Application Note

In vitro study of protein–protein interactions using antibody-coated multiple-well plates

BY LIN ZHANG

With the completion of the Human Genome Project, there is much interest in developing methods amenable to the functional characterization of proteins on a global basis. One of the major tasks in the analysis of protein functions is deciphering protein complex formation, since protein–protein interactions mediate the vast majority of physiological processes in cells. Epitope tagging, a process of fusing a very short peptide that is recognized as an antigenic determinant to a protein of interest, has been widely used to facilitate the analysis of protein–protein interactions. Tagging a protein with an epitope at the N- or C-terminus allows the rapid detection, isolation, and analysis of protein interactions with other proteins, without prior knowledge of the target protein.[1–5] Various tags have been used for the expression of recombinant proteins in many expression systems and cell-free systems. Among them, the FLAG epitope tag is most widely used. The system relies on the small FLAG octapeptide (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys), which can be detected by the anti-FLAG monoclonal antibody M2.[6,7]

The most commonly used in vitro biochemical assay to study protein–protein interaction is immunoprecipitation employing antibody coupled to resins.[8] Although this method has proven invaluable in elucidating the function of proteins on a case-by-case basis, the procedure involves laborious and time-consuming steps that limit sample throughput, and it cannot be applied to the study of protein–protein interactions on a genomic scale. In addition, aspirations required for removing the wash buffer often result in loss of the affinity resin and thus bound proteins. Obtaining quantitative data becomes quite problematic, especially when handling small quantities of samples.

This paper discusses the use of an anti-FLAG epitope antibody-coated 96-well microplate (Anti-FLAG® M2 plate, **Sigma-Aldrich Corp.,** St. Louis, MO) for capturing in vitro translated FLAG-tagged fusion protein and subsequently in-well characterization of protein–protein interactions. A pair of known proteins that interact in vivo—p53 and SV40-large T-antigen—was chosen as the model system to demonstrate the feasibility of the system for the analysis of protein–protein interactions. p53 is a tumor suppressor protein originally identified as a protein bound to large T-antigen in SV40-transformed cells.[9] The large T-antigen forms a complex with the p53 suppressor gene, resulting in its functional inactivation and thus promotion of cell transformation.[10]

COS-7 cells (SV40-transformed African Green monkey kidney fibroblasts) were used to provide the source for large T-antigen in this study. It is known that the COS-7 cells express two major SV40 translational products, including the 94-kD large T-antigen and the 21-kD small T-antigen, both of which are encoded by the early region of the SV40 viral genome.[11] In this study, p53 was in vitro translated as a FLAG-p53 fusion protein, and its interaction with SV40 large T-antigen was demonstrated by applying COS-7 cell extracts to the wells and allowing the interaction to occur in the same well used to capture FLAG-53. Bound large T-antigen was subsequently determined with a rapid and quantitative colorimetric ELISA. The results demonstrate that the Anti-FLAG M2 plate offers a much faster and simpler procedure than conventional immunoprecipitations using affinity resin. The 96-well format can greatly increase the number of samples that can be analyzed in parallel. The effective capture of FLAG-tagged fusion protein from crude in vitro translation mixture coupled with highly sensitive, specific, and quantitative detection of the interacting protein partners makes the microplate a more effective platform for high-throughput assay development for the study of molecular interactions.

Materials and methods

Unless otherwise indicated, all of the materials used in this research were from **Sigma-Aldrich Corp.**

Microplate

The Anti-FLAG M2 plate was prepared by covalently attaching the M2 antibody (mouse IgG1) to the surface of a 96-well clear plate via the Fc portion of the antibody. This attachment provides a favorable orientation for the antibody on the surface for maximum capture of FLAG-tagged fusion proteins. The maximum binding capacity per well of the plate was determined by using purified FLAG-tagged bacterial alkaline phosphatase protein (FLAG-BAP), and was estimated to be about 400 ng or 1 pmol per well. In addition, detection sensitivity (based on a standard BAP enzyme activity assay)[12] is about 1 ng or 20 fmol. The standard deviation of binding capacity from plate to plate and well to well is less than 10% (data not shown).

