METHODS IN MOLECULAR BIOLOGY™

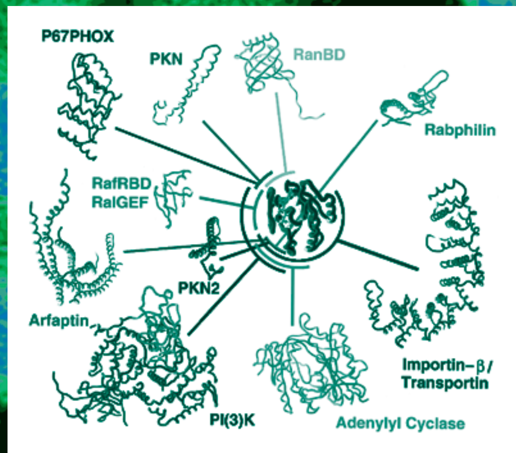
Volume 261

Protein–Protein Interactions

Methods and Applications

Edited by

Haian Fu



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Studying Protein–Protein Interactions via Blot Overlay or Far Western Blot

Randy A. Hall

Abstract

Blot overlay is a useful method for studying protein–protein interactions. This technique involves fractionating proteins on SDS-PAGE, blotting to nitrocellulose or PVDF membrane, and then incubating with a probe of interest. The probe is typically a protein that is radiolabeled, biotinylated, or simply visualized with a specific antibody. When the probe is visualized via antibody detection, this technique is often referred to as "Far Western blot." Many different kinds of protein–protein interactions can be studied via blot overlay, and the method is applicable to screens for unknown protein–protein interactions as well as to the detailed characterization of known interactions.

Key Words

Protein–protein interactions; blot overlay; Far Western blot; protein; receptor; association; nitrocellulose; SDS-PAGE; binding.

1. Introduction

During preparation for SDS-PAGE, proteins are typically reduced and denatured via treatment with Laemmli sample buffer ***(1)***. Because many protein–protein interactions rely on aspects of secondary and tertiary protein structure that are disrupted under reducing and denaturing conditions, it might seem likely that few if any protein–protein interactions could survive treatment with SDS-PAGE sample buffer. Nonetheless, it is well known that many types of protein–protein interaction do in fact still occur even after one of the partners has been reduced, denatured, run on SDS-PAGE, and Western blotted. Blot overlays are a standard and very useful method for studying interactions between proteins.

From: *Methods in Molecular Biology, vol. 261: Protein–Protein Interactions: Methods and Protocols*
Edited by: H. Fu © Humana Press Inc., Totowa, NJ

In principle, a blot overlay is similar to a Western blot. For both procedures, samples are run on SDS-PAGE gels, transferred to nitrocellulose or PVDF, and then overlaid with a soluble protein that may bind to one or more immobilized proteins on the blot. In the case of a Western blot, the overlaid protein is antibody. In the case of a blot overlay, the overlaid protein is a probe of interest, often a fusion protein that is easy to detect. The overlaid probe can be detected either via incubation with an antibody (this method is often referred to as a "Far Western blot"), via incubation with streptavidin (if the probe is biotinylated), or via autoradiography if the overlaid probe is radiolabeled with ^{32}P (also see Chapter 31). The specific method that will be described here is a Far Western blot overlay that was used to detect the binding of blotted hexahistidine-tagged PDZ domain fusion proteins to soluble GST fusion proteins corresponding to adrenergic receptor carboxyl-termini ***(2)***. However, this method may be adapted to a wide variety of applications.

2. Materials

1. SDS-PAGE mini-gel apparatus (Invitrogen).
2. SDS-PAGE 4–20% mini gels (Invitrogen).
3. Western blot transfer apparatus (Invitrogen).
4. Power supply (BioRad).
5. Nitrocellulose (Invitrogen).
6. SDS-PAGE prestained molecular weight markers (BioRad).
7. SDS-PAGE sample buffer: 20 m*M* Tris-HCl, pH 7.4, 2% SDS, 2% β-mercaptoethanol, 5% glycerol, 1 mg/mL bromophenol blue.
8. SDS-PAGE running buffer: 25 m*M* Tris-HCl, pH 7.4, 200 m*M* glycine, 0.1% SDS.
9. SDS-PAGE transfer buffer: 10 m*M* Tris-HCl, pH 7.4, 100 m*M* glycine, 20% methanol.
10. Purified hexahistidine-tagged fusion proteins.
11. Purified GST-tagged fusion proteins.
12. Anti-GST monoclonal antibody (Santa Cruz Biotechnology, cat. no. sc-138).
13. Goat anti-mouse HRP-coupled secondary antibody (Amersham Pharmacia Biotech).
14. Blocking buffer: 2% nonfat powdered milk, 0.1% Tween-20 in phosphate-buffered saline, pH 7.4.
15. Enhanced chemiluminescence kit (Amersham Pharmacia Biotech).
16. Blot trays.
17. Rocking platform.
18. Autoradiography cassette.
19. Clear plastic sheet.
20. Film.

