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Drug Receptor Identification from Multiple Tissues Using Cellular-Derived mRNA Display Libraries

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Summary

The use of display technologies to identify small molecule receptors from proteome libraries would provide a significant advantage in drug discovery. We have used mRNA display to select, based on affinity, proteins that bind to a drug of interest. A library of mRNA-protein fusion molecules was constructed from human liver, kidney, and bone marrow transcripts and selected using an immobilized FK506-biotin conjugate. Three rounds of selection produced full-length FKBP12 (FK506 binding protein 12 kDa) as the dominant clone. An analogous method was also used to map the minimal drug binding domain within FKBP12. Using this approach, it is anticipated that mRNA display could eventually play a key role in the discovery and characterization of new drug receptor interactions.

Introduction

The identification of bioactive compounds causing enzyme inhibition and perturbation of receptor or channel function has predominated as the basis for medicinal discovery. The majority of these drugs elicit their pharmacological action through binding to known protein targets. Although well-characterized biological targets continue to provide a strong foundation for therapeutic intervention, a shift in paradigm has emerged wherein genomic and proteomic technologies are being utilized to identify new protein targets for drug discovery [1, 2]. This approach, often referred to as "gene-to-screen," draws upon the wealth of human and pathogen genome sequence information now available to identify candidate genes for chemical screening. Unfortunately, genomic technologies are unable to provide definitive confirmation of a candidate's "drugability" before downstream affinity-based screens are performed. An alternative approach to drug target identification involves high-throughput screening of natural product and/or combinatorial libraries for a desired phenotypic response in cells or tissues followed by affinity-based identification of cellular proteins that bind to promising lead compounds. This "screen-to-gene" strategy would accomplish two main goals: (1) the discovery of candidate ligands capable of exerting their effect within a biological milieu, and (2) the identification of target proteins that explicate mechanism of action or toxicity. Traditionally, biochemical techniques such as affinity chromatography [3–9] and affinity labeling [10–14] have been employed to facilitate the purification and identification of drug binding proteins. However, these approaches often fail after laborious efforts to isolate and microsequence proteins that are unstable, are expressed at levels below the sensitivity of detection, or are inadequately pure for unambiguous characterization. To circumvent these problems, several expression-cloning methods have been developed to detect natural product-protein interactions [15]. Yeast-three hybrid [16], functional expression cloning in tissue culture cells [17], drug-Western [18], and phage-display cloning [19] have all allowed the encoded proteins from cDNA libraries to be probed for ligand binding activity. However, each of these techniques has inherent limitations arising from the constraints of working within an in vivo system. A methodology that combines the versatility of in vitro cDNA manipulation with drug affinity chromatography would provide significant benefits. mRNA display [20, 21], where libraries of proteins are covalently linked to their encoding mRNA, provides just such a system and has been used to select for small molecule binding proteins from a random-sequence library [22]. In addition, mRNA display libraries have been constructed from human tissues that have allowed the discovery of novel protein-protein [23] and enzyme-substrate [24] interactions. This approach has now been demonstrated to be a new tool for the identification of drug receptors using the FK506-FKBP (FK506 binding protein) interaction as a model drug-receptor complex [7]. Here, we describe the successful selection of full-length FKBP12, a ubiquitous receptor of FK506, from a mixture of human kidney-, liver-, and bone marrow-derived mRNA display libraries using an FK506-biotin conjugate. In addition, the versatility of mRNA display in defining the FK506 binding domain within FKBP12 is demonstrated through construction of a random-primed cDNA library from an FKBP12-containing template, in vitro selection against a FK506 matrix, and evaluation of the selected proteins in drug affinity binding assays.

Results and Discussion

The general strategy outlined in Figure 1 was employed for the selection of proteins that bind to a drug or small molecule of interest. Starting from a cDNA template, specific primers are used during PCR to introduce fixed sequences required for downstream transcription, puromycin linker ligation, and in vitro translation. Following translation and fusion formation, the resulting mRNA-protein fusion molecules are then purified and reverse transcribed to create a stable cDNA template for later amplification. Incubation of the cellular mRNA display library with an immobilized drug or small molecule is performed with subsequent washing to remove unbound constructs. After elution of bound species and amplification by PCR, an enriched drug binding library is generated for further selection. This approach is ame-

