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Simultaneous identification of multiple receptors of natural product using an optimized cDNA phage display cloning

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ABSTRACT

Simultaneously isolating more than one receptor of natural product remains a challenge to chemical genetics. Using cyclosporine A as an affinity probe and an optimized phage display cloning procedure, not only cyclophilin A, but also cyclophilin B was isolated as the full-length gene clone from a human brain cDNA library. This optimized protocol can be used to select protein targets of chemicals dependent on the binding affinity rather than on the relative abundance in cells.

Display cloning, developed by Austin's group using natural product FK506 as probe,[1] has been successfully employed to identify the specific receptors of bioactive chemicals exemplified by doxorubicin,[2] curcumin derivative HBC,[3] FK506 mimetic AP1497,[4] benzodiazepine Bz-423,[5] taxol,[6] and insulin mimic demethylasterriquinone B1.[7] In these screens, the biotinylated chemicals are usually applied to be immobilized on an avidin/streptavidin-coated plate or other solid-phase resins. After each round of selection, the bound phages were eluted by the buffer with biotin or sodium dodecylsulfate. Additionally, more than 10^9 plaque forming units (pfu) of phage pool are needed in order to detect potential target-binding phage when evaluating a screen. However, there are still some particular problems in some cases: (1) the non-specific phages produce false positives, which often make the screening data ambiguous; (2) it is difficult to isolate more than one target homologue from a cDNA library, especially when there is a disparity in the relative abundance and affinity among family members; (3) identification of a low-copy target protein often hindered by the large amount of homologues. In this study, using an optimized phage display cloning procedure and natural product cyclosporine A (CsA) as probe, we simultaneously isolated two receptors, cyclophilin A (CypA) and cyclophilin B (CypB), from a human brain cDNA library dependent on the binding affinity rather than on the relative abundance in cells.

The immunosuppressive drug CsA, binding to a cytosolic protein CypA with high affinity,[8,9] was selected as a natural product probe to establish the display cloning method in our laboratory. We synthesized biotinylated CsA (Supplementary Fig. S1), and confirmed it remains the capacity of binding human CypA with K_d of 0.5 μM.[10] Beginning with the immobilization of biotinylated CsA onto a monomeric-avidin agarose gel, then blocking with 1% BSA, a 100-μL aliquot of human brain T7 cDNA phage library (1.7×10^9 pfu) was used to evaluate a screening according to the described methods.[1] Analysis of individual clones from the sixth round showed that less than 10% randomly selected clones were identified as human CypA gene by PCR and DNA sequencing.

To reduce the non-specific binding events, several optimized steps were taken into account. First, immobilizing chemicals onto a solid surface with the avidin/streptavidin–biotin interaction could be replaced directly by covalent affinity matrix, which has been widely used in traditional protein-affinity chromatography. Second, the blocking step and pre-incubation with an untreated plate or resin could greatly reduce the amount of background binding.[4] Third, omitting the elution step, the natural product bound phages captured on the resin were directly used to infect *Escherichia coli* without prior release from the resin beads. This modification has been proved effectively enough to clone RNA-binding proteins from T7 cDNA phage libraries.[11] So the optimized cDNA phage display cloning procedure is shown schematically in Figure 1. The chemicals were immobilized onto the resin to directly form covalent affinity matrix, at the same time, a pre-column with similar blocked affinity matrix was prepared. At the beginning of each round of selection, the phage displayed libraries were precleared by passing through the pre-column to reduce non-specific binding events. Then after binding and washing to remove unbound phages, the remaining phages captured on the resin were directly used to infect *E. coli*. After replication, the phages can either be

