It is estimated that there are 2.0×10^8 cells/ml in a culture at OD_{600} = 0.5. The level of infectivity (LOI) is kept above 1000 (LOI = # cells/# of phage) in order to ensure complete infection. This is important since a liquid culture amplification was performed. The culture was incubated at 37°C until completely lysed. To achieve high-salt conditions of the phage supernatant, 100 ml of 5 M NaCl was added and the suspension centrifuged at 4700 rpm for 20 min at 4°C

in order to precipitate the cell debris. This supernatant solution had a final library titer of 1.8×10^{10} pfu/ml and was used directly in the affinity selections described below.

Affinity chromatography with biotinylated FK506 and cDNA phage

Three 1 ml aliquots of monomeric avidin resin (ImmunoPure Immobilized Monomeric Avidin Gel, Pierce Chemical Company, Rockford, IL) were incubated with (i) PWB, (ii) 5 mM biotin in PWB and (iii) FK506–biotin (4) with slow rotation for 30 min at 4°C. All three columns were then rinsed with PWB (30 ml). The cDNA phage supernatant (100 ml) was poured over each column, followed by washing with PWB (100 ml) and distilled water (10 ml). The elution step for each column was identical and involved treatment with 10 ml of 5 mM biotin and collecting the eluate in 0.5 ml aliquots. A small amount (10 μl) from each fraction was used for titer determination (Figure 5). The fractions from the FK506 selection were combined and used to inoculate 100 ml of log phase BLT5403 cells and incubated at 37°C until completely lysed. After lysis, 10 ml of 5 M NaCl was added and the solution centrifuged at 4700 rpm for 20 min at 4°C to precipitate the cell debris. The supernatant contains the amplified phage from the first selection, however, it still has approximately 0.5 mM biotin from the previous elution. The biotin was removed through precipitation of the phage by adding 80 ml phage precipitant (33% PEG-8000, 3.3 M NaCl) and centrifuging at 7000 rpm for 10 min at 4°C. The precipitated phage were resuspended in 30 ml PWB, and a small amount (5 ml) was stored at –78°C as an 8% glycerol stock. An FK506–biotin affinity column was prepared as described above and the remaining 25 ml of the amplified phage were poured over the column. The column was washed with PWB (100 ml) and distilled water (10 ml), then eluted with 5 mM biotin (10 ml). The eluate for both the first and second selection rounds were plated onto 2 × YT top agar and single plaques were selected for DNA analysis.

Evaluation and sequencing of cDNA inserts

Individual plaques, 20 prior to selection and five from each round of selection, were stabbed and used to inoculate log phase cultures of BLT5403 cells (10 ml) and allowed to incubate at 37°C until the culture completely lysed. Genomic DNA was isolated from the phage supernatants using standard lambda DNA purification resin (Wizard Lambda Preps DNA Purification System, Promega Corp., Madison, WI). Two primers, T7-up (5′-GGAGCTGTCGTATTCCAGTC-3′) and T7-down (5′-AACCCCTCAAGACCCGTTTA-3′) anneal to the vector sequence immediately flanking the cloning site and were used with standard PCR conditions to evaluate the relative size of the insert region of the isolated clones (Figure 4). As all of the clones from the second round of selection appeared to have the same insert size, they were subjected to DNA sequencing and were subsequently found to be identical clones (Figure 6). Sequencing reactions were performed with the T7-up primer using fluorescently labelled dideoxynucleotides and Taq FS DNA polymerase in a thermal cycling protocol, and were analyzed on an Applied Biosystems 377 DNA Sequencer.

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