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Specific Blocking to Improve Biopanning in Biological Samples such as Serum and Hybridoma Supernatants

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ABSTRACT

Screening phage-displayed peptide libraries (biopanning) is an important technique for acquiring peptide ligands and for mapping peptide epitopes recognized by antibodies (Ab). In biological samples, other materials, not only contaminants but also natural constituents, often interfere with biopanning. Capture methods use anchoring Abs that reduce the need for purification of the intended panning target from a crude sample. This capture method is analogous to sandwich ELISA. However, when the target molecule concentration is low in the initial mixture, the panning of a captured target often yields epitopes that bind to the capture antibody rather than the target of interest. We have developed a methodology that utilizes specific blocking reagents of the capture Ab to extend the sensitivity and applicability of the capture approach to phage panning. A flowchart is presented to enable the worker to begin panning with the simplest approach and then to employ sandwich capture and specific blocking reagents as necessary.

INTRODUCTION

Biopanning with phage display libraries is an important tool for obtaining ligands to molecules and surfaces of interest and for epitope mapping (10). In the case of biopanning an antibody (Ab), purified Ab can be immobilized on a solid-phase support such as polystyrene plates or, if the Ab has been previously conjugated with biotin, captured with streptavidin. However, Abs of interest are often present in complex biological samples such as serum, ascites fluid, or hybridoma supernatants. Directly coating a polystyrene plate with these fluids does not provide a

workable substrate for successful biopanning. Other proteins, such as albumin in serum, are often present at high concentrations and interfere with coating and biopanning of the Ab. Purifying the Ab represents a significant time and material investment, particularly when a large number of samples are being processed.

Sandwich ELISA assays use a capture Ab to immobilize specific targets from an impure sample (7). Crameri et al. (3) have demonstrated that such capture methods can be used for biopanning. Their innovation is often useful but sometimes fails. We have incorporated specific blocking reagents that reduce artifacts and increase sensitivity of the capture method. Sometimes the choice of specific blocking reagent is obvious *a priori*; at other times, the sequence of an appropriate blocking peptide arises from information gathered in another experiment. In a related modification of the Crameri method, we have used a high-diversity mixture of class-specific antibodies to block unused sites on a capture antibody. Many variations are available, and it is important to know how and when to use them. A flowchart is presented to facilitate experimental design for biopanning. It starts from the straightforward application of the capture method and guides the use of more complex methods of blocking when required.

MATERIALS AND METHODS

Hybridoma culture supernatants were the kind gift of Toby Rodman and James Sullivan (Rockefeller University). All peptides were purchased from Genemed Synthesis (San Francisco, CA, USA). Peptides were analyzed by mass spectrometry by the manufacturer and the spectra provided. All other reagents were purchased from Sigma (St. Louis, MO, USA).

Three phage-displayed peptide libraries were purchased from New England Biolabs (Beverly, MA, USA). The random peptide is displayed at the N-terminus of the geneIII protein (pIII). There is a small leader sequence separating the peptide from the protein: Ph.D.7, NH₂-XXXXXXXX-GGGS-pIII; Ph.D.12, NH₂-XXXXXXXXXXXX-

GGGS-pIII; Ph.D.C7C, NH₂-ACXX XXXXXC-GGGS-pIII.

These libraries were built in M13-mp18 and as such contain the *lacZα* fragment. They will not form blue plaques on *E. coli* strain S26*lacZ*- (1) when plated on media containing X-Gal. Phage stocks were grown on strain K91, an S26 derivative that has been cured of lambda.

Procedures for phage-displayed peptide library panning, analysis by eluant phage titer, sequencing, and analysis by ELISA have been described in detail elsewhere (9) and do not differ substantially from the standard protocols (11).

