A versatile method of identifying specific binding proteins on affinity resins

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Abstract

The isolation of both specific and nonspecific binding proteins on affinity matrices bearing bioactive compounds hinders the identification of drug cellular targets. Although solid-phase elution and competition methods conventionally are used to distinguish between specific and nonspecific receptor–ligand interactions, these approaches often are severely restricted by low ligand solubility and/or slow kinetic dissociation. This article describes an alternative and versatile method, termed *serial affinity chromatography*, to identify ligand receptors using affinity resins.
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The identification of target proteins for drugs, natural products, and toxins is an essential component of modern pharmaceutical research because it facilitates the development of novel drugs with greater selectivity and/or potency through the use of effective screening systems and structure-based design[1] Therefore, it is important to identify proteins that specifically bind such bioactive compounds. Although affinity chromatography matrices, bearing bioactive compounds, are a major tool toward achieving this goal [1,2], it often is difficult to identify specific binding proteins on affinity resins owing to a preponderance of nonspecific binding proteins [3]. If an "inactive derivative," with a similar structure but without bioactivity, is known, binding proteins on affinity resins, bearing either active or inactive derivative compounds, can be compared [4]. However, this approach is not commonly used because usually a great deal of effort is required to obtain inactive compounds.

Currently, there are two practical methods for identifying specific binding proteins that lack inactive derivatives. In solid-phase elution, a binding protein can be identified as a specifically eluted protein by the addition of aqueous solutions of the active ligand. However, this method often suffers from the slow dissociation kinetics of the specific binding protein and its ligand on the affinity resin (Supplementary Fig. 1 [Fig. S1]). Thus, the competition method, in which the active compound is added to a mixture (e.g., lysate) to disturb the specific interaction with the affinity resin competitively, is now widely used. However, the low solubility of the active compound in aqueous solution, which usually includes fairly high concentrations of several salts and other reagents for stabilizing proteins, limits the use of this method. For example, the addition of 1 μmol/ml or more of a synthetic drug such as dexamethasone, a well-known steroid hormone, or valdecoxib, a well-known COX-2 inhibitor, into a lysate from rat brain resulted in the appearance of insoluble material (Figs. S2 and S3). Nonetheless, high concentrations of the test solutions often are necessary in the competition method because the bait compounds usually are immobilized on affinity resins in micromolar amounts. Difficulties arising from the low solubilities
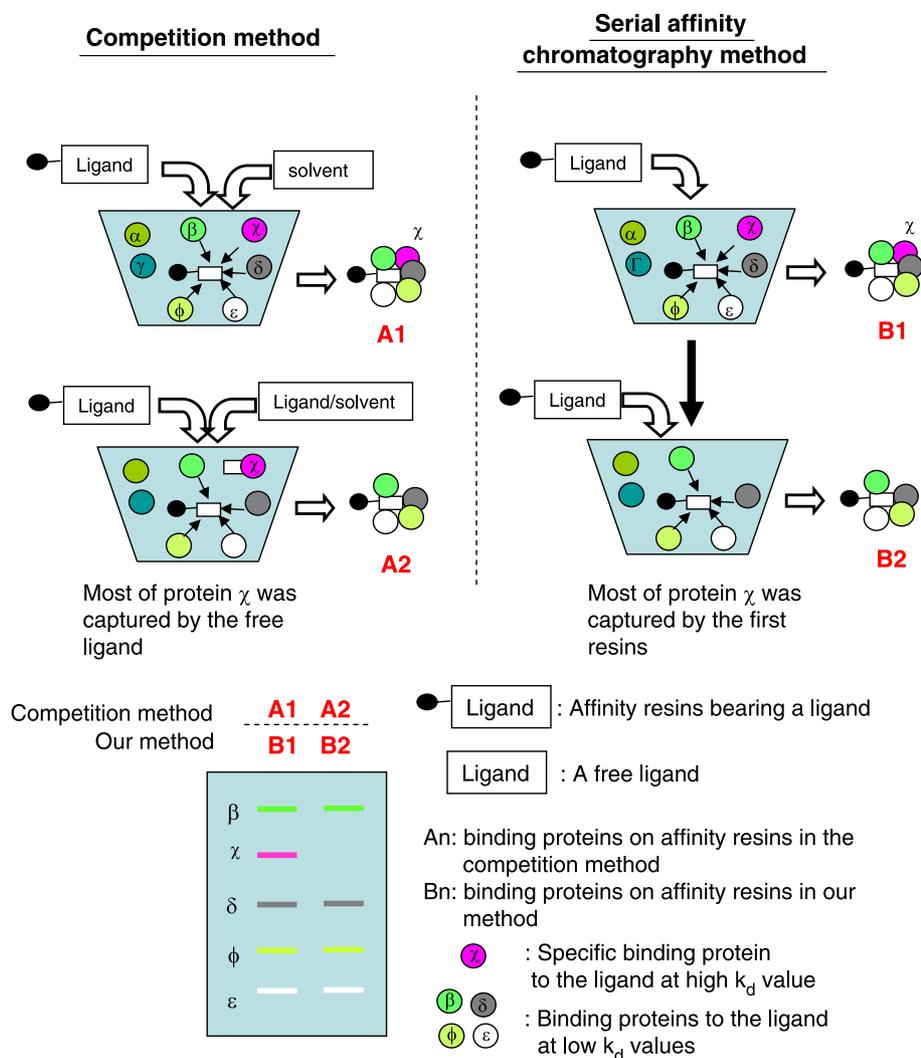

