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Clean western blot signals from immunoprecipitated samples

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Abstract

We present a strategy that overcomes the high background arising during Western blotting (WB) detection of proteins obtained through immunoprecipitation (IP). Traditional HRP-conjugated secondary antibodies, which detect the *denatured* heavy and light antibody chains, produce high background that often mask the signals of interest on WBs. Here, we show that HRP-conjugated Protein A and Protein G, which detect almost exclusively *intact* antibody molecules, can be effectively used to obtain clean and specific WB signals of target proteins.

Keywords

Heavy Ig chain; Light Ig chain; Protein A-HRP; Protein-G-HRP; Background western signals

1. Introduction

Immunoprecipitation (IP) is a well-established method where by a protein of interest is selectively purified from a complex mixture using a specific antibody. IP can be used for different purposes, including (a) determination of the abundance and molecular weight of the immunoprecipitated protein; (b) assessment of post-translational modifications (e.g. glycosylation, phosphorylation, or acetylation) of the protein of interest; (c) quantification of the rates of protein synthesis or degradation by pre-incubation with ^{35}S-radiolabeled amino acids; and (d) identification of other macromolecules such as proteins, RNA, or DNA interacting with the protein of interest [1-4]. During IP, an antibody (monoclonal or polyclonal) is allowed to form an immune complex with the antigen it recognizes, typically a protein present in a suspension (e.g. cell lysate). The immune complex is then captured on a solid support (e.g. agarose or sepharose) carrying immobilized Protein A or Protein G. After washing away any proteins not bound by the immobilized Protein A or G, the components of the immune complex (antibody and antigen) are eluted and analyzed by SDS-PAGE, often followed by Western blot analysis (WB) to visualize the protein of interest.

A frequent problem with WB using IP samples is the appearance of very high background signals. This problem arises because the same antibody (or an antibody from the same species) is often used in both IP and WB; since the heavy chain (HC, 55 kDa) and light chain (LC, 25 kDa) of the IP antibody are also eluted from the beads and size-fractionated by SDS-PAGE with the antigen, the secondary antibody used to detect the primary antibody on WBs will also recognize the HC/LC bands on WB filters. If the antigen size is comparable to either the HC or the LC, then the antigen band may be masked, since HC and LC molecules are much more abundant than the antigen. To circumvent this problem, investigators have devised a variety

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of strategies. Methodologies based on the use of ^{35}S-methionine and ^{35}S-cysteine to label cells and isotopically visualize the protein after IP and SDS-PAGE are adequate when the antigen is abundant and the antibody is highly sensitive and specific; however, this approach requires the use of high doses of radioactivity and cannot detect low-abundance proteins or proteins lacking methionine and cysteine residues [4]. In other protocols, the primary antibody is covalently crosslinked to Protein A- or Protein G-Sepharose beads before IP [5], a process that entails additional standardization and expense. Other approaches exclude reducing agents from the IP buffers and avoid boiling the samples in order to preserve the antibody tetrameric structure, which then migrates as a 160-kDa complex; the chief limitation of this procedure is that without reducing agents and heat, the antigen may not be effectively released from the antibody. Finally, investigators perform WB with antibodies from different species to avoid cross-reactivity, but such antibodies are not always available and their routine use is also more costly.

Here, we report a strategy that overcomes the aforementioned limitations. We show that horse radish peroxidase (HRP)-conjugated Protein A or Protein G can be used for WB detection instead of the traditional HRP-conjugated secondary antibody. Like a secondary antibody, Protein A and Protein G-HRP bind the Fc portion of the primary antibody [6] but, unlike a secondary antibody, which also binds the *denatured* individual HC and LC polypeptides on WB filters, Protein A-and protein A-HRP binds only (and Protein G preferentially) to the *intact* IgG molecule. Accordingly, WB analysis using Protein A-or Protein G-HRP yields clean and specific signals corresponding to the antigen.

2. Materials and methods

2.1. Cell culture and treatment

RKO cells were cultured (at 37 °C, 5% Co_2) in minimum essential medium supplemented with 10% fetal bovine serum and antibiotics. For the detection of p53, cells were irradiated with 15 J/m^2 UVC.