RESULTS AND DISCUSSION

Biopanning with phage-displayed peptide libraries failed when the supernatants of monoclonal B-cell hybridoma culture were coated directly on polystyrene plates. As these supernatants were IgM positive by ELISA, we panned against them again, first coating wells with affinity-purified goat anti-human IgM, thereby hoping to "capture" our target. This also led to frustration. The phage recovered after three rounds of panning, elution, and outgrowth only bound to the anchor anti-IgM Ab. Candidate phage were sequenced and displayed peptides mimicking a segment of the μ chain, specifically residues 205–215 with the central phe-gln (FQ) being the most common (Figure 1a). Three phage were selected and confirmed to bind specifically to the anti-IgM Ab (Figure 1, b and c). This binding could be blocked by a synthetic peptide (pIgM201) with the sequence DHRGLTFQQNAS derived from the μ chain. The binding of the phage to the Ab could also be blocked with human IgM (data not shown).

A panning cycle was performed against just the anti-IgM Ab in the presence of peptide pIgM201. After three rounds of panning, significant enrichment in eluant phage titer was seen. However, the final round eluant bound equally well to anti-IgM-coated wells with or without blocking peptide, indicating that the peptide did not prevent enrichment of anti-IgM binding clones. This is most likely due to the polyclonal nature of the anti-IgM Ab used. It

was clear that to prevent enrichment against the anchoring anti-IgM Ab, a blocking reagent that would mask all unoccupied anchor sites was required.

Pannings against hybridoma supernatants were repeated essentially as before with an additional step. Anti-IgM-coated wells were incubated with hybridoma supernatant as before. Before the addition of phage, human IgM (a high-diversity sample pooled from greater than 1000 uninfected individuals) was added at a concentration of 100 μ M for 1 h. This poly-specific IgM was intended to block any exposed binding sites on the coated anti-IgM Ab without providing a high concentration of a new target. Pannings done in this manner against two hybridoma supernatants, JSA4 and JSA5, produced significant enrichment in the third-round eluant titer. Phage clones were tested by ELISA for binding specifically to the mAb in the hybridoma supernatant.

In each case, 5 of 10 clones tested were positive and were sequenced. The positive phage selected against JSA4 all displayed IYNKGTLPTAL. Those selected against JSA5 displayed VT-SKTTPLYLFV.

Synthetic peptides based on each of the above sequences were synthesized. Representative phage clones were panned against wells coated with anti-IgM and then either the cognate hybridoma supernatant, the cognate supernatant and the cognate peptide, human IgM, or buffer (Figure 2). Each phage bound specifically to the hybridoma supernatant against which it was panned and was competed by the cognate synthetic peptide. The phage did not bind to wells coated only with the anti-IgM Ab or to anti-IgM Ab blocked with IgM.

The methods used for sandwich ELISA can be adapted to phage display library panning against unpurified Ab material such as hybridoma culture su-

Figure 1. Specificity of anti-IgM binding phage. (a) Phage-displayed peptides that bind to anti-IgM Ab are similar to human μ chain between residues 201–210. Sequences shown are the peptides displayed by phage recovered after three rounds of panning against anti-IgM Ab in the presence of hybridoma supernatant. Phage used for specificity assays are labeled (e.g., 3b). (b) Phage that were selected against the anti-IgM Ab (black bars) do not bind to wells coated with anti-IgG Ab (gray bars) made in the same species (goat) or to uncoated (white bars) wells. (c) anti-IgM binding phage only bind anti-IgM in ELISA when the phage are coated on the plate. Wells were coated with approximately 10^{11} phage (indicated in legend). After blocking with 3% bovine serum albumin (BSA), the primary Ab was put on at 10 μ g/mL. Detection was done with anti-goat IgG peroxidase conjugated Ab developed with o-phenylenediamine dihydrochloride (OPD). Phage Z37b and C1q-d are control phage from unrelated pannings, and M13mp18 is the parent vector phage with no displayed peptide.

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pernatants and mouse ascites fluid. The flowchart of the experimental design, shown in Figure 3, illustrates specific protocol decisions that are made based on the materials available and feedback from experimental results.