Constructs

Two constructs were used in this work to generate in vitro translated FLAG-tagged fusion proteins. pCITE-FLAG-p53 contains FLAG fused to the N-terminus of the wild-type C terminal domain of mouse p53, and pCITE-FLAG-BAP containing FLAG fused to the N-terminus of BAP. pCITE-FLAG-p53 was a derivative of pFLAG-CMV-2-p53. pFLAG-CMV-2-p53 is a mammalian expression vector encoding an N-terminal FLAG-tagged p53 and was constructed by amplifying DNA encoding residues 72–390 of mouse p53 from plasmid pM-53 DNA-BD vector (**Clontech,** Palo Alto, CA). pCITE-FLAG-p53 was prepared by amplifying FLAG-p53 from plasmid pFLAG-CMV-2-p53 using a pair of primers p53 forward (5′–CTAGGATATCGACTACAAAGACGATGACGACAAG-3′) and p53 reverse (5′–CTAGGAATTCGTCTGAGTCAGGCCCCACTTTCTTG-3′). The resulting polymerase chain reaction (PCR) fragments containing E*co*R V and E*co*R I in the forward and reverse primers, respectively, were then double digested with E*co*R V and E*co*R I and ligated into the E*co*R V and E*co*R I sites of pCITE-2a(+) (**Novagen,** Madison, WI). pCITE-FLAG-BAP was constructed by amplifying FLAG-BAP from plasmid pFLAG-CMV-2-BAP using oligos BAP forward (5′-CTAGGATATCGACTACAAAGACGATGACGACAAG-3′) and BAP reverse (5′-CTAGGGATCCTCAGCCCCAGAGCGGCTTTCATGG-3′). Additional bases corresponding to E*co*R V and B*am*H I were incorporated in the forward and reverse primers, respectively. The resulting PCR fragments were then double digested with E*co*R V and B*am*H I and ligated into the E*co*R V and B*am*H I sites of pCITE-2a(+). Both PCR reactions were performed with ReadyMix™ Taq PCR reaction mix. The conditions for PCR were 50 sec denature (94 °C), 1 min annealing (55 °C), and 2 min extension (72 °C) for 25 cycles. The sequence of the resulting plasmids, pCITE-FLAG-p53 and pCITE-FLAG-BAP, were confirmed using the Big dye terminator cycle sequencing kit and an ABI 373 DNA sequencer (**Applied Biosystems,** Foster City, CA).

Cells

COS-7 and HeLa cell lines were obtained from **American Type Culture Collection (ATCC,** Manassas, VA). Cells were maintained at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 µg/mL streptomycin, and 500 mg/L L-glutamine. Cells were cultured in a humidified, 5% CO_2 atmosphere tissue culture incubator and subcultured twice a week using trypsin-EDTA (ethylenediaminetetraacetic acid) (0.05%, 0.53 m*M*) solution.

Preparation of cell extract

Growth medium was aspirated from the transfected cells to be assayed and the cells were rinsed twice with ice-cold phosphate buffered saline (PBS). Ice-cold lysis buffer (1.0 mL) supplemented with mammalian protease inhibitors (10 µL per 1 mL lysis buffer) was added to a 10-cm plate. The lysis buffer contained 20 m*M* tris-HCl, pH 7.4, 1 *M* NaCl, 1 m*M* dithiothreitol (DTT), 1.0% Triton X-100. Cells were removed from the plate with cell scrapers, transferred to microcentrifuge tubes, and incubated on ice for 1 hr, followed by centrifugation at 16,000 × *g* for 10 min at 4 °C. Total protein concentration in the cell extract was determined by the Bicinchoninic acid kit for protein determination.

In vitro transcription and translation

Plasmids pCITE-FLAG-p53 and pCITE-FLAG-BAP were linearized with X*ba* I, and mRNAs were synthesized using the MEGAscript™ kit according to the manufacturer's instructions (**Ambion,** Austin, TX). The transcripts were purified with LiCl, and in vitro translation was carried out with 1 µg of the purified transcript in 25 µL reaction mixture supplemented with 25 µL methionine and 10 µL Red Nova Lysate (**Novagen**) for 1 hr at 30 °C.

Capture in vitro translated FLAG-53 and FLAG-BAP on Anti-FLAG M2 plate

The Anti-FLAG M2 plate was washed once with wash buffer containing 20 m*M* tris-HCl (pH 7.4) and 0.15 *M* NaCl to rehydrate the plate. The plate was then incubated with varying amounts of in vitro translation mixture prepared as above on a rotary shaker for 1 hr at 4 °C. Nonspecific proteins were removed by washing the plate four times with wash buffer.

Examination of the captured FLAG-p53 and FLAG-BAP in Western blotting

The captured proteins were eluted from the microplate by incubating with 60 µL of 2× sodium dodecyl sulfate (SDS) sample buffer containing 125 m*M* tris-HCl (pH 6.8), 4% SDS, 10% β-mercaptoethanol, 20% glycerol, and 0.004% bromophenol blue for 20 min at room temperature. Samples were boiled for 5 min and analyzed by Western blotting as described.