Fig. 1. A schematic comparison of serial affinity chromatography (right) with the competition method (left). In the competition method, the addition of a bioactive compound or its bioactive derivative disturbs a specific interaction. A comparison of sample without competitor (A1) and with competitor (A2) showed that the amount of a specific binding protein, χ, is greatly decreased in the latter. The competitor usually is added with solvent to accelerate its dissolution (A2). Thus, the same solvent was added to A1 without competitor to offset solvent effects. In serial affinity chromatography, affinity resins are mixed with lysate, the resins are removed, and the same amount of fresh affinity resin is added to the remaining lysate. Most of the specific binding protein χ should be captured by the first resin owing to its high affinity for the ligand. This, in turn, causes a large decrease in the amount of protein χ in the remaining lysate. Therefore, the amount of protein χ on the second affinity resin (B2) should be much less than the amounts of other proteins present in the lysate. In serial affinity chromatography, it is not necessary to add other reagents, such as solvent, into the mixture, and this should minimize artifacts.

of competitors are not uncommon because the hydrophobic properties of these compounds often are vital for their bioactivity and/or membrane permeability. If the insoluble material has little effect on the nature of the binding proteins, this is not a problem. However, such material often denatures and coprecipitates proteins. As seen in Figs. S2 and S3, the disappearance of a binding protein on affinity resins in the competition method, using insoluble material, does not always imply that it has specific binding activity; rather, this may indicate its susceptibility to being trapped in the insoluble material. Therefore, successful identification of target protein, using currently available methods, often depends on the design and synthesis of hydrophilic derivatives that maintain bioactivity. Alternatively, methods that avoid such limitations are needed.

We have developed a simple method to identify specific binding proteins on affinity matrices. A mixture of proteins, such as a lysate, is sequentially treated at least twice with affinity resins bearing bioactive ligand, and the binding proteins on each resin are comparatively analyzed, as illustrated in Fig. 1 (right half). The binding of specific binding protein χ is greatly reduced on the second resin (B2) compared with the first resin (B1), similar to the result obtained by the competition method (A1 vs. A2). Our method, termed *serial affinity chromatography*, is based on the fact that, theoretically, most of the specific binding protein is captured by the first resin due to its high affinity for the ligand, and little remains in the lysate. In contrast, the amounts of nonspecific binding proteins on the two resins should be similar because most of them remain in the lysate

due to their low affinity for the resin. This simple method is also versatile because only an iterative mixing of the components is required in the procedure, whereas experimental prerequisites, such as the high solubility of the ligands, are of little concern. It should be noted that if the lysate includes a stoichiometric or greater amount of specific binding protein compared with ligand immobilized on the affinity resin, the technology may fail. However, this kind of situation is rare because most putative target proteins are present in amounts that are much lower than the micromolar amounts of their respective ligands on affinity chromatography matrices [3]. Although some abundant proteins, such as tubulin, actin, and albumin, are present in large amounts in the lysate, they usually are not the desired target proteins. The novel method could afford confusing results when the ligand immobilized on the affinity resin has low affinity for the specific binding protein. In this case, the amount of specific binding protein on B2 could be similar to that on B1, making identification difficult because most of the specific binding protein would not be captured by the first resin and thus would remain in the lysate. In this case, synthesis of alternative affinity resins would be necessary, not only for our method but also for use in conventional methods, because it is difficult to adequately capture target proteins using such loss-of-function affinity resins.

Materials and methods

Preparation of lysate from rat brain

Rat brain (1.8 g, cat. no. J201, Funakoshi, Tokyo, Japan) was homogenized in lysate buffer A (18 ml, 0.25 M sucrose, 300 μM diethyldithiocarbamate [DDC],[2] and 25 mM Tris–HCl, pH 7.4). The homogenate was centrifuged at 9500 rpm for 10 min. The supernatant was removed, centrifuged at 50,000 rpm for 30 min, and diluted in lysate buffer to a total protein concentration of approximately 7 mg/ml. The resulting lysate was kept at −80 °C until use.

Preparation of THP-1 lysate

THP-1 cells were grown at 37 °C in RPMI 1640 medium, containing 10% fetal bovine serum (FBS), penicillin (100 μg/ml), and streptomycin (100 U/ml), until nearly confluent. The cells ($\sim 1 \times 10^9$) were washed once with phosphate-buffered saline (PBS), collected by centrifugation at 1000 rpm for 3 min, and resuspended in ice-cooled lysate

buffer B (10% [v/w], 0.25 M sucrose, 300 μM DDC, 0.1% Tween 20, 25 mM Tris–HCl, pH 7.5). The cell suspension was sonicated, and cellular debris was removed by centrifugation at 3000 rpm for 10 min. The resulting supernatant formed the lysate used in binding experiments.

Preparation of aqueous solutions of purified proteins

Human FKBP12 were cloned and expressed in *Escherichia coli* BL21(DE3) strain with pDEST17 plasmid (Invitrogen). The broken cells were subjected to His tag purification (Ni–NTA Super Flow, cat. no. 30410, Qiagen), and the elution was purified again through a gel filtration column (HiLoad 16/60 Superdex 75 pg, cat. no. 30430, Amersham Biosciences). Tubulin was purchased from Funakoshi (cat. no. TL238-B).

FKBP12 and tubulin were dissolved with PBS (pH 7.6, FKBP12 ~0.2 mg/ml, tubulin ~0.2 mg/ml) for binding experiments.

Synthesis of FK506 affinity resin

Synthesis of an FK506 derivative bearing a linker moiety at position 32 and its immobilization on a commercially available poly(methacrylate) resin Toyopearl (AF-Amino-650 M, cat. no. 08002, Tosoh) were carried out using a method similar that described previously [3].

