A solid-phase assay to screen monoclonal antibodies against DNA-binding protein

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ABSTRACT

A method is described for selecting monoclonal antibodies (mAb) against DNA-binding protein. The protocol involves a non-radioactive solid-phase DNA binding assay using a 96-well plate. Because the solid-phase assay is highly specific and sensitive, partially purified antigen is sufficient for the immunization, and mAb screening can be performed with crude cell extract as the antigen. MAbs obtained by this method could supershift the DNA–protein complex in the electromobility shift assay, and were sufficient for immunoscreening of a cDNA expression library.

Transcription, an essential process of gene expression, is regulated by DNA-binding proteins. Identification of the responsive element and its binding protein provides us with clues about the function of the gene. In addition to the conventional gene cloning procedures, such as PCR amplification with degenerated primers of the gene, many novel strategies including South-western screening (1), yeast one-hybrid screening (2) and phage display (3), were developed in the past several years. Difficulties were encountered, however, because only small amounts of DNA-binding proteins are present in cells, and relatively large amounts of them were required for the biochemical approaches. Also, these molecular biological procedures produce numerous false positives. If a method to screen monoclonal antibodies (mAb) with small amounts of antigen or crude antigen was available, it would be easy to accomplish gene cloning by immunoscreening of the expression library with these mAbs.

Here, we describe a new method to screen mAbs against DNA-binding proteins by using a solid-phase DNA binding assay in 96-well plates. The principle of the assay is depicted in Figure 1. MAbs from hybridoma culture supernatants were immobilized onto 96-well plates coated with anti-IgG (Fc). The plates were incubated with crude antigen, and the DNA binding activity trapped onto the plates was measured using a biotinylated oligo DNA probe and horseradish peroxidase conjugated streptavidin. Different from the conventional mAb screening method ELISA, which requires large amounts of purified protein, for this new assay, partially purified protein is sufficient for the immunization, because the assay is based on the detection of specific DNA binding activity. Furthermore, the assay is sensitive enough to detect mAbs using a crude cell extract as an antigen for the screening. MAbs obtained from this screening would immunoprecipitate the DNA binding complex rather than inhibit the DNA binding activity. These mAbs, therefore, can be used for identifying the protein in the DNA binding complex detected in the electromobility shift assay (EMSA) as well as for immunoscreening.

A solid-phase DNA binding assay for mAb screening was carried out using Maxi Soap 96-well plates (Nunc). One microgram of affinity-purified goat anti-mouse IgG (Fc-fragment specific; Jackson Immuno-Research Laboratories Inc.) was immobilized onto microwells by incubation in 50 µl of PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 8 mM Na2HPO4 pH 7.2) at 4°C overnight. After blocking with 200 µl 5% (w/v) skim-milk and 1% (w/v) casein in PBST [0.1% (v/v) Tween-20 in PBS], 50 µl hybridoma culture supernatant was added to each well followed by incubation for 1 h at room temperature with gentle shaking. The hybridoma culture supernatant was removed by aspiration and 50 µl of the crude cell extract (20 µg) or recombinant protein (0.1 µg) was added to each well. After 1 h incubation at 4°C, the extract was removed by aspiration and the plates were washed once with PBS. These plates were then subjected to the non-radioactive solid-phase DNA binding assay. The binding reaction mixture (50 µl) contained 0.4 nM 5’ biotinylated five tandem repeats of ENO1 RPG-box (4) and 0.1 mg/ml poly(dI-dC)-poly(dI-dC) (Pharmacia) in DNA binding buffer [10 mM Tris–HCl pH 7.5, 0.5 mM EDTA, 1 mM MgCl2, 0.5 mM DTT, 50 mM NaCl and 4% (v/v) glycerol]. After 30 min incubation at room temperature, the mixture was removed from each well, and the wells were rinsed three times with PBS over a 10 min period. After 1 h incubation with streptavidin-conjugated horseradish peroxidase (HRP, 1:500 dilution; Amersham) at room temperature, HRP activity trapped onto the well was measured as the amount of DNA binding protein by a standard method (5).

To confirm the specificity of our solid-phase assay, we performed this assay with the known Saccharomyces cerevisiae Rap1p (6). We synthesized ENO1 RPG-box, a Rap1p target sequence (4), five other known native RPG-boxes from S.cerevisiae (TPI, TEF2, RPC40, RP39A and HIS4), five eno1 RPG-box mutants (C4G, C5A, C6T, C10T and A11C) and two unrelated oligo DNAs (AP2 and NFκB) as competitor DNAs. We carried out the competition analysis by our solid-phase assay, and the quantitative EMSA in which bacterially expressed GST-Rap1p, labeled ENO1 RPG-box probe and a 10-fold molar excess of unlabeled competitor was used (Fig. 2). Nearly identical results were obtained from these two assays. This finding reveals that the specificity of our method is comparable with that of EMSA.

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Using this method followed by the immunoscreening, we cloned a DNA binding protein gene. **RBF1**, from the major human pathogen *Candida albicans* (7). Rbf1p was identified as a *S. cerevisiae* ENO1 RPG-box binding factor. *Candida albicans* crude extract had an activity that could form two complexes with ENO1 RPG-box, an Rbf1p-containing complex and a non-specific complex in the EMSA (Fig. 2b, lane 1, complexes I and II, respectively). We partially purified this complex I forming activity from *C. albicans* crude cell extract ~20 000-fold to 70% purity by a five-step column chromatographic procedure (7). Then two mice (Balb/c) were immunized twice with 3 µg of this sample. Three days after the last injection, one mouse spleen was selected according to the reactivity of their antiserum in the solid-phase assay and hybridoma cells were performed with the mouse anti-Rbf1p antiserum, and we obtained 12 independent cDNA clones. Because none of the cDNA clones expressed DNA binding activity in *Escherichia coli*, we could not confirm any true **RBF1** cDNA clone. During the primary screening, we generated anti-Rbf1p mAbs by the method described above. Then, we used these mAbs to further select **RBF1** cDNA from the 12 clones and obtained one **RBF1** cDNA clone, p12B. This cDNA clone, however, lacked the C-terminal half of the coding sequence. Therefore, we carried out a primary screening of the same library using this mAb mixture in order to isolate the full length cDNA clone. That screening, however, resulted in the isolation of the same cDNA clone, p12B (7). This suggests that our method is fully applicable for immunoscreening of expression libraries. As a result, in our experiment, 16 µg of the partially purified protein was sufficient for all the gene cloning procedures.

Our procedure for mAb generation requires only small amounts of antigen. It permits the use of partially purified protein for immunization and a crude extract for hybridoma screening and enables the isolation of supershift mAbs in EMSA. The strategy described here is also applicable for screening mAbs against nucleic acid-, protein- and small ligand-binding proteins, if their solid-phase assay systems are available.

**REFERENCES**