In vitro binding of large T-antigen to the captured FLAG-p53 on the Anti-FLAG M2 plate

Various amounts of in vitro translated FLAG-

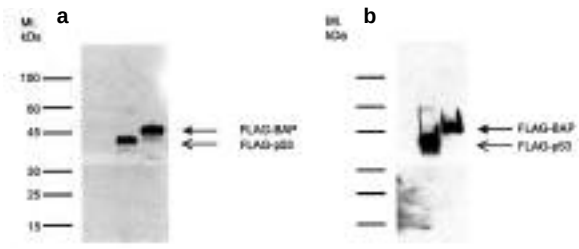

Figure 1 *Western blot analysis of in vitro translated FLAG-tagged proteins and the immunoprecipitates from the Anti-FLAG M2 plate. a) Equal amounts of in vitro translation reaction (15 µL/lane) were resolved by SDS-PAGE and analyzed by Western blotting.b) Forty microliters of in vitro translation reactions used in (A) was incubated with the Anti-FLAG M2 plate for 1 hr at 4 °C. Bound proteins were eluted with 2× SDS sample buffer at room temperature and were subjected to Western blotting with Anti-FLAG M2-HRP conjugate. Reactive bands were developed with the ECL plus detector. Lane 1: In vitro translation reaction with no RNA. Lane 2: In vitro translation reaction with in vitro transcribed FLAG-p53 RNA. Lane 3: In vitro translation reaction with in vitro transcribed FLAG-BAP RNA. In vitro translated FLAG-tagged p53 and BAP are indicated by light arrow and heavy arrow, respectively.*

tagged fusion protein were incubated with the plate as described earlier. At the end of the incubation, the plate was washed four times with 200 µL of the wash buffer. The FLAG-p53 binding reaction was performed in the wells with the immobilized FLAG-tagged fusion proteins in a final volume of 200 µL. The in vitro binding reaction was initiated by adding 50 µL (100 µg) of cell extract prepared from COS-7 cells or HeLa cells to the wells as described earlier, and the plate was incubated at 37 °C for 2 hr. The reaction was terminated by washing the plate four times with the wash buffer. To detect bound large T-antigen, biotin-conjugated mouse anti-SV40 large T- and small T-antibody (clone Pab108) (**Research Diagnostics Inc.,** Flanders, NJ) (1:10,000 in blocking buffer) was added at 200 µL/well and incubated for 3 hr at room temperature. Blocking buffer contained 3% nonfat milk in TBST (tris buffered saline with Tween 20, pH 8.0). After the plates were washed three times with the wash buffer, 200 µLof peroxidase-conjugated streptavidin (1:1000 in blocking buffer) was added. At the end of the incubation, the plates were washed four times with the wash buffer followed by incubation with peroxidase substrate 3,3′,5,5′-tetramethylbenzidine (TMB) at 200 µL/well for 30 min. Reactions were stopped with 100 µL of H_2SO_4. The absorbance was read at 450 nm (OD_{450}).

Western blotting and antibodies

Western blot analysis was carried out following standard procedures. Briefly, proteins were separated on a 4–20% gradient SDS-PAGE (polyacrylamide gel electrophoresis) gel and electrotransferred to immobilon-P polyvinyliden difluoride (PVDF) membrane. The membranes were incubated with Anti-FLAG M2 monoclonal antibody peroxidase (horseradish peroxidase, HRP) conjugate (1:2000 in blocking buffer) for 1 hr at room temperature. All subsequent washes were done in TBS buffer containing 0.05% Tween 20 at room temperature. Following the washing steps, reactive bands were detected with the ECLplus™ electrochemiluminescent detector according to the manufacturer's instructions (**Amersham Pharmacia Biotech Inc.,** Piscataway, NJ).

Results

Western blotting revealed that in vitro transcription and translation of plasmids pCITE-FLAG-p53 and pCITE-FLAG-BAP resulted in FLAG-tagged p53 and BAP fusion proteins exhibiting expected molecular weights of 42 and 52 kD, respectively (*Figure 1a*). No immunoreactive bands were evident when only Red Nova Lysate was used (lane 1). In order to determine whether the Anti-FLAG M2 plate can capture in vitro translated FLAG-tagged proteins from crude in vitro translation reaction, bound protein was eluted from the wells with 2× SDS sample buffer and was subjected to Western blot analysis using the M2 antibody. As shown in Figure 1*b*, both FLAG-tagged proteins were specifically immunocaptured by the microplate.

An important aspect of the plate for the analysis of protein–protein interactions is not only the ability to capture the fusion protein, but also the functional consequence of capturing. To this end, an in-well in vitro binding assay was developed, in which binding activity of the captured FLAG-p53 can be analyzed by incubating the COS-7 cell extracts enriched in large T-antigen in the same well followed by direct detection of bound large T-antigen in an ELISA analysis using anti-SV40 large T-antigen antibody. As for the controls, extra wells were incubated either with the highest amount of Red Nova Lysate used for the binding assay or in vitro translated FLAG-BAP. The in vitro binding reaction and detection were processed the same way as for the wells incubated with in vitro translated FLAG-p53.