Synthesis of benzenesulfonamide affinity resins

Benzenesulfonamide affinity resins were designed based on previously reported results [5].

A solution of 4-sulfamoyl-benzoic acid (402 mg, 2.0 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 420 μl, 2.4 mmol), *N*-hydroxysuccinimide (276 mg, 2.4 mmol), and acetonitrile (10 ml) was stirred at room temperature overnight. The solution was then poured into a mixture of water and ethyl acetate (AcOEt), and the separated organic layer was washed with brine, dried over MgSO₄, and filtered. The filtrate was evaporated in vacuo, and the resulting residue was recrystallized from *n*-hexane/AcOEt to give 4-sulfamoyl-benzoic acid 2,5-dioxo-pyrrolidin-1-yl ester (350 mg, 59%). ¹H NMR (dimethyl sulfoxide [DMSO]) δ: 2.91 (4H, s, –COC*H₂CH₂*CO–), 7.69 (2H, s, –N*H₂*), 8.07 (2H, d, *J* = 8.2 Hz), 8.30 (2H, d, *J* = 8.2 Hz).

A solution of 4-sulfamoyl-benzoic acid 2,5-dioxo-pyrrolidin-1-yl ester (97.0 mg, 0.33 mmol), 7-aminoheptonoic acid (47.2 mg, 0.33 mmol), and dimethylformamide (DMF, 2 ml) was stirred at room temperature overnight. The solution was then poured into a mixture of water and AcOEt, and the separated organic layer was washed with brine, dried over MgSO₄, and filtered. The filtrate was evaporated in vacuo and then purified by silica-gel column chromatography (eluted with *n*-hexane/AcOEt) to give 7-(4-sulfamoyl-benzoylamino)heptanoic acid (36 mg, 33.7%). ¹H NMR (DMSO) δ: 1.31 (4H, m), 1.47–1.54 (4H, m), 2.20 (2H, t, –C*H₂*COOH, *J* = 7.4 Hz), 3.23–3.28 (2 H, m, –NHC*H₂*–),

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7.45 (2H, s, $-NH_2$), 7.88 (2H, d, $J = 8.2$ Hz), 7.97 (2H, d, $J = 8.2$ Hz), 8.60 (1H, t, $-CONH-$, $J = 5.6$ Hz), 11.95 (1H, s, $-COOH$).

A mixture of 7-(4-sulfamoyl-benzoylamino)heptanoic acid (32.8 mg, 100 μmol), Toyopearl (250 μl, 25 μmol), EDC (21.1 μl, 120 μmol), 1-hydroxybenzotriazole (HOBt, 16.2 mg, 120 μmol), and *N*-methyl-2-pyrrolidinone (NMP, 1 ml) was shaken at room temperature overnight. The solvent was removed by filtration, and the resin was washed with NMP. The resulting resin was mixed with 20% acetic anhydride/NMP (1 ml), washed with NMP and water, and kept in an aqueous solution of 20% ethanol (EtOH) until used in binding experiments.

Synthesis of methotrexate affinity resin

The methotrexate (MTX) affinity resins were designed based on previously reported results [6]. A mixture of MTX (15.3 mg, 30 μmol, Nacalai Tesque), Toyopearl(1 ml, 100 μmol), EDC (4.7 mg, 30 μmol), HOBt (4.1 mg, 30 μmol), and DMF (5 ml) was shaken at room temperature for 16 h. The resulting resin was washed with DMF, mixed with 20% acetic anhydride in DMF (6 ml), washed with DMF and water, and kept in an aqueous solution of 20% EtOH until used in binding experiments.

Typical procedure for capturing binding proteins using affinity resins

Lysate from rat brain (1 ml) was stirred gently with FK506 affinity resin (10 μl) at 4 °C for 40 min. The resin subsequently was precipitated by centrifugation in a microcentrifuge at 12,000 rpm for 1 min and then washed five times with 1.0 ml of lysate buffer A. The washed resin was resuspended in 20 μl of sodium dodecyl sulfate (SDS) sample buffer solution (cat. no. 30566-22, Nakalai) containing 4% (w/v) SDS, 20% (v/v) glycerol, 0.01% (w/v) bromophenol blue, 10% (v/v) 2-mercaptoethanol (2-ME), and 0.125 M Tris–HCl (pH 6.8); shaken at 25 °C for 10 min; and centrifuged for 1 min. The supernatant (10 μl) was subjected to SDS–polyacrylamide gel electrophoresis (PAGE) followed by Coomassie brilliant blue (CBB) and/or silver staining.

Typical procedure for a competition experiment

To disturb the specific interaction between an affinity resin and its ligand, free FK506 (1.5 μmol in 10 μl DMSO) was mixed with lysate 40 min before carrying out the binding experiment described above. Solvent effects were offset by adding 10 μl of DMSO instead of the FK506 solution. Other protocols were carried out as described above.

Identification of proteins

FK506-binding protein 1A (FKBP12, 12 kDa), tubulin, actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH),

carbonic anhydrase II (CA2), and dihydrofolate reductase (DHFR) were identified by their tandem mass spectrometry (MS/MS) ions using electrospray ionization (ESI) ion trap MS (Thermoelectron, LTQ) after in-gel digestion. A commercially available apparatus (cat. no. 1D-SDS, Dainippon Seiki) was used for slicing off strips containing the desired protein from CBB- or silver-stained SDS gels.