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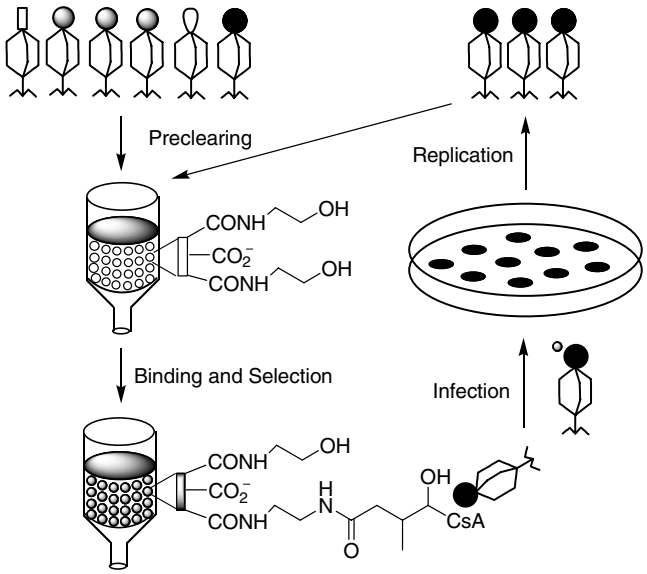
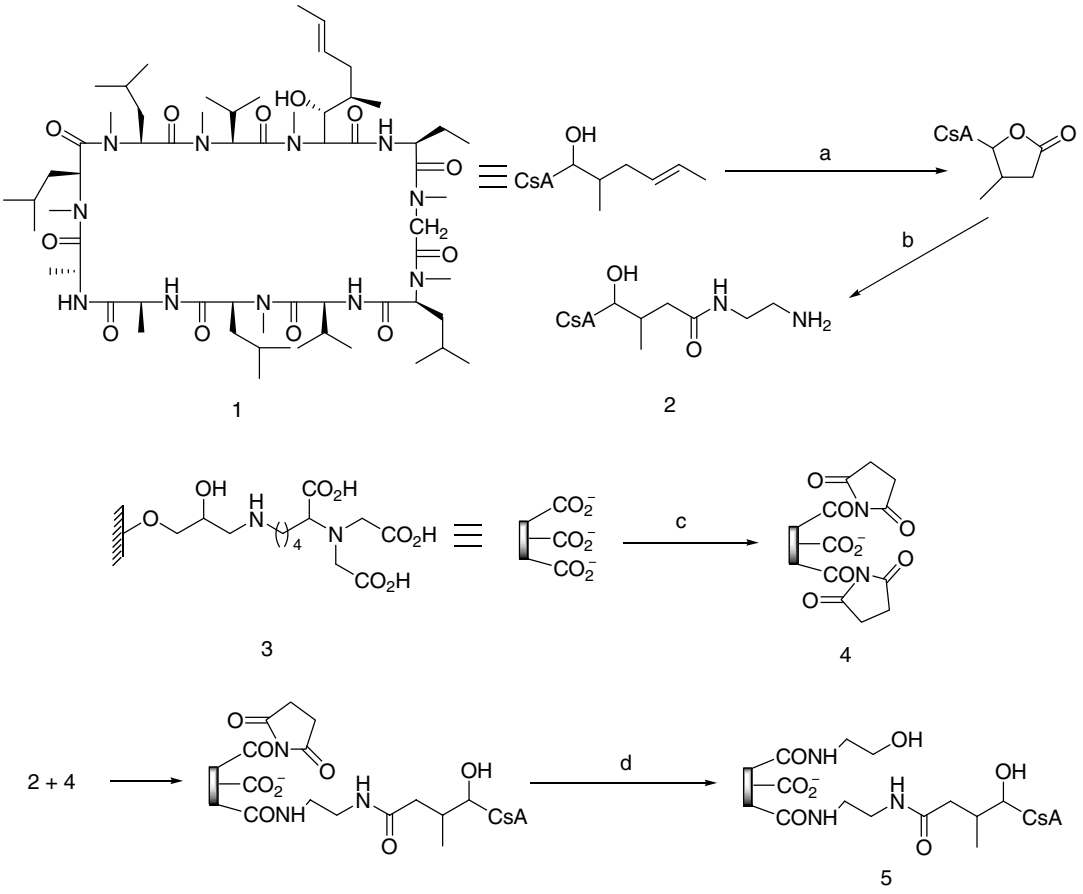


Figure 1. In vitro selection of receptors of natural product using an optimized display cloning. At the beginning of selection, a pre-column was introduced to reduce non-specific binding events. After binding and washing, the bound phages captured on the resin were used to infect *E. coli* without prior release from the resin beads. Then after replication, the phages can either be analyzed by DNA sequencing or subjected to next round(s) of selection.

analyzed by DNA sequencing or subjected to additional round(s) of selection.