As shown in *Figure 2*, large T-antigen from the COS-7 cell extracts specifically binds to the wells incubated with in vitro translated FLAG-p53. The binding is quantitative, since the amount of bound large T-antigen is dependent on the amount of captured FLAG-p53. The assay is sensitive; the signal can be detected in the wells incubated with as little as 5 µL of the FLAG-p53 translation reaction. Furthermore, the binding was very specific, since no measurable signal was detected in comparison to the controls, in which the same amount of COS-7 cell extracts was used to incubate with the wells used to capture FLAG-BAP. Specificity was further demonstrated when wells containing the captured highest amount of in vitro translated FLAG-p53 failed to generate any signals above the background when the same amount of HeLa cell extracts was used for the FLAG-p53 binding reaction (Figure 2).

Discussion

The utility of the Anti-FLAG M2 plate was demonstrated for the capture of FLAG-tagged fusion protein and subsequently in-well studying its interactions with other proteins. The results show that the microplate system is a convenient, sensitive, specific, and quantitative method for studying such interactions. Since the Anti-FLAG M2 antibody is covalently coupled to the plate and uniformly distributed throughout the wells, the uniform capture of FLAG-tagged fusion proteins is easily attained. Because the captured fusion proteins still retain the binding activity, binding to suspect binding partners can be measured quantitatively. These features make the plate an effective platform for which to design high-throughput analysis to identify potential binding proteins or particular compounds that induce or inhibit protein–protein interactions.

To further demonstrate the utility of the microplate in the analysis of protein–protein interactions, the plate-assay format was used successfully to study another well-known protein pair, p65 of the nuclear factor NF-κB complex and the NF-κB inhibitor IκBα. In that experiment, p65 was in vivo expressed as a FLAG-tagged fusion protein in HeLa cells. The Anti-FLAG M2 plate was used to capture the FLAG-tagged p65 along with its endogenous interacting protein IκBα from the FLAG-p65-transfected HeLa cells. Detection was achieved directly on the plate by ELISA analysis using a specific antibody against IκBα.

The Anti-FLAG M2 plate-based system not only can be used for the analysis of stable protein–protein interactions, but is also useful for the analysis of transient protein interactions such as protein kinase activity. The Anti-FLAG M2 plate-based kinase assay is utilized for the study of mitogen-activated protein kinase (MAPK) activity. The plate allows the rapid and quantitative determination of phosphorylation of FLAG-MAPK directly from stimulated, transfected cell lysate. Furthermore, captured phospho-FLAG-MAPK still retains its functionality to catalyze its substrate in the same well used to capture phospho-FLAG-MAPK. Finally, captured nonphosphorylated-FLAG-MAPK can be phosphorylated in the same well by upstream regulators that use MAPK as substrate.

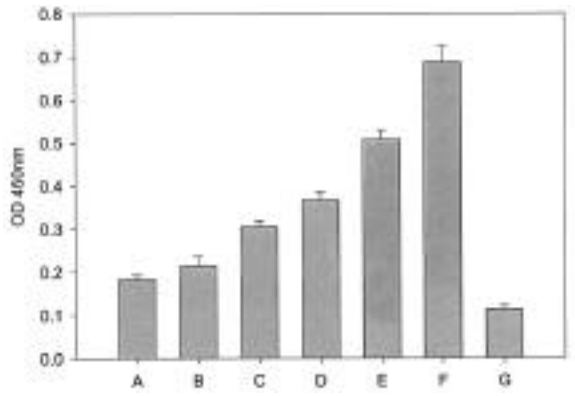

Figure 2 *In-well binding of large T-antigen by in vitro translated FLAG-p53 on the Anti-FLAG M2 plate. Varying amounts of in vitro translation reaction expressing FLAG-p53 were incubated with the Anti-FLAG M2 plate for 1 hr at 4 °C. 40 µL of Red Nova Lysate or 40 µL of in vitro translation reaction expressing FLAG-BAP was included as the control. The reaction was initiated by applying 50 µg of COS-7 cell extracts or 50 µg of HeLa cell extracts and was allowed to occur at 37 °C for 2 hr. Detection of the bound large T-antigen was obtained as described in Materials and methods. a) Forty microliters of Red Nova Lysate and COS-7 cell extracts; b) 40 µL of in vitro translated FLAG-BAP and COS-7 cell extracts; c) 5 µL of in vitro translated FLAG-p53 and COS-7 cell lysate; d) 10 µL of in vitro translated FLAG-p53 and COS-7 cell extracts; e) 25 µL of in vitro translated FLAG-p53 and COS-7 cell extracts; f) 40 µL of in vitro translated FLAG-p53 and COS-7 cell extracts;and g) 40 µL of in vitro translated FLAG-p53 and HeLa cell extracts (error bars: SD of three wells).*

Although only data on the analysis of protein–protein interactions using the Anti-FLAG M2 plate strategy are demonstrated, the system should find wider use for high-throughput applications in bioassay development and many other molecular interactions.

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