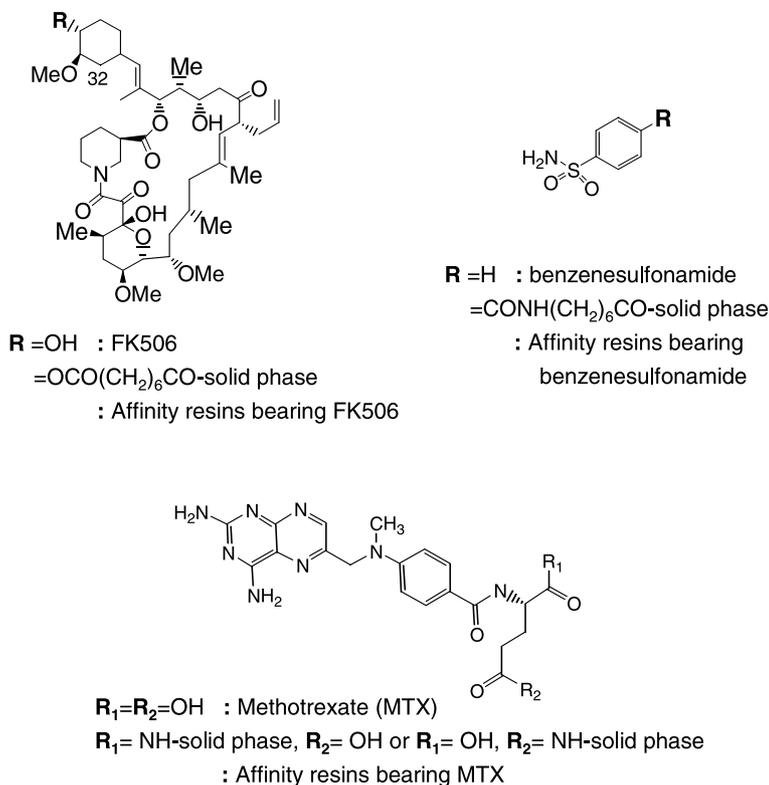### Typical procedure for identifying specific binding protein using serial affinity chromatography method

Lysate from rat brain (1.0 ml) was stirred gently with FK506 affinity resin (10 μl, ~1 μmol FK506) at 4 °C for approximately 40 min and then was precipitated by centrifugation in a microcentrifuge at 12,000 rpm for 1 min. The resulting supernatant was mixed with another 10 μl of the FK506 affinity resin at 4 °C, again for approximately 40 min. The resulting resin was washed five times with 1.0 ml of lysate buffer, resuspended in 20 μl of SDS sample buffer, shaken at 25 °C for 10 min, and then centrifuged for 1 min. The supernatant was subjected to SDS–PAGE. The resulting bands were stained with CBB and then comparatively analyzed to identify specific binding proteins. For repetition tests, the above procedures were carried out multiple times.

Results and discussion

Compatibility of serial affinity chromatography method with the competition method

The effectiveness and compatibility of the novel method, compared with the competition method, was assessed by identifying FKBP12, CA2, and DHFR using affinity resins bearing their well-known ligands: FK506 [1], a benzenesulfonamide derivative [5], and MTX [6], respectively (Scheme 1). These small compounds were chosen based on their good solubility in aqueous solution, thereby allowing the competition method to be carried out without generating insoluble material such as occurs when using dexamethasone and valdecoxib (Figs. S2 and S3).

FK506, an immunosuppressive drug, bound specifically to FKBP12 and originally was isolated using FK506 affinity resin [1]. FKBP12 (Fig. 2A, red arrow) was captured from 1 ml of rat brain lysate using 10 μl of FK506 affinity resin bearing approximately 1 μmol of FK506. Several other nonspecific binding proteins, such as tubulin, actin, and GAPDH, also bound to the resin (Fig. 2A). The specific binding protein captured by the competition method was identified by adding 1.5 μmol of FK506 to this mixture (A2), resulting in the disappearance only of FKBP12 (Fig. 2A, A1 vs. A2). This result demonstrated that the competition method was functional under these conditions. Specific binding proteins were then identified using serial affinity chromatography and the same FK506 affinity resin and lysate. First, 10 μl of FK506 affinity resin was mixed with 1 ml of lysate. Then the resin (B1) was removed, and the same amount of

R =OH   **:** FK506
  =OCO(CH$_2$)$_6$CO-solid phase
    : Affinity resins bearing FK506

R =H   **:** benzenesulfonamide
  =CONH(CH$_2$)$_6$CO-solid phase
    : Affinity resins bearing
       benzenesulfonamide

R$_1$=**R$_2$**=OH   **:** Methotrexate (MTX)
R$_1$= NH-solid phase, **R$_2$**= OH or **R$_1$**= OH, **R$_2$**= NH-solid phase
       : Affinity resins bearing MTX

Scheme 1. Bioactive compounds and affinity resins bearing them used in this study.

fresh FK506 affinity resin (B2) was added to the remaining lysate. Analysis of the binding proteins on B1 and B2 (Fig. 2A) showed a substantial decrease only in FKBP12, whereas there were few obvious changes in the amounts of proteins identified by the competition method. This result indicated the effectiveness and compatibility of serial affinity chromatography Interestingly, the amounts of some binding proteins on A1 (Fig. 2A, blue arrows) were greater than those on B1, although these two affinity resins should have captured the same amount of proteins. This difference was most likely an artifact of the competition method due to the addition of 10 μl of DMSO to the mixture during preparation of A1 to offset solvent effects; FK506 was also added as a DMSO solution (10 μl) to A2, whereas only the resins were added to B1 and B2. Therefore, the increase in the amounts of three nonspecific proteins captured by the competition method probably was caused by the addition of DMSO, indicating the superiority of serial affinity chromatography, because artifacts can be suppressed or kept to a minimum.