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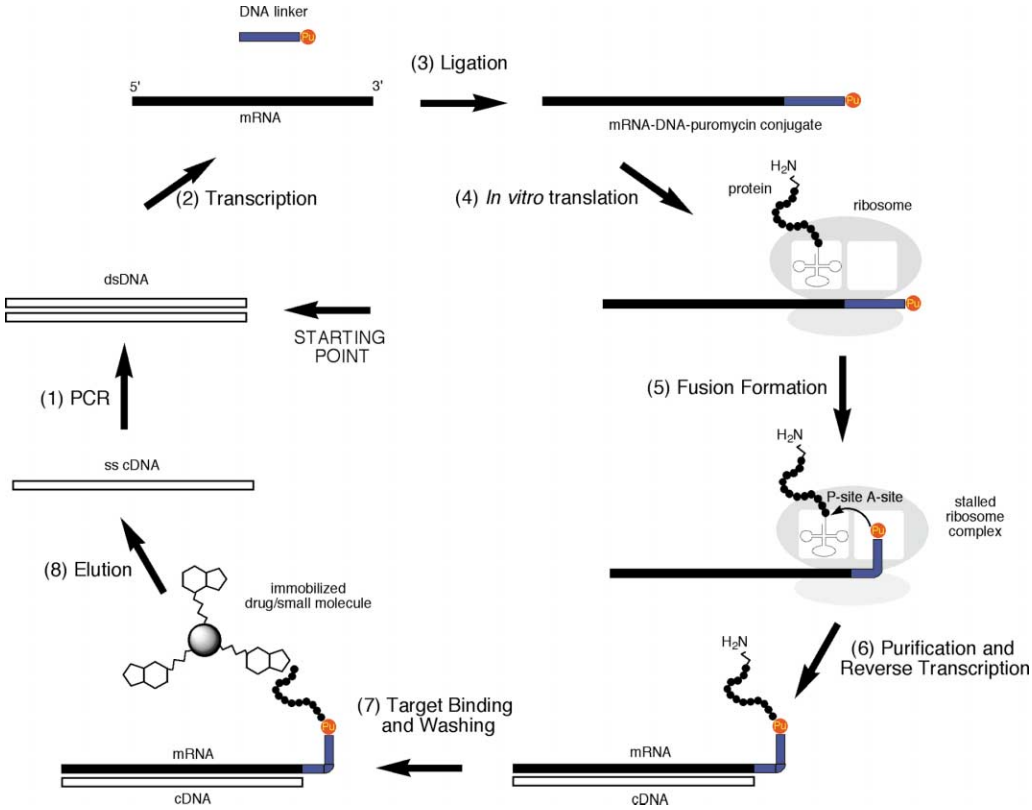

Figure 1. In Vitro Selection Using mRNA Display

(1) PCR-amplified cDNA is generated using primers that introduce the engineered sequences necessary for transcription, ligation of the corresponding mRNA, in vitro translation of the mRNA-puromycin linker conjugate, and epitope-based purification of the mRNA-protein fusion.
(2) The engineered PCR product is transcribed to produce mRNA that is then hybridized to a poly-dA-containing linker carrying a 5′-psoralen moiety and a 3′ terminus containing the transfer-RNA mimic puromycin (Pu).
(3) UV irradiation produces a covalent crosslink between the mRNA and DNA-puromycin linker [34].
(4) This mRNA-DNA-puromycin conjugate is then used as a template for in vitro translation. The ribosome translates the open reading frame and pauses at the mRNA-DNA junction.
(5) The absence of a stop codon obviates the action of release factors and allows the conjugate puromycin to enter the A-site of the ribosome. The peptidyl transferase subunit catalyzes amide bond formation between an amine group on the puromycin and the carboxyl terminus of the mature protein to give an mRNA-protein fusion.
(6) The fusion is purified after dissociation from the ribosome by virtue of its poly dA linker with oligo-dT cellulose. Reverse transcription of the fusion creates a cDNA strand that protects the mRNA against degradation and serves as a template for future PCR. Further protein-based purification of the mRNA display construct also allows the removal of unfused mRNA-DNA-puromycin conjugate.
(7) Libraries of mRNA display molecules, generated from the initial random priming of cellular mRNA, are then incubated with an immobilized drug or small molecule, and unbound material is removed by washing.
(8) Bound fusion is then eluted either specifically using excess of drug or nonspecifically using KOH. The eluted cDNA then serves as template to generate a new library enriched for drug binding proteins.

nable to any drug, small molecule, or natural product that contains or can be functionalized with an appropriate reactive moiety (e.g., hydroxyl, amine, or thiol) for direct immobilization with retention of bioactivity. In examples where little or no SAR information is available on the compound, multiple sites of modification may need to be explored to present the drug in a biologically relevant format. Biotinylation of target compounds with concomitant avidin-based capture may also be used to create an affinity matrix. The latter method of immobilization was chosen to allow more accurate control over tethered drug concentrations and to thereby facilitate downstream affinity ranking of putative selection winners in binding studies.

The FK506-biotin derivative used in this study (Figure 2A) was prepared from FK506 **1** via esterification of the less hindered C-32 exocyclic hydroxyl group with succinic anhydride to give the carboxylic acid intermediate **2**. Subsequent treatment with N-hydroxysuccinimide (NHS) and dicyclohexylcarbodiimide (DCC) activated the carboxylic acid for coupling with biotin polyethylene oxide-long chain-amine (biotin PEO-LC-amine) to yield the biotinylated FK506 product **3**, which was purified by preparative thin-layer chromatography. The extended PEO arm was chosen to impart some hydrophilic character to the FK506-biotin conjugate and thus assist in aqueous solubility. In addition, it was anticipated that the 23 Å spacer would render the FK506 component more accessible to the mRNA display libraries while anchored to an avidin-based support.