2.2. Preparation of cell lysates

Ten million RKO cells were rinsed with PBS and lysed in 500 μl RIPA buffer to obtain whole-cell lysates [7]. Whole-cell lysates (~5 μg/μl) were stored at -20 °C or used directly for IP.

2.3. Antibodies

Antibodies recognized AUF1 (rabbit polyclonal, Upstate Biotech.), procaspase-3 (rabbit polyclonal, Santa Cruz Biotech.), p53 (rabbit polyclonal, Santa Cruz Biotech.), α-tubulin (mouse monoclonal IgG2a, Santa Cruz Biotech.), or TIAR (goat polyclonal, Santa Cruz Biotech.).

2.4. Immunoprecipitation

For IP, Protein A-Sepharose beads (Sigma) were coated with the appropriate antibodies (above), then incubated with 100 μl (500 μg) of whole-cell lysate and 400 μl NT2 buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM $MgCl_2$, and 0.05% Nonidet P-40) and mixed gently for 2 h at 4 °C. Beads were washed four times (1 ml NT2 buffer each), and centrifuged at 5000×*g* for 5 min.

2.5. Western blot analysis

Proteins were eluted by adding 100 μl 2× SDS-PAGE sample buffer and heating at 95 °C for 10 min. Samples were size-separated by electrophoresis in SDS-containing (12%) polyacrylamide gels (NuPAGE Bis-Tris gels, Invitrogen) and transferred to nitrocellulose

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membranes (Invitrogen). Membranes were blocked at 25 °C for 30 min with PBST (PBS plus 0.1% Tween-20) containing 5% skim milk powder. Blots were then incubated (25 °C, 1 h) with primary antibodies recognizing AUF1 (diluted 1:1000), procaspase-3 (diluted 1:500), p53 (1:500), α-tubulin (diluted 1:500), or TIAR (diluted 1:500). Blots were washed three times (5 min each) with PBST. For standard Western blotting detection, blots were incubated with either an anti-rabbit HRP-conjugated antibody (1:10,000, Amersham), anti-mouse HRP-conjugated antibody (1:10,000, Amersham), or an anti-goat HRP-conjugated antibody (1:40,000, Sigma), for 1 h at 25 °C. For improved Western blotting detection to avoid HC and LC signals, blots were incubated (1 h at 25 °C) with a 1:5,000 dilution of Protein A-HRP (Amersham) or a 1:10,000 dilution of Protein G-HRP (Upstate Biotech.), prepared in blocking buffer. After washing three times at 25 °C with PBST (5 min each), blots were developed with ECL Plus (Amersham).

3. Results and discussion

To illustrate the usefulness of this methodology, lysates were prepared from the human colorectal cancer line RKO, and IP reactions followed by WB analyses were performed to detect proteins whose sizes overlapped with those of the HC or the LC. The proteins chosen for analysis were p53 (a protein that comigrates with the HC), AUF1 (expressed as four isoforms -p37, p40, p42, p45- that migrate between the HC and LC), procaspase-3 (which migrates close to the LC), α-tubulin (migrates close to the HC), and TIAR (migrates close to the HC). RKO cells were treated with ultraviolet light (UVC, 15 J/m^2) for p53 analysis, and were left untreated for the analysis of AUF1, procaspase-3, α-tubulin, and TIAR.

After IP with the corresponding antibodies, samples were size-fractionated by SDS-PAGE and transferred onto nitrocellulose filters. The primary antibodies used for WB analysis of the proteins of interest were the same as those used for IP. WB detection of the proteins of interest was then performed in parallel using either classical HRPconjugated secondary antibodies or HRP-conjugated Protein A or Protein G. WB signals resulting from the use of HRP-conjugated secondary antibodies showed extensive background, mainly because the secondary antibodies detected the HC (and sometimes the LC) bands of all primary antibodies: rabbit polyclonal (Fig. 1A, *left*), mouse monoclonal (Fig. 1B, *left*), or goat polyclonal (Fig. 1C, *left*). By contrast, WB signals resulting from use of Protein A-HRP were dramatically clearer than those resulting from use of anti-rabbit secondary antibodies. As shown, p53, AUF1, and procaspase-3 (Fig. 1A, *right*), as well as α-tubulin (Fig. 1B, *right*) were detected with virtually no contaminating signals from the HC or LC bands. Similarly, WB detection of TIAR was significantly more clear when using Protein G-HRP (Fig. 1C, *right*) than when using an HRP-conjugated anti-goat secondary antibody (Fig. 1C, *left*); Protein G-HRP did detect the HC, albeit with low intensity.