The CsA derivative-containing affinity matrix **5** was designed and synthesized according to the procedure outlined in Scheme 1. A CsA lactone was prepared by the oxidative cleavage of the double bond of 2-*N*-methyl-(*R*)-((*E*)-2-butenyl)-4-methyl-L-threonine residue of CsA (**1**) using $NaIO_4$ and $KMnO_4$.[10] Then the lactone was refluxed with excess 1,2-ethylenediamine in tetrahydrofuran (THF) to give the reactive amino-functionalized CsA derivative **2**, which was characterized by proton NMR and electrospray mass spectrometry. The activation of the carboxyl groups on the matrix nitrilotriacetic acid agarose (**3**) is performed by adding a mixture of succinimide (NHS) and carbodiimide (EDC·HCl) to form active esters **4**, which react with amino groups of **2** to immobilize CsA onto the surface. After coupling, excess ethanolamine was added to deactivate remaining active esters to provide the CsA containing affinity matrix **5**. Similarly, the affinity matrix of pre-column was prepared by simply coupling agarose **4** with excess ethanolamine. A 10-μL aliquot of human brain T7 cDNA phage library (1.7×10^8 pfu) was amplified and incubated with preclearing affinity matrix then allowed to pass through the pre-column to further remove any resin and background binding members in the library before it was subjected to the first round of selection. After incubation for binding, washing to remove the non-specific binding, the remaining phages bound on the resin were captured and amplified by addition of a log-phase culture of *E. coli* BLT5615. The resulting phages lysate was subjected to preclearing step and then used in the next selection round, and this cycle was repeated for a total of six rounds.

The specific binding phages increased obviously with the round of selection indicating the potential targets enrichment. The phage titer enriched more than 1400 times from 7.0×10^5 pfu/mL after



Scheme 1. Synthesis of the cyclosporine A derivative affinity matrix **5**. Reagents and conditions: (a) $NaIO_4$, $KMnO_4$, K_2CO_3, H_2O, *tert*-BuOH, rt, 2× 30 h; (b) $NH_2CH_2CH_2NH_2$, THF, reflux, 30 h, 69% (two steps); (c) NHS, EDC·HCl; (d) 1 M $NH_2CH_2CH_2OH$·HCl, pH 8.0, 10 min.

the first-round to 9.9×10^8 pfu/mL at the sixth-round, especially from the third- to the sixth-round with 21, 6, 3, and 2 times increase, respectively. To confirm the enrichment of specific phage clones, cDNAs inserted into phages of every round were analyzed by PCR with primers flanking the library construction site of the T7 phage DNA. The PCR products from first two rounds appeared as smear bands; however, enrichment was obviously found with bands of 750 bp from the third- to the sixth-round of amplifications (Fig. 2A). These results indicated that CsA-specific binding phages were selectively amplified by this screening procedure.

In order to further evaluate the selection results, specific PCR primers were also designed that recognized the CypA gene. After six successive rounds of selection and amplification, 60 phage clones were randomly picked up as template and the PCR analyses were carried out with primers annealing to the vector sequence and CypA gene, respectively. The agarose gel electrophoresis analysis of PCR products showed that 80% (48 out of 60) clones are found with single bands of 750 bp, three clones appeared in the single bands of 700 bp, and 2 clones appeared in the bands of both 700 and 750 bp. However, to our surprise, only the 5 clones with 700 bp bands responded to the CypA specific primers (Supplementary Fig. S3A). DNA sequences were determined for five clones randomly selected from the 48 phages with single bands of 750 bp, which are identical to the gene of another cyclophilin member, CypB (Supplementary Fig. S2). Then specific PCR primers were designed according to the CypB gene, and PCR analysis of the 60 clones clearly confirmed that all the 50 clones with bands of 750 bp contained full length of CypB gene (Supplementary Fig. S3A).