Benzenesulfonamide and its derivatives are known to bind specifically to CA2 [5]. Analysis of a mixture of affinity resins bearing a benzenesulfonamide derivative with lysate obtained from the human monocyte cell line THP-1 showed that CA2 was successfully captured by the resins, and their specificity was confirmed by both the competition method and serial affinity chromatography (Fig. 2B). However, CA2 was still detectable on the affinity resins after the addition of 1.5 equivalents of benzenesulfonamide (Fig. 2B, A2). A similar result was obtained using serial affinity chromatography with the same resins and lysate (Fig. 2B, B2).

These results indicated that the affinity of ligand on resins with CA2 was weaker than that of FK506 with FKBP12, consistent with the K_d values of these interactions ($K_{d\,\text{FK506–FKBP12}}$: 0.4 nM [7], $K_{d\,\text{benzenesulfonamide–CA2}}$: 0.56 μM) [5].

In another experiment, the ability of DHFR to bind specifically to MTX affinity resin was tested using both methods. DHFR is the target protein of MTX, an anticancer drug [6,8]. Serial affinity chromatography allowed the successful isolation of DHFR as a specific MTX-binding protein because the amount of DHFR on B2 was less than that on B1 (Fig. 2C). The amount of DHFR on the resin was also clearly reduced by the addition of 1.5 equivalents of free MTX (Fig. 2C, A1 vs. A2).

Repetition effects on the detection of binding proteins using serial affinity chromatography

To distinguish between specific and nonspecific binding proteins by our method, the same protocol was applied six times using the same affinity resins and lysate, followed by analyzing the binding proteins captured on each resin, as illustrated in Fig. 3A.

FKBP12 was detected only on the first FK506 affinity resin (Fig. 3B, B1), demonstrating that it was completely captured. This result indicated that the K_d value of FK506 immobilized on FKBP12 resin was quite low, consistent with the reported K_d value of 0.4 nM [7]. Interestingly, the amounts of proteins A and B, which have yet to be identified, were slightly greater on resins B2 to B6 than on resin
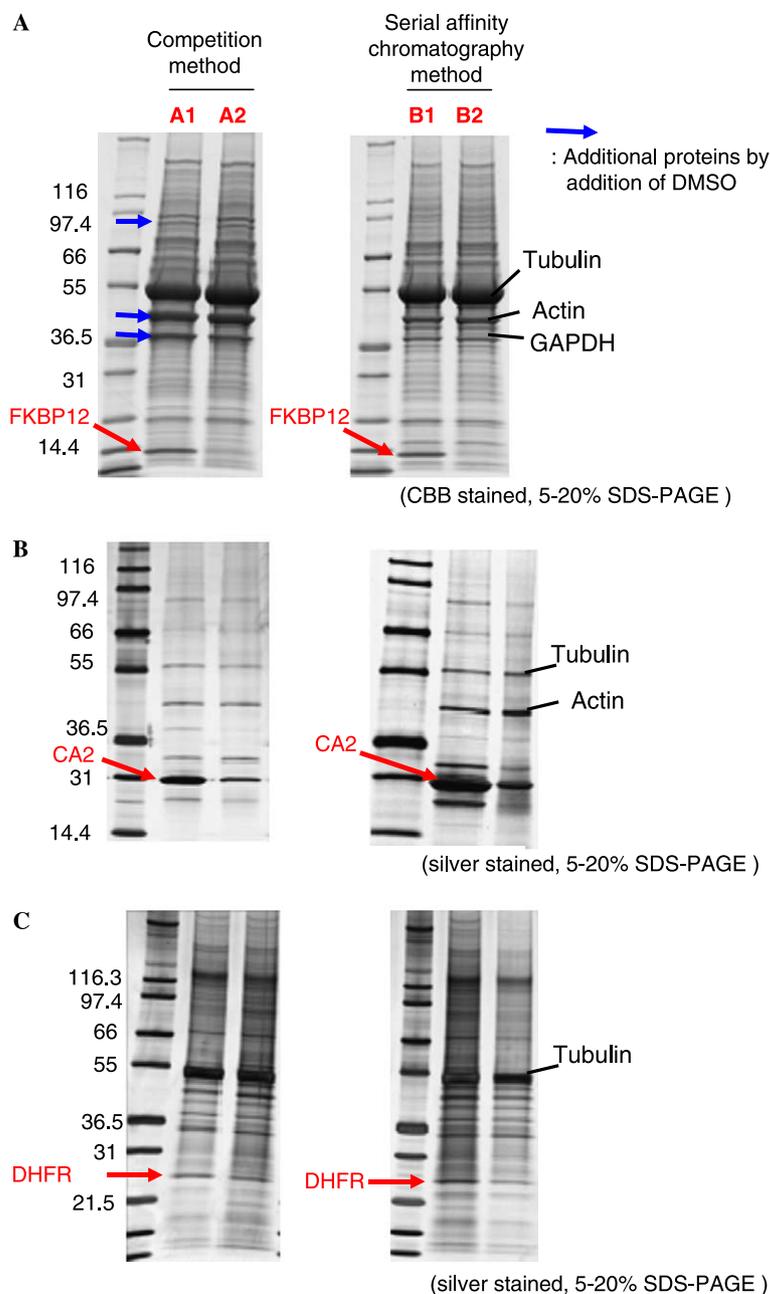
Fig. 2. Compatibility of serial affinity chromatography (right) with the competition method (left) for identifying specific binding proteins. (A) Identification of FKBP12 as a specific binding protein to FK506 affinity resin. FK506 affinity resin bearing 1 μmol of FK506 captured FKBP12 and other proteins in 1 ml of lysate from rat brain (A1 and B1). To identify FKBP12 as a specific binding protein by the competition method, a solution of FK506 (10 μl, 1.5 μmol) in DMSO was premixed with the lysate as a competitor and exposed to the resin. This resulted in the disappearance of FKBP12 alone (red arrow). In this case, the competition method was successful because no precipitate was observed in the mixture after the addition of FK506. In A1, 10 μl of DMSO was added to offset the solvent effects. To identify specific binding proteins using serial affinity chromatography, the same amount of FK506 affinity resin was mixed twice with the lysate, as illustrated in Fig. 1. A comparison of proteins bound to the first resin (B1) and second resin (B2) showed that only FKBP12 decreased significantly in amount, indicating that it was a specific binding protein. Comparison of A1 and B1 showed that the amount of some binding proteins in the lysate increased (blue arrow), indicating that the properties of those proteins were modified by the addition of 1% DMSO because the only difference between the two samples was the presence of 1% DMSO in the mixture. (B) Identification of CA2 as a specific binding protein of benzenesulfonamide affinity resin. (C) Identification of DHFR as a specific binding protein to MTX affinity resin. CA2 and DHFR were identified as binding proteins using the competition method and serial affinity chromatography, carried out as described above. See text for details. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