3. Methods

3.1. SDS-PAGE and Blotting

The purpose of this step is to immobilize the samples of interest on nitrocellulose or an equivalent matrix, such as PVDF. It is very important to keep the

blot clean during the handling steps involved in the transfer procedure, because contaminants can contribute to increased background problems later on during detection of the overlaid probe.

1. Place gel in SDS-PAGE apparatus and fill chamber with running buffer.
2. Mix purified hexahistidine-tagged fusion proteins with SDS-PAGE sample buffer to a final concentration of approx 0.1 μg/μL of fusion protein (*see* **Note 1**).
3. Load 20 μL of fusion protein (2 μg total) in each lane of the gel. If there are more lanes than samples, load 20 μL of sample buffer in the extra lanes (*see* **Note 2**).
4. In at least one lane of the gel, load 20 μL of SDS-PAGE molecular weight markers.
5. Run gel for approx 80 min at 150 V using the power supply.
6. Stop gel, turn off the power supply, remove the gel from its protective casing, and place in transfer buffer.
7. Place precut nitrocellulose in transfer buffer to wet it.
8. Put nitrocellulose and gel together in transfer apparatus, and transfer proteins from gel to nitrocellulose using power supply for 80 min at 25 V.

3.2. Overlay

During the overlay step, the probe is incubated with the blot and unbound probe is then washed away. The potential success of the overlay depends heavily on the purity of the overlaid probe. GST and hexahistidine-tagged fusion proteins should be purified as extensively as possible. If the probe has many contaminants, this may contribute to increasing the background during the detection step, making visualization of the specifically bound probe more difficult.

1. Block blot in blocking buffer for at least 30 min (*see* **Note 3**).
2. Add GST fusion proteins to a concentration of 25 n*M* in 10 mL blocking buffer.
3. Incubate GST fusion proteins with blot for 1 h at room temperature while rocking slowly.
4. Discard GST fusion protein solution and wash blot three times for 5 min each with 10 mL of blocking buffer while rocking the blot slowly.
5. Add anti-GST antibody at 1:1000 dilution (approx 200 ng/mL final) to 10 mL blocking buffer.
6. Incubate anti-GST antibody with blot for 1 h while rocking the blot slowly.
7. Discard anti-GST antibody solution and wash blot three times for 5 min each with 10 mL of blocking buffer while rocking the blot slowly.
8. Add goat anti-mouse HRP-coupled secondary antibody at 1:2000 dilution to 10 mL blocking buffer.
9. Incubate secondary antibody with blot for 1 h while rocking the blot slowly.
10. Discard secondary antibody solution and wash blot three times for 5 min each with 10 mL of blocking buffer while rocking the blot slowly (*see* **Note 4**).
11. Wash blot one time for 5 min with phosphate buffered saline, pH 7.4.

3.3. Detection of Overlaid Proteins

The final step of the overlay is to detect the probe that is bound specifically to proteins immobilized on the blot. In viewing different exposures of the visualized probe, an effort should be made to obtain the best possible signal-to-noise ratio. Nonspecific background binding will increase linearly with time of exposure. Thus, shorter exposures may have more favorable signal-to-noise ratios.

1. Incubate blot with enhanced chemiluminescence solution for 60 s (*see* **Note 5**).
2. Remove excess ECL solution from blot and place blot in clear plastic sheet.
3. Tape sheet into autoradiography cassette.
4. Move to darkroom and place one sheet of film into autoradiography cassette with blot.
5. Expose film for 5–2000 s, depending on intensity of signal.
6. Develop film in standard film developer.