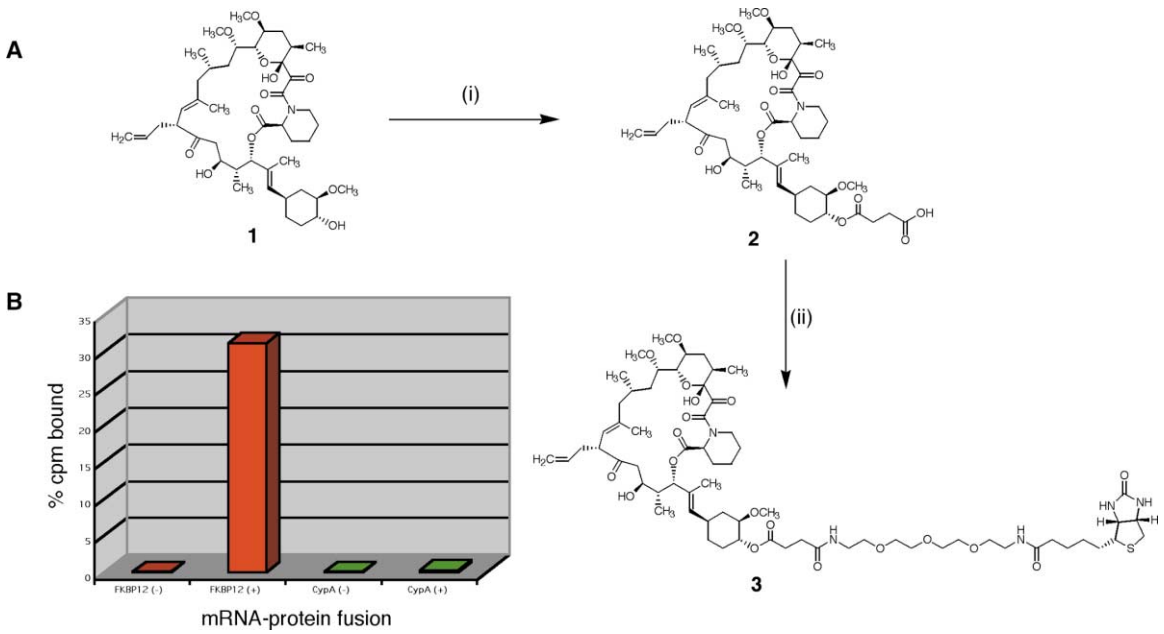

Figure 2. Synthesis and Binding Analysis of an FK506-Biotin Derivative to mRNA Display Constructs

(A) Synthesis of an FK506-biotin conjugate (**3**). (i) Succinic anhydride, DMAP, TEA, rt (ii) NHS, DCC, biotin-PEO-LC-NH_2.

(B) Binding of control mRNA display constructs to FK506. FKBP12 and cyclophilin A were prepared as mRNA-protein fusions. Each ^{35}S-labeled mRNA display construct was incubated with either FK506-biotin immobilized on streptavidin beads (+) or control beads without drug (−). Percent binding was calculated based on the amount of input material retained on the beads after washing.

The ability of a known drug receptor to bind its natural ligand (in an immobilized format) in the context of an mRNA-protein fusion was assessed as a model system to demonstrate the feasibility of selecting a drug receptor(s) from an in vitro mRNA display library. An mRNA display construct was created from a full-length cDNA encoding FKBP12 (FK506 binding protein 12 kDa) using the scheme outlined above. A cyclophilin A (CypA) mRNA-protein fusion was prepared as a control immunophilin that would not bind to FK506. Each protein contained a C-terminal sequence encoding the peptide DYKDDDDKASA to allow anti-FLAG immunoprecipitation and provide a spacer between the natural terminus of the protein and the nucleic acid tail. The ^{35}S-methionine radiolabel incorporated into the protein during in vitro translation was used to monitor the extent of binding. Each mRNA-protein fusion was mixed with streptavidin-coated magnetic beads that had been preincubated in the presence or absence of FK506-biotin. After 30 min, the beads were captured and washed with buffer several times, and the amount of bound fusion was measured by scintillation counting (Figure 2B). The results indicated that FKBP12 fusion binding was drug specific, with 31.2% of input counts being retained on the FK506 matrix compared with only 0.01% on the control beads. As expected, only 0.3% of the CypA fusion bound to the FK506 beads (cf. 0.05% control).