The use of protein A-HRP is particularly valuable for detecting p53 and α-tubulin, as these proteins would be virtually impossible to visualize after IP with a secondary antibody. This approach was also useful for elucidating the optimal amount of IP antibody necessary for WB detection. The strongest WB signal for p53 was observed with 1 μg of IP antibody and decreased with higher amounts of antibody, likely because the abundant HC masked the antigen signal [in such cases, less antibody (1 μg) or a non-reducing elution buffer may be used]. For AUF1, maximum WB signals were achieved by using 30 μg of IP antibody, whereas maximum procaspase-3, α-tubulin, and TIAR WB signals required only 10 μg of IP antibody.

HRP-conjugated Protein A yields WB signals that are virtually exclusive for the antigens of interest on WB filters (as illustrated here for p53, AUF1, procaspase-3, and α-tubulin), whereas HRP-conjugated Protein G yields signals that are strongly selective for the antigen, with some HC remaining visible. This selectivity is based on the fact that Protein A and Protein G bind

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the Fc portion of intact immunoglobulin molecules with strong preference [6]. HRP-conjugated secondary antibodies also bind the intact Fc portion of the primary antibody but, unlike Protein A and Protein G, they also bind the denatured individual HC and LC polypeptides on WB filters. The use of Protein A-or protein G-HRP as substitute for secondary antibody in WBs of IP samples is principally indicated for the detection of these and many other proteins whose molecular weights are close to those of the HC or LC, or for low abundance proteins of any size that are obscured by IP antibodies [7]. The main limitation of this approach is that neither Protein A-nor Protein G-HRP bind adequately to mouse IgG1 or rat antibodies (not shown).

In conclusion, Protein A-HRP can be used as substitute for a secondary antibody in WB analyses following IP when the primary antibodies for WB are rabbit immunoglobulin or mouse IgG2a or IgG2b immunoglobulins, all of which have high affinity for Protein A [2]. Protein G-HRP can successfully detect primary antibody from goat (Fig. 1C). It is important to point out that Protein A-HRP binds almost *exclusively* to the intact IgG molecule (Fig. 1A, B), whereas Protein G-HRP binds *preferentially* to the intact IgG molecule, but can also recognize the denatured IgG at low levels (Fig. 1C). It is also worth noting that Protein A-and Protein G-HRP can be used on standard Westerns (without IP, not shown) as well as for the detection of interacting proteins by co-IP assays (IP of one protein, WB analysis of an interacting protein using Protein A-HRP or Protein G-HRP [8]). For these widely used primary antibodies, the approach presented here affords the investigator a simple, rapid, and economical method of obtaining strong and clean WB signals from IP material.

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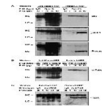

Fig. 1.

Comparison of the conventional and improved methods for Western blot analysis from IP samples. Following IP of RKO cell lysates, samples were subjected to SDS-PAGE and transferred onto nitrocellulose filters. (A) After incubation with rabbit primary antibodies recognizing p53, AUF1 or Procaspase-3, blots were washed and incubated either with a standard anti-rabbit secondary antibody (*left*) or with Protein A-HRP (*right*). (B) Following incubation with a primary mouse anti-α-Tubulin antibody, blots were washed and incubated either with a standard anti-mouse secondary antibody (*left*) or with Protein A-HRP (*right*). (C) After incubation with a primary goat-anti-TIAR antibody, blots were washed and incubated with either a standard anti-goat secondary antibody (*left*) or Protein G-HRP (*right*). All blots were developed by enhanced chemiluminescence after 1 min of exposure.