4. Notes

1. The protocol described here is intended for the in-depth study of a protein–protein interaction that is already known. However, blot overlays can also be utilized in preliminary screening studies to detect novel protein–protein interactions. For this application, tissue lysates would typically be loaded onto the SDS-PAGE gel instead of purified fusion protein samples. The blotted tissue lysates would then be overlaid with the probe of interest. The advantages of this technique are (i) many tissue samples can be screened in a single blot and (ii) the molecular weight and tissue distribution of probe-interacting proteins can be immediately determined. The disadvantages of this method are (i) due to the multiple washing steps involved in the procedure, a fairly high affinity interaction is required for the interaction to be detected, (ii) detection of probe-interacting proteins is dependent on their level of expression in native tissues, and (iii) interactions requiring native conformations of both proteins will not be detected. Tissue lysate overlays have been utilized as screening tools to detect not only the interaction of the β_1-adrenergic receptor with MAGI-2 described here ***(2)*** (**Fig. 1**), but also the interaction of the β_2-adrenergic receptor with NHERF ***(3)*** and the interactions of a number of different proteins with actin ***(4–6)***, calmodulin ***(7,8)***, and the cyclic AMP-dependent protein kinase RII regulatory subunit ***(9–12)***.
2. Because some probes can exhibit extensive nonspecific binding to blotted proteins, it is important in overlay assays to have negative controls for probe binding. When the blotted proteins are GST fusion proteins, GST by itself is a good negative control (as illustrated in **Fig. 2**). When the blotted proteins are His-tagged fusion proteins, as illustrated in **Fig. 1**, it is helpful to have one or more His-tagged fusion proteins on the same blot that will not bind to the probe. In this way, it is possible to demonstrate the specificity of binding and to rule out the possibility that the observed interaction is due to the tag.

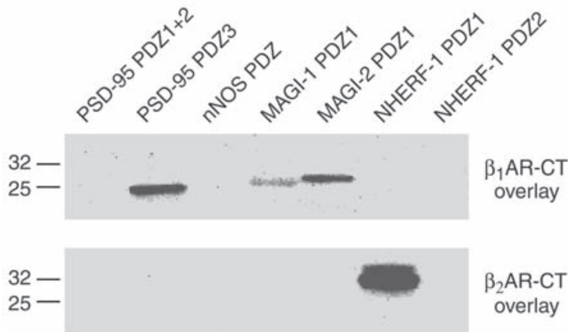

Fig. 1. Overlay of GST-tagged adrenergic receptor carboxyl-termini onto hexahistidine-tagged PDZ domains. Equal amounts (2 μg) of purified His-tagged fusion proteins corresponding to PDZ domains from PSD-95, nNOS, MAGI-1, MAGI-2, and NHERF-1 were immobilized on nitrocellulose. Overlays with the carboxyl-terminus of the β_1-adrenergic receptor expressed as a GST fusion protein (β_1AR-CT-GST) (25 n*M*) revealed strong binding to PSD-95 PDZ3 and MAGI-2 PDZ1, moderate binding to MAGI-1 PDZ1, and no detectable binding to the first two PDZ domains of PSD-95 or to the PDZ domains of nNOS or NHERF-1. In contrast, overlays with the β_2-adrenergic receptor expressed as a GST fusion protein (β_2AR-CT-GST) (25 n*M*) revealed strong binding to NHERF-1 PDZ1 but no detectable binding to any of the other PDZ domains examined. These data demonstrate that selective and specific binding can be obtained in overlay assays.

3. The blocking of the blot is a very important step in every overlay assay. The idea is to block potential nonspecific sites of protein attachment to the blot, so that nonspecific binding of the probe will be minimized. When a high amount of nonspecific background binding is observed, it is often helpful to block for a longer time or with a higher concentration of milk. Some investigators favor bovine serum albumin or other proteins in place of milk for blocking blots prior to overlay.
4. The washing of the blot is of critical importance. If the washes are not rigorous enough, the nonspecific background binding of the probe will be undesirably high. Conversely, if the washes are too rigorous, specific binding of the probe may be lost and the protein–protein interaction of interest may be difficult to detect. Thus, if a large amount of nonspecific background binding is observed, one should consider increasing the rigor of the washes, whereas, conversely, if the background is low but little or no specific binding is observed, one should consider decreasing the rigor of the washes. The rigor of the washes is dependent on (i) time, (ii) volume, (iii) speed, and (iv) detergent concentration. To make washes more rigorous, one should wash for a longer time, wash in a larger volume, increase the rate at which the gels are rocked during the washes, and/or increase the detergent concentration in the buffer used for washing.