Selection of FKBP12 from a Mixed-Tissue mRNA Display Library

The specificity of the mRNA display-drug interaction and the low background binding confirmed optimal conditions for initiating a selection using mRNA display libraries derived from cellular transcripts. Human liver, kidney, and bone marrow cDNA libraries were synthesized individually from commercially available poly(A)$^+$ purified mRNA by random priming [23]. The 5′ primer included fixed sequence that contained a T7 RNA polymerase promoter, a tobacco mosaic virus translation initiation site, an 8 nucleotide library-specific sequence tag, and a start codon directly upstream from the random 9 nucleotide priming sequence. The 3′ primer included sequence coding for the FLAG epitope and a puromycin linker hybridization-photoligation sequence in addition to the nine random nucleotides. The unique sequence tag within the 5′-UTR has the advantage of allowing specific amplification of a particular library from within a mixed pool in addition to identifying cellular origin when analyzing individual clone sequence upon completion of a selection with multiple libraries. As expected, PCR amplification of the individual libraries gave heterogeneous smears of cDNA ranging in size from 200 bp to >1 kb (Figure 3A, Round 0). A mixture of tissue-specific mRNA display libraries was prepared from these PCR products that gave a starting pool of $\sim 1.8 \times 10^{11}$ fusion molecules. The absence of in vivo transformation bottlenecks during library construction coupled with the assumption that the human genome contains ~30,000 distinct genes [25, 26] suggests that even rare transcripts are well represented in this pool. The mixed libraries were incubated with biotin-pretreated streptavidin magnetic beads to remove mRNA display constructs that bound nonspecifically to the solid phase. The precleared library was then mixed with an FK506 affinity matrix, and washed and bound material was eluted. The eluate was used as a template for PCR to generate an

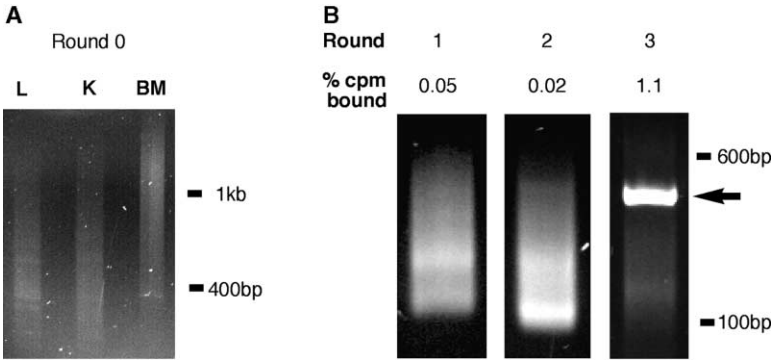

Figure 3. PCR Analysis of Human Liver, Kidney, and Bone Marrow mRNA Display Libraries before and after Selection against FK506

(A) PCR product of individual tissue-specific libraries before selection. Each library displays cDNA products ranging in size from 200 bp to >1 kb.

(B) PCR profile of mixed-tissue libraries after one, two, and three rounds of affinity selection against immobilized FK506. After three rounds, an intense band was observed migrating at approximately 420 bp, indicating that the selection had successfully enriched binding proteins of a consistent size.

enriched cDNA library (Figure 3B, Round 1). A correspondingly enriched mRNA display pool was then generated to begin the next round. The progress of the selection was monitored by comparison of the PCR product between rounds and by the extent of input radioactivity that bound the immobilized target. After three rounds, 1.1% of the selected mRNA-protein fusion molecules bound the FK506 target, compared with 0.05% in Round 1 and 0.02% in Round 2. In addition, PCR analysis of the selection showed the progression from an indistinct smear in Rounds 1 and 2 to a strong band migrating at ~420 bp in Round 3 (Figure 3B, Round 3). This band was excised, purified, cloned, and sequenced. From a total of 24 sequences analyzed, 23 belonged to a single homologous sequence cluster with two identical clones. A nBLAST sequence search showed the redundant clone to be FKBP12. Further analysis revealed all 23 sequences aligned with the native ORF of FKBP12, and 19 of those sequences contained the full-length sequence. Specific C-terminal additions were observed in all of the selected clones when compared to the native protein sequence (Figure 4). These additions arose via single (80%) or double (20%) nucleotide mutagenesis within the TGA stop codon of the FKBP12 native transcript. Such mutations are not unexpected since the library was constructed by random priming with a 9 nucleotide region, and any mispairing within these nucleotides will result in a mutation relative to the parent ORF. Therefore, mutations within the stop codon of a native transcript are necessary to create productive and functional mRNA display constructs of proteins that do not tolerate truncations at their C terminus. Inclusion of the first three nucleotides (CAG) of the FKBP12 3′-UTR within the selected message resulted in its translation as a glutamine codon. Overall, 15 of the selected clones contained a 2 amino acid (X-Gln) extension immediately following Glu107 in the parent protein where X is Arg (73%), Trp (13%), Gly (7%), or Val (7%). Additionally, 25% of these clones isolated contained modifications at the N terminus of the protein, specifically a deletion of the first two amino acids (Gly-Val) of FKBP12.

Surprisingly, the unique 5′-UTR sequence tag identified all 23 sequences as originating from the kidney library. Although FKBP12 is a ubiquitous cytosolic protein, there was no precedent to suggest that FKBP12 was present in higher abundance in kidney relative to bone marrow and liver [27]. This bias may have arisen due to preferential annealing of the kidney-specific

