Molecular Targeting

Photo-Cross-Linked Small-Molecule Affinity Matrix for Facilitating Forward and Reverse Chemical Genetics**

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Affinity purification of small-molecule binding protein has been one of the most important techniques in forward chemical genetics (Figure 1a).[1,2] Once hit compound(s) which induce a specific phenotype in a cellular system are identified, the next step is to identify the molecular target. Thus, derivatization of the small molecules and structure–activity relationship studies are conducted to prepare tagged probes (for example, biotin conjugates) that can be used in a pull-down assay. Many targets of biologically important drugs have been discovered or identified by using this technique.[3–6] However, it remains challenging and time-consuming to derivatize small molecules without a loss in biological activity, and an easy-to-use, universal approach would be of great benefit in this context.

Affinity purification can also be used in reverse chemical genetics[7] for other purposes (Figure 1b). In vitro binding assays including small-molecule microarrays have been used to identify small-molecule ligands for proteins of interest. Although these techniques have led to the successful identification of ligand candidates, they do not provide information about the specificity of the ligand–protein interaction. If the ligand could be immobilized on an affinity matrix in a manner that was independent of the functional groups present, pull-down experiments and competitive binding assays using this matrix could be used to confirm whether or not an interaction is specific. We have looked for a rapid and general approach for the immobilization of small molecules on an affinity matrix to enhance the forward and reverse chemical genetics approaches described above.

Recently, we reported a coupling method that enables the introduction of a variety of small molecules onto glass slides through a photoaffinity reaction.[8] By using this method, aryl

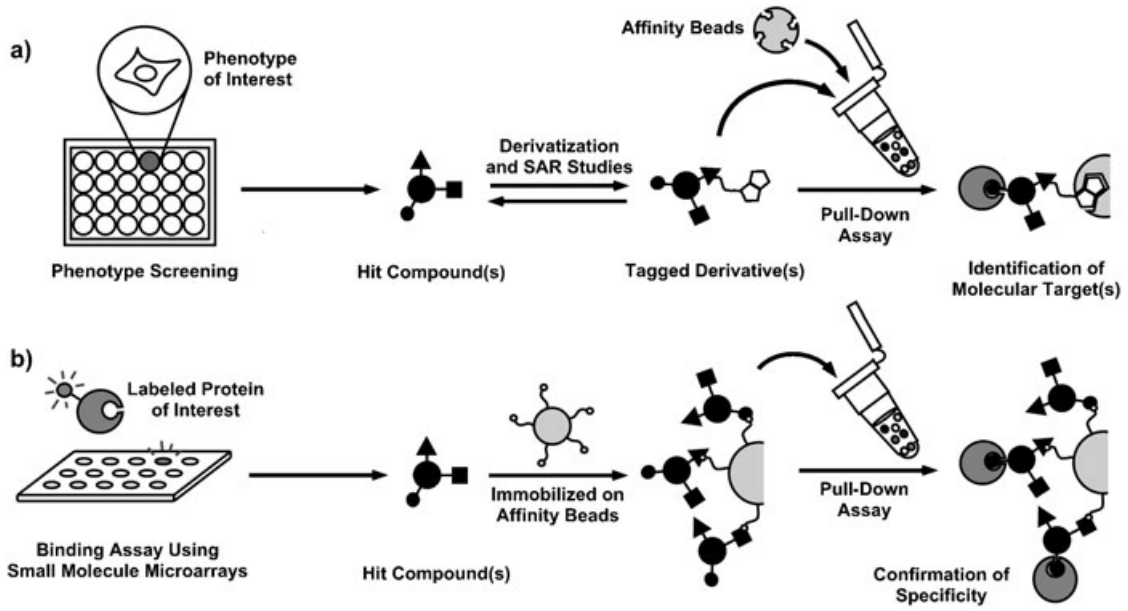

Figure 1. Affinity purification of small-molecule binding proteins in forward and reverse chemical genetics studies.

diazirine groups covalently attached to glass slides are transformed upon irradiation with UV light into highly reactive carbenes, which in turn bind to or insert irreversibly into a proximal small molecule in a manner that is independent of the functional groups.[9] This method, referred to as photo-cross-linking, has proven useful in the construction of small-molecule microarrays.[10] It is expected that a similar chemical approach could be used to introduce small molecules onto affinity gel, such as onto agarose beads. To test this hypothesis a photoaffinity linker **1** was introduced on agarose beads to prepare photoaffinity-linker-coated (PALC) agarose beads **3**, and the photo-cross-linking of complex small molecules onto **3** was demonstrated.