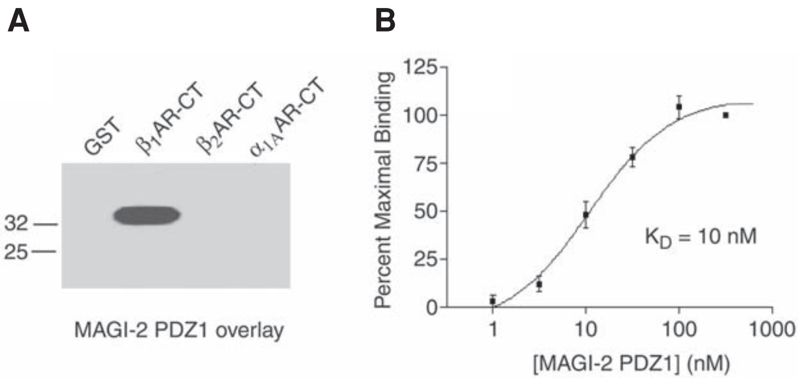

Fig. 2. Overlay of hexahistidine-tagged MAGI-2 PDZ1 onto GST-tagged adrenergic receptor carboxyl-termini. **(A)** In the reverse of the overlay experiments illustrated in Fig. 1, equal amounts (2 μg) of purified GST fusion proteins corresponding to the carboxyl-termini of various adrenergic receptor subtypes were immobilized on nitrocellulose. Overlay with MAGI-2 PDZ1 His- and S-tagged fusion protein (20 n*M*) revealed strong binding to β_1AR-CT-GST but no detectable binding to control GST, β_2AR-CT-GST or α_1AR-CT-GST. These data demonstrate that the interaction between the β_1AR-CT and MAGI-2 PDZ1 can be visualized via overlay in either direction. **(B)** Estimate of the affinity of the interaction between β_1AR-CT and MAGI-2 PDZ1. Nitrocellulose strips containing 2 μg β_1AR-CT-GST (equivalent to lane 2 in the preceding panel) were incubated with His/S-tagged MAGI-2 PDZ1 at six concentrations between 1 and 300 n*M*. Specific binding of MAGI-2 PDZ1 did not increase between 100 and 300 n*M*, and thus the binding observed at 300 n*M* was defined as "maximal" binding. The binding observed at the other concentrations was expressed as a percentage of maximal binding within each experiment. The bars and error bars shown on this graph indicate mean ± SEM (n = 3). The K_d for MAGI-2 PDZ1 binding to β_1AR-CT was estimated at 10 n*M* (*see* **Note 6**).

5. There are a number of ways to visualize bound probe in an overlay assay. The method described here depends on detection of the probe with an antibody, which is often referred to as a "Far Western blot." One alternative approach is to biotinylate the probe and then detect it with a streptavidin/enzyme conjugate ***(5–7)***. The appeal of this approach is that it can be quite sensitive, because the streptavidin–biotin interaction is one of the highest affinity interactions known. The main drawback of this approach is that biotinylation of the probe may alter its properties, such that it may lose the ability to interact with partners it normally binds to. An additional approach to probe detection is phosphorylation of the probe using ^{32}P-ATP, to make the probe radiolabeled ***(10–12)***. A primary advantage of this method is that once the probe is overlaid onto the blot, no

further detection steps are necessary (i.e., no incubations with antibody or streptavidin are required). This cuts down on the number of washing steps and may aid in the detection of protein–protein interactions that are of somewhat lower affinity. The main disadvantages of the phosphorylation approach are (i) radioactive samples require special handling and (ii) as with biotinylation, phosphorylation of the probe may alter its properties, such that certain protein–protein interactions may be disrupted.

6. As is illustrated in **Figs. 1** and **2**, detection of the interactions between adrenergic receptor carboxyl-termini and their PDZ domain-containing binding partners are completely reversible. Either partner can be immobilized on the blot and overlaid with the other. Many other protein–protein interactions can similarly be detected in a reversible manner, but some interactions can only be detected in one direction due to a requirement for the native conformation of one of the partners. As is also illustrated in **Fig. 2**, the affinity of a given protein–protein interaction may be estimated via blot overlay saturation binding curves. This method involves increasing the concentration of overlaid probe until a maximal amount of specific binding is obtained. An estimate for the affinity constant (K_d) of the interaction can then be determined from the slope of the binding curve, much as one would determine K_d values from ligand binding curves using a program such as GraphPad Prism. Estimates such as these must be evaluated with the caveat that they are derived under artificial conditions involving many hours of incubation time, washing, and detection. Nonetheless, affinity constant estimates derived via this method are useful in comparing affinities between proteins examined under the same conditions and overlaid with the same probe.

Acknowledgments

R.A.H is supported by grants from the National Institutes of Health (GM60982, HL64713) and a Faculty Development award from the Pharmaceutical and Manufacturers of America Foundation.

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