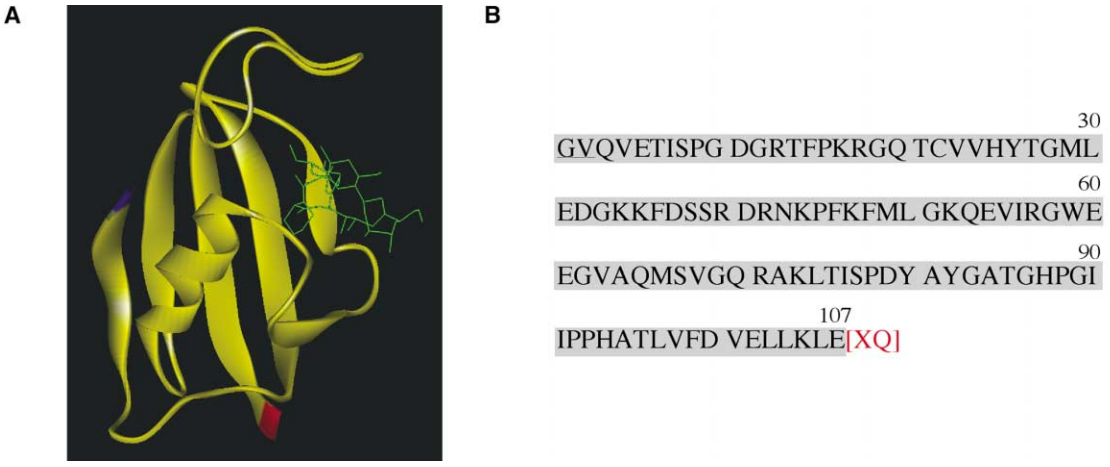

Figure 4. Sequence Analysis of Isolated FKBP12 Clones after Selection by mRNA Display

(A) Structure of the FKBP12-FK506 complex [35]. The N and C termini are shown in blue and red, respectively. FK506 is shown in green.

(B) Amino acid sequence of Round 3 clones from a mixed tissue mRNA display library after selection with FK506. Sequence homologous to FKBP12 is shaded. Out of 24 clones analyzed, 15 contained a 2 amino acid addition (X-Gln, shown in red) relative to the native protein, where X was either Arg, Trp, Gly, or Val. In addition, four of these clones contained a 2 amino acid truncation at the N terminus (underlined).

primer during PCR. To determine if the proteins from either the bone marrow or liver library were enriched by selection, library-specific reamplification of Round 3 was performed. These PCR products were recombined in equimolar amounts prior to transcription and fusion preparation. A total of 6.8% of this pool of mRNA-protein fusion molecules bound the FK506 target matrix (cf. 0.1% preclear beads), and the library-specific amplification of the recovered cDNA by PCR gave a strong band with an agarose gel mobility similar with that of the previous round (data not shown). Cloning and sequencing of these products after purification confirmed the selection of full-length FKBP12 from both bone marrow and liver tissues. The analyzed clones exhibited similar deviations in their predicted protein termini to those identified from the kidney-derived sequences.

The selection and identification of FKBP12 from a cellular-derived mRNA display library using an immobilized FK506 drug target was completed in 3–4 rounds. A similar study using T7 phage display as a selection tool required 6–7 rounds of selection to isolate an FKBP12-containing construct from a human brain cDNA library [19, 28]. As each technology typically requires 2–3 days to perform one round of selection, mRNA display presents an expeditious alternative. In addition, the presence of library-specific sequence tags within the 5′-UTR of the mRNA display libraries used in this study should prevent the problems of contamination encountered by phage display approaches.

FKBP12 was the only protein identified from our selection, although multiple isoforms of FK506 binding proteins are known to exist [29]. This may be because FKBP12 exhibits the highest affinity for FK506 ($K_d = 0.4$ nM) [30] compared to other FK506 binding proteins or because it is the smallest protein of the FKBP family in the tissues studied herein [27]. Quantitative PCR analysis of the initial cDNA libraries detected products encoding the drug binding domains of FKBP12 and FKBP52 but not FKBP36. However, examination of the cDNA pools after each round of selection against FK506 revealed that FKBP12 was the only immunophilin of the three that was enriched (data not shown). It is unclear whether this bias is indicative of differences in protein expression, folding, or drug affinity. Selection using mRNA display libraries from normalized cDNA or from the cellular mRNA of cell lines or tissues known to have a higher abundance of the larger immunophilins may promote their enrichment in an affinity selection. Additionally, the versatility of mRNA display should facilitate the application of library size fractionation, larger sequence sampling, cDNA array filter analysis, and dominant clone depletion to aid in the identification of additional binding proteins in future studies.

Mapping the Minimal FK506 Binding Domain of FKBP12

mRNA display was applied in the investigation of the nature of the FK506-FKBP interaction in more detail by constructing a cDNA library based on random priming of a homogenous mRNA template containing the FKBP12 sequence and selection of the minimal protein that mediates binding to FK506 (Figure 5A). The mRNA template, comprised of FKBP12 (50%), 5′- and 3′-UTR (40%), and vector (10%) sequence, was used to simulate a drug binding domain within a larger protein, a feature common to most immunophilins. The resulting library consisted of a heterogeneous smear of cDNA products that ranged in size from ~800 bp to <400 bp and should contain a mixture of extended, full-length, and fragmented FKBP12 sequences (Figure 5B, Round 0). This starting pool was cloned and sequenced to determine representation. Sequence analysis of 27 random clones revealed 89% contained a fragment of the FKBP12 coding sequence, 11% contained UTR sequence, and only 3% contained the full-length gene. An mRNA display library was prepared and subjected to three rounds of selection against immobilized FK506 using similar conditions to the cellular selection. After the third round, 2.2% of mRNA display fusions bound the drug target, and PCR amplification of eluted cDNA gave a dominant band migrating close to a 400 bp standard (Figure 5B, Round 3). In contrast to the cellular selection, the PCR product was cloned without gel purification, and 53 random clones were picked for sequence analysis. Twenty-one of these clones contained the full-length FKBP12 sequence, while an additional 20 sequences encoded various fragments of the full-length protein. The remaining 12 clones contained only UTR sequence, suggesting an incomplete selection, and were not examined further.