B1, whereas the amounts of other proteins were nearly constant. This difference may be due to the fact that affinities of proteins A and B for the FK506 affinity resin are higher than those of the other proteins except for FKBP12. Therefore, after most of the FKBP12 was captured by the first resin, larger amounts of the other proteins could bind to subsequent resins. Moreover, these results indicated that FKBP12 was the most specific binding protein in the lysate,
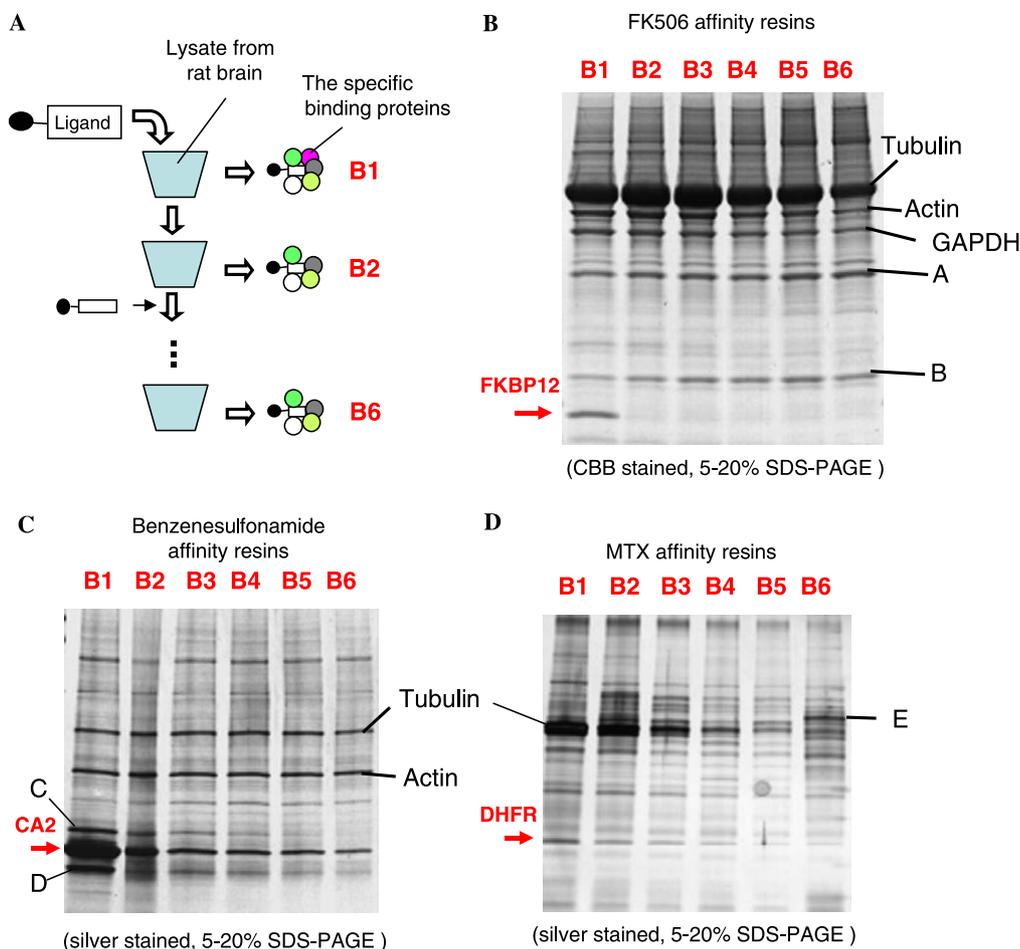
Fig. 3. Repetition effects using serial affinity chromatography. (A) Protocol for a study on the effects of repetition testing of specific binding proteins using serial affinity chromatography. The same protocol was repeated six times, and the binding proteins on each resin (B1–B6) were analyzed. (B) Binding proteins on FK506 affinity resins (B1–B6) were analyzed by SDS–PAGE. FKBP12 was found only on the first resin (B1) and was not detectable on the other resins, indicating that the compound was completely captured on B1 and thus no longer was present in the remaining lysate. Other proteins bound to the resins were of low affinity because they remained in the lysate and bound similarly to subsequent resins. Binding proteins on the series of benzenesulfonamide (C) and MTX (D) affinity resins were analyzed by SDS–PAGE. See text for details.

whereas the other components probably were nonspecific binding proteins with weaker affinities for immobilized FK506. The results also indicated that these types of repetition protocols may allow estimation of the relative rank order of proteins with respect to binding affinity for particular resins.