The preparation of **3** and the immobilization of small molecules on **3** were performed in a straightforward manner

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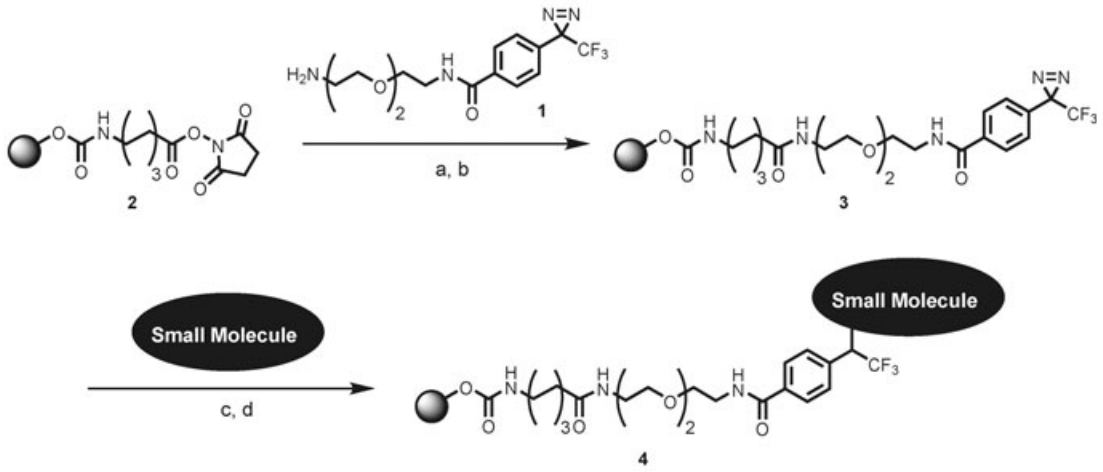

Scheme 1. Preparation of PALC agarose beads and photo-cross-linking of a small molecule. Reagents and conditions: a) **1**, $NaHCO_3$, NaCl, H_2O/dioxane (1:1), RT, 2 h; b) 1.0 M aq ethanolamine, RT, 1 h; c) 10 mM methanolic solution of small molecules, drying; d) irradiation at 365 nm, 4 J cm^{-2}; washing with organic solvent and water.

(Scheme 1). Activated CH sepharose 4B beads (Amersham Bioscience, Uppsala, Sweden; **2**) were first coupled with the photoaffinity linker **1**, and the beads were then blocked with 1.0 M aqueous ethanolamine, by using a solid-state coupling approach, to give **3**. Introduction of the photoaffinity linker **1** on the beads **2** was confirmed by high-resolution solid-state ^{19}F NMR measurements of **3** ($\delta = -65.9$ ppm, ArCF_3). To immobilize structurally diverse small molecules on **3** a methanolic solution of a small molecule was premixed with **3**, and then the mixture was concentrated and dried in vacuo. The dried beads were exposed to UV light (365 nm, 4 J cm^{-2}) from a UV cross-linker, and then washed thoroughly with methanol and water.

Figure 2 shows that the immobilization of the fluorescent rhodamine B depended on the photoaffinity linker **1** and irradiation with UV light: when agarose beads (sepharose CL-6B, Amersham Bioscience, Uppsala, Sweden) were irradiated in the presence of rhodamine B and then washed, no fluorescence was detected by fluorescence microscopy (Figure 2b). Fluorescence was observed only on PALC beads which had been irradiated in the presence of rhodamine B and then washed (Figure 2d). No fluorescence was observed on the beads in the absence of UV irradiation (Figure 2f). These results suggest that the photo-cross-linking strategy could be applied not only in studies using glass slides but also in assays with agarose beads.

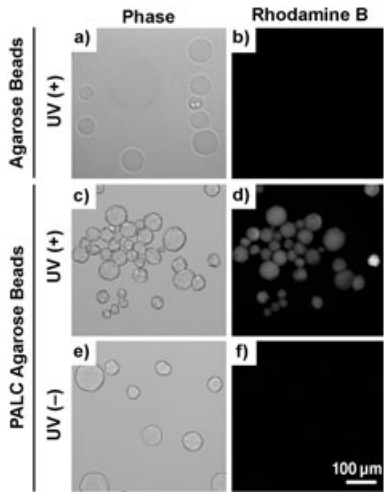

Figure 2. Observation of beads with immobilized rhodamine B by microscopy. Agarose beads and PALC agarose beads were mixed with rhodamine B and dried in vacuo. After incubation of the beads with or without irradiation, they were washed with methanol and water, and were observed under a microscope in the phase-contrast (a, c, e) or fluorescence mode (b, d, f).