Representative sequences were chosen to assess the binding affinities of the corresponding peptides or proteins to FK506 (Figure 6). All proteins were expressed in vitro using [^{35}S]methionine for radiolabeling, then purified by anti-FLAG M2 immunoprecipitation. The selected variants were incubated with a range of immobilized FK506 concentrations (10 nM–5 μM), and after washing, the bound material was quantified by scintillation counting. Bound counts were normalized and affinities were calculated based on a nonlinear regression curve fit. Surprisingly, only proteins that contained the full-length FKBP12 sequence bound to FK506. No drug binding was observed for any of the truncated constructs even at the highest FK506 concentration data point used (5 μM).

An examination of the FKBP12 framework indicates the C-terminal amino acids Leu97-Glu107, which form the backbone β strand of FKBP12's β sheet, appear necessary for structural stability of the protein, and this may explain the absence of functional C-terminally truncated FKBP12 proteins from both selections. Also, the lack of binding to immobilized FK506 observed with an FKBP12 construct devoid of the first 16 residues (K17-Q109) (Figure 6) highlights the importance of the N terminus of FKBP12 in forming and/or preserving a FK506 binding fold. Indeed, the selection of FK506 binding proteins with a Gly1-Val2 truncation supports the observation that only minimal deletion of N-terminal sequence is tolerated for drug binding. The deficiency of the shorter FKBP12 fragments in FK506 binding assays suggests that the presence of these sequences within the pool is indicative of either an incomplete selection or low-affinity binding of these peptides to FK506 below the level of assay sensitivity. Overall, this approach may

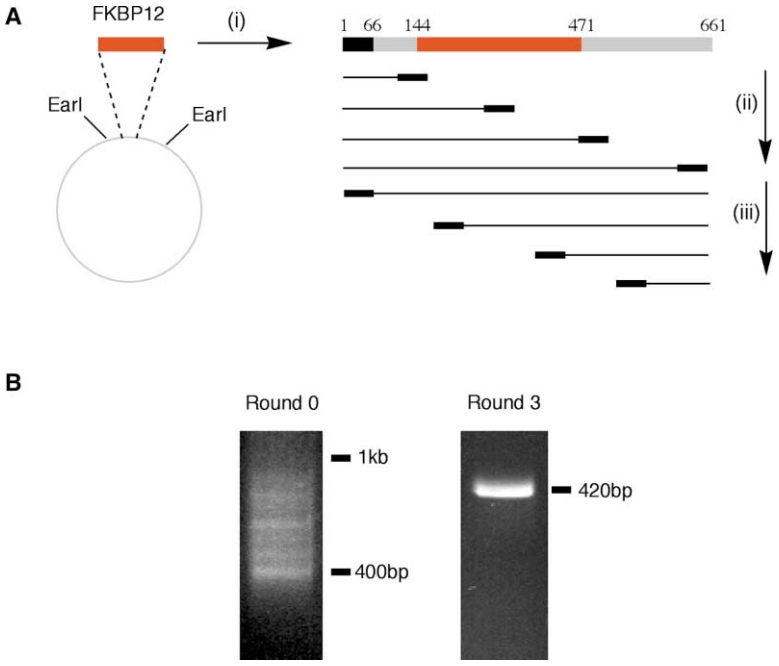

Figure 5. FKBP12 Domain Library Generation and Selection against FK506-Biotin

(A) Construction of a random-primed cDNA library from an FKBP12-containing template. (i) An FKBP12-containing plasmid was subjected to restriction digest with EarI to give a 1.1 kb segment containing the FKBP12 gene downstream from the T3 promoter. This template was used to synthesize a 661 nucleotide mRNA with T3 RNA polymerase. Vector, UTR, and FKBP12 sequences are depicted in black, gray, and red, respectively. (ii) First-strand cDNA synthesis was generated by reverse transcription of the FKBP12-containing message with a 9 random nucleotide primer encoding a C-terminal FLAG epitope and photoligation site. (iii) After RNase H treatment, random-primed second strand cDNA synthesis was completed with the Klenow fragment of *E. coli* DNA polymerase and an oligonucleotide containing fixed 5′ sequence (T7 promoter, translation initiation site, library-specific tag, start codon) in addition to nine random nucleotides. Unextended primer was removed by size-exclusion chromatography, and the cDNA product was PCR amplified to give a heterogeneous FKBP12 domain library that varied in size from <400 bp to 800 bp.