In the next experiment, the same procedure was carried out using the benzenesulfonamide affinity resins (B1–B6) and lysate from THP-1 (Fig. 3C). The amount of CA2 was markedly lower on the second resin (B2) and on subsequent resins (B3–B6) than on B1, showing that serial affinity chromatography successfully validated CA2 as the specific benzenesulfonamide-binding protein. Nonetheless, CA2 continued to be detected on affinity resins B3 to B6, albeit in decreasing amounts. This result differed from that obtained using the FK506 affinity resins, in which FKBP12 was found only on B1 (Fig. 2B). The binding of CA2 to B1 to B6 was confirmed by Western blot (Fig. S4). An explanation for this result may be that the K_d value of CA2 and the benzenesulfonamide affinity resins, 0.56 μM, was not suffi-

cient to completely capture CA2 from the lysate. The results indicated that although it may be possible to estimate the relative binding potency of ligands with their partner proteins by carrying out these types of repetition tests, the amounts of the respective proteins must be kept in mind. Fig. 3C shows that proteins C and D may be specific binding proteins because the amounts bound to B2 were lower than those bound to B1. However, these intriguing proteins remain to be identified.

The same protocol was also carried out using MTX affinity resin (B1–B6) and THP-1 lysate (Fig. 3D). The amount of DHFR was markedly less on B2 than on B1, and the amount of free protein gradually declined following repeated treatments with the resin. On SDS–polyacrylamide gel, DHFR was detectable in all lanes, although the results showed that only small amounts were present on B4 to B6. Interestingly, the amount of tubulin decreased and was hardly detected on B6, indicating that tubulin has a relatively high affinity for the MTX affinity resin, although no information on this relationship has been reported. The
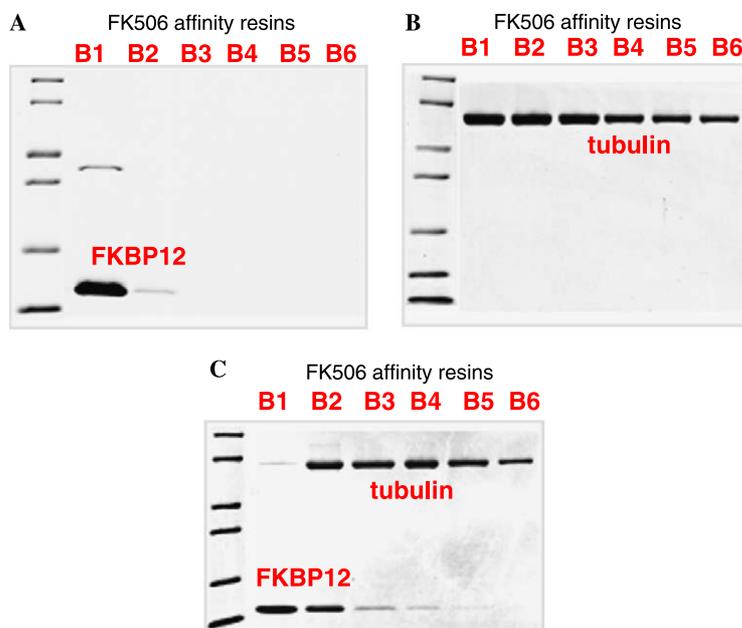
Fig. 4. Model studies of serial affinity chromatography using FK506 affinity resins and purified proteins. (A) Binding of specific binding protein, FKBP12, on FK506 affinity resins after mixing with a solution of FKBP12. (B) Binding of nonspecific binding protein, tubulin, on FK506 affinity resins after mixing with a solution of tubulin. (C) Binding of proteins on FK506 affinity resins after mixing with a mixture of FKBP12 and tubulin.

amount of protein E, as yet unidentified, increased only on B6, but this result was not investigated further.

Model study of serial affinity chromatography using purified proteins

To assess the ability of the new method for estimation of relative rank order of proteins with respect to binding affinity for particular resins, the same protocol in Fig. 3 was carried out using known proteins, FKBP12 and tubulin, instead of lysate. FKBP12 is a specific binding protein to FK506 with potent K_d (0.4 nM) [7]. Tubulin is known as a nonspecific binding protein whose K_d value to FK506 could be weaker than that of FKBP12. At first, FK506 affinity resins were mixed, with an aqueous solution of purified FKBP12 and binding proteins captured on each resin analyzed (Fig. 4A). As shown in Fig. 4A, most of the FKBP12 was captured by the first affinity resins and little was detected on other resins. This result similarly showed that FKBP12 was a specific binding protein to the FK506 affinity resins. On the other hand, tubulin was found on all resins and the amount gradually decreased (Fig. 2B). This result indicated that the majority of tubulin remained in the solution after being mixed with the FK506 affinity resins and that only a fraction of tubulin could be captured by the resin, indicating that a K_d value between tubulin and FK506 was not potent.