To validate the binding ability of small molecules that had been photo-cross-linked on affinity beads with their target proteins we used the immunosuppressive drugs cyclosporin A (CsA) and FK506 (FK) as model ligands. CsA and FK are known to bind to the cellular targets cyclophilin A[11] and FKBP12,[3] respectively. In the specific case of CsA, photo-cross-linking with 4-benzoylbenzoic acid in solution[12] and a site-selective free-radical reaction on an appropriately protected CsA derivative[13] have been reported. Both molecules were photo-cross-linked on **3**, as shown in Scheme 1. The resulting CsA beads were incubated with the cell lysates of an *E. coli* BL21(DE3)pLysS strain over-expressing GST-cyclophilin A (GST-CypA), and the FK beads were incubated with the same *E. coli* strain, but which over-expressed $(His)_6$-FKBP12 (Figure 3a). First, we were able to identify a protein band of approximately 40 kDa that co-precipitated with the CsA beads (Lane 1). To determine the specificity of this interaction we examined whether or not CsA could compete for the binding of the CsA beads to the 40-kDa protein. The binding of CsA beads to the 40-kDa protein was found to be completely blocked by the addition of 10 and 100 µM CsA (Lanes 2 and 3), although no change in binding was observed in a control experiment using DMSO (Lane 4). The 40-kDa protein was reactive with an *anti*-GST antibody, thus indicating that the protein was GST-CypA (Figure 3b, left panel).

We were also able to identify a band of approximately 16 kDa (Figure 3a, Lane 6) that was reactive with an *anti*-$(His)_6$ antibody (Figure 3b, right panel); binding between the FK beads and the 16 kDa protein was blocked by a dose-