(B) Gel analysis of library cDNA before (Round 0) and after (Round 3) three rounds of in vitro selection with FK506

provide an alternative to current mapping methods involving proteolytic dissection of a protein of interest [31]. In this first example using mRNA display to map the minimal drug binding domain within a protein, the resulting map indicates that almost the entire protein sequence is necessary for functionality. Additional drug receptor identification and mapping studies using the techniques described here are currently in progress.

Conclusions and Future Work

We have demonstrated that mRNA display libraries provide a potentially valuable tool for probing drug-protein interactions. Drugs identified in high-throughput functional screens can be used as bait in selections with libraries constructed from multiple tissue sources. If the drug receptor is already known, then mRNA display technology can potentially be used to map drug binding to a particular domain within the target protein. Moreover, the identification of additional targets for a given drug will be extremely helpful in examples of unwanted side effects or detrimental biodistribution mediated through such targets. The robust conjugation of genotype to phenotype coupled with the flexibility of the in vitro methodology of mRNA display should allow the application of automated selection protocols. Parallel selections against an array of immobilized small mole-

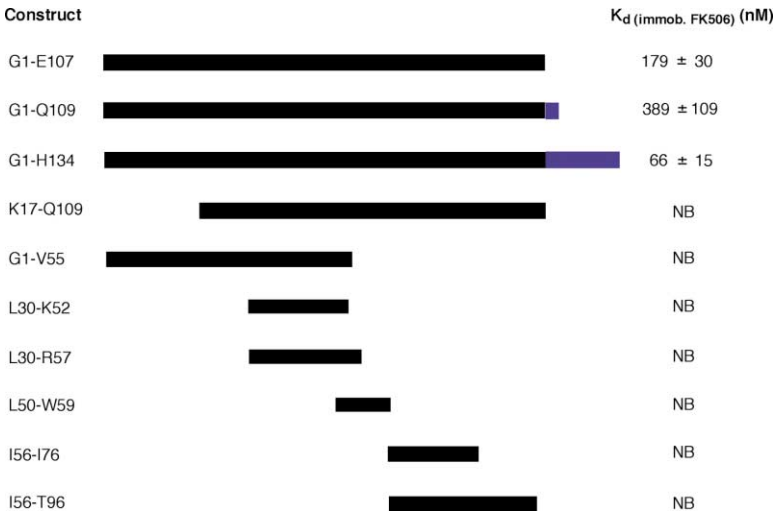

Figure 6. Schematic Alignment of Select FKBP12 Constructs Isolated from the FKBP12 Domain mRNA Display Library after Three Rounds of Selection with FK506

Each construct was expressed in vitro as a ^{35}S-labeled protein, and its FK506 binding activity was assessed in assays where the immobilized drug concentration was varied between 10 nM and 5 μM. Constructs are identified by their first and last residues. Each polypeptide sequence contained an initiator methionine residue followed by the FLAG epitope-containing sequence DYKDDDDKASA. NB denotes no binding was observed.

cules [32, 33] with densities that allow individual features to be eluted and amplified by PCR would help to simultaneously identify large numbers of small molecule-protein partners. This technology will expedite the identification of small molecule-protein interactions and advance our comprehension of disease at the proteomic level while accelerating the drug discovery process.

Significance

mRNA display is an emerging proteomic technology that allows a protein to be directly linked to its encoding nucleic acid. Construction of mRNA display libraries from cDNA allows a comprehensive repertoire of cellular proteins to be interrogated for biological interactions of medicinal importance. We have expanded the scope of mRNA display technology to include natural products as targets for in vitro selection. The successful affinity selection of an mRNA-protein construct of FKBP12, the cytosolic receptor of the immunosuppressant FK506, demonstrated that in vitro production of proteins from cellular libraries as nucleic acid-protein fusions allows for proper folding and functionality. As more compounds of pharmaceutical interest are explored, the potential for mRNA display to discover novel protein targets of therapeutic or toxicological relevance will be realized.

Experimental Procedures

Synthesis of FK506-Biotin

To a solution of FK506 (19.7 mg, 24 μmol), succinic anhydride (12 mg, 120 μmol), and 4-dimethylaminopyridine (DMAP, 5.9 mg, 48 μmol) in dichloromethane (DCM, 1 ml), we added triethylamine (TEA, 4 μl, 30 μmol), and the solution was stirred for 22 hr at room temperature. The mixture was diluted with dichloromethane (7 ml) and washed with aqueous 0.1 N HCl, distilled water, and saturated aqueous NaCl. The organic phase was separated, dried over anhydrous Na_2SO_4, filtered, and concentrated. The crude FK506 hemisuccinate was purified by preparative TLC (100% EtOAc) to give 3 mg (3.3 μmol) of a white solid. A mixture of FK506 hemisuccinate (3 mg, 3.3 μmol), N-hydroxysuccinmide (NHS, 0.7 mg, 6 μmol), and N,N′-dicyclohexylcarbodiimide (DCC, 4.4 mg, 21 μmol) in EtOAc (0.5 mL) was stirred for 20 hr at room temperature. The reaction mixture was centrifuged, and the organic supernatant was evaporated to give the crude FK506-hemisuccinic NHS ester, which was used without further purification. To a solution of the crude ester in DMF (0.5 ml) was added a solution of biotin-PEO-LC-NH_2 (5.4 mg, 13 μmol) in dry dimethylformamide (DMF) (0.3 ml). The mixture was stirred at room temperature for 0.5 hr. The solvent was evaporated, and the crude residue was purified by preparative TLC (10% MeOH/dichloromethane) to yield 0.8 mg (0.61 μmol) of FK506-biotin (**3**). MS (MALDI): *m/z* 1308 ($C_{66}H_{105}N_5O_{19}S + 3H^+$: 1307.63).