Finally, we analyzed binding proteins on FK506 affinity resins after mixing with a mixture of FKBP12 and tubulin (Fig. 4C). On the first FK506 resins (B1), a large amount of FKBP12 was found, whereas there was only a detectable level of tubulin. This indicated that most of the FK506 was

immobilized on resins occupied by FKBP12, owing to their high affinity. Tubulin and FKBP12 were found on B2 in similar amounts, but only tubulin was observed on B3–B6. A fraction of FK506 could be occupied by tubulin on the first resins, and some FKBP12 remained in the resulting mixture because FKBP12 was also found on the second resins, whereas the amount of FKBP12 on second resins was only at a detectable level, as shown in Fig. 4A. These model studies also show that the new method has the ability not only to distinguish between specific and nonspecific binding proteins but also to assess the relative rank order of binding affinity of proteins to ligand on affinity resins. Further examples, using miscellaneous known proteins and ligands, were required for quantitative assessment of K_d values only by this method.

Conclusion

Affinity chromatography matrices bearing bioactive compounds are a major tool in the identification of target proteins. Although the solid-phase elution and competition methods are widely used to distinguish specific binding proteins from nonspecific ones on affinity resins, the low solubility of some ligands in aqueous solutions of proteins and/or the slow dissociation kinetics between ligand and binding protein (Figs. S1–S3) often limit the use of these procedures. It is especially difficult to identify target proteins using synthetic compounds because they often have low solubility in aqueous solutions.

To circumvent these problems, we developed a simple and versatile method, termed *serial affinity chromatography*, to detect target proteins. A mixture of proteins, such

as those present in a lysate, is sequentially treated at least twice with affinity resins bearing ligand, and the binding proteins on each resin are then comparatively analyzed (Fig. 1). Problems, such as the denaturation of proteins induced by adding free ligand or solvents, are minimal compared with their effects when conventional detection methods are used (Fig. 2A) because only the affinity resin is added to the protein mixture. However, serial affinity chromatography could fail if the lysate includes a stoichiometric or greater amount of specific binding protein compared with the amount of ligand immobilized on the affinity resin. In addition, the results may be confusing if the affinity of the resin-immobilized ligand for its specific binding protein is weak. Nonetheless, these limitations did not restrict the application of serial affinity chromatography because, in the former case, only a few exceptionally abundant proteins (e.g., tubulin, actin, GAPDH, albumin) and, in the latter case, only loss-of-function-type affinity resins are inappropriate for detecting target proteins.

This study has demonstrated the effectiveness and compatibility of serial affinity chromatography with the competition method. This conclusion is based on three examples in which FKBP12, CA2, and DHFR were successfully identified as specific binding proteins to FK506, benzenesulfonamide, and MTX affinity resins, respectively. In these examples, the specific binding proteins were successfully identified by both methods because the small target compounds were sufficiently soluble in aqueous solution to allow their detection by the competition method. In contrast, it is difficult to identify the specific binding proteins of dexamethasone and valdecoxib using the competition method due to the poor solubility of these drugs in aqueous solution (Figs. S2 and S3). The success of our approach can be explained by the fact that it is independent of ligand solubility.

Repetition testing carried out as part of serial affinity chromatography, using lysate and a mixture of purified proteins, showed that a specific binding protein could be identified after two rounds of exposure to the affinity resin (Figs. 3 and 4) This study also demonstrated that relative rank order, with respect to the specific binding activity of a ligand in a sample mixture to the affinity resin, can be estimated by repeated exposure to the affinity resin, keeping in mind the abundance and/or potential denaturation of the ligand following addition of the affinity resin.

The simplicity and effectiveness of the novel method described here, and its few limitations, afford a wide range of opportunities to identify target proteins of bioactive compounds using affinity resins and, especially, synthetic compounds with low solubility.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2006.02.008.

References

[1] M.W. Harding, A. Galat, D.E. Uehling, S.L. Schreiber, A receptor for the immunosuppressant FK506 is a *cis–trans* peptidyl-prolyl isomerase, Nature 341 (1989) 758–760.

[2] J. Taunton, C.A. Hassig, S.L. Schreiber, A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p, Science 272 (1996) 408–411.

[3] T. Tamura, T. Terada, A. Tanaka, A quantitative analysis and chemical approach for the reduction of nonspecific binding proteins on affinity resins, Bioconj. Chem. 14 (2003) 1222–1230.

[4] Y. Oda, T. Owa, T. Sato, B. Boucher, S. Daniels, H. Yamanaka, Y. Shinohara, A. Yokoi, J. Kuromitsu, T. Nagasu, Quantitative chemical proteomics for identifying candidate drug targets, Anal. Chem. 75 (2003) 2159–2165.

[5] J. Lahiri, L. Isaacs, J. Tien, G.M. Whitesides, A strategy for the generation of surfaces presenting ligands for studies of binding based on an active ester as a common reactive intermediate: a surface plasmon resonance study, Anal. Chem. 71 (1999) 777–790.

[6] L.W. Miller, J. Sable, P. Goelet, M.P. Sheetz, V. Cornish, Methotrexate conjugates: a molecular in vivo protein tag, Angew. Chem. Int. Ed. 43 (2004) 1672–1675.

[7] J.J. Siekierka, S.H. Hung, M. Poe, C.S. Lin, N.H. Sigal, A cytosolic binding protein for the immunosuppressant FK506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin, Nature 341 (1989) 755–757.

[8] A.R. Gargaro, A. Soteriou, T.A. Frenkiel, C.J. Bauer, B. Birdsall, V.I. Polshakov, I.L. Barsukov, G.C. Roberts, J. Feeney, The solution structure of the complex of *Lactobacillus casei* dihydrofolate reductase with methotrexate, J. Mol. Biol. 277 (1998) 119–134.