In many cases, using an anchoring Ab will suffice. In these simple cases, the concentration of the target molecule in the raw sample is high enough that phage, specific for the target, will form a significant fraction of the final eluant pool. The concentration at which this occurs varies from target to target. A reasonable number of the resulting phage can be individually tested against the anchoring Ab with and without the target material present in either the panning format or in an ELISA in which the phage are coated on the plate (Figure 3, decision box A). However, if a significant number of those phage recovered are not target specific but are anchoring Ab specific, additional or alternative steps must be employed.

There are two general approaches to eliminating the problem of anchor-specific phage. The first (Figure 3, decision box B) is to change the anchoring Ab used in each round. For instance, if different mAb recognizing non-cross-reactive epitopes on the target molecule exist, then the Ab used to anchor the target could be alternated from round to round. By changing the anchoring Ab, one should prevent the enrichment of phage specific for any single anchoring Ab. This approach could also be applied when polyclonal Ab are used for the anchors. To do so, target-specific polyclonal Ab from different sources or species could be used.

An alternative approach is to block the unoccupied anchoring sites. Blocking can be accomplished with either a peptide (Figure 3, decision box C) or, when the target molecule is an Ab, another Ab of the same class or isotype recognized by the anchoring Ab (Figure 3, decision box D). The peptide approach is best when the anchoring Ab is monoclonal because a single peptide sequence will obscure all unoccupied anchoring Ab. The blocking peptide sequence can be derived from the phage previously found to be specific for the anchoring Ab (i.e., the tool for a successful experiment can be derived from a failed one).

Figure 2. Phage selected against the IgM in JSA4 and JSA5 hybridoma culture supernatants are specific for the cognate Ab. Each phage is competed by a free peptide of the same sequence as the peptide they display. The sandwich method with anti-IgM-coated wells was used with either the hybridoma culture supernatant, bulk human IgM, or no secondary coating (none).

Figure 3. Flowchart illustrating strategies for sandwich panning. Decision points referred to in the text are identified with capital letters.

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In addition, interesting biology may result from analysis of the peptide sequences found. For example, phage in this study, which bound the anti-IgM, displayed peptides similar to a region of the human μ chain. Such similarity implied the location of a specific epitope recognized by the anti-IgM Ab.

Blocking peptides may be less successful when the anchoring Ab is affinity purified or otherwise polyclonal. In those cases, several different epitope specificities may exist. This was apparently the case with the polyclonal anti-IgM used in this study. When included in a second cycle of panning, the peptide (pIgM201) derived from the initial batch of anti-IgM binding phage did not prevent enrichment of other anti-IgM binding phage. Although in principle one could apply an iterative panning and blocking strategy to find multiple peptides (8), each of which blocks a fraction of the polyclonal Ab, simpler alternatives exist.

One might use other Ab of the same class (and isotype if using isotype-spe-

cific anchoring Ab) as the target Ab to block unoccupied anchoring Ab. However, this could introduce artifacts of its own, mainly selection of phage specific for the blocking Ab. Such a problem can be avoided by two alternate strategies (Figure 3, decision box E). One can use bulk purified Ab of the proper class and/or isotype, as was done here, or one could use alternating mAb with different, non-cross-reactive specificities. Bulk IgG is superior for this application because Ab of any particular specificity will not exist as a large fraction of the total; therefore, it is unlikely they will select phage. In all of the above cases, controls are essential to determine that the selected phage are specific for the desired Ab and not the blocking or anchoring Ab.

There are examples of selection of phage that bind to the constant regions of Ab (4,5); our experience is that they are not routinely found in antibody panning. Should such phage be selected, they can be eliminated by similar blocking strategies.

The sandwich panning method is not limited to Ab and could be applied to any target molecules for which there are anchoring molecules available. The same strategies outlined here can be applied to other types of library selections including phage-displayed Ab fragments (6), nucleic acid (12), and combinatorial chemical libraries (2).

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