Affinity Selection of Cellular mRNA Display Libraries with FK506-Biotin

Streptavidin-coated magnetic beads (50 μl) (SA beads, Dynal) were washed twice with TBK buffer (50 mM Tris-HCl [pH 7.0], 150 mM KCl, 5 mM EDTA, 1 mM DTT, 0.5 mg ml^{-1} BSA, 0.1 mg ml^{-1} salmon sperm DNA, 0.05% Tween 20) (200 μl). The washed beads were suspended in 250 μl of TBK buffer, and 8.4 μl FK506-biotin (60 μM in 50% MeOH, final conc. 2 μM) was added and allowed to agitate gently at RT for 30 min. The beads were washed (2 × 200 μl) and resuspended in 250 μl of TBK buffer. We added 2.5 μl biotin (500 mM, final conc. 5 mM), and the beads were agitated at RT for 10 min. The FK506 beads were then washed with TBK buffer (2 × 200 μl) and stored in TBK buffer at 4°C until needed. Control beads for negative selection were treated similarly but without addition of FK506-biotin conjugate. Purified liver, kidney, and bone marrow mRNA display library (250 μl, ~0.3 pmol) [23] was incubated with the control beads (50 μl) at RT for 30 min. The beads were captured, and the supernatant was transferred to a 1.5 ml Eppendorf tube containing 50 μl of immobilized FK506 magnetic beads. The beads were mixed gently at RT for 30 min, captured, and washed with TBK buffer (5 × 200 μl). In the last two washes, the beads were allowed to agitate in TBK buffer for 5 min. The cDNA of bound mRNA display constructs was recovered by elution with 0.1 N KOH (2 × 100 μl). The basic eluates were neutralized with 2 μl 1 M Tris-HCl (pH 7) and 8 μl of 1 N HCl, and an aliquot was removed to determine optimal PCR amplification conditions. Scale-up PCR with mixed library-specific primers was performed on the remaining eluate using the optimized conditions to produce an enriched cDNA library. Subsequent rounds were then repeated as described above.

Cloning and Sequencing

PCR product from the final round of selection was subjected to 2% agarose gel electrophoresis. A strong band migrating around 400 bp was excised and purified by spin column (Qiagen). The purified cDNA was cloned into the TOPO-TA 2.1 vector (Invitrogen), and individual colonies were picked for direct amplification and sequencing.

Construction of an FKBP12-Derived mRNA Display Domain Library

A human FKBP12-containing plasmid was purified from I.M.A.G.E clone ID 591044 and digested with the restriction enzyme EarI. Run-off transcription of digested plasmid with T3 RNA polymerase gave a 661 nucleotide FKBP12-containing message that was purified by isopropanol precipitation. This material was used as a template for random-primed cDNA synthesis as described previously [23]. A unique library-specific tag, CTCATAAC, was introduced into the 5′-UTR during library construction. Library cDNA was amplified by PCR, and the corresponding mRNA display library was prepared as described above.

Affinity Selection of a FKBP12-Derived mRNA Display Domain Library with FK506-Biotin

The affinity selection was performed essentially as described for the cellular selection. PCR product after three rounds of selection was directly cloned into TOPO-TA 2.1 vector, and plasmids were purified (Qiagen) and sequenced.

In Vitro Translation of Selected Clones

RNA transcripts were produced from PCR templates as described. Radiolabeled peptides were purified directly from translation reactions in rabbit reticulocyte lysate (Ambion) by anti-FLAG M2 immunoprecipitation, and their purity was determined by SDS-PAGE/autoradiography.

FK506 Binding Assays

Neutravidin agarose (Pierce) was aliquoted into microcentrifuge spin columns (10 × 50 μl) and washed with TBK buffer (1 ml). The columns were capped and the beads were suspended in 250 μl of varying concentrations of FK506-biotin in TBK buffer (0, 10 nM, 50 nM, 100 nM, 200 nM, 500 nM, 1 μM, 2 μM, 3 μM, and 5 μM) and agitated at RT for 20 min. The beads were washed with TBK buffer (2 × 0.5 ml, 1000 rpm, 10 s), and ~0.1 pmol of selected [^{35}S]protein in 250 μl of TBK buffer was added to each drug matrix. The FK506-beads/protein mixture was gently agitated at RT for 30 min, and unbound material was removed by centrifugation (1000 rpm, 10 s). The beads were washed four times with 0.5 ml of TBK buffer. Inverted columns were placed in scintillation vials and centrifuged (4000 rpm, 3 min) to maximize recovery of radiolabeled beads, and bound protein was quantified by scintillation counting.

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