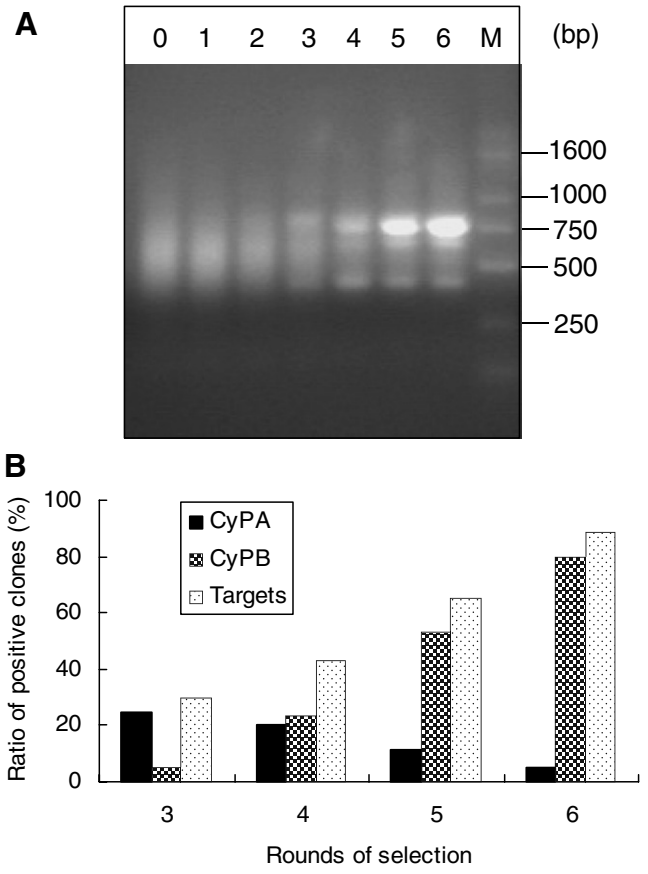


Figure 2. Analysis of the phages after each round of selection. (A) PCR analysis of amplified phages after each selection round with primers flanking the insertion sites. The PCR products from the first-, second-, third-, fourth-, fifth-, and sixth-round of selections were loaded in lanes 1, 2, 3, 4, 5, and 6, respectively, along with prior to selection (lane 0) and DNA marker (lane M). (B) The ratio of positive clones were calculated according to the PCR analysis of randomly-selected sixty clones after each selection round with primers specific for the cyclophilin A and cyclophilin B gene sequence, respectively.

In order to follow and evaluate the progress of the selection, 60 phage clones were randomly selected from the third- to the fifth-round of amplifications as template and the PCR analysis were carried out with primers annealing to the vector sequence, CypA and CypB gene specific, respectively (Supplementary Fig.S3B, C, and D). All the analysis results were summarized in Figure 2B. The selected clones containing the target proteins with gene from the third-, fourth-, fifth-, and sixth-round of amplifications enriched obviously from 30% (CypA/CypB of 5/1), 43% (CypA/CypB of 1/1), 65% (CypA/CypB of 1/5) to 90% (CypA/CypB of 1/10). Compared to CypA, human CypB shows higher affinity to CsA with K_d of 9.8 nM (CypA with K_d of 36.8 nM)[12] but relatively lower abundance in human brain.[13] So in the early selection rounds, the higher concentration of CypA conferred it as the predominantly binding target; however, the affinity played more and more important roles with the progress of selection, which resulted in the entire library of clones converging to one dominant member, CypB, in later rounds, which has higher affinity though relatively lower abundance.

Simultaneous identification of more than one receptor of natural product could provide useful information to chemical genetic research. Using a modified selection protocol, Austin's group reported the only example to simultaneously clone three homologues of the FK506-binding protein by a FK506 mimetic AP1497.[4] The CypA protein was first characterized by affinity chromatography,[8] while CypB, a second human CsA-binding protein with higher affinity, was identified by probing human cDNA libraries with CypA cDNA under reduced stringencies.[13,14] Further Northern blot analysis showed that the distribution of mRNA levels of CypA in different human tissues is always 5–10 and 50–100 times more abundant than CypB mRNA level, especially in human brain, less than 1% CypB mRNA could be detected compared to CypA.[13] It is coincident with the abundance of CypA and CypB in the human brain T7 phage library by PCR analysis (Supplementary Fig. S4). So the simultaneous identification of CypA and CypB as the full-length gene from a human brain cDNA library is really unexpected. In principle, display cloning, which benefits from consecutive rounds of selection based on affinity chromatography and phage infective ability, could isolate target protein and its gene together from cDNA library even at trace level. However, in practice, the non-specific binding interactions, a disparity in the relative abundance and affinity among family members, and the growth and infectivity advantages can baffle the progress to the dominant sequences. Recently, a cuvette type quartz crystal microbalance (QCM) device was combined with the T7 phage display system to reduce the background binding.[15] Similarly, our optimization of the selection protocol will be helpful to remove the non-specific binding, which will see the entire cDNA library of clones converging to one predominant sequence with the progress of selections, representing the highest affinity binders in the library. Following analysis of the early rounds when enrichment appeared will not only evaluate the progress of the selection, but also identify the target homologues with relatively low binding affinity.

In conclusion, the optimized phage display cloning protocol can be used to simultaneously isolate more than one protein targets of natural product from cDNA library dependent on the binding affinity rather than the relative abundance in cells. With the development of chemical genomics and phenotype-based screening, more and more active chemicals are accompanied by the challenge of identifying the molecular targets. It should be noticed that this methodology is being generally used in chemical biology studies.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.06.006.

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