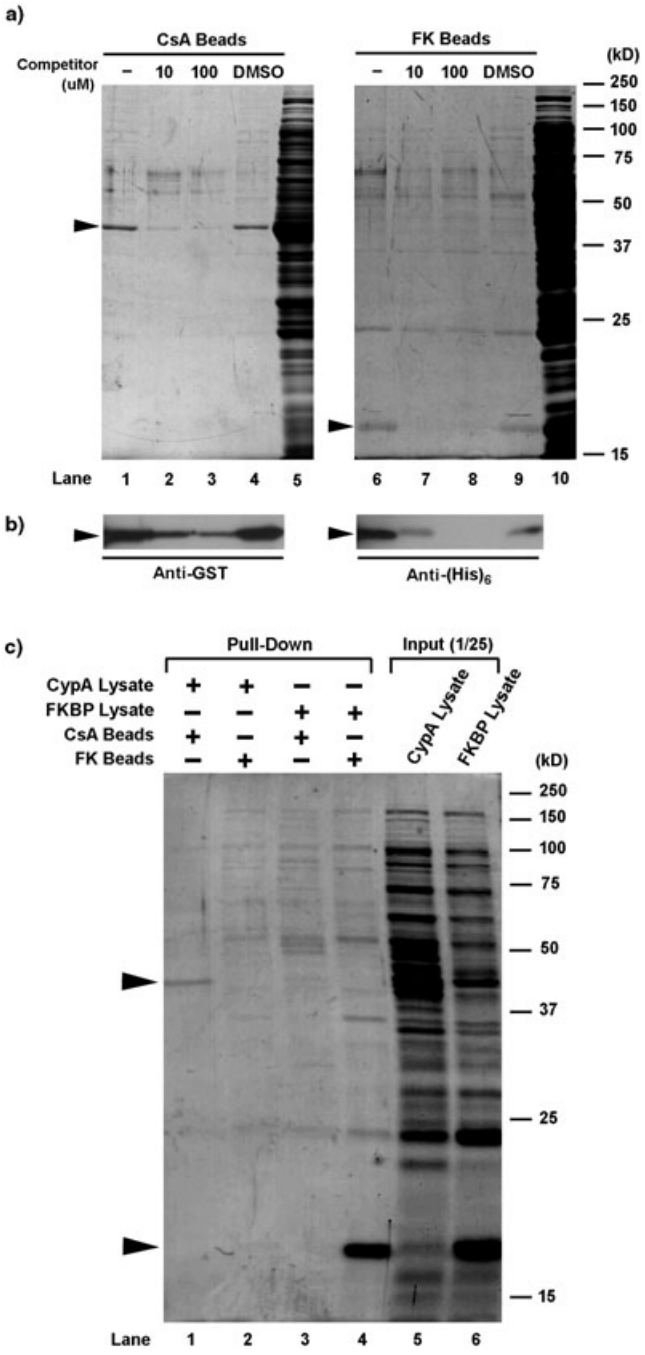

Figure 3. Detection of binding proteins for small-molecule-immobilized beads. Cell lysates of *E. coli* over-expressing GST-CypA or $(His)_6$-FKBP12 (namely, CypA lysate or FK lysate) were incubated with or without competitor. CsA beads or FK beads were then added to absorb the binding proteins. After the beads had been washed with the incubation buffer they were resuspended in SDS sample buffer, heated, and subjected to SDS-PAGE. The gels were visualized by CBB staining. a) Competition experiments. Lanes 1–4: CypA lysate plus CsA beads with CsA as a competitor; Lane 5: CypA lysate (loading control); Lanes 6–9: FK lysate plus FK beads with FK as a competitor; Lane 10: FK lysate (loading control). b) Western blot analysis. Protein bound specifically to the CsA beads was reactive to *anti*-GST antibody (left panel). Protein bound specifically to the FK beads was reactive with an *anti*-(His)6 antibody (right panel). c) Cross-experiments. Lane 1: CypA lysate plus CsA beads; Lane 2: CypA lysate plus FK beads; Lane 3: FK lysate plus CsA beads; Lane 4: FK lysate plus FK beads; Lane 5: CypA lysate (loading control); Lane 6: FK lysate (loading control).

dependent competition by the addition of FK506 (Figure 3a, Lanes 7 and 8). These results demonstrated that the protein was $(His)_6$-FKBP12 and also that the binding was specific.

The specificity of the interactions between a photo-cross-linked drug and immunophilin was further confirmed by cross-experiments (Figure 3c). Briefly, GST-CypA and $(His)_6$-FKBP12 did not co-precipitate with the FK and CsA beads, respectively (Lanes 2 and 3).

The next issue to be addressed was whether the photo-cross-linked affinity beads would be able to both purify the over-expressed proteins and also identify the cellular target(s) of the immobilized ligand. To address this question we incubated the CsA beads with Jurkat cell lysate, and the co-precipitated proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Figure 4a). This procedure led to the detection of a protein band of approximately 20 kDa by Coumassie Brilliant Blue (CBB)

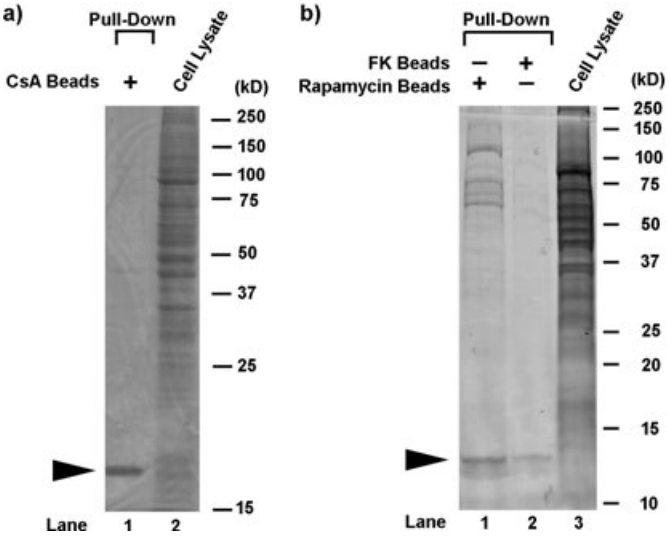

Figure 4. Detection of CsA-, FK-, and rapamycin-binding protein from the Jurkat cell lysate. The Jurkat cell lysate was first incubated with beads on which each of the small molecules were immobilized (CsA for Lane 1 of (a); rapamycin for Lane 1 of (b); FK for Lane 2 of (b)). The beads were then washed with lysis buffer containing 0.3% triton X-100, resuspended in SDS sample buffer, heated, and subjected to SDS-PAGE. The gels were visualized with CBB staining. The arrowheads indicate the bands which disappeared on addition of the respective small molecules (10 μM).

staining (Lane 1). To identify the protein the protein-containing region was excised and digested on SDS-polyacrylamide gel, and the resulting peptides were extracted and analyzed by MALDI-TOF MS (Supporting Information). The protein was identified from peptide mass fingerprinting (PMF) using the Mascot search program (Matrix science Inc., USA) and the Swiss-Prot database as human cyclophilin A with a protein score of 125—a score of greater than 52 is considered significant ($p < 0.05$). Besides being successful in the case of CsA beads, we also succeeded in purifying a human FKBP12 from Jurkat cell lysate by using both the FK beads and beads on which rapamycin was photo-cross-linked (Figure 4b).[14]

In summary, we have prepared PALC beads and demonstrated that photo-cross-linked small-molecule agarose beads can be used to identify and purify the binding protein. It was of note that complex small molecules such as FK506, rapamycin, and cyclosporin A could be introduced on the affinity matrix by using a uniform and straightforward procedure. Although the present procedure can not be applied to small molecules that degrade upon UV irradiation, technologies aimed at immobilizing ligands on an affinity matrix in a functional-group-independent manner are expected to facilitate the identification of molecular targets of small molecules in forward chemical genetics, and is also considered potentially useful for the confirmation of specificity between small molecules and proteins of interest in the field of reverse chemical genetics.

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